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# Evaluation of Gabapentin and Ethosuximide for Treatment of Acute Nonconvulsive Seizures following Ischemic Brain Injury in Rats

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## ABSTRACT

Acute seizures following brain injury have been associated with a worsening of patient outcome, but they are often undiagnosed and untreated when they occur without motor convulsions. Here, we sought to compare the antiseizure profile of ethosuximide (EXM; 125–312.5 mg/kg i.v.) and gabapentin (GBP; 0.3–50 mg/kg. i.v.) in a rat model of nonconvulsive seizures (NCS) induced by brain ischemia. Seizures were detected by continuous electroencephalographic monitoring for 24 h following permanent middle cerebral artery occlusion (MCAo). Both "preseizure" and "postseizure" treatment effects were evaluated. Control rats experienced a 91% incidence of NCS (averaging 10–11 NCS/rat), which was significantly reduced following preseizure treatment (delivered 20 min post-MCAo) with either EXM ($ED_{50}$ = 161 mg/kg) or GBP ($ED_{50}$ = 10.5 mg/kg). In contrast to preseizure treatment effects, only GBP reduced NCS when given after the first seizure event. A further, albeit nonsignificant, 20% reduction in NCS incidence was measured when given in combination postseizure. Drug treatment also reduced infarct volume, which was positively correlated to the number of NCS events ($r = 0.475$; $P < 0.001$). EXM and GBP treatment of cultured neurons exposed to neurotoxic or ischemic insults showed no neuroprotective effects, suggesting that in vivo neuroprotection can be attributed to antiseizure effects. We conclude that EXM and GBP significantly attenuate NCS in a dose-related manner and may help to improve patient outcome from brain ischemia-induced seizure activity.

Acute brain injury involves two phases of pathology: the primary mechanical or cerebrovascular insult and a variety of secondary pathologies that influence maturation of the injury and critically determine patient outcome (Bramlett and Dietrich, 2004). One source of secondary injury is neuronal hyperexcitability that can manifest in paroxysmal ictal discharges. It is estimated that 10–27% of patients with acute brain injury experience early seizures that are detectable by a clinical manifestation (Jordan, 1999), and, in most cases, are treatable with a standard pharmacotherapy regiment of antiepileptic drugs (AEDs). However, a substantial percentage of brain-injured patients also experience nonconvulsive seizures (NCS) that can only be diagnosed definitively with electroencephalography (EEG). EEG studies estimate that NCS occurs in 6 to 37% of patients, dependent upon the type of brain injury (i.e., ischemic, traumatic, hemorrhagic, and so on) (Vespa et al., 1999, 2003; Claassen et al., 2004).

The incidence of brain injury-related NCS has traditionally been underappreciated due to the subclinical nature of NCS and lack of acute EEG monitoring in most clinical centers worldwide. In addition, the incidence of NCS may be underestimated, particularly during the "hyperacute" period (within the first few hours postinjury). For example, in 94 patients with moderate to severe brain injury, a 22% incidence of electrographic seizure activity was reported, although the average delay from time of injury to EEG monitoring was 9.6 h (ranging from 3 to 24 h) (Vespa et al., 1999). In another study, diagnosis of NCS was made in the first 24 h in only 57% of patients; diagnosis in the remaining patients was delayed 1 to 5 days following hospital admittance

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**ABBREVIATIONS:** AED, antiepileptic drug; NCS, nonconvulsive seizure(s); EEG, electroencephalography/electroencephalographic; GBP, gabapentin; EXM, ethosuximide; SWD, spike-and-wave discharge; LVACC, low-voltage-activated calcium channel; MCAo, middle cerebral artery occlusion; SWS, slow-wave sleep; DIV, days in vitro; H/H, hypoxia/hypoglycemia; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium; ANOVA, analysis of variance.

(Kaplan, 1996). In contrast, experimental studies have indicated the incidence of spontaneous NCS activity to be as high as 90% following brain injury, with most seizure activity occurring within the first few hours (Hartings et al., 2003; Williams et al., 2004).

Substantial evidence now indicates a harmful role of NCS on the injured brain, and the early detection and treatment of NCS have been defined as crucial factors to improve neurological outcome (Vespa et al., 2003). To address this mounting clinical concern, we recently compared the antiseizure efficacy of seven clinically available AEDs (Williams et al., 2004) in a rat model of spontaneous NCS induced by focal cerebral ischemia (Hartings et al., 2003). Interestingly, the two most effective AED therapies seemed to be the calcium channel blockers gabapentin (GBP) and ethosuximide (EXM), whereas therapy with common first-line AEDs such as midazolam or phenobarbital was ineffective (Williams et al., 2004).

Both EXM (Zarontin) and GBP (Neurontin) are Food and Drug Administration-approved AEDs available for treatment of distinct types of epileptic conditions. Both drugs are currently formulated only for oral (i.e., capsules/tablets/syrup) but not parenteral administration. The succinimide derivative EXM is commonly used to treat generalized absence (petit mal) seizures with little effect against other forms of generalized or partial seizures (Brunton et al., 2006). In particular, EXM has been shown to effectively attenuate the 3-Hz spike-and-wave discharges (SWDs) that occur during absence seizures (Coulter et al., 1989), a condition associated with the activation of low-voltage-activated calcium channel (LVACC) currents (Coulter et al., 1989; Kostyuk et al., 1992). Gabapentin is a structural analog of GABA used in the treatment of partial epilepsy and neuropathic pain (Dougherty and Rhoney, 2001; Brunton et al., 2006). Although originally designed as a GABA mimetic, GBP has no significant activity on GABA receptors, and the biological effects of GBP are largely attributed to the selective inhibition of high-voltage-activated calcium channels containing the $\alpha_2\delta$-1 subunit (Sills, 2006). The goal of the present study was to evaluate the full dose-response efficacy of both GBP and EXM for the treatment of brain injury-induced NCS and, furthermore, to compare the antiseizure effects to a neuroprotective mechanism of action both in vivo and in vitro.

## Materials and Methods

**General Surgical Procedures.** Male Sprague-Dawley rats (280–320 g; Charles River Laboratories, Raleigh, NC) were used in all studies. Food and water were provided ad libitum pre- and post-surgery, and animals were individually housed under a 12-h light/dark cycle. For all surgical procedures, anesthesia was induced by 5% halothane and maintained at 2% halothane delivered in oxygen with body temperature maintained normothermic (37 ± 1°C) by means of a homeothermic heating system (Harvard Apparatus Inc., Holliston, MA).

