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Effects of lamotrigine on conditioned learning after global cerebral ischemia in rabbits

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Cerebral ischemia is one of the leading causes of neurologic injury after resuscitation from cardiac arrest and traumatic brain injury. The pyramidal cell layer of the hippocampus and the Purkinje cell layer of the cerebellum are particularly vulnerable to ischemia [1]. The selective vulnerability of these neurons may be related to high concentrations of interstitial glutamate that have been found in the hippocampus during transient global ischemia [2].

Eyeblink conditioned-response training in rabbits has widely been used as a model for the study of learning and memory [3]. Acquisition of a conditioned response (CR) is a complex task that may be mediated by the hippocampus [4] and cerebellum [5], which are vulnerable to ischemia. In a previous study [6], we showed that 6.5 min of global cerebral ischemia impaired the acquisition of the eyeblink conditioning response. We also showed that either hypothermia (30°C) during ischemia or pretreatment with 50 mg·kg^{-1} of a neuronal sodium channel antagonist, lamotrigine is able to attenuate the impairment of the eyeblink conditioning response using a trace interval (TI) of 300 ms.

The overall rate of conditioned response acquisition is highly sensitive to changes in the duration of the TI, which is defined as the delay between the end of the conditioned stimulus (CS) and the start of the unconditioned stimulus (UCS) [7]. Increasing the duration of the TI to 500 ms or more, decreases the rate at which the CR is acquired and makes the CR dependent on hippocampal processing [4]. Because some of the neuronal pathology associated with aging occurs in the hippocampus, some studies have shown that a long TI is more sensitive to age-related differences. In the trace-conditioning paradigm with 500 ms TI in rabbits, old animals acquired the CR significantly more slowly than young ones [8]. Because the hippocampus is also sensitive to ischemic injury [9], using 500 ms of TI may be more appropriate to evaluate ischemic brain injury and lamotrigine modulation than the 300 ms that was used in our previous study.

In this study, we evaluated the effect of the neuronal sodium channel antagonist lamotrigine on the ability of rabbits to acquire a trace-conditioned eyeblink response with 500 ms TI after 6.5 min of cerebral ischemia.

After approval by the institutional Animal Care and Use Committee, 29 experimentally naive male New Zealand white rabbits aged 3 months and weighing 3–4 kg were randomly assigned to one of four groups: the sham ischemia group (S, $n = 8$), the ischemia group (C, $n = 6$), the lamotrigine 20 mg·kg^{-1} treated group (L20, $n = 8$), and the lamotrigine 50 mg·kg^{-1} treated group (L50, $n = 7$). The animals were housed one per cage at the institutional Animal Resource Center, where they received routine veterinary care. All rabbits were anesthetized with 4% halothane in oxygen in a Plexiglas chamber. After loss of consciousness, endotracheal intubation was performed and mechanical ventilation was established. The end-tidal carbon dioxide tension and halothane concentration were monitored (Capnomac Ultima, Datex, Helsinki, Finland) and maintained at 35–40 mmHg and 0.8%–1.0%, respectively. A 22-gauge ear vein catheter was inserted and 0.9% saline was infused at 50 ml·h^{-1}. The ear artery was cannulated with a 22-gauge catheter for continuous monitoring of mean arterial blood pressure (MAP) and arterial blood gas analysis. The body temperature was measured by an esophageal probe and was maintained at 38°C with a heating lamp. In all animals, the scalp was infiltrated with bupivacaine 0.25%, incised in the midline, and

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reflected laterally to expose the skull, with the use of an aseptic technique. Four burr holes (1.2 mm in diameter) were drilled into the skull, and stainless metal screws were inserted. The metal screws did not perforate the inner table of the skull and served as fixation points for the dental acrylic, which cemented a stainless-steel bolt to the skull. This bolt was subsequently used to attach the photosensor used during the eyeblink conditioning. The scalp was closed, and electrodes were placed for continuous recording of the frontopartial electroencephalogram (EEG) (D.P.-304 Differential Amplifier, Warner Instrument, Hamden, CT, USA).

After completion of the surgical preparation and while the animals were still anesthetized with halothane, an inflatable neck tourniquet (6 cm width) was secured around the rabbit's neck. In the L20 and L50 groups, 20 $mg \cdot kg^{-1}$ or 50 $mg \cdot kg^{-1}$ of lamotrigine (Lamictal, Glaxo Wellcome, Greenville, NC, USA) was dissolved in 20 ml of distilled water and infused through the ear vein catheter over a period of 20 min, starting 40 min before the induction of ischemia. To induce cerebral ischemia, MAP was decreased to 40 mmHg with a bolus of trimethaphan (5 mg iv). Then the neck tourniquet was inflated to a pressure of 700 mmHg within 0.5 s using a regulated tank source of air. Ischemia was considered effective if EEG isoelectricity was achieved within 30 s after inflation of the tourniquet. After 6.5 min of ischemia, the neck tourniquet was deflated and MAP was restored with a bolus of phenylephrine (10 μg iv). The EEG was examined for the appearance of spontaneous activity in the postischemic period. After the ischemic period, halothane administration was discontinued. Thereafter, the animals were put into a Plexiglas chamber with supplemental oxygen until fully awake. Then the animals were moved to the institutional Animal Resource Center, where they had free access to food and water.

