

## FULL-LENGTH ORIGINAL RESEARCH

# Localized transmeningeal muscimol prevents neocortical seizures in rats and nonhuman primates: Therapeutic implications

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### SUMMARY

**Purpose:** To determine whether muscimol delivered epidurally or into the subarachnoid space can prevent and/or terminate acetylcholine (ACh)-induced focal neocortical seizures at concentrations not affecting behavior and background electroencephalography (EEG) activity.

**Methods:** Rats ( $n = 12$ ) and squirrel monkeys ( $n = 3$ ) were chronically implanted with an epidural or subarachnoid drug delivery device, respectively, over the right frontal/parietal cortex, with adjacent EEG electrodes. Recordings were performed in behaving rats and chaired monkeys. Via the implants, either a control solution (artificial cerebrospinal fluid, ACSF) or muscimol (0.25–12.5 mm) was delivered locally as a “pretreatment,” followed by the similar delivery of a seizure-inducing concentration of ACh. In five additional rats, the quantities of food-pellets consumed during epidural ACSF and muscimol (2.5 mm) exposures were measured. In a last

group of four rats, muscimol (0.8–2.5 mm) was delivered epidurally during the ongoing, Ach-induced EEG seizure.

**Results:** In contrast to ACSF pretreatments, epidural muscimol pretreatment in rats completely prevented the seizures at and above 2.5 mm. In the monkeys, subarachnoid muscimol pretreatments at 2.5 mm completely prevented the focal-seizure-inducing effect of Ach, whereas similar deliveries of ACSF did not affect the seizures. Furthermore, 2.5 mm epidural muscimol left the eating behavior of rats intact and caused only slight changes in the EEG power spectra. Finally, muscimol delivery during Ach-induced EEG seizures terminated the seizure activity within 1–3 min.

**Conclusions:** The results of this study suggest that muscimol is a viable candidate for the transmeningeal pharmacotherapy of intractable focal epilepsy.

**KEY WORDS:** Muscimol, Acetylcholine, Dura mater, Pia mater, Neocortical seizures.

Intractable focal epilepsy resistant to antiepileptic drugs (AEDs) afflicts about 500,000 people in the United States and possibly more than 10 million people worldwide (<http://www.epilepsyfoundation.org>; Brodie, 2005). The primary alternative therapy for these patients is traditional

surgical intervention, mainly tissue resection. However, a substantial subclass of this patient population is not suitable for tissue resection, owing to contraindicative medical conditions, the location of seizure foci in the eloquent cortex, or other risk factors (Silfvenius, 2000). In response to this challenge, several teams of neuroscientists, epileptologists, and bioengineers have recently started to develop intracranial therapeutic devices for the treatment of intractable focal epilepsy not amenable to traditional surgery. These devices, recently reviewed by Stacey and Litt (2008), include AED-releasing intracortical polymers (Tamargo et al., 2002), local cooling devices (Rothman &

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Yang, 2003), the Responsive Neurostimulator (RNS) system by NeuroPace, Inc. (Mountain View, CA, U.S.A.) (Worrell et al., 2005), and hybrid neuroprostheses for transmeningeal pharmacotherapy (Ludvig, 2000; Ludvig & Kovacs, 2002; Ludvig et al., 2006). Of these devices, the RNS apparatus has already been introduced into clinical trials (Stacey & Litt, 2008), whereas the others are in the preclinical phase.

Transmeningeal pharmacotherapy “offers drug deliveries directly...to the epileptogenic brain tissue, via sealed, single or multiple, regularly flushed, subdural/epidural catheter-units equipped with recording electrodes/sensors that provide feed-back from the exposed neural tissue” (Ludvig et al., 2006). The concept builds on prior demonstrations of the ability of various drugs to reduce epileptiform electrical activity in the rat cerebral cortex upon their localized administrations (Collins, 1980; Piredda & Gale, 1985; Yokoi et al., 1987; Smith et al., 1993; Eder et al., 1997; Stein et al., 2000; Ansel et al., 2004). We have shown the relevance of these animal studies to the human condition in a clinical study where lidocaine was transmeningeally delivered into the seizure focus of patients prior to tissue resection, and within minutes a clear reduction in interictal electroencephalography (EEG) spike activity was detected (Madhavan et al., 2008). Indeed, the physicochemical properties of the cerebral meninges apparently allow the diffusion of small, water-soluble molecules into the underlying neocortex to exert pharmacologic effects (Ludvig et al., 2008). The success of transmeningeal pharmacotherapy for intractable focal epilepsy will depend on: (1) the availability of reliable seizure-prediction software so that seizures can be prevented by drug deliveries before seizure onset; (2) the construction of a flawlessly functioning, subdural/subarachnoid recording/drug delivery device; (3) the development of safe techniques for the surgical placement and postoperative management of this device; and (4) the use of an ideal seizure-preventing drug or drug combination in the implant, which is addressed in this article.

Some of the most important criteria of transmeningeally applicable seizure-preventing drugs are their solubility in water at neutral pH, their potential to prevent seizures at relatively low concentrations, their negligible interference with physiologic neural functions, and their stability in body temperature. It is immediately apparent that not all AEDs meet these criteria. For example, pentobarbital solutions exert a powerful seizure-preventing effect in the neocortex (Ludvig et al., 2006), yet, considering the study of Lockard et al. (1979) on the toxicity of polyethylene glycol in monkeys, the need to use propylene glycol and other alcohol solvents in pentobarbital solutions (Lockard et al., 1979) makes this AED less than optimal for transmeningeal pharmacotherapy. Water-soluble benzodiazepines, such as midazolam, are available, but the strongly acidic environments necessary to keep these compounds

water-soluble may also limit their use for therapeutic intracranial deliveries.  $\gamma$ -Aminobutyric acid (GABA) is also problematic, as we found this neurotransmitter unable to prevent focal neocortical seizures, at least in the experimental conditions we used, although it can stop ongoing seizure activity (John et al., 2007). Lidocaine has been used routinely in clinical practice for spinal epidural treatments, such as epidural anesthesia. As a consequence, a large body of data has been accumulated on the benefits and occasional complications of these interventions. Because of this clinical experience, lidocaine is a useful tool for exploring the viability of transmeningeal drug therapy for focal epilepsy in experimental neurosurgical settings (Madhavan et al., 2008). At the same time, the broad nonspecific effects of this local anesthetic on virtually all exposed neocortical axons, dendrites, and cell bodies justifies the search for alternative transmeningeal AEDs.

Muscimol, 3-hydroxy-5-aminomethylisoxazole, is the decarboxylation product of ibotenic acid, a compound found in the mushroom *Amanita muscaria*. Muscimol is readily water-soluble at pH = 7.4 and acts primarily as a competitive GABA<sub>A</sub> receptor agonist. Upon systemic administration, it causes visual and auditory hallucinations, loss of equilibrium, and, in some cases, psychotic symptoms (Halpern, 2004; Brvar et al., 2006). Because of these psychoactive effects, orally or parenterally administered muscimol has not been, and cannot be, utilized for therapeutic purposes. In animal models of epilepsy, parenteral muscimol injections were found to be either proconvulsant (Meldrum, 1984) or anticonvulsant (Loscher, 1985), or even ineffective (Pedley et al., 1979), depending on the employed seizure model. Nevertheless, in contrast to these conflicting pharmacological data, the few prior studies analyzing specifically the effects of direct muscimol applications into the neocortex revealed clear anti-epileptic actions. Therefore, in his groundbreaking study, Collins (1980) showed in rats that muscimol can block frontal cortical epileptiform activity induced by penicillin, bicuculline, and picrotoxin, albeit not strychnine. Furthermore, Yokoi et al. (1987) demonstrated, also in rats, that muscimol applied to the frontal/parietal cortical pia mater suppresses focal EEG spiking induced by guanidinoethanesulfonic acid.

Extending the mentioned muscimol studies, the present experiments aimed: (1) to determine whether epidurally delivered muscimol can prevent acetylcholine (Ach)-induced focal seizures in the rat frontal/parietal cortex, as this specific information had not been available; (2) to investigate, for the first time, whether muscimol delivered into the subarachnoid space exerts focal seizure-preventing effect in the primate neocortex; (3) to obtain some initial insights into how transmeningeally delivered muscimol affect physiologic EEG activities and normal behavioral patterns; and (4) to reveal whether muscimol applied epidurally during an ongoing, Ach-induced EEG

seizure can terminate this epileptiform activity. We hoped the studies would answer the question of whether muscimol is a viable candidate for a drug that can prevent focal neocortical seizures upon subdural/subarachnoid delivery, safely, and without interfering with normal brain functions.

