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Natural sleep modifies the rat electroretinogram

(glia/Müller cell/ERG/cortical evoked potentials/animal)

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ABSTRACT **We show here electroretinograms (ERGs) recorded from freely moving rats during sleep and wakefulness. Bilateral ERGs were evoked by flashes delivered through a light-emitting diode implanted under the skin above one eye and recorded through electrodes inside each orbit near the optic nerve. Additional electrodes over each visual cortex monitored the brain waves and collected flash-evoked cortical potentials to compare with the ERGs. Connections to the stimulating and recording instruments through a plug on the head made data collection possible at any time without physically disturbing the animal. The three major findings are (*i*) the ERG amplitude during slow-wave sleep can be 2 or more times that of the waking response; (*ii*) the ERG patterns in slow-wave and REM sleep are different; and (*iii*) the sleep-related ERG changes closely mimic those taking place at the same time in the responses evoked from the visual cortex. We conclude that the mechanisms that alter the visual cortical-evoked responses during sleep operate also and similarly at the retinal level.**

These experiments were undertaken to compare the b-wave of the electroretinogram (ERG), which is a potential generated by retinal glial cells, with the cortical visual-evoked potential, whose origin is still the subject of debate. The first author of this paper, a participant in the debate, has speculated that cortical glial cells (astrocytes) may be involved in the generation of spontaneous and evoked electrocortical events (1–3). We report here results consistent with that position.

The ERG is the potential evoked from the retina when light falls on the eye. It is both the first evoked potential ever recorded (in 1866 by Holmgren) and the earliest in the series of them that electrophysiologists trace through the visual pathway from retina to cortex. The major ERG component, the b-wave, interests glia physiologists because it represents potassium ions moving through the cytoplasm of retinal glial cells called Müller cells (see ref. 4 for a review). The ions enter Müller cells from the extracellular space, where neuronal activity following rod and cone excitation elevates potassium ion concentration, and are transported away through a process called spatial buffering. The resulting intracellular K^+ current, returned extracellularly by other ions, appears outside the eye as the ERG. The experimental evidence supporting this explanation is strong, and we assume that the b-wave phenomenon itself, as well as the changes in it about to be documented in this report, directly reflects the synaptic activities of the retina. However, this brief summary of b-wave electrogenesis omits several qualifications often included (e.g., see refs. 5 and 6) and the hypothesis is not universally accepted (see, e.g., ref. 7).

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We have found no previous reports of sleep-induced changes in the ERG. Armington (8) discusses certain conditioning and training experiments claimed to modify the ERG pattern in humans, and Jacobson and Gestring (9) postulated on the basis of cat and monkey experiments in 1958 that "there exists in the brain a center that controls retinal function, and . . . acts to inhibit retinal activity." The earliest controversy over whether such a brain center exists was summarized by Rivers in 1900 (10). If the center exists, nothing is known about its functions, perhaps because these are not likely to be displayed by unanesthesized mammals, and because it is difficult to deliver quantifiable visual stimuli to an animal free to move its eyes. We believe we have solved both of these problems in rats by implanting a recording electrode behind the eyeball and a small light-emitting diode (LED) beneath the skin above the eye; rats tolerate both of these implants well and have consistently delivered similar b-waves for as long as 3 months.

MATERIALS AND METHODS

The LED Stimulus. The implanted LED is a transparent plastic cylinder 5 mm in diameter and 8 mm long (BI-B6334 SQD; Bright LED Electronics, Budapest). It emits red light measuring 300 mcd at its saturation current (2 mA). Its calibration curve (see Fig. 3*A*) shows its output to be proportional over 3 log units to log duration of a 4-V square pulse delivered to it. The stimuli used in the experiments were of 3 or 5 msec duration.

Implantations. Implants were placed during halothane (1% in air) anesthesia. Electrodes were stainless steel screws (1 × 5 mm) turned into the skull. The ERG electrodes entered the skull about 10 mm rostral to the bregma; they were directed laterally, and at one autopsy the tip was found within the orbit dorsal and anterior to the optic nerve. The visual cortex screws were positioned about 2 mm from the midline and 1 mm rostral to the lambdoid suture. Some animals had additional frontal and auditory cortex electrodes. The reference electrode was placed over the cerebellum ≈2 mm off the midline and behind the lambdoid suture. The LED was inserted under the skin over the left eye and fixed in place with dental cement. Insulated wires from the LED and electrodes were soldered to leads in a socket attached to the skull in the midline with dental cement.