The rabbits were neurologically assessed daily for 10 days after ischemia by an observer unaware of the treatment group, using the grading scale in Table 1. The neurologic deficit score ranged from 0 (normal) to 100 (severely impaired) [10].

On the seventh postischemic day, the animals were habituated to restraint in a padded, sound-attenuated, ventilated chamber (Model ac-3, Industrial Acoustics, Bronx, NY, USA) for 1 h. A panel 35 cm behind the animal contained a speaker that delivered an 85 dB (SPL), 6 kHz tone for 250 ms as the conditioned stimulus (CS). This was followed by a delay of 500 ms (the trace interval) after the end of the CS before the onset of the unconditioned stimulus (UCS). The UCS consisted of a puff of air, 150 ms in duration, directed at the cornea from a 0.5-mm diameter nozzle positioned 2 mm from the rabbit's eye. An opto-electronic emitter-detector sensor (Optec OPB704, TRW Electronics, Carrollton, TX, USA) was used to detect closure of the eyelid. This sensor was attached to the rabbit's head and positioned 4 mm from the cornea. Any movement of the nictitating membrane or eyelids was detected by a light-emitting diode and phototransistor combination. A conditioned response (CR) was defined as an eyelid movement that occurred during the trace interval (the time between the end of the CS and the onset of the UCS) with an amplitude exceeding 4 standard deviations of baseline fluctuations. The animals received 80 trials each day. The intertrial interval was randomized between 30 and 60 s, with an average of 45 s. The entire training period lasted for 15 days.

Learning curves were generated by calculating the percentage of CRs for each day for each group (%CRs). The mean percentages of CRs for each of the groups were plotted versus the number of training days.

A one-way analysis of variance (ANOVA) with the study group as the factor, followed by a multiple-

Table 1. Rabbit neurologic deficit grading scale

Assessment	Score	Maximum score
Level of consciousness		
NOrmal	0	
Clouded	5	
Stuporous	10	
Comatose	25	25
Respiration		
Normal	0	
Abnormal	5	
Cranial nerves		
Normal	0	
Vision	1	
Light reflex	1	
Oculocephalic	1	
Corneal	1	
Facial sensation	1	
Auditory	1	
Gag reflex	1	7
Motor and sensory function		
Normal	0	
No flexor response to pain, front	2	
No flexor response to pain, rear	2	
No righting reflex	10	14
Gait		
Normal	0	
Minimal ataxia	5	
Moderate ataxia	10	
Able to stand	15	
Unable to stand	20	
No purposeful movement	25	
Behavior		
Normal	0	
No grooming	4	
No eating or drinking	10	
No exploratory movements	10	24
Total		100

comparison test (Fisher's PLSD), was used to determine statistically significant differences in physiologic variables and neurologic deficit scores between the four study groups. A two-way ANOVA followed by a multiple comparison test (Fisher's PLSD) was performed to compare the S, L20, L50, and C groups for days 13, 14, and 15. *P* values less than 0.05 were considered statistically significant. Descriptive statistics are expressed as means ± SD when appropriate.

There were no statistically significant differences between the groups in baseline body weight, PaO_2, $PaCO_2$, pH, or hemoglobin concentration. There were no statistically significant differences between the groups in either the pre- or postischemic MAP. In all animals, the EEG became isoelectric within 30 s and remained isoelectric during inflation of the neck tourniquet. The times required to obtain an isoelectric EEG were 16 ± 6 s in the C group, 19 ± 5 s in the L20 group, and 20 ± 4 s in the L50 group. The times to reappearance of EEG spike-wave activity were 16 ± 6 min in the C group, 14 ± 8 min in the L20 group, and 14 ± 3 min in the L50 group. There were no statistically significant differences between groups in the time required to obtain an isoelectric EEG and the reappearance of EEG spike-wave activity.

The mean neurologic deficit scores on the first postischemic day were 2 ± 4 in the C group, 2 ± 2 in the L20 group, 1 ± 2 in the L50 group, and 0 in the S group. There were no significant differences between groups ($P = 0.51$) (Fig. 1).

