Dedication

I dedicate this work to the United States soldier. I hope these studies will lead to the development of new strategies to improve the recovery and preserve the quality of life of head-injured soldiers.

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14. ABSTRACT

Conantokin-G (Con-G or CGX-1007), a potent NR2B subunit selective NMDA receptor antagonist, was evaluated for its neuroprotective properties in experimental models of neuronal injury. In primary neuronal cultures Con-G was shown to decrease excitotoxic calcium responses to NMDA and provide 100% neuroprotection against hypoxia/hypoglycemia (34 μM[13-91]), NMDA (77 μM[42-141]), glutamate (819 μM[346-1937]) or veratradine (2136 μM[1508-3026]) induced injury (numbers in parentheses indicate EC50 and 95% confidence limits). Con-G (0.1-1 μM) also provided up to 80% protection against staurosporine-induced apoptotic injury (P<0.01, n = 12/group), which was linked to the NR2B subunit. For in vivo brain injury studies, middle cerebral artery occlusion (MCAo) in the rat was used as a model of transient focal brain ischemia. In this model Con-G (0.01-2.0 nmoles, i.c.v.) reduced brain infarction and improved both neurological and electroencephalographic (EEG) recovery as evaluated both 24 and 72 h post-injury. The maximal neuroprotective effect was measured with the highest dose of Con-G tested (2.0 nmol, i.c.v) with an 89% reduction of core infarct volume (P<0.05, n = 6-10/group). Post-injury time course experiments demonstrated a therapeutic window out to at least 4 h from the start of the injury. These neuroprotective effects were also associated with a 50% reduction in the early expression (i.e. 1-4 h) of the c-fos gene (P<0.05, n = 3-4/group), a preservation of Bcl-2 immunoreactivity at 24 h (P<0.05, n = 4), and with a reduction in DNA strand breaks in the ischemic hemisphere as evaluated 24 h post-injury (P<0.05, n = 6/group). Clinically relevant routes of administration were evaluated by administering intrathecal (i.t.) injections of Con-G (20-160 nmol), which provided up to 62% reduction in brain infarction (P<0.05, n = 8-9/group) along with significant neurological recovery and a therapeutic window of up to 8 h post-injury. Con-G (i.t.) treatment was also associated with fewer ischemia-induced EEG seizures with a positive albeit non-significant trend (P>0.05, n = 6-7/group) between the number of brain seizures and infarct volume. Intrathecal Con-G was not associated with changes in c-fos gene expression although, similar to i.c.v. administration, Bcl-2 immunoreactivity was preserved in cortical tissues (P<0.05, n = 3/group) and presence of TUNEL positive cells decreased at 24 h (P<0.05, n = 6/group). 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 in humans.

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Abstract:

Conantokin-G (Con-G or CGX-1007), a potent NR2B subunit selective NMDA receptor antagonist, was evaluated for its neuroprotective properties in experimental models of neuronal injury. In primary neuronal cultures Con-G was shown to decrease excitotoxic calcium responses to NMDA and provide 100% neuroprotection against hypoxia/hypoglycemia (34 μM[13-91]), NMDA (77 μM[42-141]), glutamate (819 μM[346-1937]) or veratradine (2136 μM[1508-3026]) induced injury (numbers in parentheses indicate EC_{50} and 95% confidence limits). Con-G (0.1-1 μM) also provided up to 80% protection against staurosporine-induced apoptotic injury ($P<0.01$, n = 12/group), which was linked to the NR2B subunit. For *in vivo* brain injury studies, middle cerebral artery occlusion (MCAo) in the rat was used as a model of transient focal brain ischemia. In this model Con-G (0.01-2.0 nmoles, i.c.v.) reduced brain infarction and improved both neurological and electroencephalographic (EEG) recovery as evaluated both 24 and 72 h post-injury. The maximal neuroprotective effect was measured with the highest dose of Con-G tested (2.0 nmol, i.c.v) with an 89% reduction of core infarct volume ($P<0.05$, n = 6-10/group). Post-injury time course experiments demonstrated a therapeutic window out to at least 4 h from the start of the injury. These neuroprotective effects were also associated with a 50% reduction in the early expression (i.e. 1-4 h) of the *c-fos* gene ($P<0.05$, n = 3-4/group), a preservation of Bcl-2 immunoreactivity at 24 h ($P<0.05$, n = 4), and with a reduction in DNA strand breaks in the ischemic hemisphere as evaluated 24 h post-injury ($P<0.05$, n = 6/group). Clinically relevant routes of administration were evaluated by administering intrathecal (i.t.) injections of Con-G (20-160 nmol), which provided up to 62% reduction in brain

infarction ($P<0.05$, n = 8-9/group) along with significant neurological recovery and a therapeutic window of up to 8 h post-injury. Con-G (i.t.) treatment was also associated with fewer ischemia-induced EEG seizures with a positive albeit non-significant trend ($P>0.05$, n = 6-7/group) between the number of brain seizures and infarct volume. Intrathecal Con-G was not associated with changes in *c-fos* gene expression although, similar to i.c.v. administration, Bcl-2 immunoreactivity was preserved in cortical tissues ($P<0.05$, n = 3/group) and presence of TUNEL positive cells decreased at 24 h ($P<0.05$, n = 6/group). 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 in humans.

Neuroprotection Profile of the High Affinity NMDA Receptor Antagonist Conantokin-G

by

Anthony J Williams

Thesis/Dissertation submitted to the Faculty of the

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Table of Contents (alternate thesis format)

Introduction:

Ischemic brain injury accounts for a leading cause of death and disability worldwide every year (STAIR, 1999). Although the human brain only comprises 2% of total body weight it consumes 25% of the available blood glucose and 20% of systemic oxygen. Nearly half of the central nervous system's energy supplies are utilized to drive Na^+/K^+-ATPase ion pumps. This high energy demand is due in large part to neuronal membrane repolarization following an action potential and the maintenance of ionic balance in glial cells (Magistretti et al., 1999). Brain ischemia is a disease process involving the loss of cerebral blood flow and the ensuing brain damage that follows. One of the initial consequences of an ischemic attack is the loss of mitochondrial metabolism resulting in a decrease of high-energy phosphate compounds available to the affected cells (Lipton and Rosenberg, 1994; Koroshetz and Moskowitz, 1996). In turn, cellular ion pumps shut down and a loss of ionic homeostasis ensues, leading to a complex series of events underlying cellular damage.

To further exacerbate the consequences of a loss of cellular membrane potential following a focal disturbance of blood flow, increased levels of the excitatory neurotransmitter glutamate are released into the synaptic cleft due to the influx of presynaptic neuronal Ca^{++} (Garcia et al., 1993; Martin et al., 1998). Reverse operation of the Na^+/glutamate transporter also increases extracellular glutamate levels, while glial cells become unable to absorb the excess glutamate (a process dependent on the Na^+ gradient created by Na^+-K^+-ATPase pumps). The resulting rise in extracellular glutamate overstimulates the postsynaptic neuron inducing the phenomenon termed excitotoxicity, leading to a further influx of intracellular sodium and calcium through glutamate

receptors. Increases in intracellular calcium can then lead to the activation of cellular destructive agents such as phospholipases, proteases, and both reactive nitrogen and reactive oxygen intermediates (Lipton and Rosenberg, 1994; Koroshetz and Moskowitz, 1996; Nicotera and Lipton, 1999). Glutamate levels have been shown to increase 6-fold in rats and up to 50-fold in human brain following injury (Koura et al., 1998). Furthermore there is a significant relationship between high glutamate levels and poor patient outcome as well as increased intracerebral pressure in head injury patients (Koura et al., 1998).

In the core of the infarcted brain regions an irreversible loss of mitochondrial membrane potential results in the early necrotic death of cells (Garcia et al., 1993; Lipton and Rosenberg, 1994; Martin et al., 1998). The result is the necrotic rupturing of cells due to osmotic swelling associated with the loss of ionic homeostasis (Carter, 1998). An evoked inflammatory response then ensues due to release of necrotic cellular debris into the extracellular space (Zhang et al., 1994; Pantoni et al., 1998; Nicotera and Lipton, 1999). Surviving cells that maintain at least some mitochondrial function may still undergo delayed cell death due to activation of ATP-dependent apoptotic processes (Eguchi et al., 1997; Barinaga, 1998; Martin et al., 1998). The classical indicators of apoptotic death include chromatin condensation, formation of apoptotic bodies, and DNA strand breaks as the cells slowly and methodically deconstruct. The induction of apoptosis may occur through a variety of pathways including; extrinsic (i.e. receptor mediated caspase activation), intrinsic (i.e. activation of "default" apoptosis pathways due to removal of growth factors), or Ca^{++}-mediated induction (i.e. influx of Ca^{++} through the NMDA receptor) (Lipton, 1999; Bredesen, 2000). In effect, an apoptotic/necrotic

continuum of cellular death is induced following brain ischemia and is potentially influenced by glutamate toxicity (Cheung et al., 1998; Martin et al., 1998).

In addition to the chemical inducers of cell death (described above) following ischemic injury our lab has also recently characterized the presence of electroencephalographic (EEG) disturbances inherent to this type of injury in rats. The presence of non-convulsant seizure (NCS) activity is of particular concern, which has been documented in brain injury patients as well (Vespa et al., 1999). The role of ischemia-induced electrical disturbances such as NCS on the outcome of brain injury has not been completely characterized to date. However, we have shown that treatment with an experimental sodium channel blocker RS100642 is able to block NCS and was associated with a neuroprotective reduction of brain infarct volume and improved neurological recovery following MCAo in rats (Williams and Tortella, 2002).

Early explorations utilizing neuronal culture models revealed that blocking the NMDA receptor with a variety of specific antagonists was a powerful method of altering glutamate-stimulated signaling as well as to reduce intracellular Ca^{++} influx. NMDA antagonists have thus been extensively studied as a therapeutic intervention to ameliorate the damaging cascades associated with glutamate toxicity following ischemic injury. However, initial studies with several NMDA antagonists showing efficacious effects in *in vivo* animal models have been terminated in clinical trials due to insignificant results or the emergence of toxic side effects (Bullock, 1995; Muir and Lees, 1995). Despite these failures, NMDA receptors remain a potential target for neuroprotection drug development (Whetsell, 1996). A search for agents specific for individual NMDA receptor subtypes may avoid the unwanted side effects and offer better therapeutic intervention as

neuroprotective treatments. In particular, NR2B is an NMDA receptor subunit largely restricted to cortical forebrain regions while other NMDA receptor subtypes are either not present in the cortex, i.e. NR2C and NR2D, or located throughout the brain, i.e. NR1 and NR2A (Wenzel et al., 1995). In addition to addressing the toxicity of potential neuroprotective drugs, the therapeutic profile of these compounds must be better assessed in the laboratory. In particular, it is important to define delayed post-injury treatment effects since many clinical patients may not receive therapy for several hours following brain injury (STAIR, 1999). Clinical trials are also being designed to better evaluate the neuroprotective effects of these and other candidate drugs as related to pre-clinical findings (Muir and Grosset, 1999).