Subjects. Five young adult Wistar rats, two of the WAGRij strain, served as subjects. Rat 1, implanted on Oct. 1, 1993, was killed by anesthetic overdose 112 days later when the electrode plug separated from the skull; data collected from this animal on 17 experimental days included 13 sessions (as defined below) and numerous calibrations and tests. Rat 4

Abbreviations: ERG, electroretinogram; LED, light-emitting diode; EEG, electroencephalogram; SWS, slow-wave sleep; REM, rapid eye movement.

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delivered data during 16 sessions and died on day 45 after testing under anesthesia. Three additional rats yielded data in 49 sessions and were still active and producing data 21, 55, and 61 days after implantation.

Response Acquisition and Analysis. Four physiological channels (two retrobulbar and two cortical) were amplified by Grass model 8B amplifiers (gain, 7 μV/mm; bandpass, 0.01–10,000 Hz), delivered to the A/D converters of the Cambridge Electronic Devices (Cambridge, U.K.; CED 1401) signal analysis system, and averaged using its SIGAVG 6.0 program. Samples 500 msec long containing 50 sweeps were converted at 5000 Hz and averaged beginning 10 msec before the stimulus. Data were plotted out on a Hewlett–Packard plotter and stored as ASCII text files for statistical analysis.

RESULTS

Part I: Cortical and Retinal Potentials Evoked During Sleep and Wakefulness. Fig. 1 assembles waking (on the left) and sleeping records from a rat stimulated by flashes emitted by a LED implanted over the left eye. Its four lines display, from top down, the ERGs recorded from electrodes behind the left and right eyeballs and the evoked potentials (EPs) recorded through electrodes over the left and right visual cortexes. The ERGs and EPs are averaged responses to ≈50 flashes presented manually at intervals no shorter than 10 sec. Note that two tracings are superimposed in every case; the data in the figure were collected in four different 50-flash experimental sessions (two while the rat was awake, and two while the rat was asleep) and the results of all four are consolidated in this single display.

The decision to segregate responses by whether the animal was awake or asleep came about more or less fortuitously; the laboratory conducts sleep experiments on rats for other purposes, and so it is routine to monitor animals continuously for behavioral and brainwave (electroencephalogram; EEG) signs of sleep. The records in Fig. 1 were among the first to be collected after an observer noted that responses appeared to be larger when the animal slept.

Fig. 2 shows response averages segregated by whether the rat was awake, in rapid eye movement (REM) sleep, or in slow-wave sleep (SWS). Shown below are EEG samples that define these states (11) and shown above, in the boxes, are the averages collected by an experimenter who triggered the flash only when the ongoing EEG trace was seen to match one of the samples. For instance, the session that yielded the averages in the REM boxes consisted of 50 flashes triggered while the EEG was displaying the ≈8 per sec EEG waves characteristic of REM sleep. (Records overlapped in the boxes are the left and right side averages.) Because rats move back and forth between sleeping and waking states unpredictably, the sessions varied in length from the shortest possible, 8–9 min, to an hour or longer.

This report is based on the analysis of >80 sessions of the sort just described collected by four different observers. A statistical comparison of the difference between 24 waking, 36 REM, and 96 SWS ERGs (collected in 48 of these sessions) was performed. After extracting the first three principal components, which eliminated the undesirable muscle artifact from the waking record, they were compared by one-way multifactorial ANOVA (CSS STATISTICA 3.1, Statsoft, Hungary). The Wilks' Lambda was 0.4649 ($P > 0.0001$). The difference between SWS and waking waveshapes and the difference between SWS and REM sleep were both significant ($P \geq 0.0001$).

Part II: Calibrations, Comparisons, and Controls. *LED calibration.* Fig. 3*A* plots the amplitude (*Inset*, between dots) of an averaged visual cortical-evoked response as a function of the duration of the square wave applied to the LED. The response threshold was reached when a 4-V, 20-μsec square pulse was applied; this light level was about 3 log units below that produced by the pulse durations used in the experiments.