Indwelling i.v. cannulas (polyethylene-50) were placed into the right jugular vein of all animals for drug delivery, and EEG electrodes were implanted in the skull (Tortella et al., 1999). Bipolar EEG recordings were obtained from both the left and right parietal cortex using two electrodes positioned 5 mm lateral to midline, at 0 and 4 mm posterior to bregma. A fifth ground electrode was implanted posterior to lambda over the transverse sinus/cerebellum. EEG electrodes consisted of stainless steel screws (0-80 × 1/8 in.) soldered to insulated nichrome wire (0.2 mm in diameter). The screws were implanted epidurally and fixed to the skull using dental acrylate cement. Free ends of the wires were soldered to a multipin connector (March Electronics, West Hempstead, NY), also secured by dental acrylate.

This research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the *Guide for the Care and Use of Laboratory Animals*, National Research Council Publication (1996; U.S. Government Funding).

**In Vivo Brain Injury Model.** After 3 to 4 days of recovery from the above-mentioned procedures, animals were subjected to permanent focal ischemia using the filament method of middle cerebral artery occlusion (MCAo) as described previously (Tortella et al., 1999). In brief, the right external carotid artery was isolated, and its branches were coagulated. A 3-0 uncoated monofilament nylon suture with 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 permanently occluding the origin of the middle cerebral artery. Neurological scoring (see below) was performed before injury and 1 h and 24 h post-MCAo. Rectal temperatures were recorded before injury and 20 min and 1, 6, and 24 h postocclusion. At 24 h, rats were deeply anesthetized, euthanized by decapitation, and brains were harvested for quantification of infarction (see below). Animals not exhibiting maximal neurological deficit at 1 h postocclusion, not surviving to 24 h, or having intracranial hemorrhage at post-mortem examination were excluded from the main data analysis.

**Continuous EEG Recording.** All animals were housed in custom-designed Plexiglas EEG recording chambers (Dragonfly Inc., Ridgeley, WV) equipped with multichannel gold contact swivel commutators (Plastics One, Roanoke, VA) for continuous EEG monitoring (Tortella et al., 1999). The multipin connector on the rat skull was connected to the swivel system via a flexible shielded cable, allowing free movement of the animals during recordings. The swivel commutators were interfaced with a digital EEG amplifier and recording system (Harmonie software; Astromed Inc., West Warwick, RI). Baseline EEG signals were recorded for 30 min before the start of the experimental protocol (see below) and continuously throughout the 24-h recovery period.

**Treatment of Seizures Induced by MCAo.** The antiseizure efficacies of both EXM (125–312.5 mg/kg i.v.) and GBP (0.3–50 mg/kg i.v.) were assessed following a single bolus injection delivered at a defined postinjury time point. Two separate treatment protocols were assessed: 1) acute "pre-NCS treatment," where each injection was given at 20 min postocclusion before the onset of NCS activity; and 2) delayed "post-NCS treatment," where injections were given immediately following onset of the first NCS event as detected from real-time monitoring of the EEG record (see below).

**Assessment of AEDs in Normal (NonInjured) Rats.** Changes in cortical EEG activity induced by drug administration were assessed as described previously (Tortella et al., 1999). In brief, each animal was given a vehicle injection followed 1–2 h later by a single bolus injection of either EXM (250–312.5 mg/kg i.v.) or GBP (25–50 mg/kg i.v.). Using this protocol, each animal served as its own control. For the duration of the experiment each animal was observed for signs of abnormal behavioral activity, including sedation, ataxia, enhanced locomotion, stereotypic activity (including head weaving, grooming, preening, and scratching), and signs of clonic (convulsant) muscle activity. All EEG experiments routinely began between 9:00 AM and 11:00 AM with continuous EEG recordings collected for 6 h postdrug injection. Off-line analysis of each EEG record was used to quantitate changes in latency to slow-wave sleep (SWS) (Tortella et al., 1999) and EEG spectral parameters induced by drug treatment (see below).

**Seizure Detection.** Electrographic cortical seizure events were identified and quantitated by off-line review of all EEG records at a

display resolution of 1 mm/s and subsequently verified at a recording speed of 30 mm/s (Hartings et al., 2003; Williams et al., 2004). Criteria for identifying NCS events were as follows: 1) the occurrence of repetitive spikes or sharp wave discharges recurring at a rate of >1/s, or continuous polyspiking; 2) spike amplitude greater than background activity; and 3) duration of continuous seizure activity (defined by 1 and 2) greater than 10 s. Seizures could be either generalized or focal, and consecutive seizure episodes were considered a single event if not separated by more than 10 s. Based on the onset/offset times of each NCS event as defined by the above-mentioned criteria, several descriptive parameters were computed for each treatment group. "NCS/rat" and "total duration of NCS" were calculated as the mean value from all animals in each group. "Average duration of NCS" and "latency of onset" were calculated as the mean values from only those animals exhibiting NCS in each group. EEG recordings were also visually evaluated for other EEG abnormalities, including depressed baseline amplitude, focal slowing, polymorphic delta activity, periodic lateralized epileptiform discharges, and interictal spikes, sharp waves, polyspikes, or spike/slow-wave complexes. Animals were also visually monitored for the first 8 h postinjury for signs of behavioral convulsive activity.

**EEG Spectral Analysis.** Computer-assisted spectral analysis was performed on selected 60-s epochs of cortical EEG to evaluate changes in EEG power due to drug treatment (Lu et al., 2001). Changes in power were assessed across four frequency bands: delta (0–4 Hz), theta (4–8 Hz), alpha (8–12 Hz), and beta (12–30 Hz). The respective power scores across each frequency band were evaluated at 2 min, 15 min, 1 h, and 6 h postdrug treatment and compared with the corresponding control, vehicle injection. Compressed spectral arrays, spectral trend graphs, and spectrographic analyses were also used to evaluate EEG changes following MCAo.

**Infarct Analysis and Neurological Scoring.** Triphenyltetrazolium chloride (Sigma-Aldrich, St. Louis, MO) was used to visualize and quantitate the area of brain infarction from seven coronal brain slices, which were integrated to obtain a final infarct volume (Inquiry Digital Analysis System, Loats Associates, Westminster, MD) (Tortella et al., 1999). Quantitative metrics of injury outcome were infarct volume, hemispheric infarct size (percentage of infarction compared with total hemispheric volume, to account for brain swelling), and hemispheric swelling (percentage of increase in size of the injured over the contralateral hemisphere). Core infarct was defined as brain tissue completely lacking triphenyltetrazolium chloride staining, whereas total infarct included both the core and surrounding peri-infarct regions (Williams et al., 2000). Neurological scoring was based on a weighted 10-point scale, giving a positive score for each neurological deficit, including forelimb flexion, shoulder adduction, reduced resistance to lateral push, and contralateral circling (Tortella et al., 1999).