Although the primary focus of NMDA antagonist therapy following ischemic brain injury has been on their ability to reduce the excitotoxic damage that leads to necrotic type cell death there are several studies linking the NMDA receptor to induction of delayed cell death cascades, possibly through modulation of calcium influx into neurons (Lipton, 1999). The Ca^{++}-induced expression of the immediate early gene (IEG) *c-fos* has been shown to precede programmed cell death or apoptosis both in *in vitro* and *in vivo* models of cellular injury (Smeyne et al., 1993a; Walton et al., 1999). Although the role of IEGs following brain in injury has not been definitively determined there is evidence that several IEGs are involved in the injury process (Akins et al., 1996). In particular, NMDA agonists, traumatic brain injury, as well as seizure induction *in vivo* have been shown to increase mRNA levels of *c-fos* in many brain regions including the cerebral cortex (Morgan and Linnoila, 1991; Dragunow and Preston, 1995; Dave et al., 1997). The widespread induction of Ca^{++}-induced expression of *c-fos* throughout the

ipsilateral cortex following ischemic brain injury has been suggested to be due to post-ischemic spreading depression, which induces cellular depolarization and intracellular Ca^{++} flux (Welsh et al., 1992). Importantly, several studies have shown that MCAo induced in rats significantly increases mRNA levels of *c-fos* up to 12 h following the injury and is inhibited with the NMDA receptor antagonist dizocilpine (MK-801) (Uemura et al., 1991; Welsh et al., 1992; Collaco-Moraes et al., 1994; Kinouchi et al., 1994a; Kinouchi et al., 1994b). Thus, NMDA antagonists possess the potential to reduce *c-fos* upregulation and possibly modulate the induction of apoptosis.

Alteration of *c-fos* expression post-MCAo has also been associated with changes in the expression of the apoptotic genes *bcl-2* and *bax* (Gillardon et al., 1996). Evidence suggests that these genes are involved in balancing pro- and anti-apoptotic induction in response to a variety of cell death or cell survival signals including neurotrophins, glucocorticoids, viral agents, or ischemic injury (Graham et al., 2000). Overexpression of bcl-2 protects cells from apoptotic death while bax induces apoptotic cell death (Zhong et al., 1993; Graham et al., 2000). Following MCAo in rats low levels of bcl-2 mRNA have been reported while bax mRNA levels increased in expression ipsilateral to the injury (Gillardon et al., 1996), which was also correlated to protein levels for both these genes. The bcl-2 family of genes (including bax and bcl-2) are located on multiple sub-cellular sites including mitochondria, nuclear membrane, and the endoplasmic reticulum (Lithgow et al., 1994). Apoptosis associated with the expression of these genes has been kinked to the control of cytochrome C release from mitochondria into the intracellular space. Intracellular cytochrome C release can interact with APAF-1 and induce apoptosis through activation of proteolytic caspases (Graham et al., 2000).

My hypothesis for this project was that delayed therapeutic intervention with Con-G (a high affinity, NR2B subunit selective NMDA antagonist) following transient forebrain ischemia in rats would reduce brain infarct volume, improve functional recovery as well as block ischemia-induced brain seizure activity. Concurrent with the neuroprotective effects, Con-G may alter functional expression of genes associated with the induction of delayed cell death (i.e. *c-fos, bcl-2, bax*) following brain injury. To test this hypothesis I utilized *in vitro* and *in vivo* models of neuronal injury to assess both the neuroprotective efficacy and changes in gene expression associated with ischemic brain injury.

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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[1]

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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 penumbral 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

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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; $[Ca^{2+}]_i$, 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 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 excitoxicity, 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 normothermic (37 ± 1°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 (AALAC). 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 × ⅛ 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 referenced 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-triphenyl tetrazolium 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.

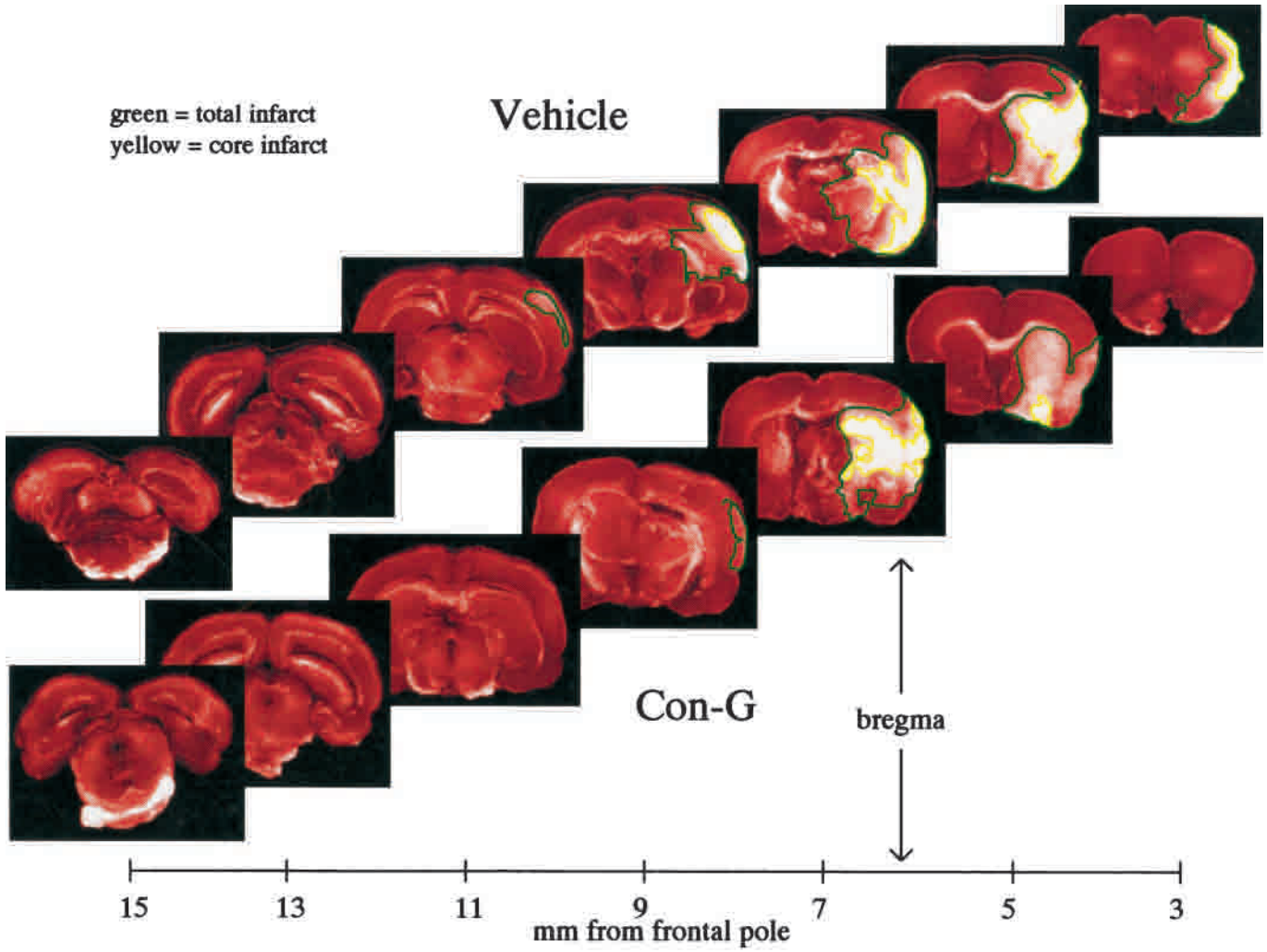

Fig. 1. Representative forebrain images of vehicle versus Con-G (0.5 nmol) administration after 2 h of MCAo and a 22-h reperfusion. Brain sections were stained with TTC, and total infarct volume (green outline) and core infarct volume (yellow outline) were defined.

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 5×10^5 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-dimethylthiazol-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% CO_2 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 μm^2) were collected every 10 s to construct the kinetic profiles of the effects of NMDA on $[Ca^{2+}]_i$ 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 ED_{50} values, the criteria for identifying a positive responder were defined as previously described (Tortella et al., 1999). Potency and ED_{50} 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 1
Post-MCAo occlusion treatment protocol for 2-h MCAo and reperfusion
All injections were given i.c.v.

Protocol	Injection Schedule	Dose Range	End Point
	h	*nmol*	*h*
Dose-response	0.5, 2, 4, 6	0.001–2.0	24
Therapeutic window	0.5, 2, 4, 6	0.5	24
	2, 3, 4, 6	0.5	24
	4, 5, 6, 8	0.5	24
	8, 9, 10, 12	0.5	24
Single-dose study	0.5, 2, 4, 6, 24, 48	0.5	72

Results

MCAo Injury. Control vehicle-treated rats subjected to 2 h of MCAo followed by 22 or 70 h of reperfusion exhibited striatal and cortical brain infarction in the right hemisphere from approximately 3 to 11 mm from the frontal pole (Figs. 1 and 2). At the 24-h end point (Fig. 3), total infarct volume in control rats was 302 ± 24 mm^3 and core infarct volume was 194 ± 23 mm^3, with the core infarct representing approximately 18% and the total infarct representing approximately 28% hemispheric infarction. After 72 h of MCAo (see Fig. 6), the total infarct volume in control rats was 390 ± 30 mm^3 and core infarct volume was 231 ± 26 mm^3, with the core representing 24% and the total 41% representing hemispheric infarction. MCAo resulted in significant hemispheric edema representing an approximately 9 and 5% increase in cerebral volume compared with the contralateral, uninjured hemisphere for the 24- and 72-h end points, respectively. Neurological function, which was severely impaired at 2 h after MCAo (score, 10 ± 0) exhibited a significant degree of spontaneous recovery (score, 8.8 ± 0.6 at 24 h and 3.0 ± 0.0 at 72 h). However, none of the injured vehicle-treated animals completely recovered neurological function (score, 0), with at least contralateral forelimb flexion and shoulder adduction (score, 3) still evident in all rats examined at both 24 and 72 h after injury.

All MCAo animals lost approximately 11 to 15% of body weight during the 24-h recovery period and a 23 to 27% loss

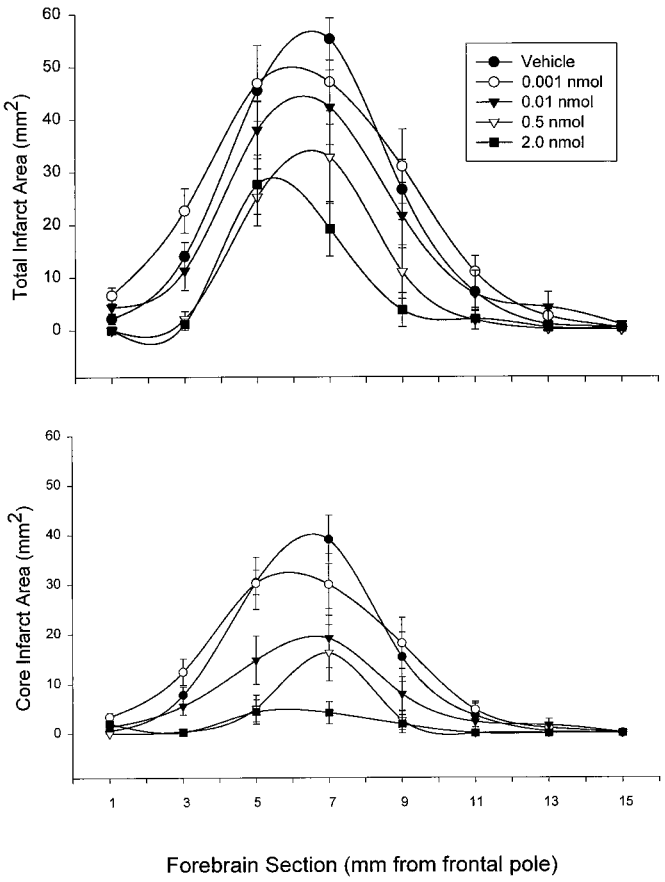

Fig. 2. Dose-dependent decrease in cerebral infarction of both total and core areas for each coronal brain section. Data are presented as mean ± S.E.

