Modulation of Mitochondrial Function and Autophagy Mediates Carnosine Neuroprotection Against Ischemic Brain Damage

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Background and Purpose—Despite the rapidly increasing global burden of ischemic stroke, no therapeutic options for neuroprotection against stroke currently exist. Recent studies have shown that autophagy plays a key role in ischemic neuronal death, and treatments that target autophagy may represent a novel strategy in neuroprotection. We investigated whether autophagy is regulated by carnosine, an endogenous pleiotropic dipeptide that has robust neuroprotective activity against ischemic brain damage.

Methods—We examined the effect of carnosine on mitochondrial dysfunction and autophagic processes in rat focal ischemia and in neuronal cultures.

Results—Autophagic pathways such as reduction of phosphorylated mammalian target of rapamycin (mTOR)/p70S6K and the conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II were enhanced in the ischemic brain. However, treatment with carnosine significantly attenuated autophagic signaling in the ischemic brain, with improvement of brain mitochondrial function and mitophagy signaling. The protective effect of carnosine against autophagy was also confirmed in primary cortical neurons.

Conclusions—Taken together, our data suggest that the neuroprotective effect of carnosine is at least partially mediated by mitochondrial protection and attenuation of deleterious autophagic processes. Our findings shed new light on the mechanistic pathways that this exciting neuroprotective agent influences. (Stroke. 2014;45:00-00.)

Key Words: autophagy ■ carnosine ■ mitochondria

Despite the high prevalence and the increasing global burden of ischemic stroke, there are no approved neuroprotective agents in clinical use. The only approved therapy is thromboly- sis with tissue-type plasminogen activator, which has a narrow therapeutic window and hemorrhagic side effects that limit clinical use. There have been extensive efforts to develop novel therapeutic candidates for ischemic stroke. However, numerous promising candidates have failed in clinical trials because of a number of factors, including poor preclinical study design, illogical clinical translation of preclinical data, poor efficacy, and serious side effects. Moreover, understanding the precise mechanisms through which candidate agents exert their protective effects is an important and critical part of therapy development. Agents that influence multiple deleterious pathways are more likely to be efficacious clinically.

There is increasing evidence that autophagy, a highly regulated cellular process that involves degradation of cellular proteins and organelles, can contribute to neuronal death during brain ischemia. Enhancement of autophagic processes was observed in brain after hypoxic ischemia, and the occurrence of autophagy measured by conversion of microtubule-associated protein 1 light chain 3 (LC3)-I to LC3-II during brain ischemia has been confirmed by in vivo imaging. Although controversy exists whether autophagy contributes to cell death or cell survival, recent observations using inhibitors or modulators of autophagy revealed that autophagy mediates neuronal cell death during ischemia. Wen et al observed autophagy in focal cerebral ischemia and demonstrated that treatment with inhibitors of autophagy significantly reduced brain damage. Data also exist showing that neuronal death during ischemia is mediated...
by oxidative stress generated from autophagosomes and mitochondria that are participating in the autophagic process.\textsuperscript{15}

Activation of autophagic pathways is associated with perturbations in mitochondrial function.\textsuperscript{16} Mitochondrial damage is known to result in activation of mitophagy, a specific type of autophagy that eliminates dysfunctional mitochondria.\textsuperscript{17,18} under normal and pathological conditions including cerebral ischemia.\textsuperscript{19} Despite the increasing attention on autophagy as a novel target for stroke therapy development, studies on agents that modulate autophagy and that could be used clinically are still limited.

Carnosine, an endogenous dipeptide, is a pleiotropic agent that exhibits diverse activities including antioxidant, antimatrix metalloproteinase, heavy metal chelating, and antixcitotoxic properties.\textsuperscript{20,21} We recently showed that carnosine robustly reduced brain damage after ischemic stroke.\textsuperscript{22–25} Post-treatment with carnosine protected against histological brain damage both in permanent- and transient-ischemic rat models, with a wide clinically relevant therapeutic window of 9 and 6 hours, respectively, along with improvements in functional outcomes.\textsuperscript{23} Carnosine did not exhibit any side effects or organ toxicity.\textsuperscript{22,25} Along with our observation, others have also reported the robust neuroprotective activity of carnosine.\textsuperscript{26–28} However, it is not known whether carnosine can influence autophagy in the ischemic brain.