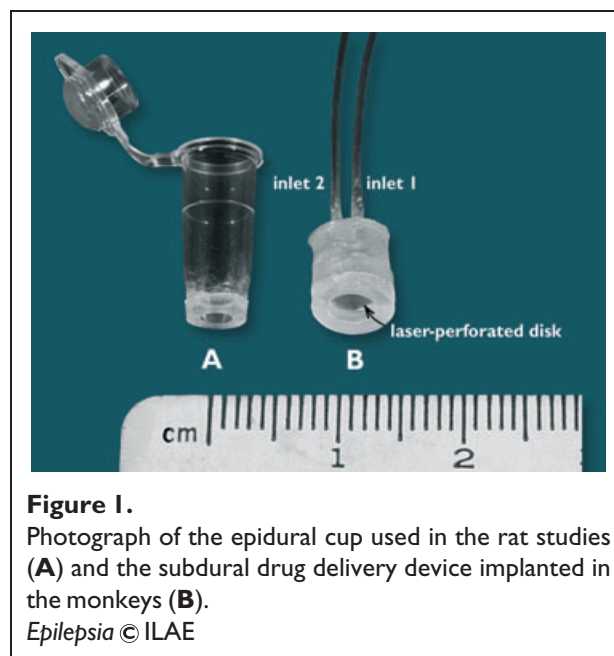
## MATERIALS AND METHODS

### Animals

Twenty-one male Long-Evans rats, weighing 350–400 g, and three male squirrel monkeys (*Saimiri sciureus*), weighing 850–1,100 g, were used. They were subjected to experimental protocols previously approved by the institutional animal care and use committees at NYU School of Medicine and SUNY Downstate Medical Center.

### Surgical procedures in rats

Following previously described procedures (Ludvig et al., 2006), 16 rats were subjected to the implantation of EEG electrodes and an epidural cup. Each rat was anesthetized with 50 mg/kg pentobarbital, i.p. (intraperitoneal). Additional injections of 0.1 mg/kg atropine, s.c. (subcutaneous), and 150,000 units/kg penicillin G Benzathine/penicillin G procaine, i.m. (intramuscular), served to reduce secretion in the respiratory system and prevent infection, respectively. The rat was placed in a stereotaxic apparatus, the skull exposed, and a 4.5-mm diameter craniotomy was drilled in the border area of the right parietal and frontal bones with its center 2.5 mm posterior to the bregma and 2.5 mm lateral to the midline, according to the rat brain atlas of Paxinos and Watson (1998). An epidural cup (Fig. 1A) was placed in the craniotomy, touching, but not pressing, the intact dura mater and the underlying somatosensory cortex (Fig. S1). Sterile bone wax was used to seal the gap between the cup and the bone. After completing this procedure, two epidural screw-electrodes prepared from TX2-4-C stainless steel screws (Small Parts, Inc., Miami Lakes, FL, USA) were placed in the skull 2 mm from each other and 1 mm from the posterior edge of the epidural cup. Two similar screw electrodes were placed over the contralateral cortical dura mater, and an additional screw was placed in the left occipital bone to serve as the grounding electrode. A Mill-Max socket, connected to the electrodes via insulated wires was also secured to the skull. The surgical procedures were completed by anchoring the entire assembly to the skull with dental acrylic and then approximating the skin, treated with Animax antibiotic ointment, with a suture. An additional group of five rats were subjected to a similar surgical protocol, except that in each of these animals only an epidural cup was implanted for a study dedicated solely to behavioral measurements. The postoperative recovery period lasted for 2 days, after



**Figure 1.** Photograph of the epidural cup used in the rat studies (A) and the subdural drug delivery device implanted in the monkeys (B).  
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which the rats were subjected to the experimental sessions.

### Surgical procedures in monkeys

Each monkey underwent overnight fasting prior to the surgical procedures, which were performed under general anesthesia, as described (Ludvig, 2000). Therefore, pre-operative medication included ketamine (11 mg/kg), xylazine (0.5 mg/kg), atropine (0.05 mg/kg), carprofen (4 mg/kg), and penicillin G benzathine/penicillin G procaine (150,000 units/kg). Monitors [electrocardiography (ECG), pulse oximetry, and noninvasive blood pressure monitor] were placed, an intravenous catheter was inserted into the femoral vein for fluid supply (lactated Ringer's solution at a rate of 4 ml/kg/h), and the monkey's body was covered with warming blankets to prevent hypothermia. Using endotracheal intubation, anesthesia was maintained with isoflurane (1–2%) inhalation in oxygen (99–98%). The monkey's head was placed in a stereotaxic apparatus, the skull exposed, and a 7-mm diameter craniotomy was drilled over the right motor cortex, with its center 2-mm anterior to the interaural line and 6-mm right to the midline, according to the squirrel monkey brain atlas of Gergen and MacLean (1962). The dura mater was opened, and a subdural/subarachnoid drug delivery device, made of silicone rings and two inlet tubes (Fig. 1B), was placed on the surface of the pia mater, above the motor cortex (Supporting Information Fig. S2). This device contained a 0.025-mm thick and 6.3-mm diameter stainless steel disk with 5- $\mu$ m diameter laser-drilled perforations with 20- $\mu$ m spacing by Lenox Laser, Corp. (Glen Arm, MD, U.S.A.) to prevent clogging by blood-clot and tissue debris. The total volume of the space

between the inlet tubes and the contacted pia mater was 40  $\mu$ l. Dental acrylic was used to seal the gap between the device and the bone and to glue the device to the skull. Two epidural screw electrodes were placed 1 and 3 mm anterior to this subdural implant, still in contact with motor cortical area, followed by the placement of similar epidural electrodes over the contralateral motor cortex. An additional epidural screw electrode was placed in the contralateral parietal bone for grounding the animal. In an attempt to also record local multineuronal activity, an array of 50- $\mu$ m diameter nichrome wires was introduced into the motor cortical tissue underneath the drug delivery device, with the reference microelectrode array placed in the contralateral white matter. All electrodes were connected to a 16-pin Mill Max socket with insulated wires. The entire assembly was secured to the skull with anchoring screws and dental acrylic, leaving exposed only the nonconnected side of the Mill-Max socket and an 8-cm segment in each drug-delivery device tubing. Each externalized tube was plugged, and a plastic protective cap was placed around the assembly. The skin was approximated with a suture and treated with Animax antibiotic ointment. Experiments on the monkeys started after a 10- to 14-day postoperative recovery period. During this period, the implanted drug delivery device was flushed with sterile saline, whereas the monkey was briefly anesthetized with the inhalation of 2% isoflurane in oxygen.

### Experimental sessions in rats

In the first study, conducted on four freely moving rats, the animal was subjected to epidural drug delivery/EEG recording sessions similar to our previous experiments (Ludvig et al., 2006; John et al., 2007). Briefly, the rat was placed in a 35  $\times$  35  $\times$  35 cm wooden box equipped with a acrylic glass door and was connected to an EEG recording apparatus. The cortical EEG signals, detected with the bipolar epidural electrodes, were amplified (10,000 $\times$ ) and filtered (using a band-pass of 0.1–100.0 Hz). Operational amplifiers, built in the recording cable, were used to eliminate movement-related artifacts. The analog data were digitized at 1,000 Hz with a National Instruments (Austin, TX, U.S.A.) PCI-MIO-16E-4 (12-bit) A/D board and stored in a binary data structure using proprietary software on a personal computer. The behavior of the animal in the test chamber was continuously monitored with a JVC Compact VHS camcorder (GR-AX940) facing the acrylic glass door. Notes were made on the rat's behavior before, during, and after drug applications. Each experimental session lasted for 20 min, and five consecutive sessions were conducted in the following schedule: a morning and an afternoon session on both day 1 and day 2, followed by a final session in the morning of day 3. The morning (a.m.) and afternoon (p.m.) sessions were separated by 4–5 h. Each experimental session comprised three phases. Phase 1 was a 5-min session to collect background EEG

activity. At the fifth minute, either artificial cerebral spinal fluid (ACSF) (pH = 7.2–7.6; volume = 50  $\mu$ l; temperature = 20–24 $^{\circ}$ C) or 12.5-mm muscimol (muscimol hydrobromide from Sigma (St. Louis, MO, U.S.A.); dissolved in ACSF; pH = 7.2–7.6; volume = 50  $\mu$ l; temperature = 20–24 $^{\circ}$ C) was delivered manually, over a 5-s period, into the epidural cup. This pretreatment period (phase 2) lasted for 3 min. At the eighth minute, 548 mM Ach (acetylcholine chloride from Sigma; dissolved in ACSF; pH = 5.2; volume = 50  $\mu$ l; temperature = 20–24 $^{\circ}$ C) was delivered into the epidural cup in the same manner as the pretreatment solution. At this concentration epidurally delivered, Ach causes focal seizures (Ludvig et al., 2006; John et al., 2007). The post-Ach EEG activity was monitored for 12 additional min (phase 3) to determine whether the pretreatment was effective in preventing the Ach-induced seizures. Importantly, each session was followed by the immediate removal of all solutions from the epidural cup and the subsequent rinsing of the cup twice with ACSF.

The second study, conducted on four rats, was performed in the same manner as the first one, except that the concentration of the epidurally delivered muscimol was 1.25 mM.