Retinal and cortical response latencies. Fig. 3*B* shows the onsets of the waking retinal and cortical records of Fig. 1; only one ERG is displayed because the other is almost identical. The retinal a-wave and the earliest cortical deflection have approximately the same latency. What appear to be cortical oscillating potentials (≈100 Hz), in phase at the two electrodes, introduce the cortical responses, and a hint of the retinal oscillating potentials appears in the retinal record. By two-way *t* test the differences between the waking ERG latency shown here and those in sleep are insignificant [for wakeful vs. SWS, $P = 0.38$ ($n = 12$); for wakeful vs. REM sleep, P = not significant ($n = 10$)].

Corneal and retrobulbar ERGs compared. Fig. 3*C* displays ERGs simultaneously recorded from the front and back of the eye. To obtain these, the rat was anesthetized with intramuscular ketamine in 10% xylazine (80 mg/kg) and mounted

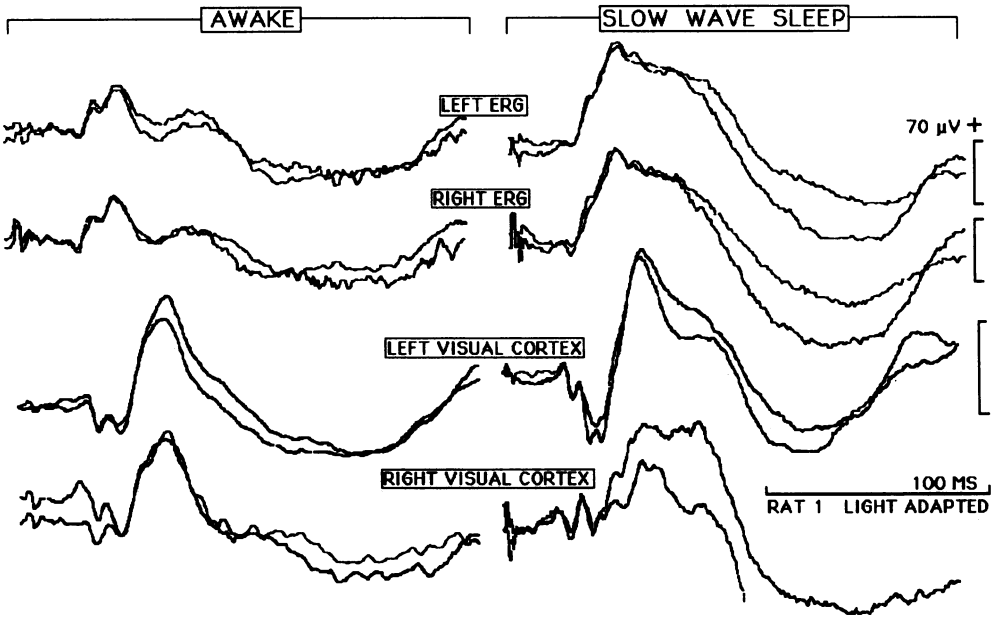

FIG. 1. Retinal and cortical responses evoked by flashes from a LED implanted above the left eye of a rat free to move.