In this study, we investigated whether carnosine has the ability to modulate autophagic processes in the ischemic brain using both in vitro and in vivo approaches. We extended our studies to mitochondria and showed that carnosine has a significant and profound effect on autophagy and associated mitochondrial perturbations that occur during ischemia. Our findings support the pleiotropic multimodal action of carnosine and provide, for the first time, proof of its influence on autophagy.

Methods

More details are provided in the online-only Data Supplement.

Animals

All animal experiments were conducted using adult male Sprague-Dawley rats weighing 250 to 300 g (Harlan; Koatech, Korea) and performed in accordance with the National Institutes of Health Policy and Animal Welfare Act under approval by the Institutional Animal Care and Use Committee at Hanyang University.

Blinding and Randomization

Treatment groups were allocated in a randomized fashion. Investigators were blind to the allocation of treatment during surgeries and outcome evaluations.

Treatments

Carnosine was obtained from Sigma and dissolved in saline. Carnosine (1000 mg/kg) was administered into the lateral tail vein at 6 hours after ischemic onset both in permanent and transient models. The choice of this dose and time window is based on previous dose finding studies.\textsuperscript{22–25}

Ischemic Stroke in Rats

Permanent or transient focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO).\textsuperscript{23} Ischemia was initiated by a silicone-coated 4-0 monofilament nylon suture (Doccol Co.) as described previously.\textsuperscript{23,29}

Calculation of Infarct Volume

At 24 hours after onset of ischemia, rats were euthanized by isoflurane overdose, and the isolated brains were cut into 2-mm sections. The infarct volume for each section was calculated by 2% triphenyl-tetrazolium chloride.\textsuperscript{30}

Assessment of Neurological Function

Deficit in neurological function was evaluated by behavioral tests, including the adhesive tape removal test and a Rota Rod test, at 24 hours after transient MCAO (6-hour ischemia).\textsuperscript{23,31} All rats were trained to the tests for 5 consecutive days before focal ischemia.

Brain Homogenization and Mitochondria Isolation

Brain samples between bregma levels +2 and –4 mm, which include ischemic core and penumbra, were rapidly isolated at 24 hours after MCAO, and brain homogenates were obtained by homogenization in isolation buffer. Brain mitochondria were further isolated using Percoll gradient centrifugation.\textsuperscript{32}

Western Blot of Brain Homogenate or Isolated Brain Mitochondria

Processed brain homogenates or brain mitochondria were examined in Western blot using Tris-HCl SDS-PAGE.\textsuperscript{23,32} Detailed information on primary antibodies is described in the online-only Data Supplement.

Complex I Activity

Complex I activity in isolated brain mitochondria was measured using colorimetric method as described previously with 2,6-dichloroindophenol.\textsuperscript{33}

In Vitro Culture of Primary Cortical Neurons

Primary cortical neuronal cultures were established as described previously.\textsuperscript{34} Neuronal cultures were maintained in a CO\(_2\) incubator at 37°C and used between in vitro days 7 and 11.

N-Methyl-D-Aspartate–Induced Excitotoxicity

Ischemic neuronal damage was examined by N-methyl-D-aspartate (NMDA)-induced excitotoxicity.\textsuperscript{34} NMDA-induced cytotoxicity was measured at 24 hours after NMDA exposure by leakage of lactate dehydrogenase. Alterations in cellular proteins were assessed by Western blot as described previously, with cell lysates extracted from neuronal cells using radioimmunoprecipitation assay lysis buffer (Thermo Scientific). To examine carnosine protection, cells were pretreated with carnosine for 30 minutes before NMDA stimulation.

Statistics

We calculated the mean and SEM for all treatment groups. Differences in values were analyzed using Student\(\text{t}\) test or ANOVA, as appropriate, using SPSS software (Chicago, IL). Multiple comparisons were made using 1-way ANOVA followed by Tukey test. Two-tailed Student\(\text{t}\) test analysis was used for comparing values between 2 groups. In all cases, a \(P\) value of <0.05 was considered significant.