Because by this time the seizure-preventing effect of muscimol had been firmly established (see Results), the third study, also performed on four rats, used a paradigm in which each rat was subjected to two muscimol pretreatments: 0.25 mM in the day 1 p.m. session and 2.5 mM in the day 2 p.m. session, whereas the day 1 a.m., day 2 a.m., and day 3 a.m. sessions utilized ACSF pretreatments.

A fourth study was dedicated solely to determine the effects of a 3-min exposure to epidurally delivered 2.5 mM muscimol solution on gross behavior in a separate group of five rats. We used this separate group of rats not subjected to Ach-deliveries to exclude the possible confounding effects of pre-muscimol Ach-seizures. These tests were conducted in a test chamber similar to the one used in studies 1–3, but this time the rats' behavior was recorded with the VideoBench 2.0 software of DataWave Technologies (Berthoud, CO, USA). Prior to these tests, the rats were trained for 2 weeks to continuously locate and consume food-pellets (25–35 mg fragments of fruit-loops) scattered on the floor of the chamber, over a 12-min period. Once this behavior was established, each rat was subjected to a 12-min experimental session, where the first 4-min period was used to record the baseline eating behavior, the second 4-min period was used to record the eating behavior following ACSF delivery into the epidural cup, and the third 4-min period was used to record the eating behavior following the muscimol delivery into the cup. In the initial 1 min of each 4-min period, the rat was gently restrained to promote subsequent movement in the chamber. Therefore, each of the actual eating periods lasted for 3 min. At the end of the session, the epidurally delivered solutions were removed, the cup was washed with ACSF,

and the rat was returned to the home cage. One hour later, an additional 4-min period (with the initial 1-min gentle restraint) was used to record the possible late effects of the muscimol deliveries on eating behavior. For each of these periods, the number of consumed food pellets was counted and documented.

Finally, a fifth study on rats #17–21 was used to reveal whether muscimol applied epidurally during an ongoing, Ach-induced EEG seizure can terminate this epileptiform activity. In each experiment, first the background EEG activity was collected for 5 min. At the fifth minute, a 548-mM Ach solution was delivered manually, over a 5-s period, into the epidural cup. The Ach-induced EEG seizures were monitored over a 3-min period, and at the eighth minute, ACSF (control solution) was delivered in the same way into the cup. The EEG effects of ACSF delivery were monitored until the end of the experimental session at the 15th minute, after which all solutions were removed from the epidural cup and the drug-exposed area was rinsed. One day later, this experiment was repeated in the same rat, with the exception that instead of ACSF, muscimol (0.8 mM) was delivered into the cup. In one rat, where the 0.8 mM muscimol did not stop the Ach-seizure, an additional experimental session was conducted in a similar way, 3 days later, delivering 2.5 mM muscimol into the epidural cup during the Ach-seizure. As in the other studies, Ach and muscimol were dissolved in ACSF (pH = 5.2 and 7.4, respectively; volume = 50  $\mu$ l; temperature = 20–24°C).

After the end of the studies, the rats were euthanized according to methods consistent with the American Veterinary Medical Association Panel on Euthanasia. Following a described protocol (Ludvig et al., 2008), in one rat we delivered 50  $\mu$ l of a 1% methylene blue solution into the epidural cup for 15 min prior to euthanasia to mark the location of the device (as shown in Fig. S1). We note that the used muscimol solutions were dilutions from 25 and 10 mM stock solutions, with the latter one used from early January 2008 through early May 2008.

### Experimental sessions in monkeys

The monkey was subjected to a brief (1–2 min) anesthesia induced by isoflurane (2%) inhalation in oxygen to avoid stress, and placed in a primate chair. The cover of the protective cap was removed, the inlet tubes (Fig. 1B) unplugged, and the subdural device was flushed with sterile saline. A lightweight plastic shield was secured to the monkey's headpiece to allow subsequent manipulations with the extracranial implant components. Once awake, the animal was transported to the EEG recording/drug delivery setup and connected to the recording cable. EEG recordings from the seated, awake monkey were conducted using the same recording parameters as in rats. Inlet tubes 1 and 2 of the subdural implant (Fig. 1B) were connected to an ACSF- or muscimol-containing syringe

and an Ach-containing syringe, respectively, with both syringes driven with a Bioanalytical Systems (BAS) microperfusion pump. Similarly to the recording/drug delivery sessions in rats, the experimental sessions comprised an initial 5-min baseline EEG collection phase, a subsequent 3-min ACSF or muscimol pretreatment phase, and a final Ach-seizure test phase, using the delivery of 548-mM Ach at the eighth minute. Instead of manual deliveries, the monkey experiments employed drug deliveries with the syringe pump. The delivery volume for ACSF, muscimol, and Ach was 20  $\mu$ l, which was delivered over a 10-s period in every experiment. Each experimental session lasted for 12–15 min (shorter than in rats to prevent skin abrasions and injuries in the trunk of the seated animal). After the end of the session, the delivered solutions were removed from the subdural device. Then the monkey was disconnected from the recording cable and the syringe pump, the protective cap was closed, and the animal was transported back to the home cage. In each monkey only one recording/drug delivery session was performed on a given experimental day, and the entire study comprised three consecutive daily sessions. On day 1, ACSF was used for pretreatment, on day 2 the pretreatment solution was 12.5 mM in the first monkey and 2.5 mM in the second and third monkeys, whereas on day 3 the ACSF-pretreatment was repeated. In two monkeys the EEG data were stored in the same way as in the rats; in one monkey the EEG data were stored with the SciWorks 5.1b data acquisition system by DataWave Technologies (Berthoud, CO, U.S.A.). Notes were made on the monkey's behavior, including reactions to acoustic stimuli (claps) and food (fruit-loops) offered by the experimenter.

After the end of the experiments, one monkey was sacrificed for examining the localization of the implanted subdural device (Fig. S2), whereas the remaining two monkeys were introduced into other studies. One study examined whether the subdural drug delivery device retained its functional integrity after the cessation of the regular flushing with saline or drug solutions. Another study examined whether the implanted microelectrodes are adequate for the recording of neuronal activity.

### Data analysis

Fast Fourier Transform (FFT) was used to assess the EEG power of the recorded waves, as described (Ludvig et al., 2006). Briefly, the Average Spectral Intensities ("power";  $\mu$ V<sup>2</sup>) of the delta (1–3.9 Hz), theta (4–7.9 Hz), low-alpha (8–9.9 Hz), high alpha (10–12.9 Hz), low beta (13–19.9 Hz), high-beta (20–29 Hz), and low-gamma (30–49.9 Hz) frequency bands were computed for each of the three phases of each 20-min data file. The computed power values, representing the average of 20-s epochs, were introduced into statistical analyses, focusing on the 13–19.9 Hz band, of which increase has been proved to be a good indicator of the development of a focal neocortical

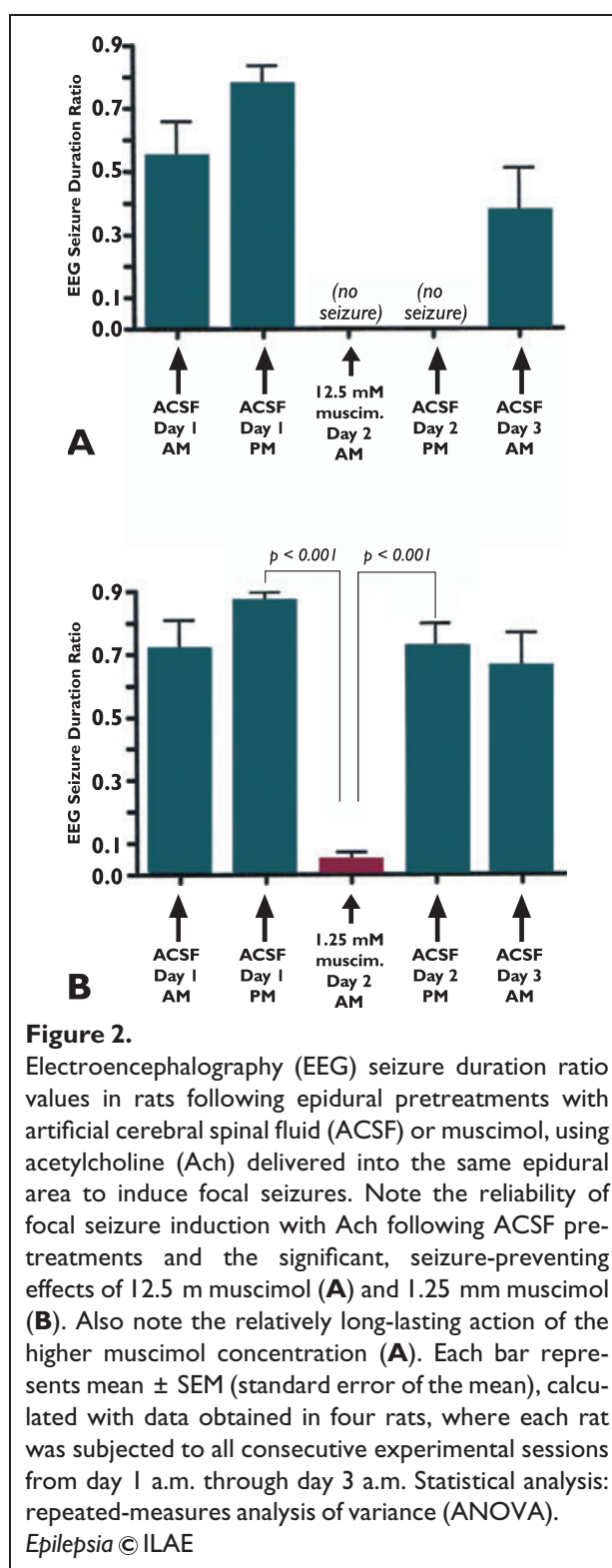
seizure (Ludvig et al., 2006; John et al., 2007). In addition, data playback was used for determining the duration of each distinct seizure episode. Using these values, EEG seizure duration ratio was calculated for each phase, by dividing the total duration of EEG seizure episodes (s) by the length of the phase (s). This yielded a value ranging from 0 (=no seizure) to 1 (=seizure throughout the entire phase). Data playback was also used to identify the latency of the Ach-induced EEG seizures and comparing the spread of EEG spike activity to the contralateral site. Behavioral data analysis obtained in the fourth group of rats compared the number of food pellets consumed during these experiments. For statistical analyses, the SPSS 11.0 (SPSS, Chicago, IL, U.S.A.) and PRISM 4.0 (GraphPad Software, La Jolla, CA, U.S.A.) program packages were both used. These analyses used three dependent variables collected in the rat study: average spectral intensity, EEG seizure duration ratio, and EEG seizure latency. The dependent variables were subjected to regular one-way analysis of variance (ANOVA) with Dunnett's post hoc test, ordinary repeated measures ANOVA with Tukey's post hoc test, and factorial repeated measures ANOVA with least significant difference (LSD) test for pairwise comparisons, as appropriate.