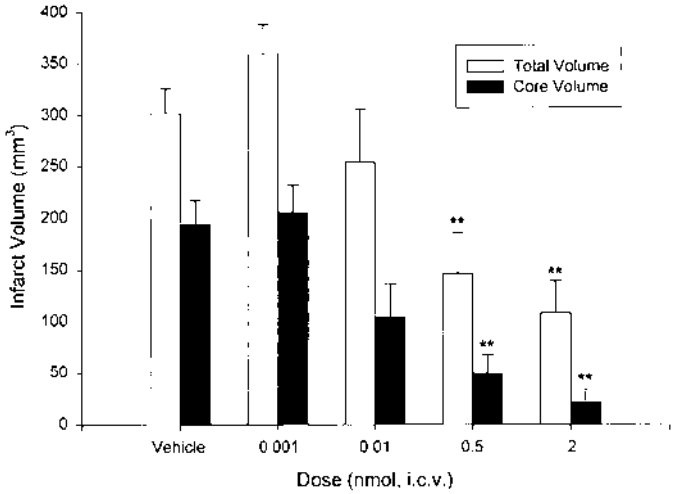

Fig. 3. Effect of increasing doses of Con-G, compared with vehicle group, to reduce infarct volume after 24 h of temporary MCAo. Data presented as mean ± S.E. *$P < .05$, **$P < .01$ compared with vehicle-treated group (independent t test).

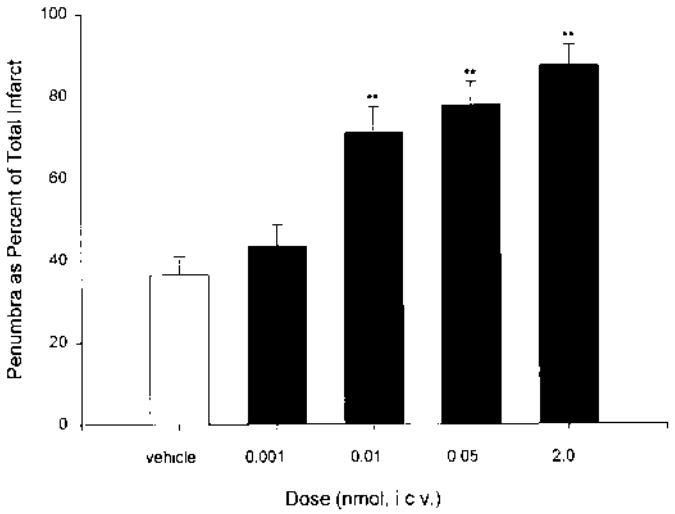

Fig. 4. Percentage of total infarct volume that is penumbra as opposed to core injury, with increasing doses of Con-G compared with vehicle group. Data are presented as mean ± S.E. *$P < .05$, **$P < .01$ compared with vehicle-treated group (independent t test).

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 ED_{50} 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.

TABLE 2

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

Values are presented as mean ± S.E.

Con-G	EEG Recovery[a]	Neurological Score
nmol i.c.v.	%	
Vehicle	23 ± 9	8.8 ± 0.6
0.001	33 ± 8	7.7 ± 1.5
0.01	52 ± 16	6.0 ± 0.9*
0.5	81 ± 30	5.2 ± 1.0*
2.0	93 ± 18**	4.7 ± 1.5*

[a] Percentage recovery of EEG power compared with pre-MCAo recording.
* $P < .05$, ** $P < .01$, compared with vehicle-treated group (independent t test).

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

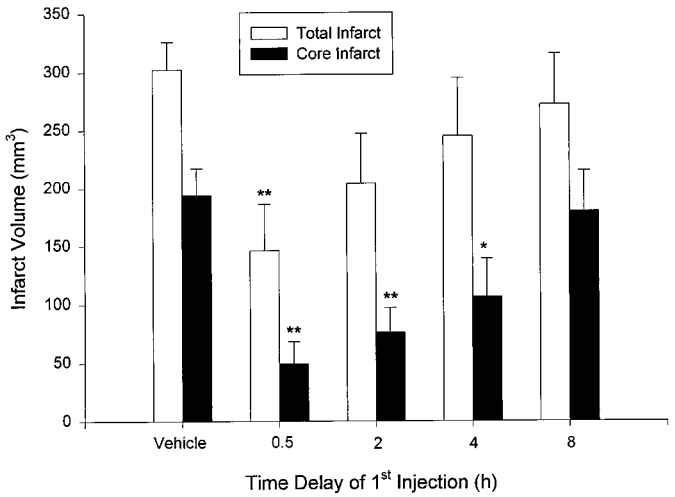

Fig. 5. Effect of delayed treatment (from time of MCA occlusion) with Con-G, compared with vehicle group, to reduce infarct volume after 24 h of temporary MCAo. Data are presented as mean ± S.E. *$P < .05$, **$P < .01$ compared with vehicle-treated group (independent t test).

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 ± 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 ± 49 and 105 ± 39 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 (pO_2 and 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

Effect of delayed treatment with 0.5 nmol Con-G i.c.v. on percentage penumbral infarct, percentage EEG recovery, and neurological score after 2 h of MCAo and 24-h recovery

Values are presented as mean ± S.E.

Time Delay of First Injection	Penumbral Infarct Volume[a]	EEG Recovery[b]	Neurological Score
		%	
Vehicle	37 ± 5	23 ± 9	8.8 ± 0.6
30 min	78 ± 6**	81 ± 30	5.2 ± 1.0*
2 h	70 ± 6**	63 ± 9**	5.9 ± 1.1*
4 h	63 ± 8*	82 ± 19*	6.0 ± 1.5
8 h	36 ± 5	29 ± 7	3.0 ± 1.5**

[a] Percentage penumbral infarction compared with the total infarct volume.
[b] Percentage recovery of EEG power compared with pre-MCAo recording.
* $P < .05$, ** $P < .01$, compared with vehicle-treated group (independent t test).

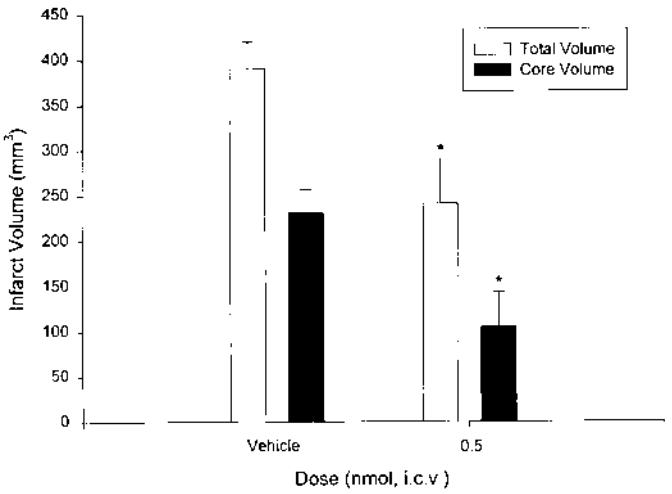

Fig. 6. Effect of Con-G (0.5 nmol), compared with vehicle group, to reduce infarct volume after 72 h of temporary MCAo. Data are presented as mean ± S.E. *$P < .05$, compared with vehicle-treated group (independent t test).

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 (EC_{50} and 95% confidence limits) were different for the respective injury mechanisms. Con-G exhibited extremely potent and similar levels of neuroprotection against NMDA [77 μM (42–141)] and H/H [34 μM (13–91)]. However, much lower neuroprotection potencies were measured when the injuries was induced by glutamate [819 μM (346–1937)] or veratridine [2136 μM (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-formazon 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-

TABLE 4

Physiological parameters for (0.5 nmol) Con-G and vehicle-treated rats with and without MCAo

Injections were given at 30 min and 2, 4, and 6 h after occlusion, and measurements were taken at 30 min after each injection. Values presented as mean ± S.D.

Time Point	MABP	HR	pO_2	pCO_2	pH
h	*mm Hg*	*beat/min*	*mm Hg*		
Con-G-treated rats, no MCAo					
Baseline	95 ± 17	267 ± 26	97 ± 15	31 ± 3	7.45 ± 0.02
1	90 ± 11	236 ± 111	105 ± 17	29 ± 11	7.43 ± 0.02
2.5	109 ± 13	280 ± 75	92 ± 7	30 ± 5	7.41 ± 0.03
4.5	122 ± 18*	316 ± 179	106 ± 26	24 ± 3	7.43 ± 0.04
6.5	125 ± 10*	250 ± 93	82 ± 10	26 ± 1	7.47 ± 0.04
Vehicle-treated rats, 2-h MCAo and reperfusion					
Baseline	98 ± 8	282 ± 59	94 ± 13	29 ± 7	7.43 ± 0.03
1	136 ± 10**	349 ± 41	99 ± 21	23 ± 4	7.44 ± 0.03
2.5	132 ± 4*	291 ± 98	87 ± 18	25 ± 9	7.45 ± 0.02
4.5	120 ± 11	349 ± 40	79 ± 3	33 ± 3	7.42 ± 0.04
6.5	102 ± 14	298 ± 88	79 ± 7	33 ± 3	7.42 ± 0.02
Con-G-treated rats, 2-h MCAo and reperfusion					
Baseline	95 ± 2	196 ± 55	91 ± 2	32 ± 4	7.48 ± 0.01
1	112 ± 15	256 ± 116	75 ± 4	33 ± 4	7.47 ± 0.04
2.5	101 ± 2	305 ± 100	75 ± 4	33 ± 3	7.43 ± 0.02
4.5	124 ± 23	271 ± 81	83 ± 22	35 ± 6	7.44 ± 0.02
6.5	115 ± 18	314 ± 129	72 ± 9	32 ± 3	7.46 ± 0.03

* $P < .05$, ** $P < .01$, compared with baseline value (Dunnett's post hoc test).

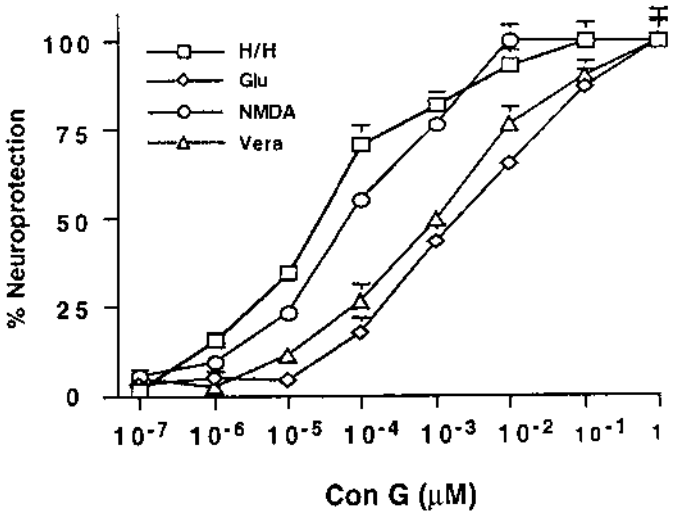

Fig. 7. Dose-dependent neuroprotection of Con-G-treated cerebellar neurons against H/H, glutamate (Glu), NMDA, or veratradine (Vera), as determined by MTT assay. Data are presented as mean ± S.E.

ical stains, such as H&E (Bederson et al., 1986; Park et al., 1988). We have also attempted to demarcate the perifocal infarcted region of light pink TTC-stained tissue. By definition (i.e., reduced staining indicative of reduced mitochondrial enzyme activity), we consider this to represent the "penumbral" area of injury.

To further analyze ischemic brain damage in our model, we also measured cortical EEG activity immediately proximal to the injured hemisphere, as well as behavioral neurological performance. In our rat model, we observed a positive correlation between the reduction in infarct volume and the recovery of EEG power (cortical function in the injured hemisphere) and improved neurological status not unlike that described previously in a rabbit MCAo model (Andrews et al., 1988).

