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The effects of immunolesions of nerve growth factor-receptive neurons by 192 IgG-saporin on sleep

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Abstract

Low-affinity nerve growth factor (NGF) receptors are present on the cholinergic neurons of the basal forebrain. We studied the effects of 192 IgG-saporin, a specific immunotoxin for the NGF receptor-positive, cholinergic basal forebrain neurons, on sleep, the power spectrum of the electroencephalogram (EEG), and body temperature. After 3 d baseline recordings, 12 male rats were injected intracerebroventricularly with 4 μg 192 IgG-saporin. EEG, motor activity, and brain temperature were recorded for 23 h on the first, third, fifth, and seventh day after the treatment. 192 IgG-saporin did not affect the total daily amounts but altered the circadian distribution of sleep. On days 1 and 3 after the injection of the immunotoxin, the amount of non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) increased during the dark period, whereas during the light both NREMS and REMS decreased. On day 5, these changes were less pronounced and sleep completely returned to the baseline by day 7. The EEG was suppressed in each frequency band and each vigilance state, and, in contrast to sleep, these changes in EEG persisted for 7 days. Brain temperature was decreased from day 3. These results suggest that NGF receptor-positive, cholinergic basal forebrain neurons are not necessary for the maintenance of total sleep time but contribute to the generation of normal EEG and the maintenance of brain temperature.

Keywords: Sleep; Nerve growth factor; Cholinergic basal forebrain; EEG power spectrum; 192 IgG-saporin; Rat

1. Introduction

Several growth factors, e.g. growth hormone-releasing hormone, growth hormone, fibroblast growth factor, prolactin, insulin, insulin-like growth factor I, and interleukin-1, have the capacity to induce sleep (reviewed [18]). Conversely, inhibition of the production or actions of several of these substances inhibits sleep. These and other growth factors and their receptors are found in brain. Often they are colocalized with specific neurotransmitter systems, the production of some growth factors, e.g. interleukin-1, is influenced by neurotransmitters. In fact, neuronal-use-influenced production of growth factors and their effects on neuronal populations are regarded as a major mechanism of sleep regulation. Further, the functional resculpturing of synaptic populations induced by the local effects of the growth factors is hypothesized to be a primary function of sleep [17].

Nerve growth factor (NGF) is a well-characterized neurotrophic factor. It is essential for the survival of certain peripheral neurons during normal development, and it promotes the differentiation and survival of cholinergic basal forebrain (CBF) neurons under a variety of experimental conditions. Basal forebrain cholinergic mechanisms have been implicated in the regulation of vigilance. In the present experiment, we studied the effects of immunolesioning brain NGF-receptive neurons on spontaneous sleep, electroencephalogram (EEG) power, and brain temperature by using the immunotoxin 192 IgG-saporin. The monoclonal antibody 192 IgG recognizes the p75 or low-affinity

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NGF receptor and, following intracerebroventricular (icv) injection, is specifically internalized in CBF neurons [27,28]. When coupled to the plant toxin saporin and delivered to the cerebral ventricle of the rat, the immunotoxin 192 IgG-saporin specifically abolishes cholinergic parameters in the basal forebrain [4,35] by killing the CBF neurons without affecting other neuronal types [5,10]. We now report that immunolesioning central NGF-receptive neurons by 192 IgG-saporin does not affect the total time spent in sleep but causes a transient impairment of the circadian distribution of sleep, a long-lasting decrease of brain temperature, and vigilance state-dependent suppression of EEG power across all frequency bands.

2. Materials and methods

2.1. Materials

192 IgG-saporin was prepared as previously described [4]. For injection, 192 IgG-saporin was dissolved in isotonic NaCl; 4 μg 192 IgG-saporin was administered into a lateral cerebral ventricle in a volume of 5 μl.

2.2. Animals

Male Sprague-Dawley rats (250–350 g) were implanted with cortical EEG electrodes, a brain thermistor, and an icv guide cannula using combined ketamine (87 mg/kg) and xylazine (13 mg/kg) anesthesia. The EEG electrodes were placed over the frontal, parietal, and occipital cortices; the thermistor was placed upon the dura over the parietal cortex. The cannula (22 GA) was implanted into a lateral cerebral ventricle. The placement of the cannula was verified before the experiments by the angiotensin-induced drinking test and after the experiments by histological examination as previously described [16]. After a 1-wk recovery period, the animals were placed into sound-attenuated individual sleep recording cages for adaptation to the experimental conditions. During this 5–7-d habituation period, the animals were connected to recording cables. The animals were kept on a light-dark cycle of 12:12 h (light onset at 0600 h) and at 22 ± 1°C ambient temperature for at least 2 wk before surgeries and during the recovery, habituation, and experimental periods. Water and food were available ad libitum throughout the experiment.