## RESULTS

### Studies in rats

Consistent with our previous experiments (Ludvig et al., 2006; John et al., 2007), epidural Ach delivery following pretreatment with localized ACSF application caused focal EEG seizure activity in all rats, within  $86.2 \pm 19.0$  s [mean  $\pm$  standard error of the mean (SEM);  $n = 12$ ; data reflecting Ach-seizures in each rat prior to muscimol tests]. Based on visual analysis of the replayed ipsilateral and contralateral EEG recordings, the EEG spikes spread into the contralateral site within a few (2–4) seconds. The ictal EEG was associated initially with left-side hindlimb and forelimb clonic convulsions, which became bilateral as the EEG seizure spread, without culminating into rearing, falling, or tonic convulsions.

In the first group of rats ( $n = 4$ ), the seizure duration ratio values following ACSF pretreatment were  $0.54 \pm 0.11$  and  $0.77 \pm 0.06$  on day 1 a.m. and day 1 p.m., respectively, which did not differ significantly ( $p > 0.05$ ). On day 2 a.m., the epidural delivery of 12.5 mM muscimol completely prevented the development of seizures in these rats, and this effect apparently persisted for 4–5 more hours, as the Ach seizures could not be induced even in the p.m. sessions following ACSF pretreatment. However, the ability of epidural Ach to induce seizures was restored by the last, day 3 a.m. session, yielding a seizure duration ratio of  $0.37 \pm 0.13$ , which, nevertheless, was still significantly ( $p < 0.05$ ) lower than that of the last seizure ( $0.77 \pm 0.06$ ) prior to muscimol delivery (Fig. 2A).

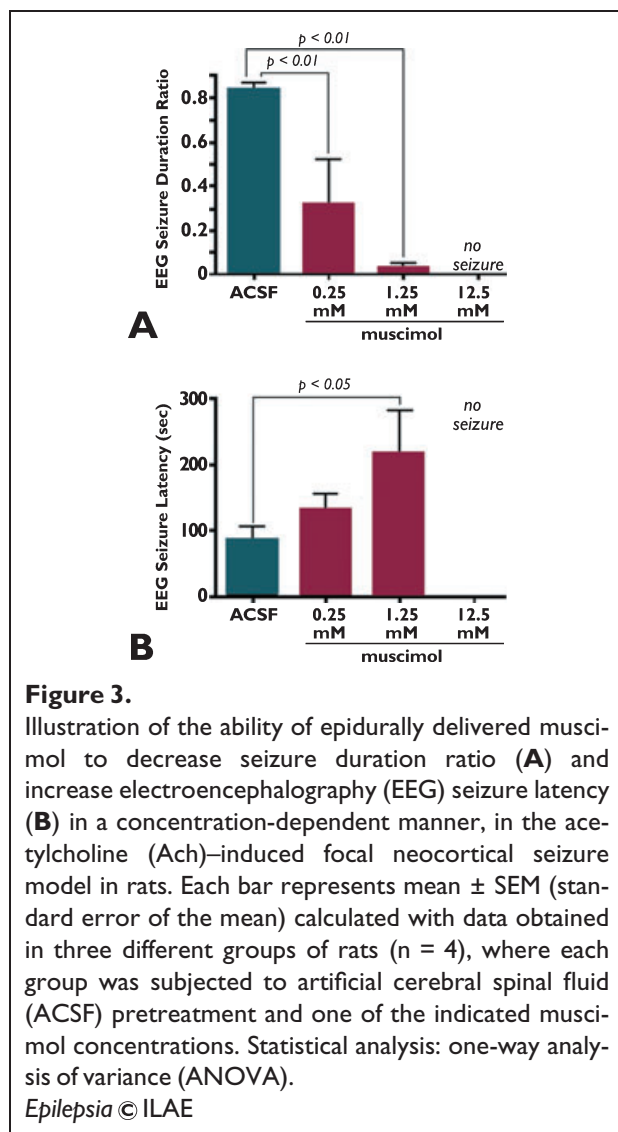


**Figure 2.**

Electroencephalography (EEG) seizure duration ratio values in rats following epidural pretreatments with artificial cerebral spinal fluid (ACSF) or muscimol, using acetylcholine (Ach) delivered into the same epidural area to induce focal seizures. Note the reliability of focal seizure induction with Ach following ACSF pretreatments and the significant, seizure-preventing effects of 12.5 mM muscimol (A) and 1.25 mM muscimol (B). Also note the relatively long-lasting action of the higher muscimol concentration (A). Each bar represents mean  $\pm$  SEM (standard error of the mean), calculated with data obtained in four rats, where each rat was subjected to all consecutive experimental sessions from day 1 a.m. through day 3 a.m. Statistical analysis: repeated-measures analysis of variance (ANOVA).

*Epilepsia* © ILAE

The data obtained in the analogous experiment in the second group of rats ( $n = 4$ ) are represented in Figs 2B and 3. Thus, the seizure duration ratio values following