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 nonsedative 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 out circumferentially from the core of the infarct with an increase in the extracellular levels of excitatory amino acids (Dyker and Lees, 1998). On occlusion of the MCA, there exists a limited time interval within which neuroprotective drug intervention can alter the outcome of the injury. For example, MK801 has a short therapeutic window of 30 min, and dextrorphan treatments offer neuroprotection only when given up to 2 h after the onset of focal cerebral ischemia (Steinberg et al., 1995; Margaill et al., 1996). As our data demonstrated, there still was a significant (i.e., 47%) neuroprotection in the core infarction after a treatment delay of at least 4 h, with significant improvements in neurological recovery still observed with treatment delays of 8 h

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-isoxazolepropionic acid ligands, antioxidants, *N*-acetylated alpha-linked acidic dipeptidase inhibitors, and anti-inflammatory treatments, reducing cerebral damage and/or improving functional deficits. (Clemens and Panetta, 1994; Kawasaki-Yatsugi et al., 1998; 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 maxi-

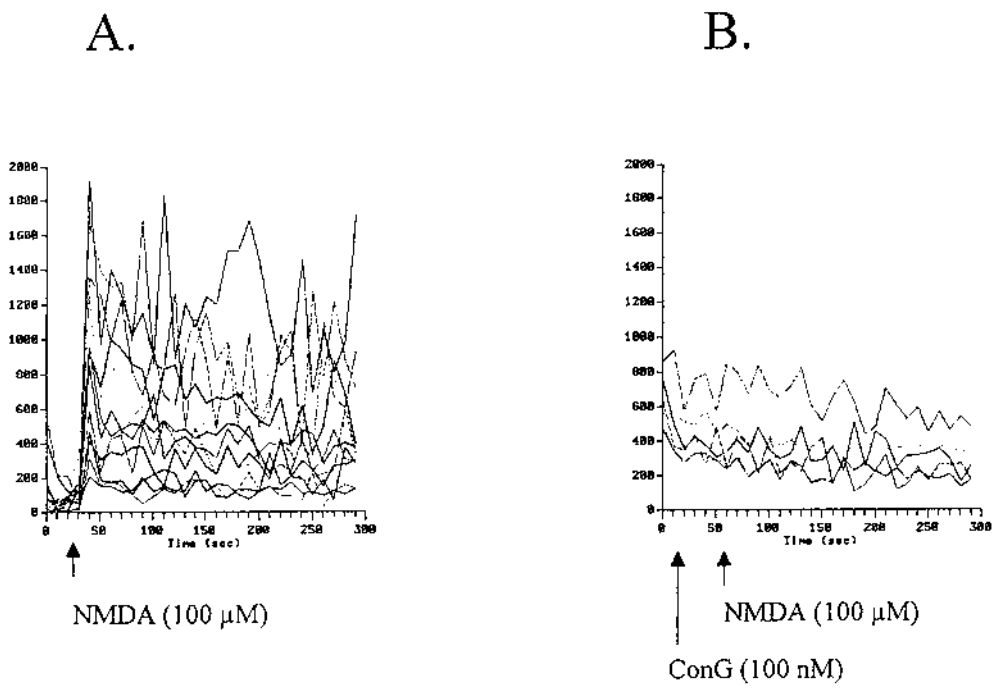

Fig. 8. Time-dependent $[Ca^{2+}]_i$ levels after NMDA (A) or Con-G and NMDA (B) treatment. Each line represents the calcium response in an individual neuron.

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 (Margaill 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 (IC_{50} = 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 Ca^{2+} mobiliza-

tion with on-off kinetics similar to that reported for other NMDA antagonists (Klette 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.

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Treatment with the snail peptide CGX-1007 reduces DNA damage and alters gene expression of *c-fos* and *bcl-2* following focal ischemic brain injury in rats

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General Discussion:

The conantokin family of peptides derived from marine snail venom has recently been characterized as the first group of naturally occurring and biochemically active molecules that are specific and potent inhibitors of the NMDA receptor (Jones and Bulaj, 2000). Studies with these compounds have revealed potent anticonvulsant and neuroprotective properties with a high protective index in several animal seizure models as compared to clinically available compounds (Bialer et al., 2002). Furthermore, studies targeting the intrathecal safety and tolerability of Con-G have recently been completed (T. McCabe, personal communication). Clinical trials have now been planned for the treatment of intractable epilepsy using the SynchroMed® infusion system. The peptide structure of conantokins has been associated with the poor blood brain barrier penetrability of these compounds thus efficacious treatment of CNS disorders requires a direct delivery approach. The SynchroMed® system is an FDA approved drug delivery system designed for continuous intrathecal drug infusions, currently available to treat chronic pain in cancer patients (Bialer et al., 2002).

Con-G is a potent NMDA antagonist derived from the marine snail *Conus geographus.* Con-G is unique in that it contains 5 γ-carboxyglutamate (Gla) residues and has no disulfide bonds involved in its secondary structure. These Gla residues near the N-terminus, along with other subunits, are involved in the inhibitory binding of Con-G the NMDA receptor (Chandler et al., 1993; Zhou et al., 1996; Blandl et al., 1998). The α-helical structure of Con-G, which is due to interactions with divalent cations including Ca^{++}, Mg^{++}, and Zn^{++}, may also be involved (Zhou et al., 1996; Chen et al., 1998; Prorok and Castellino, 1998). Con-G has been previously shown to inhibit NMDA but not

kainate induced increases of cyclic GMP in rat cerebellar granular cells. In these cells, as well as in human brain cell cultures, Con-G has exhibited a potent inhibition of polyamine enhanced [^{3}H]MK801 binding (IC_{50} = 500-950 nM) (Skolnick et al., 1992; Nielsen et al., 1999). Polyamines have been reported to have modulating effects on glutamate receptors (NMDA and AMPA receptors) as well as potassium channels although little is known about the overall physiological role or distribution of polyamines in brain injury (Johnson, 1998). However, increased polyamine levels have been measured following experimental brain seizures and in brain ischemia models in rats (Carter et al., 1995; Hayashi and Baudry, 1995). Polyamines have also been shown to enhance Con-G antagonist properties (Donevan and McCabe, 2000). This activity or injury-dependent release of polyamines may thus enhance Con-G block following brain injury.

As well as being a potent NMDA antagonist, Con-G has recently been reported to be a selective for the NR2B subunit (Donevan and McCabe, 2000). Using Xenopus oocytes to control the expression of specific recombinant NMDA receptors, Donevan and McCabe (2000) reported the selective affinity of Con-G exclusively for NMDA receptors containing the NR2B subunit and a loss of affinity if other NR2 subunits were substituted for NR2B (i.e. NR2A, NR2C, or NR2D). Con-G is also highly selective for the NMDA receptor without significant activity at any other receptor or binding site as determined from *Novascreen*® profiling studies (Bialer et al., 2002). Furthermore, the NR2B subunit is largely restricted to forebrain structures with disperse representation in the spinal column or cerebellum of the mammalian central nervous system (CNS) (Tolle et al., 1993; Hollmann and Heinemann, 1994; Wenzel et al., 1995; Sundstrom et al., 1997). In

effect, Con-G possesses a distinct binding affinity for the NMDA receptor (specifically targeting forebrain CNS) over any other NMDA inhibitor described to date.

Functional NMDA receptors are composed of several subunits that organize into heterooligomeric protein complexes. There are two major subunits of NMDA receptors, namely, NR1 and NR2. The NR1 subunit exists as one of eight splice variants while the NR2 subunit exists as one of four isoforms, NR2A, NR2B, NR2C and NR2D (Monyer et al., 1992; Chazot et al., 1994; Chazot and Stephenson, 1997; Thompson et al., 2000). The exact subunit composition of native NMDA receptors is not well defined but recent reports indicate that two NR1 and two NR2 subunits are likely to participate in the formation of tetra or pentameric complex (Dingledine et al., 1999; Hawkins et al., 1999). The regional distribution of various NMDA receptor subunit proteins in rat brain is well documented (Monyer et al., 1992; Wenzel et al., 1995; Khan et al., 2000; Thompson et al., 2000). While NR2A immunoreactivity has been shown to occur in almost all regions of the rat CNS, NR2B reactivity is restricted to forebrain, NR2C in cerebellum, thalamus and olfactory bulb and NR2D in diencephalic, mesencephalic and brain stem structures (Wenzel et al., 1995). The lack of NR2B subunit expression in spinal cord and cerebellum may indicate a potential for reduced toxicity such as motor impairment following treatment with NR2B subunit specific antagonists.

Our *in vitro* analysis of Con-G revealed complete neuroprotective efficacy in all four models of neuronal injury studied with a rank order of potency of H/H > NMDA > veratradine > glutamate (paper 1). These data are evidence for the potent neuroprotective properties of Con-G against *in vitro* ischemic damage (H/H), which correlate with the *in vivo* neuroprotective properties described below. Furthermore, Con-G is an effective

modulator of excitotoxic injury in all three of our other models with the highest potency against NMDA-induced injury as would be expected from its nanomolar binding affinity for the NMDA receptor (Skolnick et al., 1992). As we have shown, the effects of Con-G to ameliorate neuronal Ca^{++} influx, due to NMDA administration, is further support not only of the site of action of Con-G but also its potent ability to antagonize the NMDA receptor. All four of our neuronal injury models have been well characterized (Lysko et al., 1994; Dave et al., 2000; Tortella et al., 2000), as has the kinetics of the NMDA induced ($[Ca^{++}]_i$) mobilization (Klette et al., 1997). Collectively, the results of our initial *in vitro* experiments demonstrate that Con-G offers strong resistance to excitotoxic, membrane-depolarizing agents and that this property extends to H/H-induced injury as well.

The use of *in vitro* culture models for assessment of the neuroprotective properties of a an experimental compound is somewhat limited due to the fact that *in vivo* brain injury involves a host of cell death mechanisms, which may not be accounted for in culture. For instance, cell culture models lack a blood brain barrier or a circulatory immune system, which have been shown to be critical mediators of the overall progression of brain injury (Phillips et al., 2000; Berti et al., 2002; Williams et al., 2003). Furthermore, ischemic brain injury involves both necrotic as well as apoptotic cell death. The excitotoxic models described above induce primarily a necrotic type of cell death characterized by early loss of membrane integrity and cellular swelling, leading to a rapid degeneration of cell morphology. To characterize other forms of cell death, in particular apoptosis, we used a staurosporine-induced injury in primary neuronal cultures (paper 2). Staurosporine induces a predominately apoptotic injury in cell cultures as shown by

induction of chromatin condensation, DNA fragmentation, and formation of apoptotic bodies. Interestingly, we found that Con-G was highly effective against this type of injury and that importantly, the NMDA receptor antagonist MK801 had no effect at any dose tested. In this context it is worth mentioning that MK-801 is recognized for its non-selectivity for NMDA receptor subtypes, binding to NMDA receptors containing varying receptor subunits (Bresink et al., 1996). We further evaluated another NR2B specific NMDA receptor antagonist (ifenprodil) and found that it was effective against staurosporine-induced apoptosis. Ifenprodil is also selective for the NR2B receptor but has been reported to possess other biological activity such as inhibition of ornithine decarboxylase activity responsible for the formation of polyamine products (Badolo et al., 1998). In vitro it has been reported that both CGX-1007 and ifenprodil allosterically modulate polyamine binding to the NMDA receptor (Skolnick et al., 1992; Kew and Kemp, 1998). However CGX-1007 does not displace [^{3}H]ifenprodil binding (Zhou et al., 1996), indicating that each compound binds a distinct region of the NMDA receptor. Thus, a novel anti-apoptotic mechanism appears to be involved with selective antagonism of the NR2B receptor. In effect, although culture models of neuronal injury are limited in several aspects as to their correlation to whole brain injury, they are important tools available to assess effects of neuroprotective compounds on different aspects/mechanisms of the brain injury process.