**Neuronal Cultures.** Primary cultures of cortical neurons were prepared from the brain tissue of 17- to 18-day-old Sprague-Dawley rat embryos as described previously (Chen et al., 2005). In brief, the cortex was isolated and incubated for 30 min in 6 ml of neuronal conditioned medium without serum and glutamine, but containing 200 $\mu$l of 0.25% trypsin (Sigma-Aldrich). After 20 triturations with a 10-ml pipette, separated cells were suspended in serum-containing Neuronal conditioned medium, which contained 50% Ham's F-12K (Biofluids, Camarillo, CA), 50% basal medium Eagle (Sigma-Aldrich), 10% fetal bovine serum, and 10% horse serum (Invitrogen, Gaithersburg, MD), 3% 2 mM glutamine, 1% penicillin and streptomycin, and 0.6% glucose. Arabinoside cytosine (Sigma-Aldrich) was added to the culture at 3 DIV to the final concentration of 10 $\mu$M to suppress growth of non-neuronal cells.

**In Vitro Injury Model.** Cultured cortical neurons were pretreated with EXM or GBP before exposure with glutamate (Sigma-Aldrich), staurosporine (Sigma-Aldrich), or hypoxia/hypoglycemia (H/H) (Chen et al., 2005). In brief, at 7 DIV the culture medium was replaced with Locke's buffer (154 mM NaCl, 5.6 mM KCl, 3.6 mM $NaHCO_3$, 2.3 mM $CaCl_2{\cdot}H_2O$, 5.6 mM glucose, and 5 mM HEPES, pH 7.4). Neuronal toxicity was induced by addition of glutamate (to a final concentration of 100 $\mu$M) or staurosporine (1 $\mu$M, dissolved in dimethyl sulfoxide and added directly to the culture media). After 2-h incubation at 37°C, Locke's buffer was replaced with original culture medium. For H/H treatment, cells (7 DIV) kept in Locke's buffer were placed into a sealed chamber filled with 95% nitrogen plus 5% $CO_2$ gas mixture and incubated at 37°C for 3 h. Following exposure, cell viability was measured at 24 h by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT; Sigma-Aldrich) as described previously (Chen and Chuang, 1999). In brief, 30 $\mu$l of 0.025% MTT solution in phosphate-buffered saline was added to each well. After 30-min incubation at 37°C, the precipitate was dissolved in dimethyl sulfoxide and quantified spectrophotometrically at 540 nm.

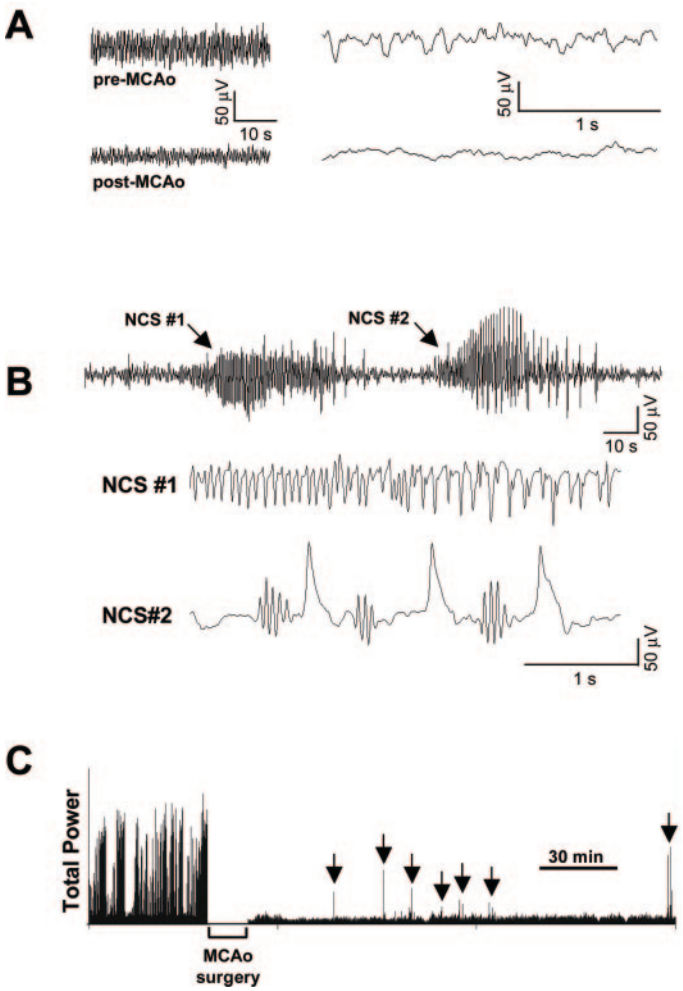
**Data Analysis.** All experimental procedures were performed by an experimenter blinded to the treatment group. For in vivo studies, an analysis of variance (ANOVA) was performed on each data set followed by a Dunnett's post hoc test for comparison of each treatment group to the corresponding vehicle control or by Tukey's post hoc analysis for between-group comparisons. Chi-square analysis was used for evaluating a trend in proportions of quantal data. Estimates of $ED_{50}$ values for seizure control and neuroprotection were calculated using PharmTools Pro data analysis software (The McCary Group, Elkins Park, PA) (Tallarida, 2000). The $ED_{50}$ for seizure control was based on a reduction in NCS incidence, and the $ED_{50}$ for neuroprotection was based on number of positive responders (i.e., animals with infarct volumes at least 1 S.D. below the mean of the vehicle-treated group) (Tortella et al., 1999). Pearson's correlations were used to determine the strength in relationship between histological outcomes and NCS parameters. For comparison of latency to SWS, paired $t$ tests were used to evaluate significant differences between vehicle and drug treatment with each animal serving as its own control. Neuronal cell culture viability was compared by one-way ANOVA followed by a Bonferroni-Dunn post hoc test. $P$ values $<0.05$ were considered significant.

**Compounds.** Ethosuximide (2-ethyl-2-methylsuccinimide; Sigma-Aldrich, St. Louis, MO) and gabapentin [1-(aminomethyl)-cyclohexane-acetic acid, Sigma-Aldrich] were dissolved/diluted in a vehicle of 0.9% physiological saline (1 ml/kg) and delivered over 1 min (i.v.) as a single bolus injection to awake, freely behaving animals.

## Results

**Nonconvulsive Seizures.** As shown in raw EEG tracings (Fig. 1, A and B) and spectral trends analysis (Fig. 1C), occlusion of the middle cerebral artery induced an immediate attenuation of the baseline EEG amplitude and intermittent epochs of NCS activity. Individual NCS events presented as repetitive spike and/or sharp wave discharges with associated increases in high-frequency (10–15 Hz) power (Fig. 1, D and E) and evolution in discharge frequency and amplitude (Fig. 1B). Overall, 91% of vehicle-treated animals exhibited NCS following MCAo. Across vehicle groups, the average duration of an individual NCS ranged from 59 to 71 s with mean latency to onset of 50 to 64 min. The majority of NCS occurred within the first 3 h postinjury, with a mean total duration of NCS activity, from onset to offset, ranging from 107 to 149 min. No signs of convulsive motor activity were observed during NCS in any animal within the first 8 h postinjury (period of visual observation).