Results

Carnosine Protects the Ischemic Brain in Focal Stroke

First, we examined the neuroprotective effect of carnosine in rat focal ischemia. All physiological variables, including body temperature and cerebral blood flow, were maintained in the reference range. Induction of focal ischemia was attained by MCAO and verified by monitoring of cerebral blood flow. Post-treatment with carnosine (1000 mg/kg) at 6 hours significantly reduced brain infarct volume (Figure 1A),
measured by triphenyltetrazolium chloride staining. Similarly, we found that carnosine improved functional outcomes after 6-hour transient MCAO, using a variety of tests, including the latency for removal of adhesive tape placed on forelimbs and the latencies to fall off from the accelerating rotarod, respectively.23,31 (Figure 1B and 1C).

Carnosine Reduced Autophagy in Brain Homogenates
To investigate whether autophagic processes are involved in carnosine-mediated protection, we examined the extent of conversion of LC3-I to LC3-II, an important marker of autophagy that is responsible for formation of autophagosome.35 A significant increase in LC3-II formation was observed in the ipsilateral hemisphere after ischemia. However, this increase in LC3-II formation was attenuated by treatment with carnosine (Figure 2A). It is also well established that inhibition of the mammalian target of rapamycin (mTOR) pathway plays a key role in autophagy.36 To investigate the effect of carnosine on the autophagic signaling pathway, we measured the levels of phospho-mTOR and phospho-p70S6K, a representative downstream target of mTOR,37 in brain homogenates after ischemia. Carnosine did not affect the basal activity of mTOR; similar levels of phospho-mTOR were observed in hemispheres contralateral to the ischemia in both saline- and carnosine-treated rats (Figure 2B). Ischemia inhibited the phosphorylated levels of mTOR, but this inhibition was blocked by carnosine. Similarly, reductions in the levels of phospho-p70S6K in ischemic brain were also reversed by carnosine (Figure 2B). Taken together, these findings support the modulating role of carnosine on autophagy in the ischemic brain. Although mTOR–autophagy pathways were significantly influenced by ischemia and reversed by carnosine, the level of phosphorylated extracellular signal-regulated kinases 1/2 was not changed by either ischemia or by carnosine treatment (Figure 2B), showing that the modulation of autophagic proteins by carnosine is not a nonspecific epiphenomenon.

Carnosine Attenuates Ischemic Injury to Mitochondria
We previously reported that carnosine reversed the impairment of mitochondrial permeability transition in primary neurons and astrocytes. Because it is well established that mitochondrial...
dysfunction contributes to autophagy induction, we examined whether carnosine protected against mitochondrial damage and mitophagy. Ischemia resulted in decreased activity of complex I in isolated brain mitochondria suggesting impairment in mitochondrial respiratory function. Ischemic mitochondrial dysfunction was significantly reversed in mitochondria isolated from carnosine-treated rats (Figure 3A). To determine whether there is a link between mitochondrial dysfunction and autophagy, we examined the levels of p-Drp1 (dynamin-related protein 1) and Parkin, which play key roles in mitochondrial fragmentation and mitophagy during cell death, respectively. The mitochondrial levels of p-Drp1 and Parkin were significantly increased by ischemia, but the increases of p-Drp1 and Parkin were attenuated by carnosine treatment (Figure 3B).

Although the levels of p-Drp1 and Parkin were increased by ischemia, the levels of cytochrome C and apoptosis-inducing factor (AIF) were significantly decreased in brain mitochondria after ischemic insult. Because cytochrome C and AIF are released from mitochondria to the cytosol during mitochondrial damage, these results were consistent with mitochondrial dysfunction. Carnosine potently inhibited the release of AIF and cytochrome C, demonstrating its protective activity on mitochondrial damage (Figure 3B).

**Carnosine Protects Against Neuronal Autophagy in Culture**

Primary cortical neurons were transiently exposed to toxic levels of NMDA, and cytotoxicity and autophagic signaling pathways were examined. As shown in Figure 4A, NMDA induced significant cytotoxicity in primary cortical neurons, and NMDA cytotoxicity was reduced by carnosine treatment. Interestingly, autophagic signaling pathways including LC3-II formation and mTOR dephosphorylation were significantly enhanced by NMDA exposure, and carnosine reversed these changes (Figure 4B), confirming the protective effect of carnosine against ischemia-induced neuronal autophagy.
Discussion