For the assessment of the neuroprotective effects of Con-G *in vivo* we have used a model of focal cerebral ischemia originally described by Longa et al. (Longa et al., 1989), which closely resembles clinical stroke pathology in humans (Ringelstein et al., 1992). Using this model we induced a 2 h temporary occlusion of the middle cerebral artery

(MCA) in the rat followed by restoration of blood flow to the ischemic area. Although reperfusion has been shown to enhance injury due to production of reactive oxygen species and increased edema (Kuroiwa et al., 1988; Lipton and Rosenberg, 1994), it also allows for restoration of metabolic activity in the compromised areas (Hallenbeck and Dutka, 1990; Halsey et al., 1991). Critically, this model is well established and been shown to be highly sensitive to pharmacological intervention with neuroprotective drugs as a means to reduce the resulting brain infarction (Clemens and Panetta, 1994; Britton et al., 1997; Kawasaki-Yatsugi et al., 1998; Tatlisumak et al., 1998; Tortella et al., 1999). The resulting lesion induced in this model includes a core area of infarction initiating from the origin of the MCA and a surrounding "penumbral" or "peri-infarct" region of less compromised tissue (Gill et al., 1995), similar to the pathology seen in clinical stroke patients (Kaufmann et al., 1999).

The actual delineation between core and peri-infarct areas depends on the physiological marker being measured including altered blood flow dynamics, metabolism, gene expression or histology (Lo et al., 1996). For example, histopathological brain damage was measured in our model using a TTC stain, which is reduced to a red-formazon product in the presence of active mitochondrial oxidative enzymes and has been shown to correlate to other staining techniques, 6 hours and beyond, following injury (Bederson et al., 1986; Park et al., 1988). The resulting pathology includes a "core" of infarcted tissue (indicated by white, non-TTC stained tissue) consistent with the necrotic cell damage as evaluated with other histopathological stains such as hematoxylin and eosin (H&E). In our neuroprotection studies with Con-G we have also demarcated the peri-focal infarcted region of light pink TTC stained tissue

as compared to the normal contralateral hemisphere. By definition, we consider this remote area of the brain lesion to define the peri-infarcted region. These outer regions of peri-infarcted tissue may still be salvageable and are a prime target for treatments targeting delayed cell death mechanisms, such as apoptosis and inflammatory-cell mediated phagocytosis, occurring in these regions.

Initially, a dose-response assessment of the effect of Con-G to decrease brain infarction was completed to establish neuroprotection potency and efficacy in our MCAo model (paper 1). The neuroprotective effects were also correlated to improvements in both the EEG activity of the injured hemisphere, and neurologic recovery in the rat. Not only did Con-G dose-dependently decrease infarct volume but 1) there was a clear separation between behavioral sedation and neuroprotection (e.g. doses < 0.5 nmol were non-sedative), with only the highest dose of Con-G tested (2.0 nmol) exhibiting mild behavioral sedation, 2) with increasing doses of Con-G a greater amount a peri-infarct tissue was present with the concomitant reduction in core brain infarction, and 3) it is unlikely that these neuroprotective effects of Con-G were due to simply to hypothermia, as the rectal temperatures never fell below the normal range during or after the MCAo surgery.

Once the potency and efficacy was established, an optimal neuroprotection dose of 0.5 nmol of Con-G was chosen for further studies. This was the dose that maintained highly efficacious effects, with a 75 percent neuroprotection in the core infarcted region, without any behavioral side effects. Due to the delayed injury processes involving apoptosis and the detrimental effects of inflammation we tested this dose of Con-G in the 72 h recovery model to establish the effects of Con-G to permanently reduce, and not

merely delay, the extent of brain injury. Although physiological changes may be occurring for several weeks following ischemic injury, studies utilizing permanent MCAo in rats have reported that brain infarction reaches a maximal volume and spread by 72 h post-injury (Garcia et al., 1993). A similar progression of infarct volume was seen in human stroke patients using diffusion-weighted imaging, with a maximal infarct size occurring 70 h following the ischemic attack (Schwamm et al., 1998). Our results indicated that 72 h post-MCAo a significant 54% neuroprotection in the core infarcted region was obtained with Con-G as compared to vehicle treatment. This neuroprotective reduction of infarction correlated with a significant recovery in both EEG function and neurological score.

Following an ischemic attack brain infarction tends to progress circumferentially from the core of the infarct region in conjunction with an increase in the extracellular levels of excitatory amino acids (EAA) (Dyker and Lees, 1998). Importantly, upon occlusion of the MCA, a limited time interval exists within which neuroprotective drug intervention can occur. For example, MK801 has a short therapeutic window of 30 min and dextrorphan only offers a 2 h treatment window in rat and rabbit models of focal cerebral ischemia, respectively (Steinberg et al., 1995; Margaill et al., 1996). Clinically, the therapeutic window is important for the stroke patient who may not receive treatment for an extended time following the initial ischemic attack (Dyker and Lees, 1998). As our data show there is still a significant (47%) reduction of core infarction following a delay of at least 4 h from the induction of MCAo until the first treatment with Con-G. Although there was no significant reduction of infarct volume when Con-G injections were delayed for 8 h, significant improvements in neurological recovery were measured.

We also evaluated the effects of Con-G treatment on cardiovascular and blood gas changes in both normal and MCAo injured animals. In general, MCAo induced a transient increase in MABP but no significant differences were measured between vehicle and Con-G treated animals. Other physiological parameters including, heart rate, blood gases and pH were not statistically different from the baseline values in vehicle or Con-G treated animals.

In initial experiments designed to explore more clinically applicable routes of administration we evaluated the neuroprotective effects of Con-G using an intrathecal catheter (allowing multiple injections into the cauda equina of the spinal cord) and injecting Con-G (20 nmol) at 30min, 2, 4, and 6 h following MCAo. The result was a significant, 48% reduction of core infarcted brain tissue (data not shown). Neurological recovery was inconclusive because of behavioral interference induced by the cannula itself. For further studies we used direct acute intrathecal injections in the cauda equina of the spinal column. Initial studies with dye injections (10 μL) via this route have revealed presence of the dye in the cranial cavity upon post-mortem inspection. Furthermore, single intrathecal injections of Con-G (80 nmol) produced low nanomolar brain concentrations in normal and MCAo injured rats. Importantly, we have shown that 0.5 nmol concentrations of Con-G delivered i.c.v. are neuroprotective (paper 1).

The results from the intrathecal neuroprotection studies (paper 3) indicated that Con-G (20-160 nmol) dose-dependently decreased infarct volume and improved neurological recovery. A progressive transformation of the infarct to include a more peri-infarct type injury with a reduced core infarction with increasing dose was also measured, similar to effects following i.c.v. Con-G administration (paper 1). For subsequent studies

we used an intrathecal dose of 80 nmol, which was not associated with any behavioral side effects. Using this dose we measured significant neuroprotection (i.e. 32% reduction of core injury) following a delayed treatment of at least 8 h from the induction of MCAo. Similar to the i.c.v. experiments, it is unlikely that the neuroprotective effects of Con-G were due simply to hypothermia as brain temperatures never fell below the normal range during or after the MCAo surgery (paper 2).

In addition to neuroprotection achieved with intrathecal administration of Con-G we also measured a reduction in NCS activity (Appendix figure 1), an effect similar to the anti-seizure effects of the sodium channel blocker RS100642 (Williams and Tortella, 2002). Induction of NCS activity occurred in nearly 80% of all animals tested, generally within the first 30 min following occlusion of the MCA. Treatment with i.t. Con-G delivered 30 min post-MCAo was associated with 44% fewer seizures as well as 49% less time spent in seizure. Although the direct role brain seizures have on the progression of injury has not been determined, the hyperactive state induced by brain seizure activity further depletes the cells energy reserves (Meltzer et al., 2000) and may exacerbate ischemic injury. In this study, we measured a low but positive correlation between infarct volume and presence of NCS activity (correlation coefficients = 0.230-0.363, Appendix figure 2). The correlation between infarct volume and the number of NCS events or the total time in seizure did not reach significance however ($P > 0.05$). Further studies involving a larger group of animals may be needed to determine if a direct correlation does exist between the induction of NCS and severity of brain injury.

Following brain injury a release of excitatory neurotransmitters including glutamate and acetylcholine occurs (Hayes et al., 1992), which can induce intracellular

calcium influx. Increases in intracellular calcium activate the CREB pathway to induce *c-fos* gene expression as described above. Injury-induced increases in *c-fos* can then induce expression of a variety of "late" genes (Mocchetti et al., 1989; Yang et al., 1994). For example, AP-1 binding sites on the genes such as neuronal growth factor (NGF) indicate that *c-fos* may influence NGF expression (Mocchetti et al., 1989), which could effect neuronal survival. Continuous *c-fos* expression has also been shown to precede programmed cell death *in vivo* in *fos-lacZ* transgenic mouse cells undergoing apoptosis (Smeyne et al., 1993b).

Our time course studies of *c-fos* expression revealed that the early expression of *c-fos* mRNA was associated with strong nuclear immunoreactivity throughout the ischemic region of the ipsilateral cortex and eventual resolution upon appearance of necrosis. Importantly, C-Fos protein was highly expressed in the ischemic region destined for necrosis by 24 h. Thus, *c-fos* upregulation was verified in brain regions that undergo cell death at later time points. However, the presence of increased C-Fos protein levels in the areas surrounding the necrotic lesion, particularly at delayed time points (> 4 h), has led to the hypothesis that C-Fos is neuroprotective in these cells, since necrotic regions lack C-Fos (Kiessling and Gass, 1994; Akins et al., 1996). Others have suggested that the increased *c-fos* expression in peri-infarct regions may only be a consequence of altered brain function such as cortical spreading depression that emanates from the ischemic core (Menniti et al., 2000). Currently, our lab is exploring the role of spreading depression as it relates to the progression of MCAo injury in rats (Hartings et al., 2003).

C-fos upregulation following an ischemic injury includes a potential array of effects. *C-fos* in conjunction with other IEGs are able to heterodimerize and effect the

expression of genes involved in the regulation of delayed cell death cascades such as apoptosis (Dragunow and Preston, 1995; Preston et al., 1996). It has been reported in several injury models that *c-fos* is indeed activated prior to cell death (Smeyne et al., 1993a; Gillardon et al., 1996; Preston et al., 1996; Kumari and Alvarez-Gonzalez, 2000). However, the role of *c-fos* as a neurodegenerative molecule is not clear and the activation of *c-fos* may only be a consequence of cellular over-excitation with no direct role in the induction of further injury to ischemic cells. We measured a differential effect of *c-fos* expression depending on the route of administration of Con-G. Direct injection of Con-G into the brain (i.c.v.) 30 min post-injury effectively reduced *c-fos* mRNA levels by 50% at 1 and 4 h post-injury in both cortical and subcortical brain regions (paper 4). In contrast, intrathecal injection of Con-G had no significant effect on *c-fos* mRNA levels (Appendix figure 3) or protein immunoreactivity (Appendix figure 4). Although both injection routes were given 30 min post-MCAo, the intrathecal route may take longer to induce respective brain levels of Con-G. Another possibility is that the measured increases in *c-fos* expression were at least partially due to Ca^{++} independent mechanisms. Regardless, both treatment routes provided significant brain neuroprotection as assessed by reduction of infarct volume as well as reducing presence of TUNEL positive cells (Appendix figures 5 and 6). Thus, although Con-G potently inhibits *c-fos* expression when delivered directly to the forebrain, the reduction does not seem to be crucial for reducing the injury since intrathecal treatment was equally effective but did not reduce *c-fos* expression. If active *c-fos* expression does in fact provide a neuroprotective effect (for instance by inducing cell proliferation) then the intrathecal route of administration of Con-G may infer an added advantage over i.c.v. administration or other NMDA

antagonists such as MK801, which reduce *c-fos* expression. In support of this hypothesis is that i.t. administrations of Con-G were associated with a wider therapeutic window for treatment of MCAo injury in rats. To further determine the exact role of *c-fos* expression following MCAo we are currently evaluating the effects of i.c.v. treatment with *c-fos* ASOs, which may potentially reduce the injury if *c-fos* itself possesses neurotoxic properties.