**Acute Treatment of NCS with EXM and GBP.** Figure 2 shows the effects of EXM and GBP (delivered before onset of NCS) to attenuate NCS activity, reduce brain infarction, and improve neurological outcome. Both EXM and GBP significantly reduced NCS incidence, NCS/rat, and total time in NCS (Fig. 2, A and B; ANOVA, $P < 0.05$) with no significant



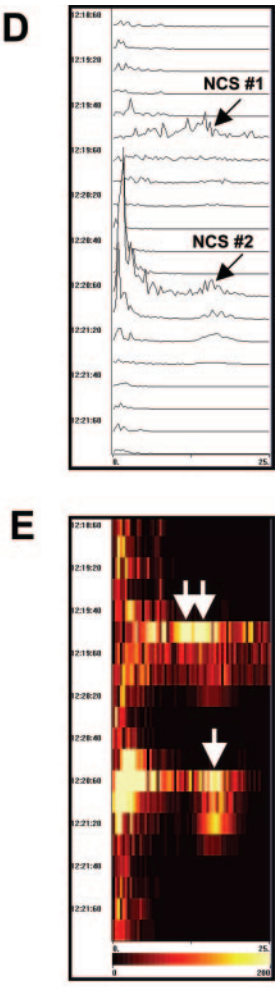




**Fig. 1.** Continuous monitoring of seizure activity following permanent MCAo in a representative vehicle-treated rat. A, reduction in amplitude of the cortical EEG signal in the ipsilateral brain hemisphere following MCAo. B, typical NCS events (an average of 10–11 intermittent episodes occurred over 24 h in vehicle-treated rats). C, spectral trend graph, indicating the reduction in total EEG power due to MCAo and interruption by episodes of high power due to NCS activity (indicated by arrows). Power fluctuations before MCAo reflect natural shifts in sleep/awake activity of the animals. D, compressed spectral array of EEG power changes during the two NCS events shown in B. E, EEG spectrograph demonstrating the increase in high-frequency power due to NCS, typically occurring in the 10- to 15-Hz range (white arrows). All EEG power analysis was done on 2.5-s segments and plotted in 10-s bins.

differences in the average duration or latency to onset of NCS (data not shown; ANOVA, $P > 0.05$). Respective $ED_{50}$ values for seizure control are given in Table 1. In comparison, GBP was approximately 15-fold more potent than EXM with a superior therapeutic dose range (3.125–25 mg/kg) compared with EXM (250 mg/kg only).

The antiseizure effects of EXM were dose-related across all NCS parameters at the lower doses (125–250 mg/kg), although the effects were nearly completely lost at the highest dose tested (Fig. 2, A and B). In contrast, GBP treatment showed significant efficacy to reduce NCS incidence at four of the six doses tested (Fig. 2A) with a dose-related effect on NCS/rat and total time in NCS (Fig. 2B). Previous results indicated that the reduction in NCS incidence with a 12.5-mg/kg dose of GBP was not significant (Williams et al., 2004), although using a larger group size in the current study, we verified that GBP was effective at this dose with a similar overall effect (i.e., 36–45% reduction in NCS incidence). In comparison, the maximal efficacious dose of EXM (250 mg/kg) was slightly more effective than GBP (12.5–25 mg/kg) at reducing the average number of NCS/rat (EXM = 0.1 ± 0.1 and GBP = 1.9 ± 1.2) and total time in NCS (EXM = 2.8 ± 2.8 s and GBP = 37 ± 36 s), although these values were not statistically significant between groups ($P > 0.05$).

In vehicle-treated animals, permanent MCAo induced a severe brain infarction (Fig. 2C) corresponding to core and total infarct volumes of 267 to 320 and 371 to 413 $mm^3$, respectively, and near maximal neurological deficit scores at 24 h postinjury (i.e., neuroscores = 7.5–7.6 out of 10; Fig. 2D). Although EXM was effective at reducing total brain infarct volume (ANOVA, $P < 0.05$), GBP offered statistically significant reductions in core and total brain infarct volume as well as hemispheric swelling (Fig. 2C; ANOVA, $P < 0.05$). In addition, GBP was approximately 33-fold more potent than EXM (Table 1), and, although both AEDs were associated with reductions in brain infarction, only GBP was associated with statistically significant reductions in neurological deficit scores (ANOVA, $P < 0.05$) (Fig. 2D).

No significant differences in weight loss or body temperature were measured between treatment groups (ANOVA, $P > 0.05$). Across treatment groups, animals lost an average 37 of 48 g of body weight by 24 h. As in other reports (Williams et al., 2000), all rats exhibited a mild hyperthermia of 1–2°C by 1 to 6 h postinjury followed by a return toward baseline values (37.6–37.9°C) by 24 h.

**Delayed Treatment of NCS with EXM and GBP.** To determine whether treatment after seizure presentation was as effective as prophylactic treatment, we delayed administration of the most efficacious dose of EXM (250 mg/kg) and GBP (25 mg/kg), alone or in combination, until onset of the first NCS. With this protocol, average injection times were 43.3 ± 5.5 min for EXM, 33.7 ± 3.7 min for GBP, and 29.3 ± 2.2 min for EXM + GBP.