Figure 2 shows the mean percentage of CRs over a 15-day period for each group. On the 15th training day, the percent CRs in the S, C, L20, and L50 groups were 44% ± 22%, 20% ± 29%, 32% ± 28%, and 55% ± 26%, respectively. The results in the S and C groups were lower than our previous results (65% ± 19% in the S group and 36% ± 38% in the C group) [6], in which we used a shorter CS and TI (100 and 300 ms, respectively). The cumulative CR counts for 15 days were 316 ± 153 in the S group, 163 ± 122 in the C group, 346 ± 227 in the L50 group, and 239 ± 179 in the L20 group. There were no statistically significant differences in the cumulative CR counts between groups ($P = 0.12$).

A two-way ANOVA to compare the percentage of CRs between the S, L50, and C groups for days 13, 14, and 15 revealed significant effects for group [$F(2, 18) = 3.7$, $P = 0.04$]. The S ($P = 0.03$) and L50 ($P = 0.02$) groups had significantly greater percent CRs than the C group by Fisher's test.

The eyeblink response in rabbits is a neurobehavioral test that has been extensively used to study learning and memory. Acquisition and retention of this response has been shown to be impaired in aged as compared with young rabbits [11]. The hippocampus, cerebellum, ventrolateral thalamic nucleus, and nucleus interpositus have all been implicated as the neuronal substrates serving this response [12–15]. Because the hippocampus and cerebellum are particularly sensitive to ischemic injury [11], it seemed reasonable to use the rabbit eyeblink response as a test of neurobehavioral outcome in our model of transient cerebral ischemia.

In our previous study [6], we showed that 6.5 min of transient global ischemia impaired the acquisition of

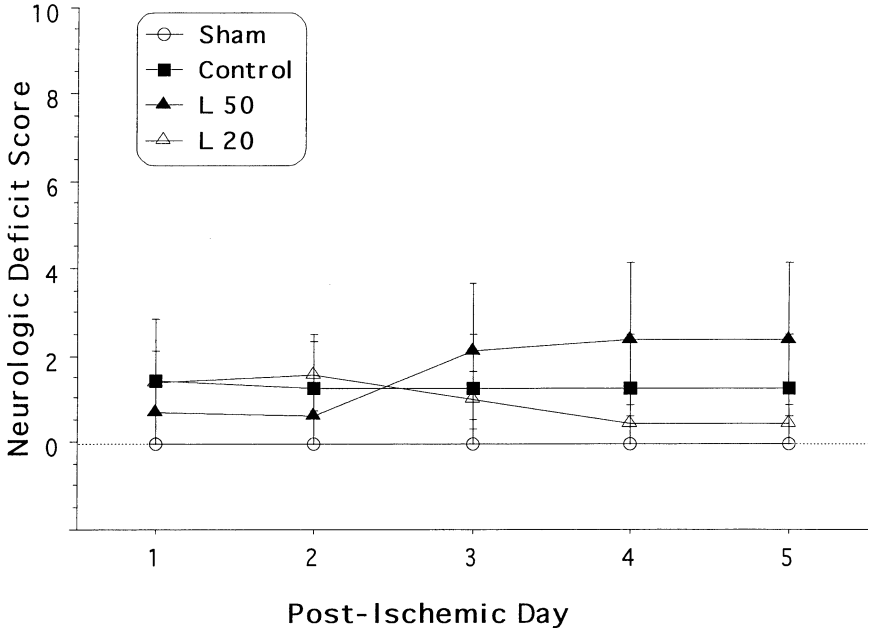

Fig. 1. Neurologic deficit scores (mean ± SEM) for each of four groups on each of the first five post-ischemic days. There were no significant differences between groups. *Control* Normothermic ischemia group; *L 20*, lamotrigine 20 mg·kg^{-1} treated group; *L 50*, lamotrigine 50 mg·kg^{-1} treated group