Discovery of the *bcl-2* family of genes was reported as early as 1984 in B-cell lymphomas (Tsujimoto et al., 1984) and were found to regulate apoptosis not only in cancer cells but a variety of other cells as well. These gene products have also been shown to play a role in the induction of apoptosis following brain injury, specifically through the induction of caspases (Bredesen, 2000; Graham et al., 2000). Manipulation of brain injury induced apoptotic signaling has become an intense area of research particularly because of concerns that delayed cell death may override the effects of neuroprotective strategies aimed at reducing the excitotoxic component of brain injury (i.e. with the use of ion channel modulators). The use of bcl-2 ASOs has revealed that down-regulation of *bcl-2* increases cell death by 50% following hypoxic treatment of cultured rat cortical neurons (Banasiak et al., 1999). Furthermore, Chen et al (Chen et al., 2000) reported that i.c.v. treatment with bcl-2 ASOs reduced expression of *bcl-2* and increased the volume of brain infarction following 60 min of temporary brain ischemia in rats. In this study, we were particularly interested in how Con-G treatment effects the expression of the anti-apoptotic gene *bcl-2* and the pro-apoptotic gene *bax* as well as the possible relation to *c-fos* expression.

From our gene expression studies we found that the early activation of *c-fos* post-MCAo was not associated with an alteration of either *bcl-2* or *bax* mRNA expression at 1 or 4 h post-injury (paper 4, Appendix figure 6). Other studies have reported that changes in *bcl-2* and *bax* do occur but not until approximately 5-6 h post-injury (Gillardon et al., 1996; Prakasa et al., 2000). By 24 h post-injury, we found that mRNA levels of *bcl-2* and *bax* were not significantly different from normal levels (paper 4, Appendix figure 6). We did measure an increase in Bcl-2 immunoreactivity in Con-G as compared to vehicle treated animals, detectable at 24 h in the ischemic injury (Paper 4, Appendix figure 7, Appendix Table 1), which may have been induced by an early but transient upregulation of the corresponding mRNA. No apparent differences were observed in Bax immunoreactivity between vehicle and Con-G treated animals (data not shown), however the number of immunopositive cells in the ischemic brain were not quantitated. The number of Bcl-2 immunoreactive cells were significantly lower in vehicle treated animals in the caudate nucleus and cortical brain regions except for the cingulate cortex (Appendix Table 1). The brain regions exhibiting reduced Bcl-2 staining corresponded to regions showing a high density of TUNEL positive cells as well (see Paper 4 and Appendix figure 6). Furthermore, Con-G treatment was associated with an increased Bcl-2 immunoreactivity when delivered either i.c.v. or i.t. Thus, the increased expression levels of the anti-apoptotic gene *bcl-2* may contribute to the neuroprotective properties of Con-G. This effect is similar to studies reporting the neuroprotective effect of hypothermia to increase Bcl-2 immunoreactivity following transient MCAo in rats (Prakasa et al., 2000). Interestingly, the increase in Bcl-2 immunostaining appeared to be independent of alteration in *c-fos* since both i.c.v. and i.t. Con-G treatments were

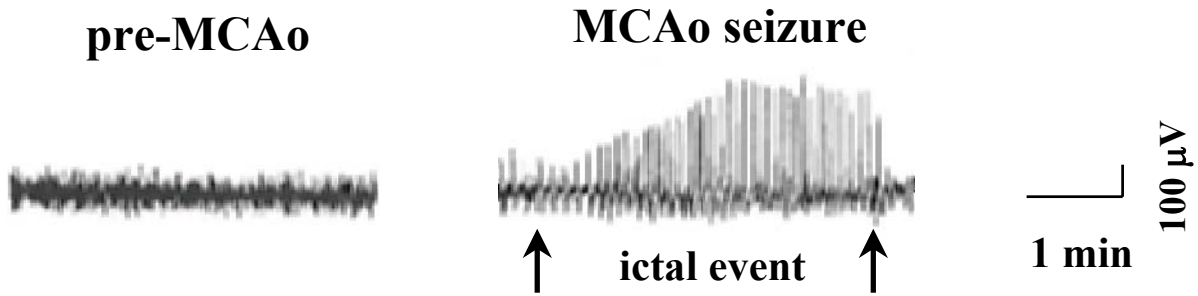
associated with increasing Bcl-2 immunoreactivity. Thus, similar to our *in vitro* results, Con-G may have anti-apoptotic effects that are not linked to alteration of calcium induced signaling but do appear to be linked to the maintenance of bcl-2 expression.

In conclusion these data have shown that *in vivo* administration of Con-G reduces infarct volume and increases both neurological and EEG recovery when administered up to 8 h following occlusion of the MCA. These effects appear to be independent of change in body temperature, cardiovascular dynamics, or behavioral side effects. In support of this data we have shown that Con-G possesses potent neuroprotective properties against multiple forms of neuronal injury *in vitro* and that this ability is due to NMDA antagonism. Con-G has been also shown to possess a novel anti-apoptotic mechanism linked to the NR2B subunit and that this effect may involve regulation of the anti-apoptotic gene *bcl-2* although a reduction of *c-fos* expression does not appear to be a prerequisite for neuroprotection with Con-G. Furthermore, higher doses of Con-G produced more of a peri-infarct type injury as opposed to a core necrotic injury (papers 1 & 2). Thus, Con-G treatment could potentially be enhanced with agents targeting the peri-infarct region such as anti-inflammatory compounds. Taken together these data suggest that clinically relevant routes of administration of Con-G can be delivered several hours following ischemic brain injury with a potent neuroprotective profile far superior to other non-subunit selective NMDA antagonists such as MK801.

Appendix: Additional Figures. Methods and Materials for all following studies have been previously described (Paper 4).

Figure 1. Effect of Con-G to reduce MCAo-induced non-convulsant seizures (NCS). No behavioral convulsions were observed during brain seizure activity and in general, animals were conscious and ambulatory although occasionally wet dog shake (WDS) behavior was observed during NCS. The NCS were generalized spike/slow-wave complexes occurring at a frequency of 1-2 per second and were recorded in both ipsilateral and contralateral brain regions. Initiation of NCS activity was delayed by 9 minutes and the total number of ictal events and time spent in seizure were reduced by half following Con-G treatment.

Figure 1



	Vehicle(i.t.)	Con-G(i.t.)
# of Animals w/ Seizures	5 in 7	5 in 6
Mean Seizure Time	35.9 ± 5.6 s	32.4 ± 6.1 s
1st Seizure (post-MCAo)	25 ± 8 min	34 ± 7 min
Total # seizures (over 24 h)	12.2 ± 3.4	6.8 ± 1.9
Total Time of Seizures	6.3 ± 3.1 min	3.2 ± 2.3 min

Figure 2. Correlation analysis between infarct volume and presence of NCS activity following permanent MCAo and 24 h of recovery. A low but positive correlation was measured between infarct size and the presence of NCS however none of the correlation values were statistically significant ($P > 0.05$).

Figure 2

Correlation	Pearson's Correlation Coefficient	P-value
Core Infarct Volume vs. Average # of Seizures	0.319	0.288
Core Infarct Volume vs. Total Time in Seizure	0.230	0.449
Total Infarct Volume vs. Average # of Seizures	0.363	0.223
Total Infarct Volume vs. Total Time in Seizure	0.299	0.321

Figure 3. Timecourse of *c-fos* mRNA level expression following MCAo and treatment with Con-G (i.t). Levels of *c-fos* mRNA are presented from 4 different brain regions at each post-injury time point as compared to sham animals (upper panels). Direct comparisons between *c-fos* levels of vehicle and Con-G treated animals (n = 6-10/group) are shown in both the injured cortical and subcortical tissues (lower panels). No significant differences were measured between vehicle and Con-G treated animals and any post-injury time point in cortical or subcortical brain regions ($P>0.05$).

Figure 3

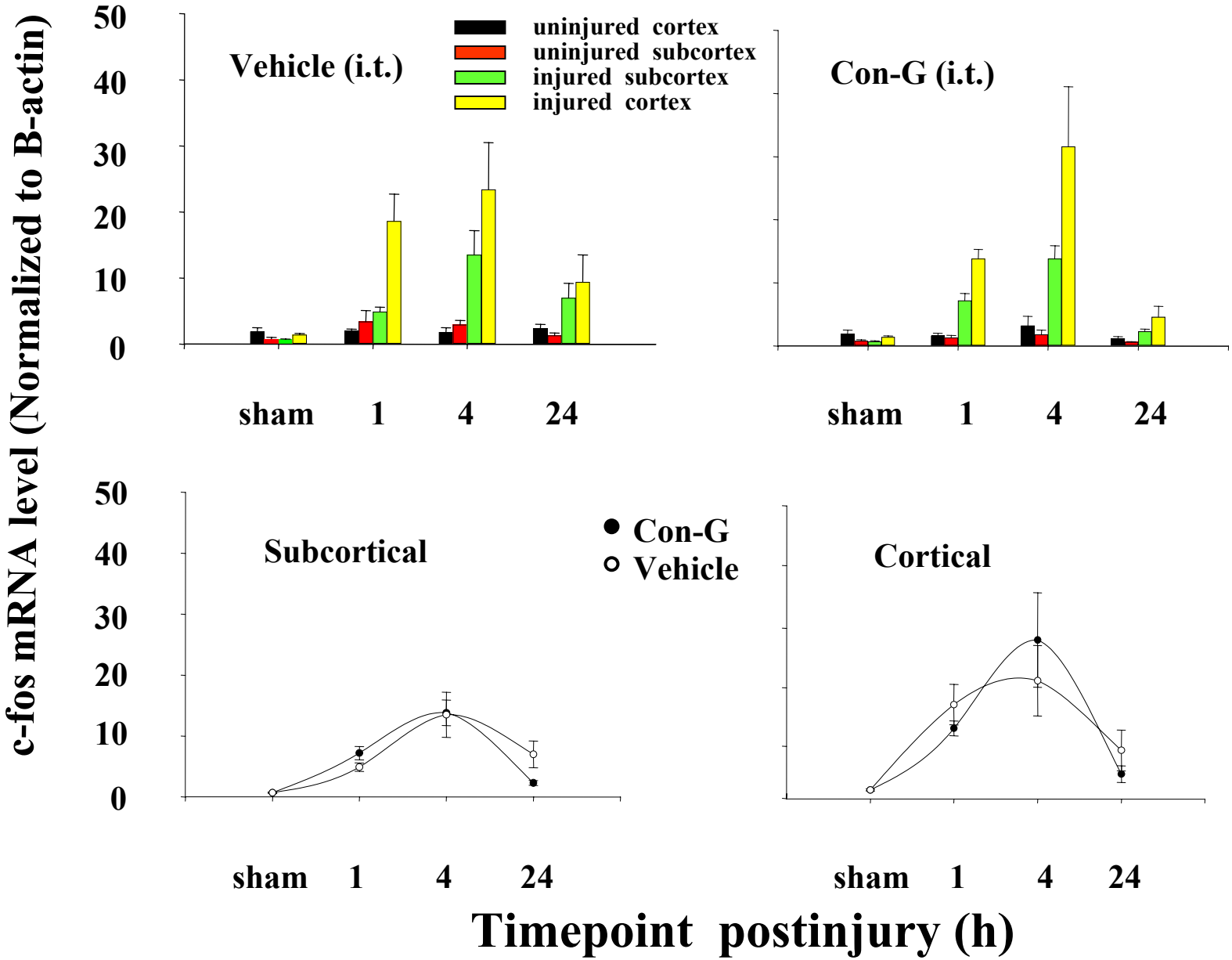

c-fos mRNA level (Normalized to B-actin)
50
40
30
20
10
0
Vehicle (i.t.)
uninjured cortex
uninjured subcortex
injured subcortex
injured cortex
Con-G (i.t.)
sham
1
4
24
Subcortical
Con-G
Vehicle
Cortical
Timepoint postinjury (h)

Figure 4. Representative sections from the cingulate cortex displaying C-Fos immunoreactivity between vehicle and Con-G treated animals at 24 h post-MCAo. C-Fos immunoreactivity was upregulated as early as 1 h post-injury in both cortical and subcortical brain tissues although the visible progression of infarction at 4 h was associated with a loss of C-Fos reactivity (data not shown). By 24 h, C-Fos immunoreactivity had largely disappeared except for along the border of the infarct as well as the cingulate cortex. No apparent differences were observed between vehicle and Con-G treated animals at any post-injury time point measured. Bar = 25 μm.