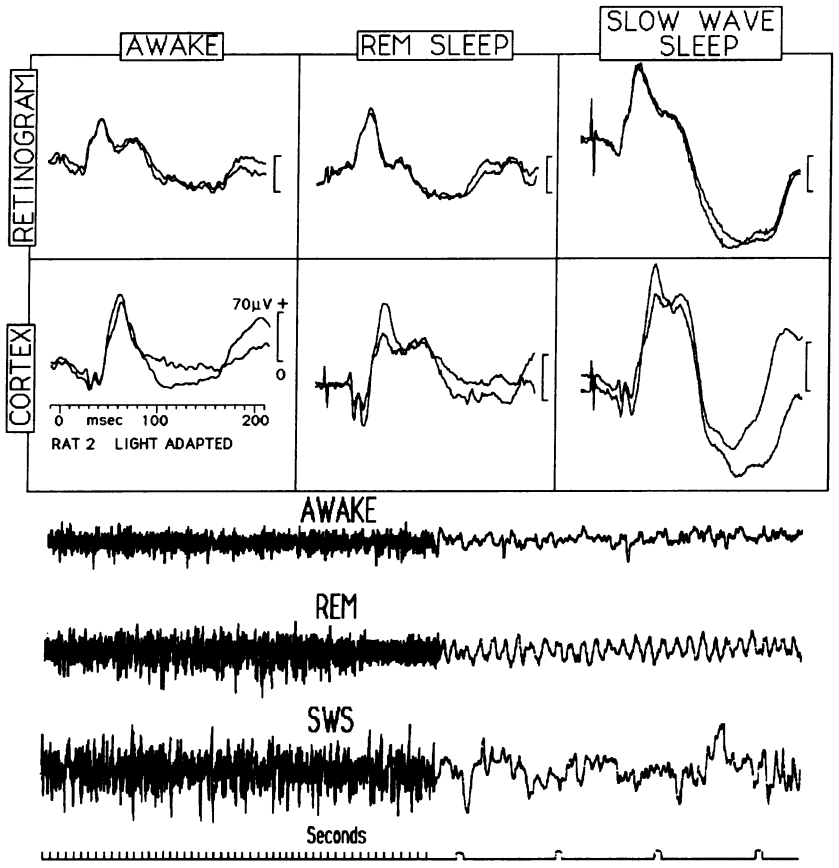

FIG. 2. Similar to Fig. 1, except, in addition, the flashes were triggered according to whether the ongoing brain wave traces matched one of those in the samples shown below. Note the transition from SWS to REM sleep beneath the word REM in the middle record.

in a headholder; the responses to 50 flashes at 1 per 2 sec were then recorded both through a stainless steel wire placed on the right cornea and through the implanted right retrobulbar electrode, the indifferent for both channels being the screw over the cerebellum. The simultaneously recorded ERGs are similar in morphology, the retrobulbar amplitudes are nearly double the corneal, and in both instances the b-wave deflections are positive at the recording electrode.

Light- and dark-adapted responses compared. Fig. 4 compares the light- and dark-adapted ERGs of one rat. During light adaptation, the condition under which Figs. 1 and 2 were obtained, the test cage was a few meters away from two 60-W fluorescent tubes, but of course the ambient level tells little about the retinal level of a rat free to move about its cage or curl up, close its eyes, and fall asleep. In Fig. 4, the ERGs collected in a typical light-adapted SWS session defined in this way appear above those collected immediately thereafter following 2 hr spent in total darkness. The left and right light-adapted ERGs (upper traces) show almost identical a-, b-, and c-waves, as in Figs. 1 and 2. However, the right