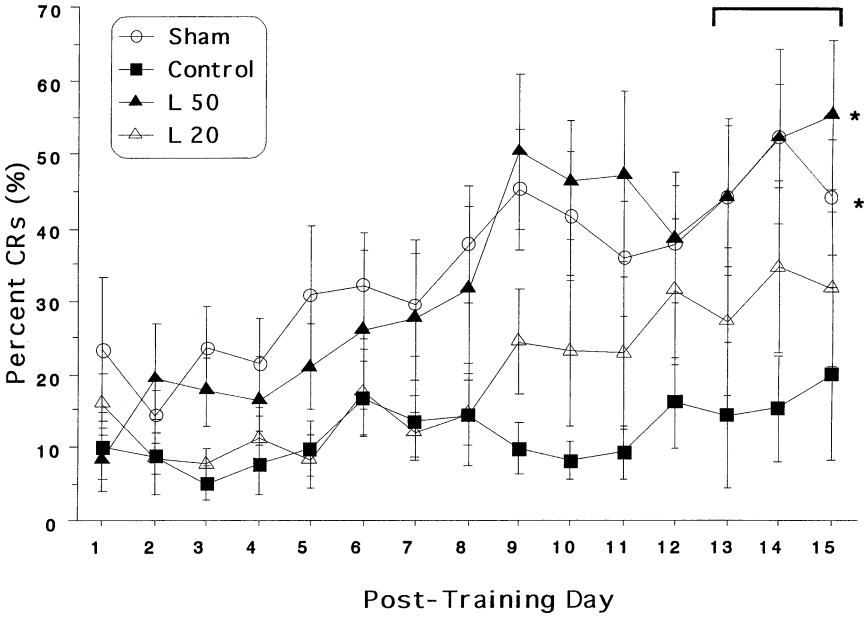

Fig. 2. Percentage of conditioned responses (*CRs*) (mean ± SEM) for each of the four groups over the course of the study. Values for the S and L 50 groups were significantly greater than for the C group. $*P < 0.05$ vs C group by ANOVA followed by Fisher's test. *Control*, Normothermic ischemia group; *L 20*, lamotrigine 20 mg·kg^{-1} treated group; *L 50*, lamotrigine 50 mg·kg^{-1} treated group

eyeblink response with a trace interval of 300 ms. We also showed that both 30°C of hypothermia before and during ischemia and pretreatment with 50 mg·kg^{-1} of lamotrigine were able to attenuate the impairment of eyeblink response produced by cerebral ischemia. Histologic evidence of neural injury in the cerebral cortex, basal ganglia, hippocampus, thalamus, cerebellum, and pons was greatest in the normothermic control group.

In this study, we compared the neuroprotective effect of 20 mg·kg^{-1} and 50 mg·kg^{-1} of lamotrigine using a 500-ms trace interval. Lamotrigine 50 mg·kg^{-1} was able to attenuate the impairment of eyeblink response to the point that there was no difference between the percent CRs in the 50 mg·kg^{-1} group and those in the sham ischemia controls. However, the neuroprotective effect of 20 mg·kg^{-1} of lamotrigine was not obvious in this study.

The trace eyeblink-conditioning response is sensitive to TI effects. Harvey et al. [7] reported that acquisition of CR was absent with a 0-ms TI, increased at the 100-ms interval, reached a maximum at the 200-ms interval, and then declined at longer intervals. Increasing the TI to 500-ms (as was done in the present study) makes the conditioning more difficult, because participation of the hippocampus is thought to be necessary for acquisition of the CR [4]. Because some of the neuronal pathology associated with aging occurs in the hippocampus, some studies have shown that a long TI is more sensitive to age-related differences. In the trace conditioning paradigm with 500-ms TI in rabbits, old animals acquired the CR significantly more slowly than young ones [8]. Compared with young cats, old cats had a marked deficit in eyeblink conditioning with 800- or 1500-ms TIs but were not significantly impaired with a 400-ms TI [15]. In this study, we used a longer TI (500 ms) than in the previous study (300 ms). The outcome with a 500-ms TI was clearly improved, as assessed by the CR neurobehavioral task in animals pretreated with high doses (50 mg·kg^{-1}) of lamotrigine. However, we could not show any advantage of using 500-ms TIs rather than 300-ms TIs. Both 300-ms and 500-ms TIs were appropriate to evaluate the neuronal damage and neuroprotective effect of a drug after cerebral ischemia.

Lamotrigine was found to attenuate the release of excitatory neurotransmitters through its neuronal sodium channel-blocking properties and has been released for use as adjuvant therapy in the treatment of partial seizures [16]. We investigated the effects of lamotrigine on in vivo glutamate accumulation during 10 min of transient global ischemia [17]. Pretreatment with 20 mg·kg^{-1} of lamotrigine partially inhibited glutamate accumulation; however, 50 mg·kg^{-1} of lamotrigine prevented the ischemia-induced increase in glutamate concentration. We have also shown that 50 mg·kg^{-1} of lamotrigine can reduce the histologic abnormality in the hippocampus after 6.5 min of transient global ischemia [6]. Glutamate accumulation in the hippocampus occurring during transient ischemia is a well-known phenomenon [2], and there is substantial evidence that glutamate plays an important role in inducing neuronal cell death after ischemia [11].

In conclusion, we evaluated the effects of lamotrigine on conditioned learning after global cerebral ischemia in rabbits. A brief episode of cerebral ischemia resulted in the impairment of this test of neurobehavioral function. Lamotrigine 50 mg·kg^{-1} was able to attenuate the

impairment of neurobehavioral outcome after cerebral ischemia as measured by acquisition of the conditioned eyeblink response. We used a longer TI (500 ms) than the previous study (300 ms) to evaluate the hippocampally mediated neuroprotective effect of lamotrigine. No advantage of using 500-ms rather than 300-ms TIs was observed in this study.

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