Overall, delayed treatment with either EXM or GBP alone

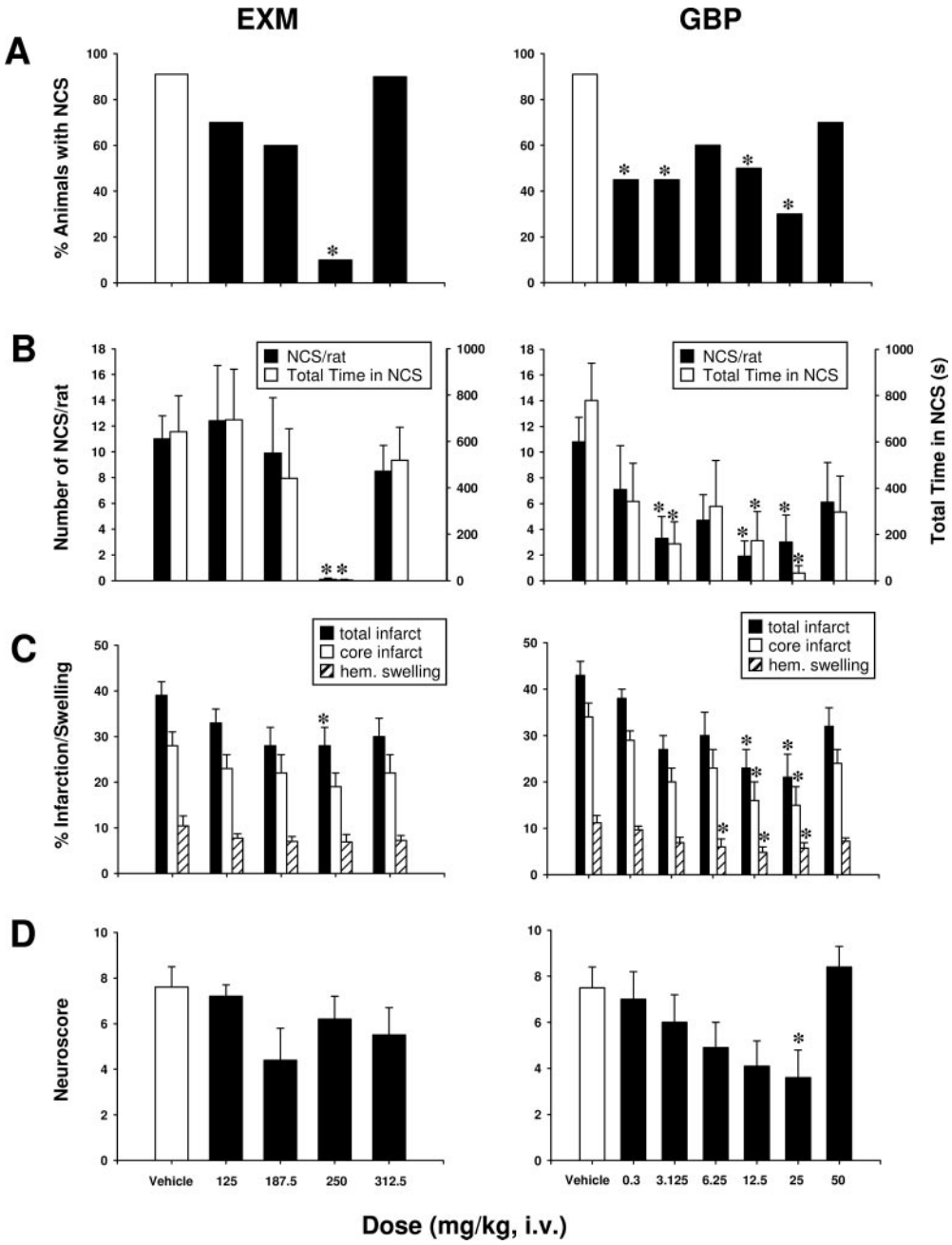



**Fig. 2.** Dose-response effect of acute EXM and GBP treatment (delivered 20 min postocclusion, before first NCS event) to attenuate NCS and improve outcome. A, incidence of NCS activity. B, number of NCS/rat and total time in NCS. C, percentage of brain infarction and hemispheric swelling (24 h postinjury). D, neurological deficit score (24 h postinjury). Data presented in A to D represent the same group of animals (mean ± S.E.M.; $n$ = 10–11/dose). ∗, $P < 0.05$ compared with vehicle (Dunnett's post hoc or chi-square analysis).

TABLE 1

Neuroprotection and seizure control $ED_{50}$ values (95% confidence limits) and maximal protective response observed at optimal doses of each AED studied

The percentage of seizure control was calculated as a percentage of reduction in NCS incidence, and the percentage of neuroprotection was calculated as a percentage of reduction of core infarction at maximal effective doses compared with vehicle controls.

| Drug | % Seizure Control | | % Neuroprotection | |
|---|---|---|---|---|
| | Protection | $ED_{50}$ | Protection | $ED_{50}$ |
| | | *mg/kg* | | *mg/kg* |
| EXM | 82 | 161 (0–249) | 32 | 26 (177–908) |
| GBP | 61 | 10.5 (0–17) | 55 | 7.86 (0–25) |

did not significantly reduce NCS incidence (Fig. 3A). Indeed, delayed treatment with EXM was ineffective at significantly reducing NCS/rat or total time in seizure (Fig. 3B). In contrast, delayed treatment with GBP significantly reduced the NCS/rat by 52% and total time in NCS by 71% compared with vehicle controls (Fig. 3B). Combination treatment with EXM and GBP did provide a further reduction of 20% in NCS incidence, compared with either drug alone (Fig. 3A; $P > 0.05$ between treatment groups). However, this combination did not further improve NCS/rat and total time in NCS (Fig. 3B; $P > 0.05$ between groups).

Similar to antiseizure efficacy, delayed treatment with 250 mg/kg EXM did not significantly reduce brain infarction or hemispheric swelling (Fig. 3C), whereas delayed treatment with GBP maintained a significant neuroprotective effect, reducing brain infarction by nearly 50% (Fig. 3C). Combination treatment with EXM and GBP did not provide a further reduction of brain infarct volume compared with GBP alone ($P > 0.05$ between groups), but it did offer a significant