2.3. Recordings

Motor activity was detected by using an ultrasonic sensor as previously described [16]. The EEG, brain temperature (T_{br}) and motor activity were recorded using a computer. The EEG was filtered below 0.1 and above 40 Hz. The amplified signals were digitized at the frequency of 128 Hz for EEG, and at 2 Hz for motor activity and T_{br}. Single T_{br} samples were saved on the hard disc in 10-s intervals; for statistical analysis, T_{br} values sampled in 1-h intervals were used. On-line Fourier analysis of the EEG was also performed every 10 s by averaging five 2-s epochs of the EEG in 0.5 Hz bands in the 0.5–30 Hz frequency range. The vigilance states were determined off-line in 10-s epochs. EEG, T_{br}, and the motor activity were displayed on the computer monitor in 10-s epochs and also simultaneously in a more condensed form, in 12-min epochs. Wakefulness (W), non-rapid-eye-movement sleep (NREMS) and rapid-eye-movement sleep (REMS) were distinguished as described before in detail [16]. Briefly, W was characterized by low-amplitude, desynchronized EEG activity, frequent gross body movements, and gradually increasing T_{br} when W was entered from NREMS; NREMS was characterized by synchronized EEG activity, high-amplitude slow waves, lack of body movement, and gradually decreasing T_{br}; REMS was indicated by desynchronized EEG activity, low-amplitude, fast EEG waves with characteristic regular theta rhythm, lack of overt body movement interrupted by occasional twitches, and steeply increasing T_{br} upon entering the state. Time spent in each vigilance state was calculated in 1-h intervals. The EEG power density values were summed in four frequency bands (delta (0.5–4 Hz), theta (4.5–8 Hz), alpha (8.5–12 Hz), and beta (12.5–30 Hz)) for each 10-s epoch. These spectral data were paired with the vigilance states, and EEG power was computed in each of the four bands separately for each vigilance state. Hourly average delta, theta, alpha, and beta activities were then calculated for W, NREMS, and REMS. The animals were also monitored by a closed-circuit video system.

2.4. Experimental protocol

After the habituation period, sleep was recorded on three consecutive days for 23 h each; the third day was regarded as baseline day. On the fourth day, the rats ($n = 12$) were injected with 4 μg 192 IgG-saporin. The injections were done between 1300 and 1500 h. Recordings started at 1800 h (dark onset) on the injection day (day 1). Twenty-three hour recordings were repeated on days 3, 5, and 7 after 192 IgG-saporin injection. Due to the loss of several EEG electrodes and malfunction of brain thermistors during the course of the experiment, the number of animals decreased across the 7-d experimental period. For sleep on day 5, $n = 9$; on day 7, $n = 4$. For T_{br} on day 1, $n = 9$; on day 3, $n = 8$; on day 5, $n = 5$; and on day 7, $n = 3$.

2.5. Histology

Animals were sacrificed 14–16 d after 192 IgG saporin injections; of the 12 animals injected, 7 were examined histologically. The animals were evaluated by immunohistochemistry for loss of cholinergic neurons. Deeply anesthetized rats (ketamine/xylazine, 87 mg/kg and 13

mg/kg, respectively) were transcardially perfused with 100 ml of isotonic NaCl followed by 250 ml of 4% buffered paraformaldehyde. The brains were removed and equilibrated with 30% buffered sucrose, and 40-μm coronal sections of brain were obtained using a vibratome. Sections were processed for choline acetyltransferase (ChAT) immunohistochemistry using a monoclonal antibody against ChAT (Boehringer-Mannheim) and subsequent localization using anti-rat immunoglobulin and an avidin-biotin-horseradish peroxidase kit (Vector Laboratories). Sections were developed using diaminobenzidine and hydrogen peroxide, mounted, dried, and coverslipped. The effect of 192 IgG-saporin on the complement of ChAT-positive neurons in the basal forebrain was assessed by one of the authors without knowledge of the sleep data. Comparable sections from unmanipulated animals were available for reference.

2.6. Statistical analysis

Sleep and T_{br} values on the baseline day and postinjection days were compared by ANOVA for repeated measures followed by the paired t-test. ANOVA was performed across 23 h on sleep and T_{br} values averaged in 1-h intervals. For sleep, ANOVA was also performed on hourly averages separately across the dark and light periods. In addition to the hourly averages, T_{br}, EEG power, and the amount of sleep were also calculated for the entire dark and light periods; average nighttime and daytime sleep were compared between the baseline day and postinjection days by paired t-test.