Figure 4

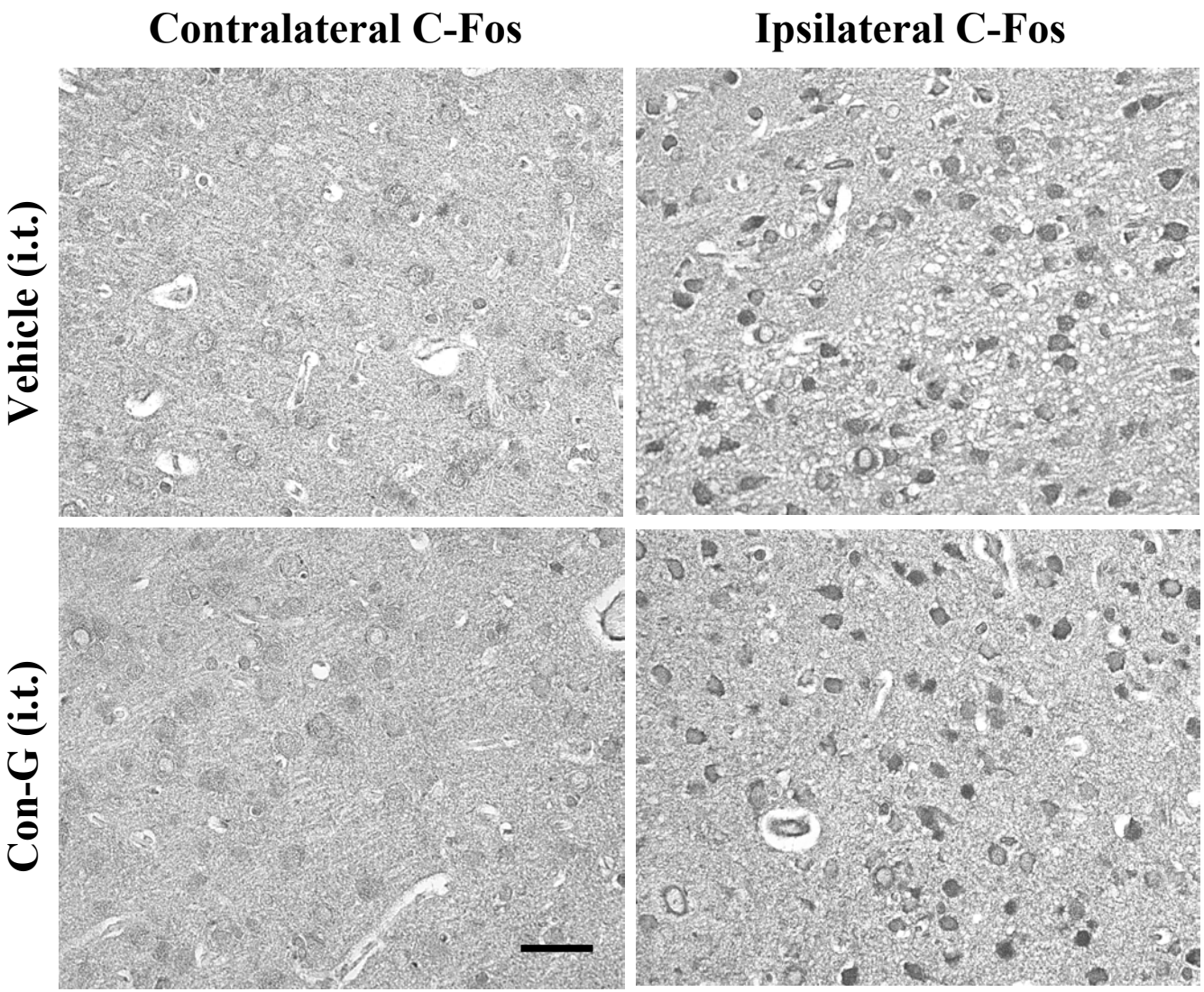


Figure 5. Comparison of H&E (A) and TUNEL (B) stained slides from representative vehicle and Con-G (i.t) treated animals, 24 h post-MCAo. Presence of infarcted tissue as determined from H&E slides (outlined regions) corresponded to brain regions expressing TUNEL positive cells (outlined regions). Lower panels show a high magnification of cortical brain regions of the slide shown directly above. TUNEL positive cells are indicated by dark black nuclei (hematoxylin counterstain, light gray, was used for identification of cellular structures). Con-G was highly effective in reducing presence of infarction and TUNEL positive cells in cortical but not subcortical brain tissues. Bar = 25 μm

Figure 5

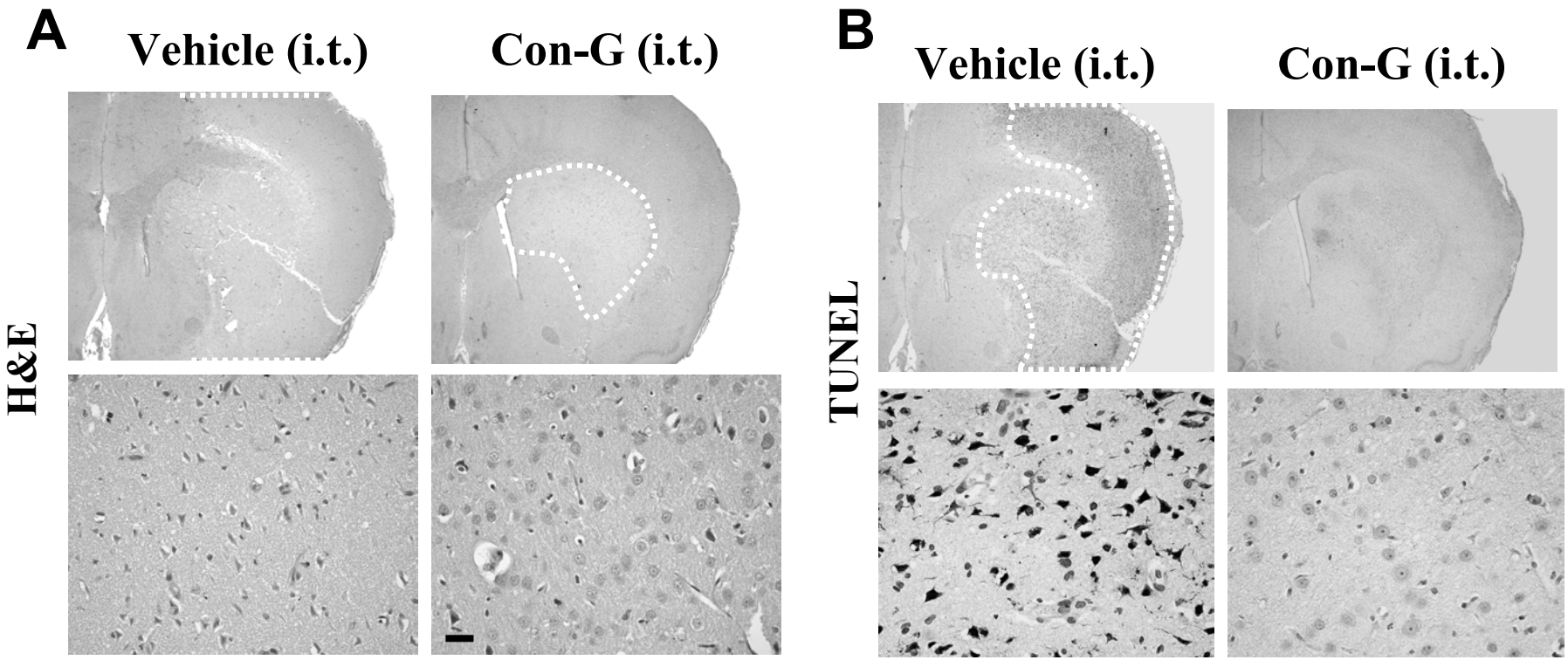
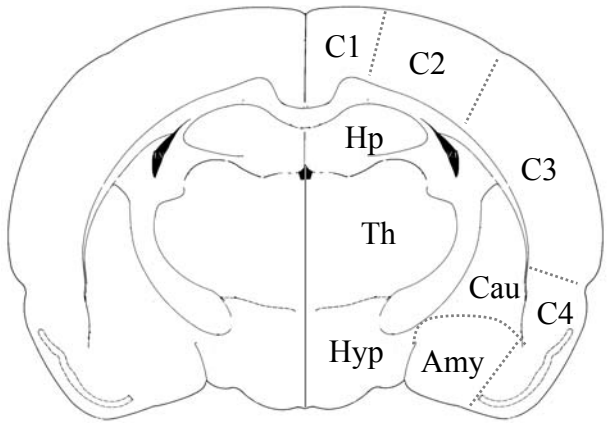
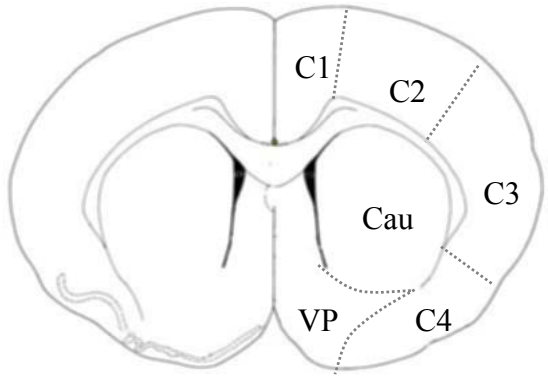
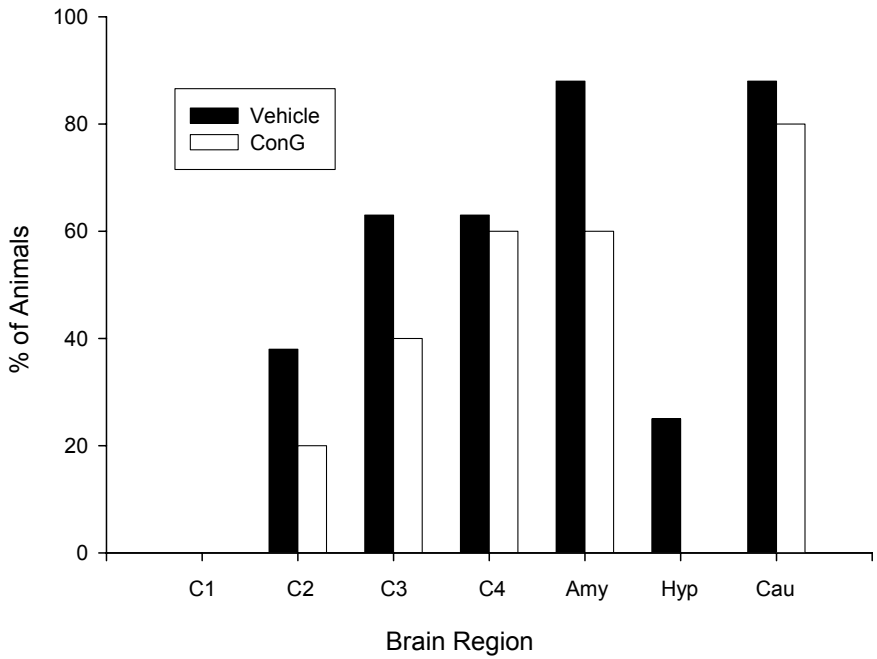


Figure 6. Brain regions expressing TUNEL positive cells were compared between vehicle and Con-G treated animals (n = 6/group). Criteria for inclusion included any evidence of TUNEL positive cells in each respective region. Data are presented as the percent of animals in each treatment group exhibiting TUNEL staining in each brain region. Hippocampus (Hp), Thalamus (Th), or Cingulate Cortex (C1) regions did not exhibit TUNEL staining in either treatment group. Con-G treatment was most effective in reducing presence of TUNEL positive cells in cortical brain regions (C1-C4) although the hypothalamus (Hyp) and ventral pallidum (VP) regions were also "TUNEL free". * $P<0.05$.