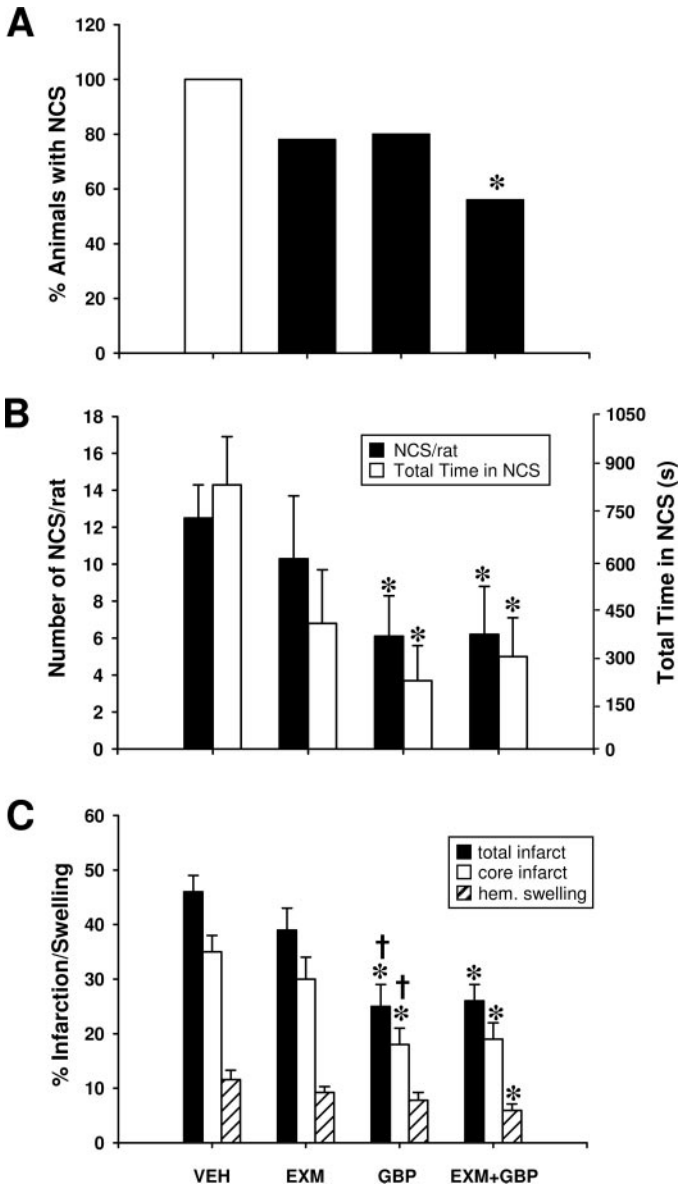



**Fig. 3.** Effects of delayed EXM (250 mg/kg i.v.) or GBP (25 mg/kg i.v.) treatment, alone or in combination, when administered immediately following the first NCS event (average time of injection = 20–55 min postocclusion). A, incidence of NCS activity. B, number of NCS/rat and total time in NCS. C, percentage of brain infarction and hemispheric swelling (24 h postinjury). Data presented in A to C represent the same group of animals (mean ± S.E.M.; $n$ = 9–10/dose). * $P < 0.05$ compared with vehicle; †, $P < 0.05$ compared with EXM-only group (Tukey's post hoc or chi-square analysis).

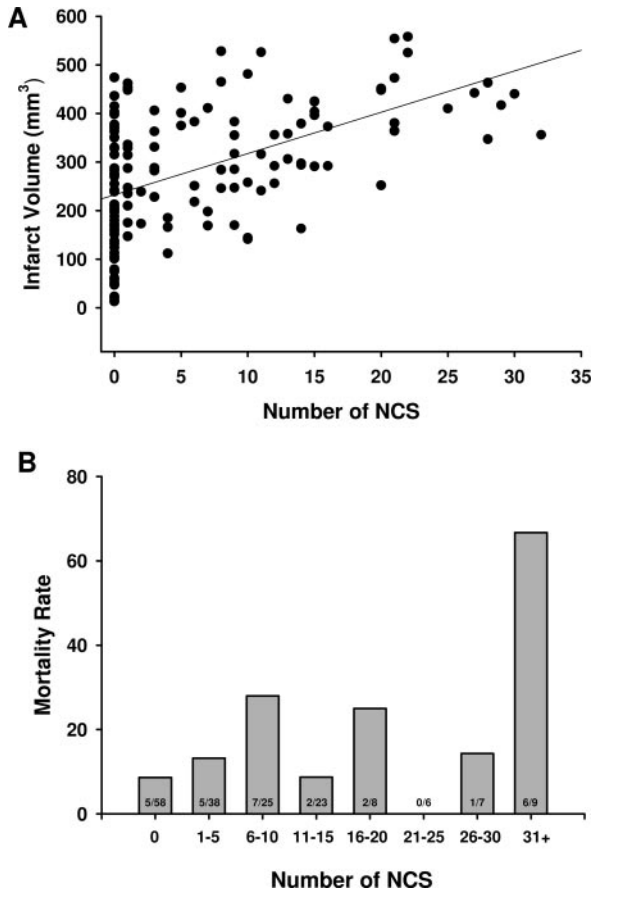



**Fig. 4.** Relationship of NCS to infarct volume and mortality rate (data pooled across treatment groups). A, correlation between total infarct volume and number of NCS events (Pearson's correlation coefficient = 0.475; $P < 0.001$). B, mortality rate compared with number of NCS events. Numbers shown on bar graphs indicate the total number of deaths over the total number of animals in each bin.

reduction of hemispheric swelling compared with vehicle (Fig. 3C).

**NCS and Brain Infarction.** A significant correlation was observed between the number of NCS events and total brain infarction across all acute and delayed treatment groups (Fig. 4A). In the absence of NCS, a full spectrum of infarct volumes was observed, ranging from 0 to 474 $mm^3$ (Fig. 4A). However, brain infarct volume always exceeded 100 $mm^3$ (~11% hemispheric infarction) in the presence of at least one NCS, and it always exceeded 200 $mm^3$ (~22% hemispheric infarction) if more than 15 NCS occurred (Fig. 4A).

**NCS and Mortality.** Across groups, the mortality rate of vehicle-treated rats ranged from 21 to 23% and did not significantly differ from drug-treated rats (0–38% mortality rate). To examine the direct association of mortality with NCS, mortality data from all experiments and treatment groups were combined and divided into bins based on the number of NCS/rat (increments of five) (Fig. 4B). Across bins, mortality rate ranged from 0 to 29% when 30 or fewer NCS events occurred (Fig. 4B). Although mortality was encountered even in the absence of NCS activity (i.e., 5/58 animals without NCS did not survive 24 h), mortality was sharply increased to 67% in the presence of 30 or more NCS events (Fig. 4B).

**EEG and Behavioral Toxicity Evaluation.** In normal (noninjured) rats, no evidence of EEG seizure activity was observed following a single acute administration of either 250 to 312.5 mg/kg EXM or 25 to 50 mg/kg GBP. EXM did produce a moderate but transient EEG slowing and sedation (1–2 min in duration) at the highest dose tested (312.5 mg/kg) (Fig. 5). GBP, on the other hand, was void of any noticeable effect on the EEG, and animals showed no stereotypical behaviors up to 50 mg/kg. Similar to qualitative assessments of EEG waveforms, spectral analysis did not reveal significant quantitative changes in EEG power for EXM or GBP except for the highest dose of EXM tested (312.5 mg/kg) (data not shown). Consistent with qualitative analysis, this dose induced a 6-fold increase in power in the delta frequency

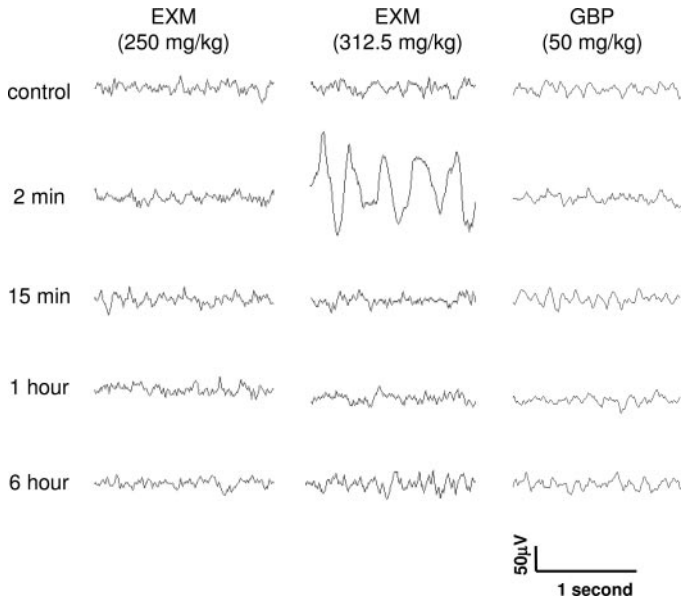



**Fig. 5.** Effect of high doses of GBP and EXM on "quiet awake" EEG activity in normal, noninjured rats.

band at 2 min postinjection, with an average peak frequency of 2.71 ± 0.16 Hz.