3. Results

3.1. Sleep

After the icv injection of 192 IgG-saporin, the animals did not show any gross behavioral abnormalities recognizable through the video system. Varying degrees of motor deficiency were noticed, however, when the rats were handled; the animals kept their heads tilted to one side. 192 IgG-saporin did not affect the total amount of daily sleep, but it significantly altered the circadian distribution of NREMS and REMS. These changes were the most pronounced on the first postinjection day, and the circadian distribution of sleep gradually returned to normal by day 7 (Figs. 1 and 2). On the first night after the injection, when rats normally are active, the amount of NREMS increased above the baseline level by $\sim 37\%$ ($P < 0.05$, paired t-test). In contrast, during the following light period, when rats normally sleep the most, NREMS was suppressed by $\sim 24\%$ ($P < 0.05$, paired t-test). REMS was also suppressed below baseline during the light period of the first postinjection day. On the third and fifth postinjection days, similar sleep patterns were observed. For example, during the night on day 3, both NREMS and REMS increased above baseline by 39 and 48% ($P < 0.05$ for both, paired t-test), whereas during the day NREMS and REMS were suppressed by 10 and 25%, respectively ($P < 0.05$ for both, paired t-test). On day 5, the total amount of NREMS was 22% above baseline during the dark period and 17% below control during the light period ($P < 0.05$ for both, paired t-test). On postinjection day 5, REMS was still

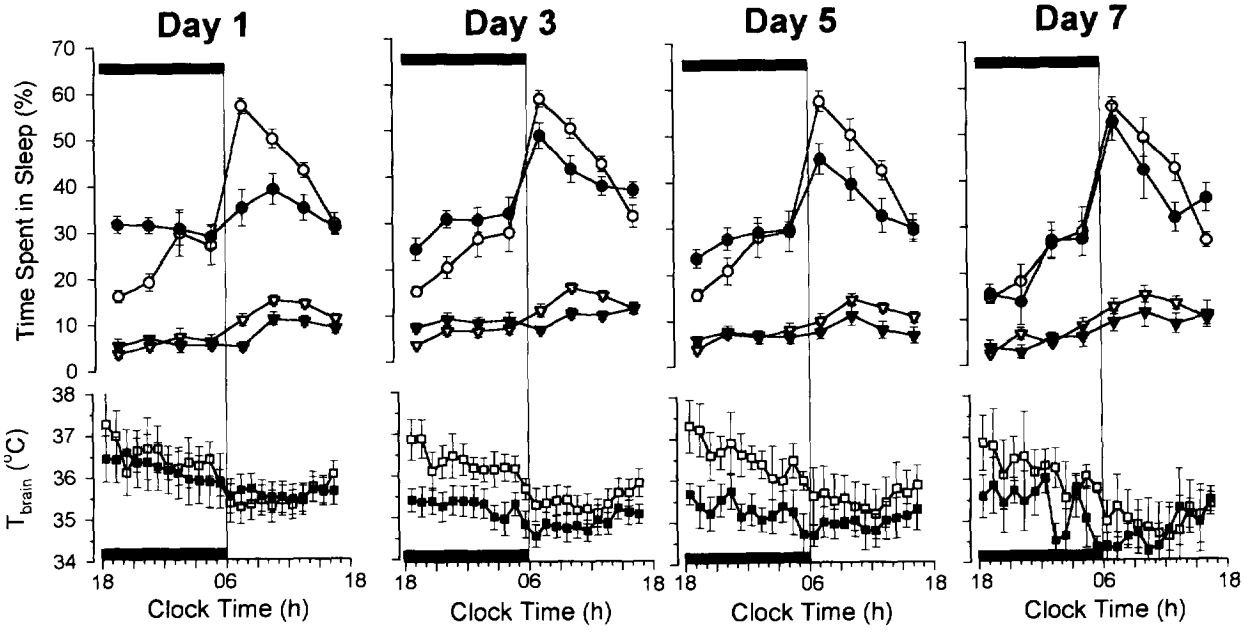

Fig. 1. The effects of 192 IgG-saporin on non-rapid-eye-movement sleep (NREMS) (circles), rapid-eye-movement sleep (REMS) (triangles), and brain temperature (T_{br}; squares), 1, 3, 5, and 7 d after treatment. Open symbols: preinjection baseline values; solid symbols: post-treatment days. 192 IgG-saporin had been injected 3–5 h before the recordings started at 1800 h on day 1.

elevated by 8% during the dark and decreased by 27% during the light period, although these changes were not significant (N.S. for both, paired t-test). By day 7 NREMS had returned to baseline, although REMS was below baseline level by 13% during the dark (N.S., paired t-test) and by 26% during the light period ($P < 0.05$, paired t-test).