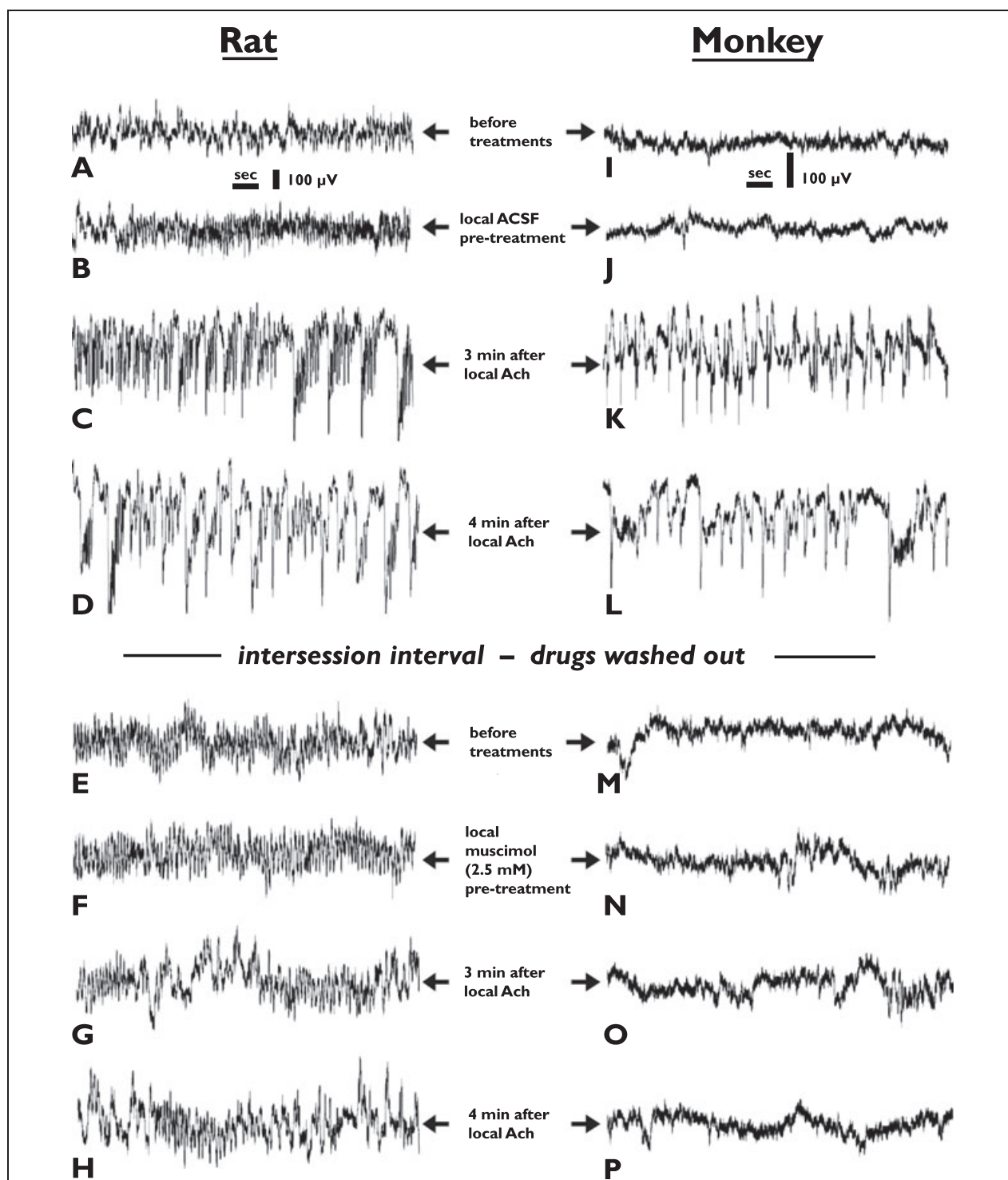


ACSF pretreatment were  $0.71 \pm 0.09$  and  $0.87 \pm 0.02$  on day 1 a.m. and day 1 p.m., respectively, which did not differ significantly ( $p > 0.05$ ). On day 2 a.m., the epidural pretreatment with the lower concentration, 1.25 mM, muscimol completely prevented the Ach seizures in two of the four rats. In the remaining animals, the Ach-seizures developed only after a long,  $216.0 \pm 64.0$ -s latency (Fig. 3B), and yielded a seizure duration ratio value of as low as  $0.04 \pm 0.02$ , which was significantly lower ( $p < 0.001$ ) than that of the corresponding ACSF pretreatment values. In contrast to the relatively long-lasting seizure-preventing action of the 12.5 mM muscimol concentration, the 10-fold less concentration used in this second study lost its ability to influence the Ach-induced seizures 4–5 h later. Therefore, in the day 2 p.m. and day 3 a.m. sessions, when ACSF was delivered again into the epidural cup prior to Ach, the seizure duration ratios,

$0.72 \pm 0.07$  on day 2 p.m. and  $0.65 \pm 0.11$  on day 3 a.m., were similar ( $p > 0.05$ ) to those obtained before the muscimol test, although significantly higher than the muscimol pretreatment value ( $p < 0.001$ ; Fig. 2B).

In the third study, the concentration of epidurally delivered muscimol in the day 1 p.m. session was further decreased to 0.25 mM. At this muscimol concentration the seizures developed in three of the four rats with an average latency of  $131.7 \pm 23.18$  s (not different from control;  $p > 0.05$ ; Fig. 3B), but yielded a significantly lower ( $p < 0.01$ ) seizure duration ratio ( $0.31 \pm 0.2$ ) than the corresponding average value ( $0.83 \pm 0.02$ ) obtained after ACSF pretreatments in all 12 rats before the muscimol test (Fig. 3A). It was this study in which the seizure-preventing action of a second epidurally delivered muscimol solution, 2.5 mM, was also tested, in the day 2 p.m. session. As shown in Fig. 4A–H, muscimol pretreatment at this concentration completely prevented the development of ictal EEG events: a phenomenon observed in all rats ( $n = 4$ ). The seizure duration ratio values for the day 1 a.m., day 2 a.m., and day 3 a.m. sessions were:  $0.87 \pm 0.04$ ,  $0.86 \pm 0.03$ ,  $0.63 \pm 0.12$ , respectively, with the day 3 a.m. value significantly ( $p < 0.05$ ) lower than those obtained after ACSF pretreatment prior to muscimol deliveries in this group. Fig. 5A–C illustrates the course of EEG power changes in three consecutive experimental sessions conducted in one of the rats from this group, confirming the robust seizure-preventing action of epidural muscimol, even at the concentration of 2.5 mM. The concentration-dependent effects of epidural muscimol pretreatment on seizure duration ratio and seizure latency values in the employed Ach focal seizure model are summarized in Fig. 3A,B, respectively. The final dataset from this study revealed the impact of 2.5 mM transmeningeal muscimol on the power of local EEG bands (Table 1). The factorial repeated-measures ANOVA for this analysis used two factors: epidural solution with two levels (ACSF and muscimol) and EEG band with seven levels (each corresponding to one of the EEG bands indicated in Table 1). The analysis showed that during the 3-min of epidural muscimol pretreatments, no significant change occurred in the power of delta, theta, and alpha frequency bands. However, the power of beta and low-gamma frequency waves was slightly but significantly ( $p < 0.05$ ) reduced, a change that was not apparent by the visual inspection of the recordings (Fig. 4E,F).

The behavioral study performed on the fourth group of rats showed that the eating behavior of the rats during the presence of 2.5 mM muscimol in the epidural cup was virtually indistinguishable from the control condition, where the cup contained ACSF, or from the condition where the cup was empty. Therefore, the number of food pellets consumed before epidural fluid deliveries, during ACSF exposure, and during muscimol exposure ( $25.2 \pm 3.3/3$  min;  $28.6 \pm 3.7/3$  min, and  $27 \pm 4.1/3$  min,



**Figure 4.**

Comparison of 15-min electroencephalography (EEG) recording segments obtained in the right somatosensory cortex of a rat (**A–H**) and the right motor cortex of a monkey (**I–P**) before epidural drug deliveries, following localized artificial cerebral spinal fluid (ACSF) and muscimol pretreatments, and after the delivery of acetylcholine (Ach) into the pretreated area. The intersession interval was 5 h in the rat experiment and 24 h in the monkey experiment. Note that muscimol was as effective in preventing the Ach-induced focal seizures in the monkey as it was in the rat. Calibrations as indicated.

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respectively) did not differ significantly ( $p > 0.05$ ; Fig. 6). In fact, a similar quantity of food pellets,  $21.6 \pm 5.4/3$  min, was consumed by the rats 1 h after muscimol exposure (Fig. 6). Because the food pellets were scattered on the entire floor area of the chamber, and thus the rat needed to move around to collect the pellets, the experiment also provided information on the movement of the animals. We detected no apparent movement abnormalities in the rats during muscimol exposures or 1 h after the drug was washed from the epidural cup.

The seizure termination study in the fifth group of rats revealed that the epidural application of ACSF during an ongoing Ach-seizure, which developed within  $44.5 \pm 7.5$  s after Ach delivery, exerted no influence on the course of the EEG seizure activity (Fig. 7A–7F). These seizures, associated with clonic convulsions, lasted throughout the rest of the 15-min recording session. In contrast, in the subsequent recording sessions, where the focal EEG seizures developed within  $46.0 \pm 7.4$  s after Ach delivery, the epidural application of 0.8 mM muscimol fully terminated the ongoing EEG seizures within 1–3 min in three rats (Fig. 7G–7L), and within 36 s after the similar application of 2.5 mM muscimol in the fourth rat. The local EEG activity returned to normal after these muscimol administrations, whereas the convulsions stopped.

It is worth noting that after washing out muscimol from the epidural cup, withdrawal-seizures such as those described by Brailowsky et al. (1990) following long-term cortical GABA infusions, were not observed in any of the described experiments.