Stroke involves a cascade activation of multiple deleterious pathways,\(^2\) and therefore, a drug candidate that specifically modulates a single pathway is not likely to show clinical efficacy against ischemic brain damage. Many therapeutic candidates, including neuroprotectants that had strong protective activity preclinically, have failed in clinical trials.\(^3,4\) One major reason for this is that past strategies have focused on targeting one pathway. We have shown that carnosine is an exciting candidate for development as a stroke therapy.\(^23,25\) It is safe and efficacious, with a large clinically relevant therapeutic time window. Moreover, it is a pleiotropic agent that favorably modulates several deleterious pathways that contribute to cell injury and cell death during and after ischemia.\(^5,6\) We show here, using in vitro and in vivo approaches, that carnosine has a profound and significant effect on autophagy, a recently identified noxious pathway in ischemic stroke. We think that this study underlines the translational importance of carnosine as a therapeutic candidate against ischemic stroke in which multiple deleterious pathways aggravate neuronal damage.

Autophagy is the cellular process that mediates degradation of cellular proteins and organelles and maintains homeostasis.\(^5\) Despite its essential role in normal cellular physiology, excessive activation of autophagic pathways is also reported to be highly associated with many disease states, including brain damage.\(^46,47\) Autophagic cell death has been referred to as type II cell death, which is one of the major types of cell death along with apoptotic (type I) and necrotic (type III) cell death.\(^48,49\) Although necrotic and apoptotic cell deaths have long been considered the main pathological events in ischemic stroke,\(^50,51\) autophagy has been recognized recently as a possible deleterious event as well. Activation of autophagic signaling was observed in ischemic brain,\(^52\) mediating ischemic neuronal death.\(^53\) Notably, autophagic cell death was found to be the most important contributing pathway in neonatal cerebral ischemia relative to apoptosis and necrosis.\(^54\) Autophagy inhibitors such as 3-methyladenine significantly reverse ischemic brain damage,\(^14\) and inhibition of autophagy was suggested to be the main mechanism of ischemic post-conditioning neuroprotection.\(^54\) Conversely, it has also been reported that autophagy may play a dual role in neuronal survival and death during ischemia,\(^10\) and further studies on the exact molecular targets that switch beneficial autophagy to detrimental autophagy would give valuable insights for development of treatments that modulate autophagy.

The role of mitochondrial dysfunction has been proposed as a contributor to autophagy.\(^6\) We and others have previously shown that ischemic insults to the brain induced mitochondrial permeability transition, resulting in damage to mitochondrial function in neurons.\(^23,41\) Onset of mitochondrial dysfunction is closely linked to initiation of autophagy in ischemia/reperfusion injured myocytes,\(^6\) in rat hepatocytes,\(^55\) and in neurons.\(^15\) Damaged mitochondria release cytochrome C, AIF, and reactive oxygen species,\(^17\) which promote mitophagy, a form of autophagy that is involved in the removal of dysfunctional mitochondria. Recent data suggest that Parkin, an ubiquitin ligase that mediates mitophagy,\(^40\) is recruited to the damaged mitochondria.\(^36,56\) In this report, we observed the increased recruitment of Parkin to the mitochondria and loss of AIF and cytochrome C from mitochondria in ischemic brain, which were significantly attenuated by carnosine, demonstrating its protective effect against mitophagy and ultimately autophagic neuronal death. Similarly, Mehta et al\(^7\) showed that selenium conserved mitochondrial function and stimulated mitochondria biogenesis, along with reduced autophagy in glutamate-induced neuronal toxicity.

Interest in the development of carnosine as an endogenous pleiotropic molecule for therapeutic use clinically has been increasing.\(^20,44,58-60\) Here, we focused on the potential of carnosine against ischemic stroke. Several previous reports showed that carnosine also had beneficial activities in neurodegenerative diseases such as Alzheimer’s disease\(^61\) and dementia.\(^62\) Of note, dysregulation of autophagic processes have been recently recognized to contribute to the progress of these neurodegenerative diseases.\(^53,54\) Further elucidation of the effects of carnosine on autophagy in these neurodegenerative diseases is needed.

In summary, we have demonstrated that carnosine inhibits ischemia-induced autophagy and mitochondrial damage. This novel action of carnosine adds to the other body of compelling data that support the development of carnosine as a therapeutic agent against ischemic stroke.

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Disclosures

None.