EXM also induced a delay to onset of SWS. Normal SWS latency, following control i.v. injections, ranged from 2.5 to 5.3 min across experimental groups. In GBP-treated rats, no significant increases in the latency to SWS ($P > 0.05$) were observed at the 25-mg/kg (3.9 ± 0.9 min) or 50-mg/kg dose (2.9 ± 1.1 min), whereas in EXM-treated rats, the time to SWS was modestly increased at both 250 mg/kg (8.2 ± 1.0 min; $P < 0.05$) and 312.5 mg/kg (15.3 ± 2.8 min; $P < 0.05$).

**Effect of EXM and GBP on In Vitro Neurotoxicity.** In vitro models of neuronal injury were used to examine whether EXM and GBP have direct neuroprotective effects independent of seizure protection. Exposure of cortical neurons to glutamate (Fig. 6A) or H/H (data not shown) induced typical morphological changes indicative of necrotic cell death, including cell shrinkage, fragmented cellular membranes, and loss of neuronal processes. Exposure to staurosporine (data not shown) produced apoptotic cell death as defined by chromatin condensation and appearance of apoptotic bodies. Across all injury models, a 40% reduction in cell viability was observed, as assessed by MTT assay at 24 h postexposure (Fig. 6B). Regardless of injury model or dose (up to 500 μM), treatment with EXM or GBP did not improve cell survival (Fig. 6B). However, no neurotoxicity was associated with drug treatment alone, because cell viabilities with concentrations of either EXM or GBP (data not shown) up to 500 μM were similar to control values (e.g., ~100% survival).

## Discussion

Here, we report the dose-related effects of EXM and GBP to attenuate spontaneous electrographic seizures induced by focal cerebral ischemia. Both EXM and GBP were effective at treating NCS with prophylactic administration initiated preseizure (i.e., before onset of NCS). However, GBP also re-

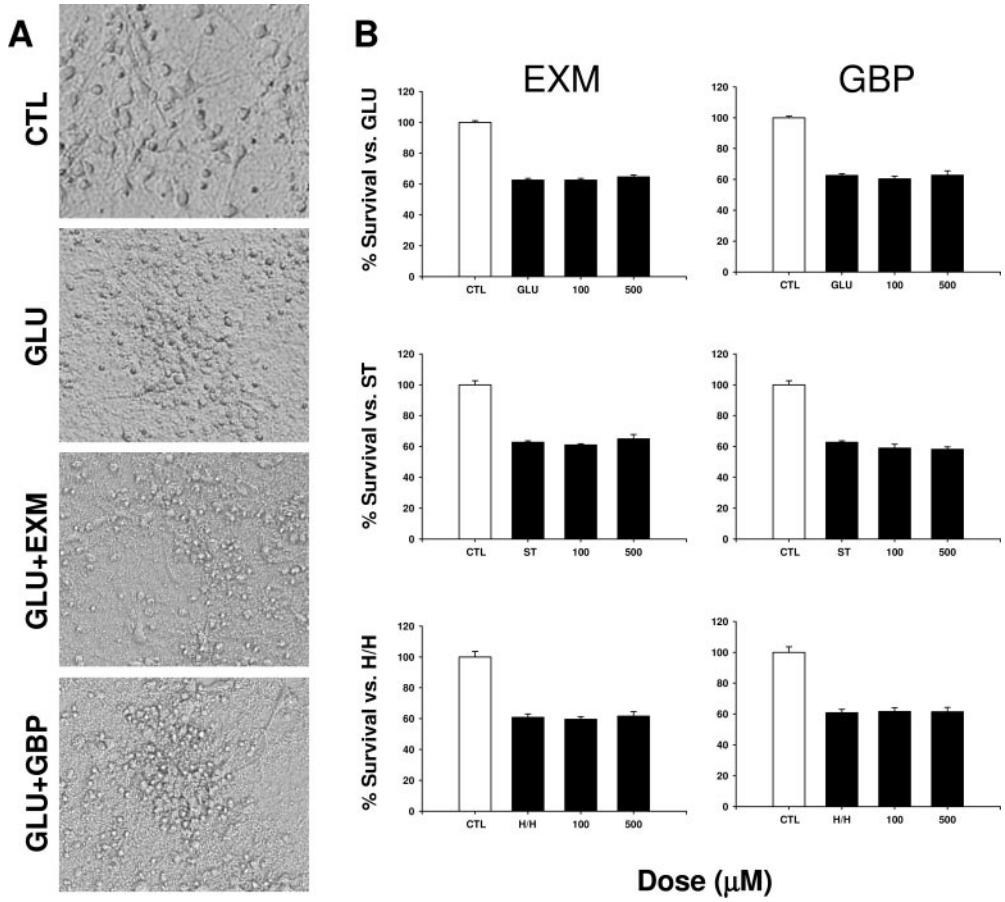



**Fig. 6.** A, representative images of primary neuronal cultures (7 DIV) indicating the lack of effect of EXM or GBP against in vitro neurotoxicity. B, percentage of survival following exposure to glutamate (GLU), staurosporine (ST), or H/H as assessed by MTT assay at 24 h postexposure. Each graph in B represents an individual experiment (mean ± S.E.M.; $n$ = 6/group).

tained significant antiseizure activity against MCAo/NCS when treatment was delayed postseizure (i.e., after onset of NCS), whereas EXM was ineffective.

In human epileptic patients, both EXM and GBP have proven to be well tolerated and safe AEDs. In comparison, both drugs have been reported to raise the threshold for seizure induction induced by pentylenetetrazol, but only GBP is effective against maximal electroshock in rats (Beydoun et al., 1995). Effective doses of EXM for control of absence-like SWDs in animal models range from 62.5 to 250 mg/kg (Mares, 1998; Matejovska et al., 1998) with no observable toxicity, aside from sedation, with daily doses of 400 mg/kg in rats (Leite and Cavalheiro, 1995). Antiseizure efficacy with GBP has been observed from 10 to 25 mg/kg in other rodent seizure models with motor deficits observed at doses of 50 mg/kg or higher (Lado et al., 2001). No other side effects have been reported with doses of GBP up to 300 mg/kg (Wheeler, 2002). Based on these data and the effects EXM and GBP on MCAo-mediated NCS (MCAo/NCS) as reported here, the estimated therapeutic index (minimal toxic dose/$ED_{50}$ for seizure control) is approximately 2.45-fold higher for GBP (4.77) than EXM (1.94).