### Studies in monkeys

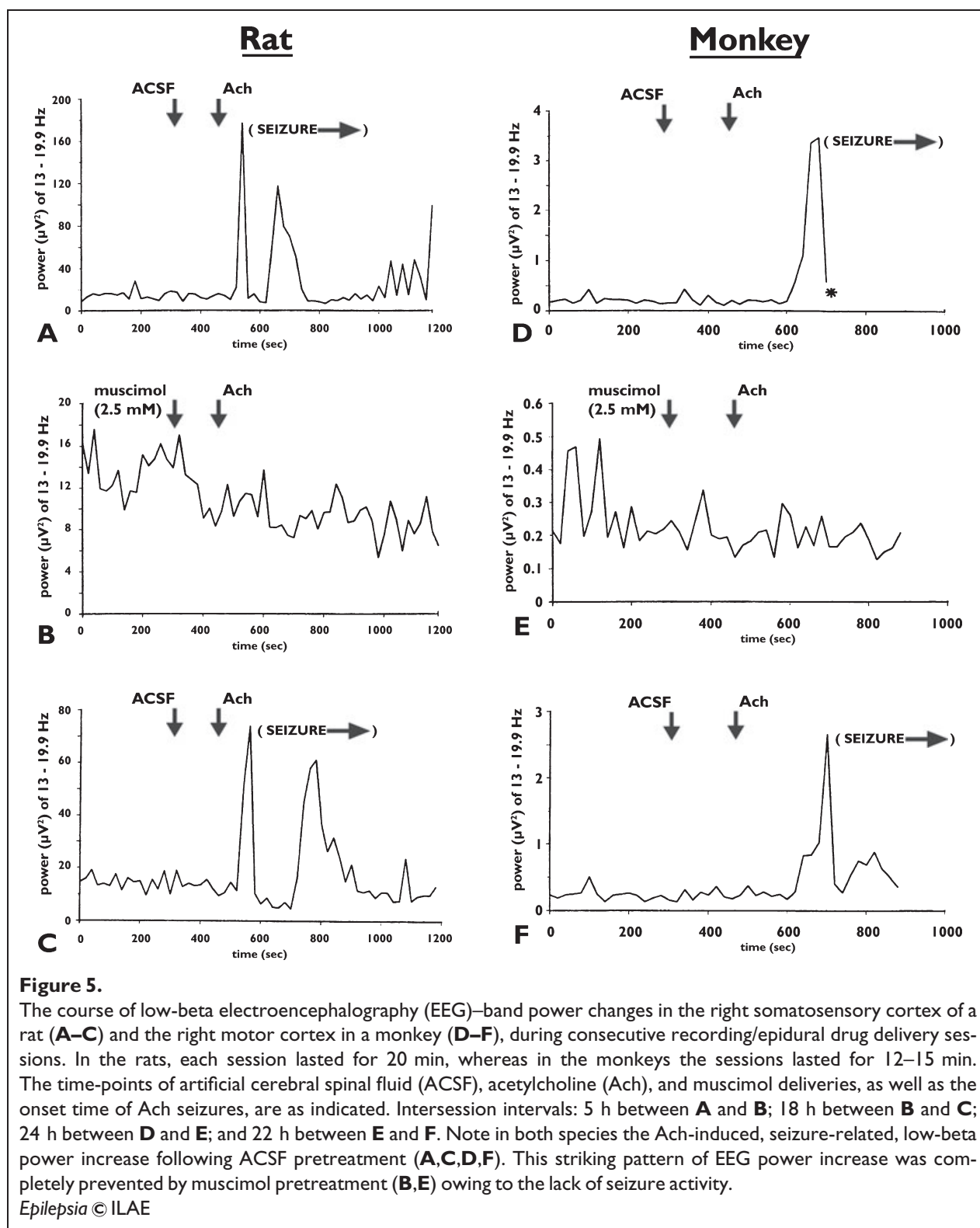
Ach application over the motor cortical pia mater following ACSF pretreatment in the same area caused EEG seizures in all three monkeys examined. The latency of EEG seizure onsets were 606, 144, and 168 s in monkeys 1, 2, and 3, respectively. The epileptiform EEG spikes spread from the ipsilateral to the contralateral motor cortex within 11, 1, and 14 s in the first, second, and third animal, respectively. The EEG seizures were accompanied within 4–8 s with clear clonic convulsions in the left leg, and with no apparent motor signs in the right leg and the rest of the body. During seizures, the monkeys did not take food pellets from the experimenter but reacted to acoustic stimuli (clapping) with turning their head toward the source of stimulus. After washing out the Ach solution from the subdural space, the EEG activity normalized within 5–10 min. Because the animals were transported back to their home cage immediately, it could be observed that after the described experiments the monkeys moved around and climbed without apparent difficulties.

Subdural pretreatment of the motor cortical area with either 12.5 or 2.5 mM muscimol completely prevented the occurrence of EEG and behavioral seizures. In fact, during the periods when both muscimol and Ach were present in

the subdural space, the monkeys both took food pellets from the experimenter and reacted normally to acoustic stimuli. When the experiment was repeated on the next day with ACSF pretreatment instead of muscimol pretreatment, both EEG and behavioral seizures occurred, as in the initial ACSF pretreatment session. Figure 4(I–P) shows a representative series of EEG recordings from monkey #2, before and after ACSF, muscimol and Ach deliveries in the subarachnoid space. The ability of subdural muscimol pretreatments to prevent Ach-induced EEG seizures was also reflected in the absence of seizure-related increase in low-beta EEG power during muscimol–Ach coexposures (Fig. 5D). Because we used three monkeys in this study, statistical analysis of the EEG power spectrum was not performed. However, qualitative examination of the recordings showed no gross alterations or abnormalities in the EEG waves during the 3-min muscimol pretreatments. After the completion of this experimental series, the subdural drug delivery device was no longer flushed regularly with saline; this led to the clogging of the apparatus. Neuronal recording was successful in only one monkey (monkey #1). Just as in rats, withdrawal seizures after removing muscimol from the subdural space were not detected in the monkeys. Importantly, during the course of this study, no neurologic symptoms or behavioral abnormalities were observed in the implanted monkeys (Fig. S3).

## DISCUSSION

This study proved that transmeningeally delivered muscimol can completely prevent the development of focal, Ach-induced neocortical seizures in both rats and nonhuman primates. The rat studies confirmed and extended prior observations by Collins (1980) and Yokoi et al. (1987) on the ability of this compound to suppress penicillin-, bicuculline-, picrotoxin- and guanidinoethanesulfonic-acid-induced epileptiform events in the neocortical circuitry, whereas the monkey studies showed for the first time that this potential is relevant to primates and thus, likely, to humans. Five facts support our conclusion that the demonstrated effects of muscimol were neurobiologically significant, specific pharmacologic actions exerted on the neocortical seizure focus. First, the Ach applications, even when separated with 4–5 h intervals, reliably induced similar ictal episodes. Therefore, the observed muscimol effects could not be caused by technical problems related to seizure induction. Second, the seizure-preventing action of muscimol was concentration-dependent and clearly different from the effects of the control treatment (ACSF delivery). This excluded the possibility of attributing nonspecific, nonpharmacologic effects to muscimol. Third, the seizure-generating capacity of the drug-exposed neocortical regions always recovered after washing-out the muscimol solutions from the epidural/subdural area. Therefore, the absence of neocortical seizures in



the presence of local muscimol was not the consequence of lost epileptogenicity, impaired Ach diffusion, or structural damage in the examined tissue. Fourth, as the normal eating

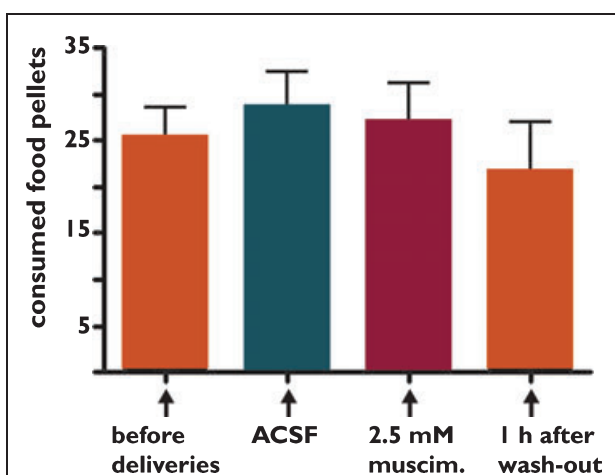
behavior of the rats and the unchanged arousal level of the monkeys during muscimol exposures showed, the primary pharmacologic actions of this drug must have taken place in

**Table 1. Average spectral intensity (“power”;  $\mu V^2$ ) of delta, theta, low-alpha, high-alpha, low-beta, high-beta, and low-gamma electroencephalography (EEG) frequency bands, before and after the epidural delivery of artificial cerebral spinal fluid (ACSF) (control solution) and 2.5 mM muscimol in freely behaving rats ( $n = 4$ ), as indicated**

Epidural treatment	Pre-ACSF	ACSF	Pre-muscimol	Muscimol
EEG frequency band (Hz)				
1.0–3.9	40.4 ± 13.8	47.8 ± 25.0	28.0 ± 10.1	32.0 ± 10.8
4.0–7.9	30.0 ± 12.5	37.1 ± 19.8	43.2 ± 21.8	38.8 ± 21.3
8.0–9.9	8.0 ± 4.3	11.1 ± 6.8	7.0 ± 3.6	8.1 ± 4.4
10.0–12.9	5.7 ± 3.2	4.5 ± 2.1	4.3 ± 1.9	3.8 ± 1.8
13.0–19.9	7.2 ± 3.0	6.8 ± 3.0	7.4 ± 2.9	5.6 ± 2.6 <sup>a</sup>
20.0–29.9	4.7 ± 1.7	4.4 ± 1.8	4.9 ± 1.7	3.5 ± 1.4 <sup>a</sup>
30.0–49.9	3.6 ± 1.5	4.0 ± 1.7	4.4 ± 1.3	3.0 ± 1.3 <sup>a</sup>

The data for each pre-ACSF and pre-muscimol value (mean ± SEM) were collected over a 5-min period before the delivery of ACSF and muscimol, respectively. The data for each ACSF and muscimol value (mean ± SEM) were collected in a 3-min period that followed the delivery of ACSF and muscimol, respectively. Factorial repeated-measures analysis of variance (ANOVA) was used for statistical analysis.