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SUPPLEMENTAL MATERIAL

Modulation of mitochondrial function and autophagy mediates carnosine neuroprotection against ischemic brain damage

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Supplemental Materials and Methods

Animal treatments

All animal experiments were conducted using adult male Sprague-Dawley rats weighing 250 to 300 g (Harlan) and performed in accordance with the NIH Policy and Animal Welfare Act under the approval by Institutional Animal Care and Use Committee (IACUC) at Hanyang University. Treatment groups were allocated in a randomized fashion using a Researcher Randomizer Program (http://www.randomizer.org/). Investigators were blind to the allocation of treatment during surgeries and outcome evaluations. Carnosine was obtained from Sigma and dissolved in saline. Carnosine (1,000 mg/kg) was administered into the lateral tail vein at 6 hr after ischemic onset both in permanent and transient models. The choice of this dose and time window is based on previous dose finding studies.1-4

Ischemic stroke in rats

Permanent or transient focal cerebral ischemia was induced by intraluminal middle cerebral artery occlusion (MCAO) according to previous reports.2 After induction of general anesthesia by isoflurane inhalation, rats were maintained under anesthesia throughout the surgical period. Rectal temperature was maintained at 37°C and the cerebral blood flow (CBF) was measured with laser Doppler (Perimed, North Royalton, OH). The left common carotid artery (CCA) and the external carotid artery (ECA) were exposed and ligated by a suture, and the occipital artery of the ECA was coagulated. The internal carotid artery (ICA) was exposed and the pterygopalatine artery was ligated. Ischemia was initiated by a silicone-coated 4-0 monofilament nylon suture (Doccol Co.) as described previously.2,5 The monofilament was inserted into the CCA and advanced into the ICA to the origin of the MCA (18.0 mm from the bifurcation). The filament was left in place for the permanent model, while reperfusion was produced by withdrawal of the monofilament 6 hrs after occlusion in the transient model. Rats were excluded from the study when the CBF was not decreased below 30% of baseline after occlusion, or CBF was not restored above 80% of basal CBF after reperfusion.

Calculation of infarct volume

Rats were euthanized by isoflurane overdose, and the brains were rapidly isolated. Brains were cut into 2 mm sections, and stained with 2% triphenyltetrazolium chloride (TTC).6 After fixed in 4% paraformaldehyde, each section was scanned to a digital image, and analyzed using the NIH ImageJ software. The infarct volume for each section was calculated and edema correction was performed by the measurement of the ipsilateral and contralateral hemisphere.

Assessment of neurological function

Deficit in neurological function was evaluated by behavioral tests at 24 hr after
tMCAO (6 hr ischemia), based on the previous literature. Somatosensory deficit was measured using the adhesive tape removal test before and after surgery. All rats were trained to the tests for 5 consecutive days before focal ischemia. As bilateral tactile stimuli, small pieces of adhesive were applied on the wrist of each forelimb. The time to remove each tape from forelimbs was recorded on 3 trials (maximum record of 180 sec). Motor–ambulatory function was assessed with a Rota Rod test. Rats were trained for 3 consecutive days before ischemia. Rats were placed on the Rota Rod and then tested with acceleration speeds. Latencies to fall off or the 395 sec cutoff were recorded for analysis with 3 trials.

**Brain homogenization and mitochondria isolation**

Brain samples between bregma levels +2 and -4 mm, which include ischemic core and penumbra, were rapidly isolated at 24 hrs after MCAO, and brain homogenates were obtained by homogenization in isolation buffer (215 mM mannitol, 75 mM sucrose, 0.1% BSA, 20 mM HEPES, and 1 mM EGTA, pH 7.2). The protein concentrations were determined using BCA assay (Thermo Scientific, Rockford, IL) after brain tissue homogenate was collected, and used for western blot.

For further isolation of mitochondria, 30% Percoll in isolation buffer was added to brain homogenates. The resultant homogenate was layered on a discontinuous Percoll gradient with the bottom layer containing 40% Percoll solution, followed by a 24% Percoll solution, and finally the sample in a 15% Percoll solution. The density gradients were centrifuged at 30,400g for 10 minutes. Following centrifugation, band 3 (non-synaptic mitochondria) were separately removed from the density gradient. The final mitochondrial pellet was re-suspended in isolation buffer without EGTA. Protein concentration was determined using the BCA protein assay.