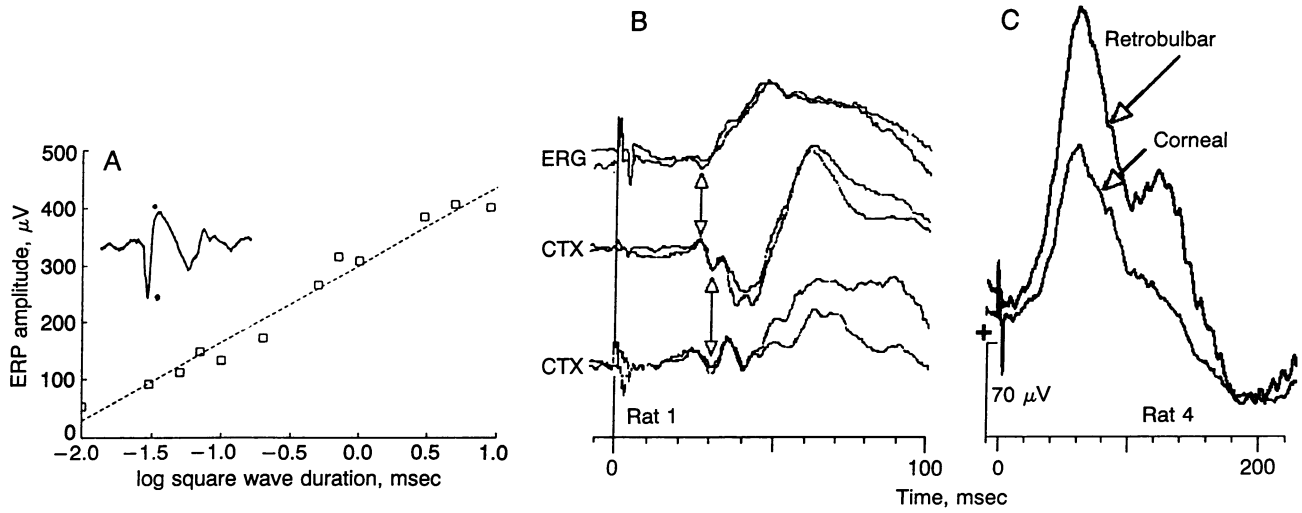

FIG. 3. (*A*) Output of the LED. Amplitude of the evoked cortical response, ERP, is a linear function of log duration of the square wave applied to the diode. (*B*) Onset latencies of the SWS records in Fig. 1. (*C*) Simultaneous recording of the ERG through two electrodes, one on the cornea, the other behind the eyeball; they have the same polarity and similar waveshapes, and the corneal response is smaller in amplitude.

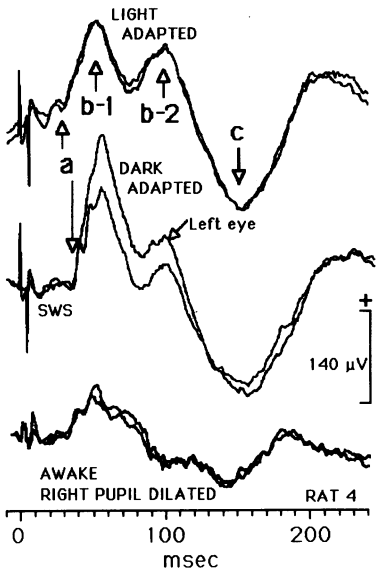

FIG. 4. Light- and dark-adapted ERGs during SWS (top traces) and ERGs (bottom traces) from the same rat, awake, after maximal dilation of the right pupil. ERG amplitudes vary with state (awake/asleep) and light/dark adaptation, but not with pupil size.

dark-adapted ERG shows smaller b-1 and b-2 waves than the left, and its b-1 displays oscillatory potentials; furthermore, the a- and b-1 dark-adapted latencies are longer than the light-adapted ones by ≈8 msec. Statistical statements about these differences await collection of more samples.

The stimulus and the light path to the retina. When the flash is presented in darkness the entire rat's head glows red, which is the color of the LED. However, the light reaching the retina probably approximates the spectrum of oxygenated rat blood, especially when the rat sleeps with eyes closed. We have no estimate of its intensity at the retina, but the closely matched ERGs in most light-adapted records suggests both eyes receive similar amounts. The relative amounts entering via pupil and sclera are also unknown.

To estimate the effects of pupil size, rat 4 was awakened immediately after the dark adaptation session in Fig. 4, a few drops of 1% tropicamide was instilled in the right eye, and the right pupil was seen under magnification to be dilated maximally some minutes later. The bottom trace in Fig. 4 shows left and right ERGs to 50 flashes spaced 2 sec apart delivered to this wide-awake animal. The two ERGs, which are as small as any ever recorded, are also virtually identical, which suggests that pupil size and ERG amplitude are not importantly related. We also infer from this result that pupil size, or changes in it, cannot explain the large response amplitudes and response-amplitude differences seen a few hours earlier in the dark- and light-adaptation sessions.

DISCUSSION

The ERG. We identify, tentatively, three components in the rat LED red-flash ERG: an a-wave; a frequently doubled, highly labile b-1–b-2 complex; and the c-wave, which is thought to arise in retinal pigment cells (12). In what follows the b-wave–c-wave complex is called the b-wave.

Anatomical and Physiological Origins of the ERG. The rodent retina is dominated by rods, and the connections these make within the retina are like those found in other mammals (13, 14). Its two types of cones, which make up <1% of the photoreceptors, are green and blue sensitive (15), which suggests that this retina responds to shortwave and middle-to-longwave light through cones and to red light through rods. Since, as already noted, the flash energy reaching the retina is probably at the wavelengths transmitted by oxygenated rat blood, it is surprising to find a large difference between the light- and dark-adapted SWS ERGs (Fig. 4). Armington (8) discusses the contributions an albino rat's limited photopic system might make to its dark-adapted ERG.