<sup>a</sup>Muscimol value significantly different from pre-muscimol value.



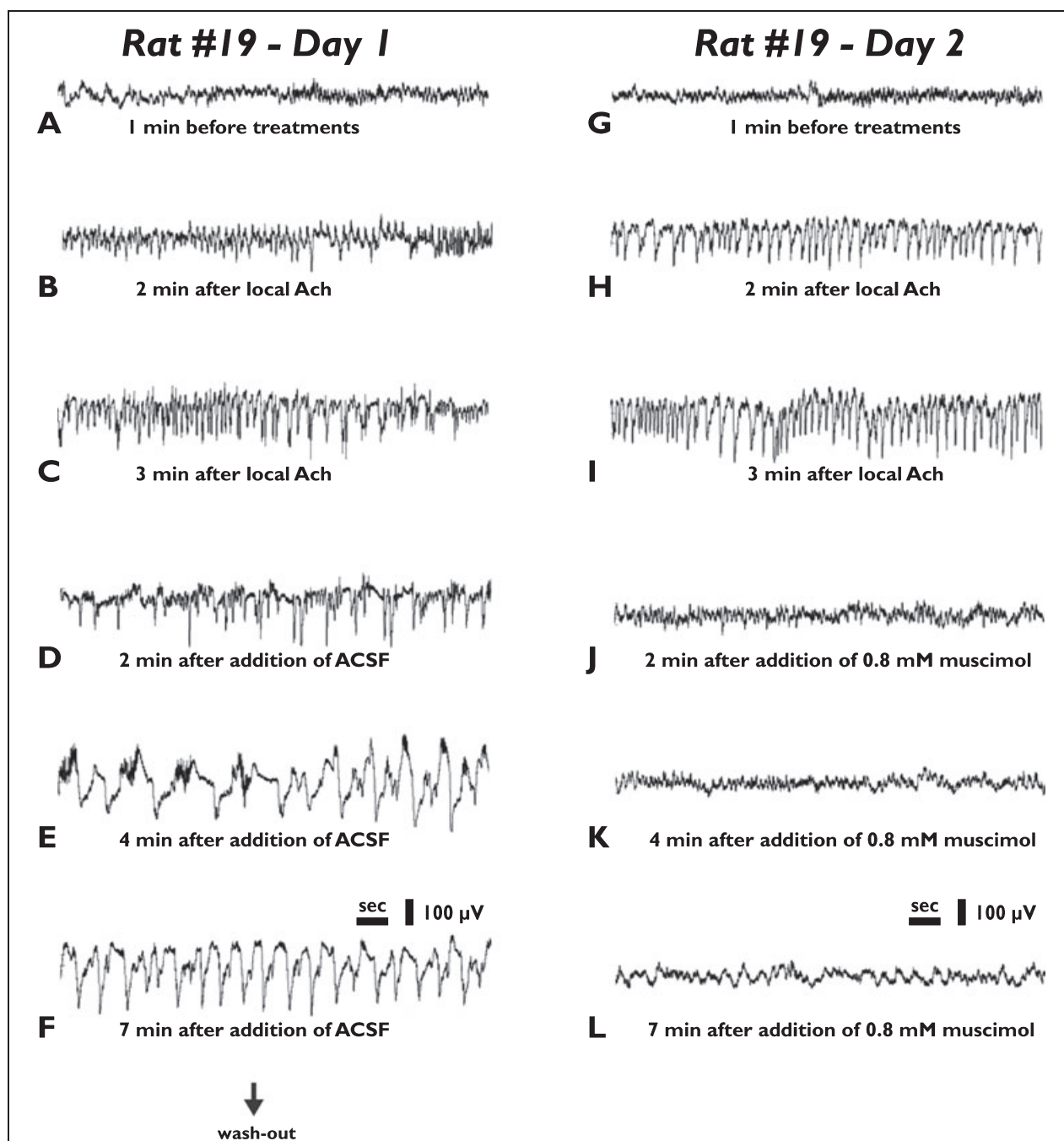
**Figure 6.**

Characterizations of eating behavior in rats, before and during epidural exposures to artificial cerebral spinal fluid (ACSF) and muscimol, as well as 1 h after the wash-out of these solutions from the epidural area. No differences were found in the number of consumed food pellets in control conditions and during muscimol exposures. Each bar represents mean ± SEM (standard error of the mean), calculated with data obtained in five rats, where each rat was subjected to all consecutive epidural treatments on a single day. Statistical analysis: repeated-measures analysis of variance (ANOVA). *Epilepsia* © ILAE

effects not apparent to the observers). Indeed, the limited behavioral consequences of transmeningeal Ach deliveries, restricted to contralateral clonic movements, are consistent with the quite localized actions of the employed drugs. Fifth, the applications of 0.8–2.5 mM muscimol into the seizure focus via the subdural/subarachnoid space terminated the focal epileptiform EEG activity and the accompanying clonic convulsions in all examined rats, again indicating the specific seizure-controlling action of muscimol in the neocortex.

The clear seizure-preventing/terminating efficacy of epidural/subarachnoid muscimol highlighted an important point. Namely, it confirmed that by solely evaluating the effects of a systemically administered drug on epileptiform events it is difficult to predict the action of the same compound on focal neocortical seizures upon transmeningeal delivery, at least in primates, since our monkey experiments proved a marked seizure-controlling action for subarachnoid muscimol: an antiepileptic action that did not surface in prior monkey studies testing intravenous muscimol (Pedley et al., 1979; Meldrum, 1984). In fact, we think that the appropriate way to determine whether an AED or other drug is a suitable candidate for transmeningeal epilepsy therapy is to test the selected compound with intracranial drug delivery methods similar to those described in this report or, ideally, with more advanced integrative neurobiologic techniques. In this context, the importance of nonhuman primate studies, not as substitutes for rodent experiments but as complementary tests, should not be overlooked, since, unlike rodents, monkeys can readily be implanted with subdural/subarachnoid drug delivery devices and can yield more complex sets of electrophysiologic and behavioral information, directly applicable to clinical trials, than subprimate species.

the circumscribed neocortical area of transmeningeal delivery (even though a fraction of muscimol molecules likely spilled over to neighboring brain areas and perhaps even passed the blood–brain barrier, causing minor systemic