Considering the efficacy of EXM against MCAo/NCS and its loss of efficacy at doses that produce sedation and high-voltage delta oscillations, LVACC currents, which are reduced by EXM (Coulter et al., 1989), may play a key role in generation of brain ischemia-induced seizures. Neurons from nearly all regions of the thalamus possess LVACC currents, which underlie the thalamic oscillatory behavior for large-amplitude rhythmic waves such as sleep spindles (Steriade and Llinas, 1988; Coulter et al., 1989). Disruption of cortical influences on thalamic circuits and associated LVACC currents can result in a transition of the intrinsic thalamic spindle oscillation to paroxysmal 3-Hz SWDs (Blumenfeld and McCormick, 2000), a mechanism thought to underlie the generation of absence-like seizures. Electrophysiological studies in both humans (Williams, 1953), and in animal models of absence seizures (Vergnes et al., 1987) indicate the rhythmic interaction of cortical and thalamic brain regions are responsible for SWD oscillations. Because of the permanent depolarization of the core middle cerebral artery territory following occlusion, silencing of corticothalamic inputs might similarly trigger SWDs in the thalamus by altering the balance of inhibitory and excitatory tone. Like absence seizures, MCAo/NCS is typically generalized, nonconvulsive in nature, and includes SWD activity, although the ictal waveforms can also include fast, high-frequency, rhythmic spike or polyspike components with clear evolution in spike frequency (as opposed to the metronomic 3-Hz frequency of absence seizures) (Hartings et al., 2003).

Interestingly, the complete attenuation of MCAo/NCS with EXM treatment at 250 mg/kg was nearly completely lost with an increase in dose of 25%, producing a U-shaped dose response. The loss of antiseizure efficacy may be related to the induction of EEG slow-wave activity and sedation seen at the higher dose (312.5 mg/kg). A similar lack of antiseizure effect in this model was reported with the AEDs midazolam and phenobarbital at doses that also induced sedation (Williams et al., 2004). Drug-induced EEG slowing and sedation is a common side effect of many AEDs. In particular, sedative doses of barbiturates such as pentobarbital induce slow-wave EEG oscillations at delta (1–4-Hz) and spindle (7–14-Hz) frequencies and further implicate LVACC currents through the involvement of synchronized reticular and dorsal thalamic neuronal activity (Ran et al., 2004).

The loss of antiseizure efficacy of EXM at high doses may be due to a paradoxical enhancement of intrinsic delta and spindle oscillatory thalamic activity. The unmasking of epileptic paroxysms have been shown to occur spontaneously during natural states of slow-wave activity, including SWDs in monkeys (Steriade, 1974), and in association with sleep spindles during stage 2 human sleep (Kellaway, 1985). Sleep spindles (7–14 Hz) that are generated in the thalamus can develop into SWDs due to enhanced excitability of neocortical neurons (Steriade and Amzica, 2003), a phenomenon that could be mimicked in the MCAo/NCS model due to drug-induced slow-wave activity coupled with the "excitotoxic" state of peri-infarct cortex. Cortical hyperexcitability induced by penicillin injection is well known to cause the transformation of normal physiological spindle activity to pathological SWDs (Kostopoulos et al., 1981). In effect, drug-induced slow-wave activity may be a confounding variable in the treatment of brain injury-associated NCS activity and may translate into an important treatment consideration for stroke or neurotrauma patients. To date, the exacerbation of NCS due to sedative concentrations of AEDs or other drugs has not been explored in clinical brain injury.

The current data support clinical reports of a worsening of outcome in the presence of seizures induced by acute brain injury. In particular, the number of NCS events was positively correlated with brain infarct volume. In addition, mortality rate for rats with 0 to 30 NCS was 0 to 30%, whereas the mortality rate for rats with >30 NCS was 67%. Although this difference was not significant, clinical data indicate that the occurrence and duration of NCS in brain-injured patients are critical determinants of outcome (Jordan, 1995). Indeed, both the time to diagnosis and duration of seizure activity are independent predictors of poor outcome (Jordan, 1995; DeLorenzo et al., 1998). Likewise, seizure duration and delay in time to seizure diagnosis and treatment were found to be significant variables increasing mortality in brain injured patients (DeLorenzo et al., 1998). The presence of seizures, particularly in cases of status epilepticus, also increased mortality in studies of stroke (Waterhouse et al., 1998) and traumatic brain injury (Vespa et al., 1999).

It is noteworthy that the present data indicate that the brain infarction and neurological deficits associated with MCAo were partially reversible with doses of EXM and GBP that attenuated NCS. In particular, the neuroprotection dose-response followed a similar pattern as the dose-response effect for seizure control of both EXM and GBP, with overlapping $ED_{50}$ confidence intervals for neuroprotection and antiseizure efficacy. In comparison, however, GBP provided better overall neuroprotective efficacy with statistically significant improvements in both the reduction of brain infarction and neurological deficit score.

In contrast to the neuroprotective effects observed in the MCAo model, no detectable neuroprotection was observed against a variety of insults to cultured rat neurons. This model is highly sensitive to neuroprotective intervention with other compounds, including sodium channel blockers (Dave et al., 2000), *N*-methyl-D-aspartate antagonists (Williams et al., 2000), iron chelators (Chen et al., 2005), and sigma ligands (De Coster et al., 1995). One plausible expla-

nation for this dichotomous effect between injury models with EXM and GBP is that the in vivo neuroprotection afforded by these AEDs is directly attributable to the antiseizure effects and not to a direct cellular neuroprotective mechanism. It would be informative to further test this hypothesis in oxygen-glucose-deprived thalamocortical slices that are capable of generating network seizure activity (Lee et al., 2005).

In summary, focal ischemic brain injury in the rat induced spontaneous NCS activity that was positively correlated to the volume of brain infarction as evaluated 24 h postinjury. Treatment with either EXM or GBP provided a dose-related reduction in NCS activity and improvement in outcome. Although complete blockade of NCS was achievable with EXM, the effects were limited by a narrow therapeutic dose range and U-shaped dose response. In contrast, GBP offered a wider therapeutic dose range than EXM and offered a superior neuroprotection profile. NCS activity was more refractive with delayed postseizure treatment, and only GBP significantly reduced the number of subsequent NCS events. The neuroprotective effect of both drugs seemed to be directly related to their antiseizure efficacy, because neither drug was neuroprotective in in vitro models of injury to cortical neurons. These data indicate that brain injury-induced NCS is a valid therapeutic target and that i.v. dosing with AEDs targeting voltage-activated calcium channels may improve recovery from ischemic brain injury. Future studies are needed to determine the applicability of this approach to other types of acute brain injury (e.g., traumatic or hemorrhagic).

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