The b-Wave: The Physiological Basis of Its Dynamic Changes. Is the difference between sleeping and waking b-wave complexes mediated by neural or by humoral events? Neural mediation is certainly possible because axons are known to leave the brain, reach the retina, and terminate there. Until recently, the evidence for these efferents in mammals was equivocal (see refs. 16 and 17 for reviews), but the newer methods clearly show axons passing from the midbrain reticular formation (and several other places) to the retina in monkey, rat, and gerbil (18–23). Their retinal destination in the rat is the inner nuclear area at least (20), and in the gerbil it is the outer plexiform layer (24). The fact that the cortex receives axons from these same midbrain reticular regions makes it plausible to suppose the same central mechanism simultaneously modulates the evoked response amplitudes in both retina and cortex.

As for humoral mediation, the retinal response to agents brought by the blood can be irreversible, as in the case of methyl alcohol, or transitory, as in the case of transmitters and metabolite level differences: for instance, the latency and amplitude of human a- and b-waves have been correlated with serum concentrations of glucose and ammonia (25, 26). Some reports appear contradictory: epinephrine injections are said to increase b-wave amplitude in the dog (27) and to decrease it in the cat (9). Whether such blood-borne substances, including pharmacological agents, act at the retinal synaptic level or on the intracellular transport of potassium ions by Müller cells continues to be actively investigated (e.g., see ref. 28).

As for the function of these retinal efferents, the hypothesis that they modulate the human optic nerve input during selective attention has been both claimed (16) and denied (17). In animals, Jacobson and Gestring (9), who, as just noted, injected epinephrine into cats, also cross-circulated two of them via the carotid arteries and reported "The reticular formation of one animal was [electrically] stimulated and, while the ERG of the stimulated cat, the donor, diminished immediately, changes in the ERG of the second cat, the recipient [of arterial blood from the donor], occurred about forty seconds later." This experiment appears to have demonstrated both neural and humoral action upon the retina in the same two-animal preparation, and the authors postulated from this and other results "a center that controls retinal function, and that we can measure its activity by its effect on the ERG."

Summary. Anatomical facts make reticular formation mediation of the ERG changes in sleep possible, but the actual mechanism remains to be described. As for our evidence, we believe it permits three firm conclusions.

(*i*) *The preparation.* To date five implanted rats have survived a total of 294 days and participated in at least a hundred sleep and waking experiments during which visual-evoked events were measured. The implanted visual stimulator can be counted on to illuminate the retina the same way every time regardless of direction of gaze, position of the lids, size of pupil, or behavioral state. The implanted LED generates no heat, illuminates both retinas about equally, and evokes large, stable, and easily measured responses. The waveshapes appearing on the right and left sides of the head are similar, and superimpositions of the results from different experimental sessions show that the results can be replicated. Evidently the stimulus delivered is indeed constant, the recording signal/noise ratio is large, and the phenomena being investigated are robust.

(*ii*) *Retinogram dynamics.* What these experiments teach cannot be learned from retinas isolated in tissue culture or examined in anesthetized animals. The retina of the rat free to move can respond to light from the front and to neural and humoral influences entering from the rear along with the optic and ciliary nerves. Demonstrations that phototransduction is not the only way to activate a retina prompt many interesting questions: for instance, to what extent is the intervention from the rear mediated by humoral as opposed to neural processes, and does the intervention alter retinal synaptic activities or the properties of the Müller cells? It should be possible to settle experimentally whether b-wave amplitudes increase during SWS because a visual stimulus now produces more potassium ions in the synaptic regions, or because the Müller cells transport potassium ions away from the synaptic regions in a different manner, or for some other reason.

(*iii*) *Central control of retinal- and cortical-evoked potentials and their similar waveshapes.* The facts illustrated in Fig. 2, where the retinal and cortical responses change together in REM and in SWS, suggest two generalizations that should be possible to test experimentally: (*a*) the (presumably reticular formation) brain mechanisms responsible for the well-known sleep–waking differences in cortical-evoked responses are also responsible for the sleep–waking differences in the ERG, and (*b*) the flash-evoked cortical patterns mimic the retinal b-wave patterns because both are generated in similar synaptic regions by identical physiological interactions taking place between glial and neuronal membranes across the fluid in the extracellular space.

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