**Western Blot of brain homogenate or isolated brain mitochondria**

Processed brain homogenates or brain mitochondria were separated in Tris-HCl SDS-PAGE Ready Gels (Bio Rad) and were transferred to PVDF membrane (Millipore). After blocking with 5% BSA, membranes were incubated overnight at 4°C with primary antibodies. Primary antibodies against phospho-mTOR (#2971), mTOR (#2972), phospho-p70S6 kinase (#9205), p70S6 kinase (#9202), phospho-p44/42 MAPK (Erk1/2) (#9101), p44/42 MAPK (Erk1/2) (#9102), phospho-DRP1 (#3455), AIF (#4642), and parkin (#4211) were purchased from Cell Signaling Technology (Danvers, MA). Anti-LC-3B (L7543), anti-β-actin (A5441), and anti-β-tubulin (P4026) antibodies were from Sigma. Anti-VDAC1 (ab154846) and anti-cytochrome C (#556432) antibodies were obtained from Abcam (Cambridge, UK) and BD Bioscience (Franklin Lakes, NJ), respectively. Anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (MAB374) was purchased from Millipore. Horseradish peroxidase-conjugated antibodies were used as secondary antibodies. The immune complexes were visualized by enhanced chemiluminescence using SuperSignal West Pico Chemiluminescent Substrate. Bands were quantified by NIH Image J program, and normalized by the corresponding loading controls. The extent of
phosphorylation was normalized by the total amount of the target proteins. All immunoblots were repeated for four independent experiments.

**Complex I activity**

Complex I activity was measured using colorimetric method as previously described with 2,6 dichloroindophenol (DCIP). Isolated brain mitochondria was mixed in a reaction buffer containing 25 mM potassium phosphate, 3.5 g/L BSA, 60 μM DCIP, 70 μM decylubiquinone, 1.0 μM antimycine-A, and 0.2 mM NADH (pH 7.8). Complex I oxidizes NADH generating electrons which reduce decylubiquinone, and the reduced decylubiquinone subsequently delivers the electrons to DCIP. The extent of DCIP reduction was monitored spectrophotometrically at 600 nm.

**In vitro culture of primary cortical neurons**

Primary cortical neuronal cultures were established as described previously. Cell culture media and reagents including Neurobasal A, B27, DMEM, glutamine, and penicillin/streptomycin were purchased from Invitrogen (Carlsbad, CA). At post-natal day 0, cerebral cortices were isolated from newborn C57BL/6J mice, and cells were dissociated in the presence of 4 mM L-cysteine, 10 U/ml papain (Worthington), and 1000 U/ml DNase (Roche) for 30 min at 37°C. Dissociated cells were washed and triturated with a pipette, and plated onto poly-D-lysine-precoated plates. Three days after plating, 50% of the medium was changed with Neurobasal A supplemented with 2% B27, and subsequently replaced every three days. Neuronal cultures were maintained in a CO₂ incubator at 37°C, and used between days in vitro (DIV) 7 and 11. These cultures contained >90% neurons as revealed by NeuN/ beta tubulin-immunohistochemistry.

**NMDA-induced excitotoxicity**

Ischemic neuronal damage was examined by N-methyl-d-aspartate (NMDA)-induced excitotoxicity. Primary neuronal cells were treated with NMDA-containing media and incubated at 37°C for 20 min on DIV 9. Exposure to NMDA was terminated by replacement with the original media collected before NMDA treatment. NMDA-induced cytotoxicity was measured at 24 hr after NMDA exposure by leakage of lactate dehydrogenase (LDH). Alterations in cellular proteins were assessed by western blot as described earlier, with cell lysates extracted from neuronal cells using RIPA buffer (Thermo Scientific). To examine carnosine protection, cells were pretreated with carnosine for 30 min prior to NMDA stimulation.

**Statistics**

We calculated the means and standard errors of means (SEM) for all treatment groups. Differences in values were analyzed using Student t-test or analysis of variance (ANOVA), as appropriate, using SPSS software (Chicago, IL). Multiple comparisons were made using one-way ANOVA followed by Tukey test. Two-tailed Student’s t-test analysis
was used for comparing values between two groups. In all cases, a p value of < 0.05 was considered significant.

Citations in the Supplemental Material