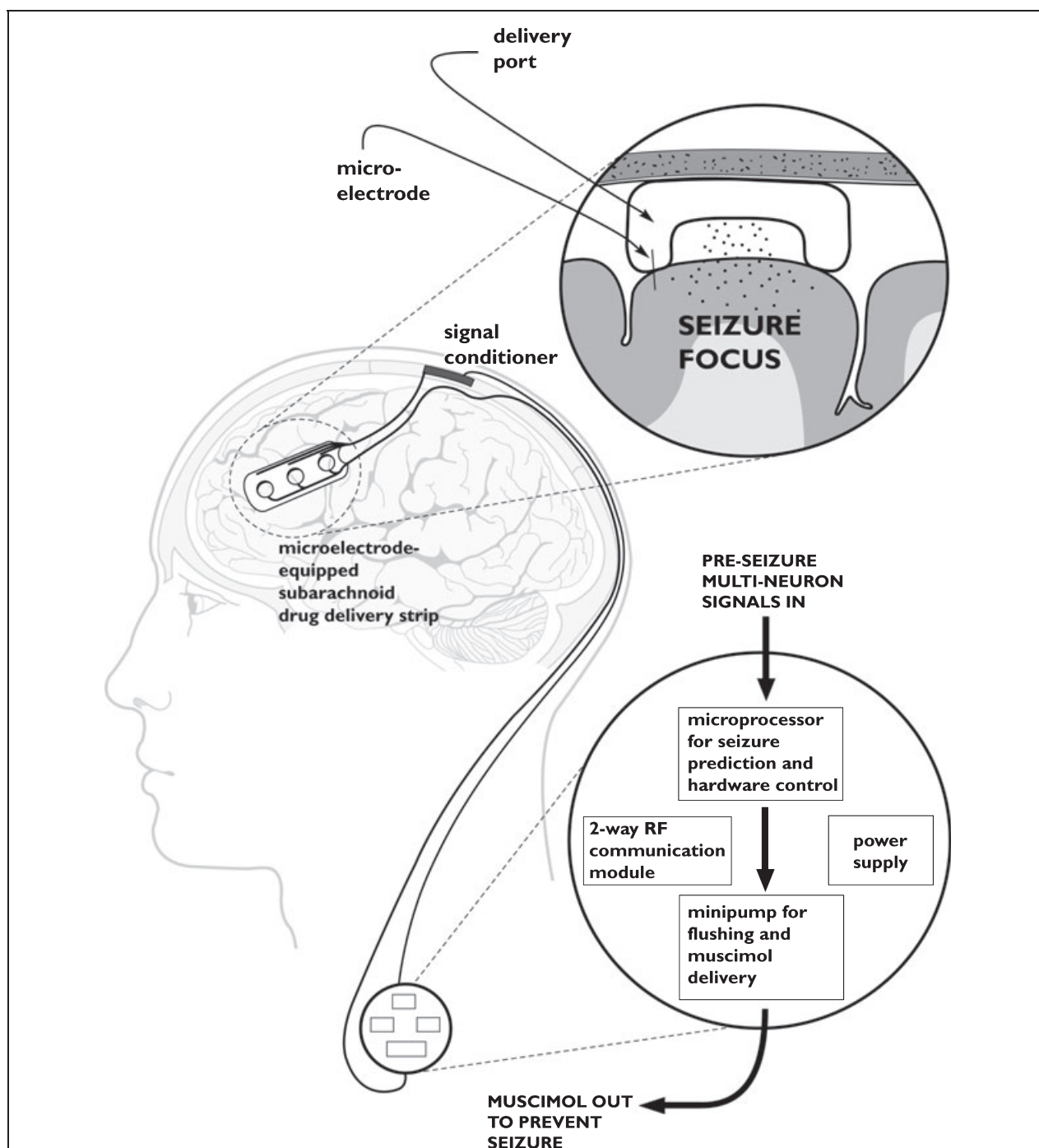
**Figure 7.**

Electroencephalography (EEG) traces to demonstrate the ability of epidural muscimol to terminate an ongoing acetylcholine (ACh)-induced focal seizure in a freely moving rat. Calibrations as indicated. Traces **A–F**: EEG recordings from the somatosensory cortex before (**A**) and during (**B,C**) an ACh-induced seizure and after the addition of artificial cerebral spinal fluid (ACSF) into the epidural cup (**D–F**). Traces **G–L**: EEG recordings from the same rat in a similar experiment 1 day later, where 0.8 mM muscimol was added to the epidural cup during an ongoing ACh-induced seizure. Note the cessation of EEG seizure activity 2 min after muscimol delivery (**J–L**).

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The properties of the seizure-preventing muscimol solutions in this study were remarkable. As mentioned in the Methods section, these solutions at pH = 7.2–7.6 and

concentrations ranging from 0.25–12.5 mM were stable, as their seizure-preventing effect did not diminish for at least 4 months. Furthermore, the minimum concentration



**Figure 8.**

Schematic diagram of the microelectrode-equipped subdural/subarachnoid hybrid neuroprosthesis (patent pending), designed to deliver muscimol into the neocortical epileptogenic zone(s), transmeningeally, upon the detection of pre-seizure multi-neuron signals. Unlike other neuroprostheses that use electrical stimulations, this device applies a drug solution, such as muscimol, to prevent focal seizure activity. The subdural/subarachnoid component of the device can be placed over large, multiple epileptogenic zones without spatial limitation. The microelectrodes are suitable for electroencephalography (EEG) recordings, as well. The implanted minipump can be refilled transcutaneously, allowing the physician to flexibly modify the transmeningeal treatment solution, even to switch from muscimol to other AEDs, if needed.

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of muscimol for completely preventing Ach-induced neocortical seizures was found to be 2.5 mM, in both rats and monkeys, and even the lowest tested concentration, 0.25 mM, reduced the severity of these seizures significantly (Fig. 3A). In fact, 0.8 mM muscimol could stop an ongoing focal seizure in three of four rats. This is consistent with the previous study by Collins (1980), who identified a muscimol concentration range of 0.1–1.0 mM as effective against bicuculline- and picrotoxin-induced neocortical seizures in rats. This suggests that muscimol, should it emerge as a candidate drug for transmeningeal epilepsy therapy, can be used in concentrations as low as 1–2 mM or less, which should not perturb the physiologic CSF and cortical extracellular osmolarity of 295 mOsmol/L. Although the present study did not collect information on the size and geometry of the neocortical areas penetrated by muscimol, in a previous rat study using similar drug delivery conditions we did provide histologic evidence for the localized diffusion of another water-soluble small molecule, *N*-methyl-D-aspartate (NMDA), through the subdural/subarachnoid space into the underlying cortical tissue (Ludvig et al., 2008). In those experiments the delivered NMDA molecules diffused from the pial surface through layer V, although they remained underneath the epidural delivery site, as it was assessed by their histologically detectable neurodegenerative effects.

Can transmeningeally delivered muscimol exert seizure-preventing action safely, without inducing unwanted side-effects in the affected neocortical circuitry? This is a critical question, since transmeningeal muscimol, and for that matter any agent selected for this type of intracranial pharmacotherapy, must exert clinically beneficial antiepileptic effects without disrupting the normal sensory, motor, and mental functions governed by the neocortex. That a 2.5 mM muscimol solution caused only slight changes in the EEG power spectrum (Table 1), induced no withdrawal-seizures, and exerted no effect on eating behavior (Fig. 6) suggest that at this seizure-preventing concentration transmeningeally delivered muscimol leaves cerebral cortical functions largely intact. In addition, the subdurally positioned drug-delivery devices were well tolerated by the monkeys for many months, and they did not develop behavioral or neurologic abnormalities. However, recent experiments in the field of cognitive neuroscience revealed that in both rats and monkeys, at least in the frontal and cingulate cortices, the local, intraparenchymal microinjections of 2.6–10.4 mM muscimol caused cognitive deficits measurable in some, albeit not all, learning/memory tests (Takehara-Nishiuchi et al., 2005; Amiez et al., 2006; Marquis et al., 2007; Ragozzino & Rozman, 2007). These muscimol concentrations were higher than the 0.25–2.5 mM range that achieved seizure prevention in our study. Nevertheless, we are aware that extensive new studies are needed to go beyond the determination of the

antiepileptic properties of transmeningeal muscimol and clarify whether the difference between the seizure-preventing and cognition-impairing concentrations of cortically applied muscimol is large enough to assure its safe therapeutic use. Such studies are underway in our laboratory.

Upon evaluating the data of this report regarding the powerful, and apparently safe, seizure-preventing effects of water-soluble, stable, neutral-pH, and low-concentration muscimol solutions on subdural/subarachnoid deliveries in animals including primates, and placing this dataset into the context of previous demonstrations by others of the ability of neocortically applied drugs to interfere with focal ictal events in rats (Collins, 1980; Piredda & Gale, 1985; Yokoi et al., 1987; Smith et al., 1993; Eder et al., 1997; Stein et al., 2000; Anshel et al., 2004), which, as we have shown (Madhavan et al., 2008), seems to be a pharmacologic effect relevant to human focal epilepsy, we conclude that sufficient experimental data have been accumulated to recognize that transmeningeal pharmacotherapy is, indeed, a viable therapeutic approach for the treatment of intractable focal epilepsy. Therefore, we propose to move from the exploratory phase of this emerging epilepsy therapy to the next level of careful preparations for clinical trials. Our vision for the preparatory phase of clinical trials includes the completion of the subdural version of the hybrid neuroprosthesis and optimizing its safe operation parameters in animals, while also testing the safety of its individual components in humans, as it is this device and its various versions with which transmeningeal pharmacotherapy can be implemented. The architecture of this implant, based on US patent 6,497,699 (Ludvig & Kovacs, 2002) and subsequent pending patents by the present authors, is shown in Fig. 8.

Based on their careful examination of the local distribution and toxicity of prolonged hippocampal infusion of muscimol in rhesus macaques, Heiss et al. (2005) already proposed that “targeted modulation of neuronal activity is a reasonable research strategy for the investigation and treatment of medically intractable epilepsy.” Although the authors used prolonged hippocampal infusions for several days, an approach different from our short-lasting, few-minute transmeningeal muscimol exposures in the neocortex, both their study and the present one point in the same direction: toward the development of novel therapeutic implants, and possibly the use of intracerebral muscimol, for otherwise intractable epilepsy.

## ACKNOWLEDGMENTS

We confirm that we have read the Journal’s position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Conflict of interest: None of the authors has any conflict of interest to disclose.

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## SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

**Figure S1.** Localization of the epidural cup over the right somatosensory cortex in a rat, as marked by methylene blue delivered into the epidural cup at the end of the experiment.

**Figure S2.** Localization of the subdural drug delivery device (arrowheads) over the right motor cortex. Positions of the used electrodes are also indicated. Note the correct localization of the subdural/subarachnoid device anterior to the central sulcus.

**Figure S3.** One of the examined squirrel monkeys (monkey #3) chronically implanted with a subarachnoid drug delivery device and adjacent epidural electroencephalography (EEG) electrodes over the right motor cortex (with an array of microelectrodes also inserted in the underlying superficial cortical layers). Photograph was taken on July 1, 2008. Arrow points to the plastic cover that protects the extracranial components (inlet tubes and electrode-connector) of the implant in between experimental sessions. The animal has showed no neurologic symptoms and displayed behaviors indistinguishable from normal, except during the acetylcholine (ACh)-induced

seizures described in this article. (In fact, since the completion of the seizure/muscimol study, the monkey has been involved in a series of behavioral experiments, including one that required the investigator to secure a 20-mm diameter plastic container to the front of the protective cover, as visible on the photographs.)

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