Figure 6


C1
C2
Hp
C3
Th
Cau
C4
Hyp
Amy


C1
C2
C3
Cau
VP
C4


100
80
60
40
20
0
% of Animals
Vehicle
ConG
C1
C2
C3
C4
Amy
Hyp
Cau
Brain Region

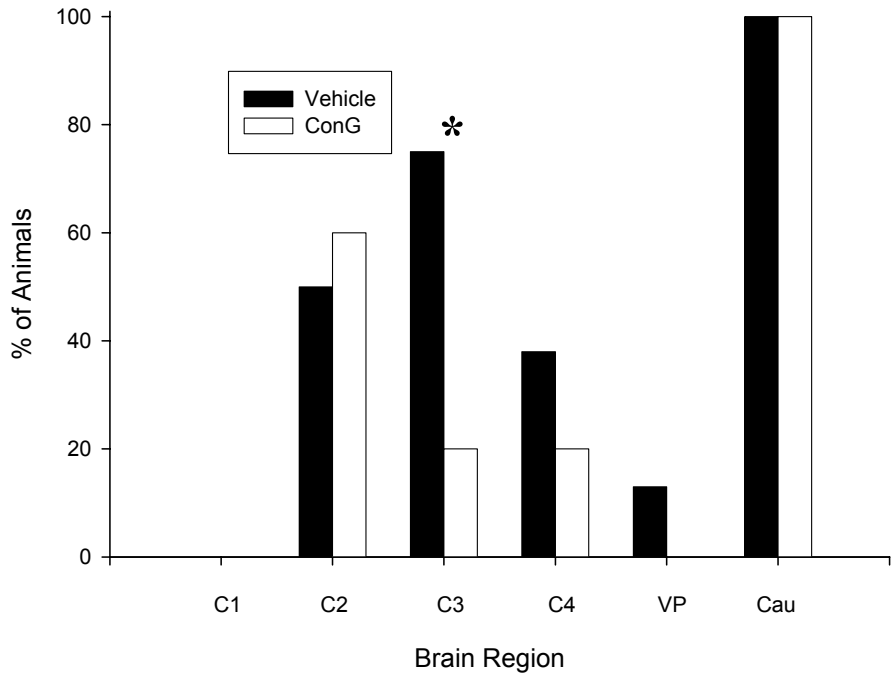

100
80
60
40
20
0
% of Animals
Vehicle
ConG
*
C1
C2
C3
C4
VP
Cau
Brain Region

Figure 7. Timecourse of *bax* (upper panels) and *bcl-2* (lower panels) mRNA levels following MCAo and treatment with Con-G (CGX-1007). Levels of *c-fos* mRNA are presented from 4 different brain regions at each post-injury time point as compared to sham animals. Messenger RNA levels ranged from 1-5% of β-actin levels but there were no significant differences between vehicle and Con-G treated animals at any time point measured ($P>0.05$, $n = 6$-10/group). Values are presented as the mean ± S.E.M.

Figure 7

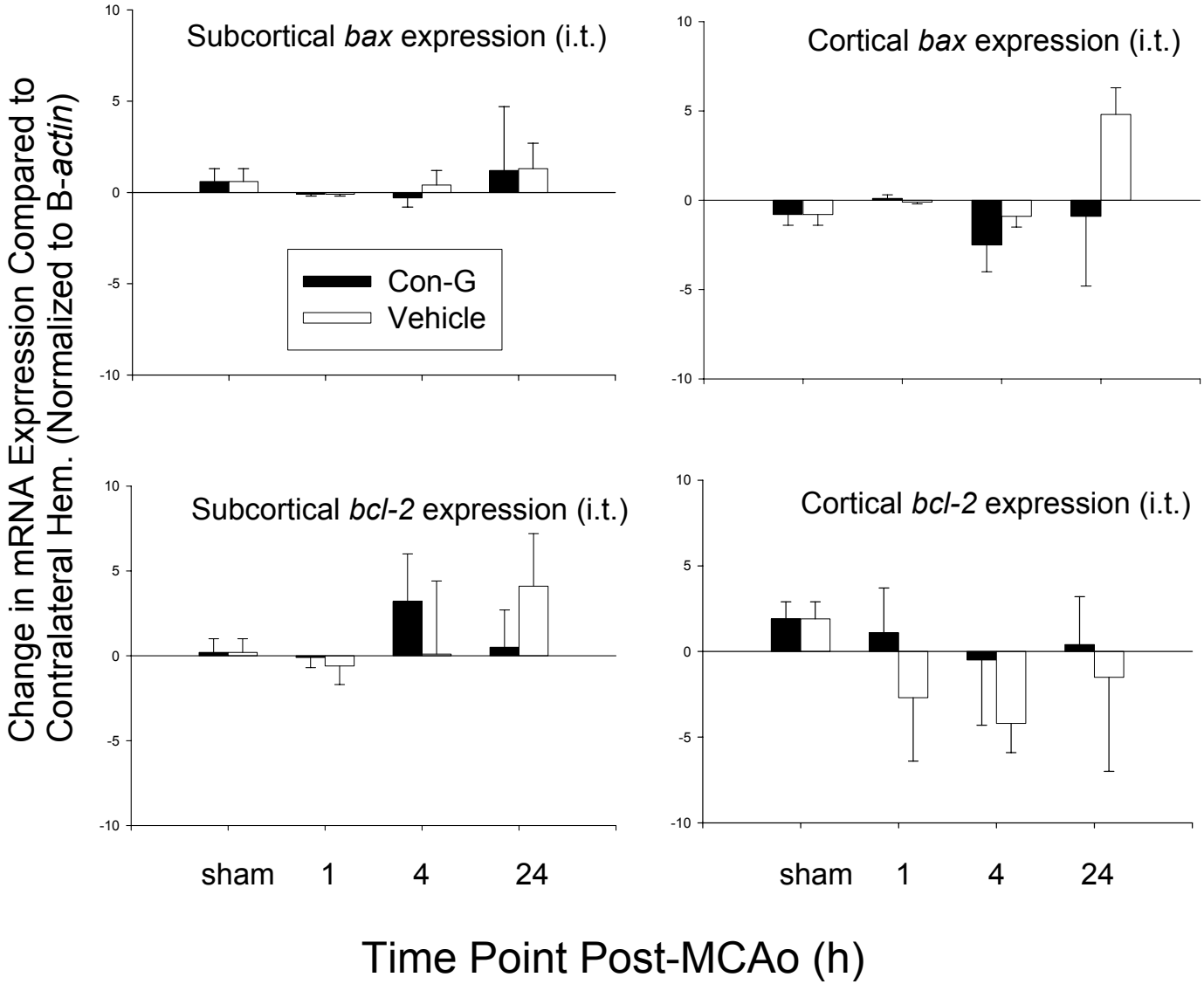

Subcortical *bax* expression (i.t.)
Cortical *bax* expression (i.t.)
Subcortical *bcl-2* expression (i.t.)
Cortical *bcl-2* expression (i.t.)
Con-G
Vehicle
Change in mRNA Exprression Compared to Contralateral Hem. (Normalized to B-*actin*)
10
5
0
-5
-10
sham
1
4
24
Time Point Post-MCAo (h)

Figure 8. Representative sections from the parietal cortex contralateral and ipsilateral to the injury are shown displaying Bcl-2 immunoreactivity at 24 h post-MCAo. No change in Bcl-2 staining was observed at 1 or 4 h post-injury (data not shown). By 24 h, Bcl-2 immunostaining was absent from infarcted brain tissue of vehicle-treated animals (A) while Con-G treated animals displayed an upregulation of Bcl-2 in cortical brain regions spared from infarction (B). Bar = 12 μm.

Figure 8

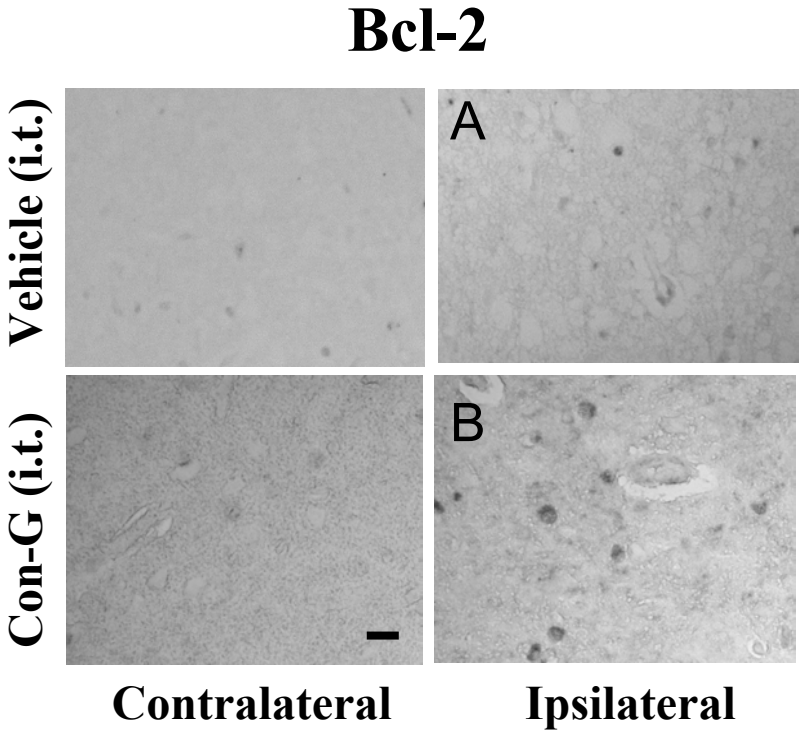


Table 1. Quantitation of BCL-2 immunopositive cells from five brain regions. Vehicle (Veh) is compared to Con-G treatment with either i.c.v. (n = 4/group) or i.t. (n = 3/group) administration. Five 40x fields were scanned from each brain region and counts were made by an observer blinded to the treatment group. Values are presented as the mean ± S.E.M. of cells per field. * $P < 0.05$ as compared to corresponding vehicle group, ANOVA followed by Fisher's post-hoc analysis.

	Vehicle (i.t.)	**Con-G (i.t.)**
Uninjured Cortex	**7 ± 1**	**8 ± 1**
Uninjured Subcortex	**7 ± 1**	**5 ± 1**
Caudate Nucleus	**5 ± 1**	**24 ± 4***
Cingulate Cortex	**12 ± 3**	**14 ± 2**
Parietal Cortex	**4 ± 1**	**11 ± 2***
Temporal Cortex	**5 ± 1**	**11 ± 1***
Piriform Cortex	**5 ± 1**	**21 ± 5***

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