3.2. EEG power spectrum

The power of the EEG was suppressed in each frequency band after 192 IgG-saporin treatment. Significant changes were apparent as early as day 1, and they lasted up to the end of the 7-d recording period (Fig. 2). During NREMS, the suppression of EEG activity was most pronounced during the dark phase on postinjection days 1–3 in the slow (delta-theta) frequency ranges. From day 5, the EEG power was equally suppressed in the four frequency ranges day and night. During REMS, the EEG power suppression was the strongest in the light period and in the faster (alpha and beta) frequency ranges. On days 1 and 3, theta power was also decreased during the dark periods. During W on days 1 and 3, slow frequencies were suppressed throughout the entire experimental day, whereas alpha and beta activities were suppressed predominantly during the light period. On day 5, the suppression became more generalized across the different bands and the entire day. On day 7, EEG power was still significantly suppressed in each vigilance state.

3.3. Brain temperature

192 IgG-saporin did not cause immediate changes in T_{br} on the first postinjection day. From day 3, however, profound changes in T_{br} occurred that lasted throughout the rest of the experiment (Fig. 1). T_{br} was suppressed below the baseline level during both the dark and the light periods. During the dark phase, when T_{br} is normally higher, the decrease in T_{br} was more pronounced than during light; this resulted in a strongly attenuated amplitude of the ultradian T_{br} fluctuation. On day 3, average T_{br} during the dark period was about 1°C below baseline, whereas during the light the difference between baseline and experimental days was 0.5°C ($P < 0.05$ for both). On day 5, the differences between the baseline and experimen-

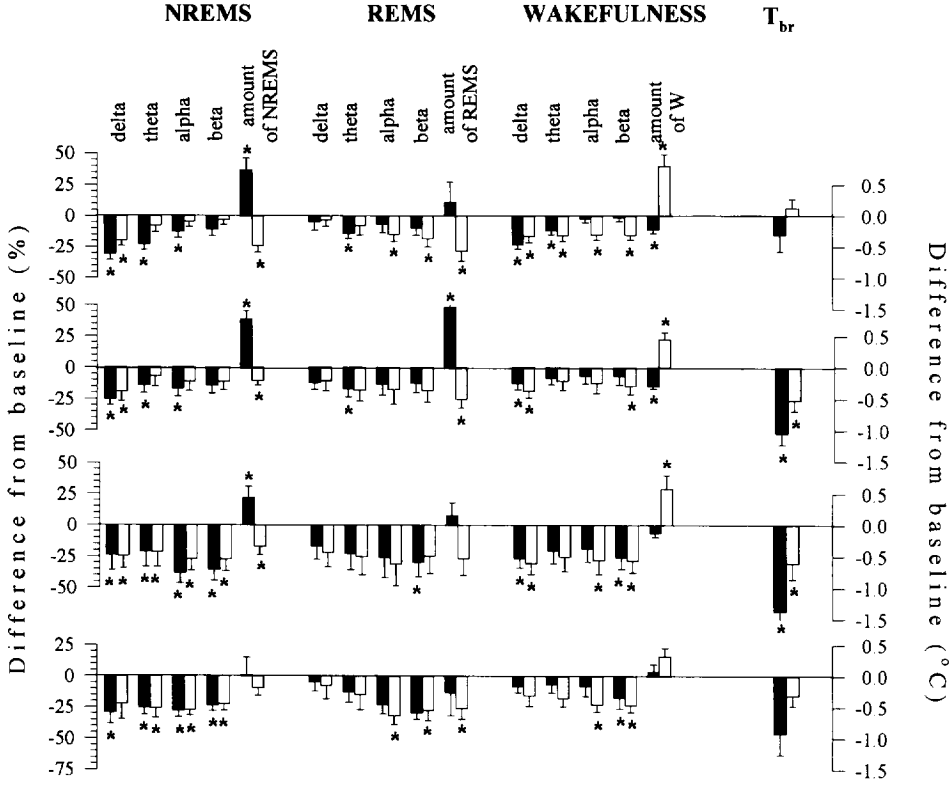

Fig. 2. The effects of 192 IgG-saporin on (a) EEG delta, theta, alpha, and beta power during NREMS, REMS, and wakefulness (W); (b) time spent in NREMS, REMS, and W; and c) T_{br}. EEG power, sleep time, and T_{br} values are averaged for 12-h dark and 11-h light periods on the baseline day and the postinjection days. The effects of 192 IgG-saporin on EEG power and vigilance states are expressed as percent change from the baseline levels (baseline: 100%) during the dark (solid columns) and light periods (open columns), 1 (upper panel), 3 (second panel from the top), 5 (second panel from the bottom), and 7 d (bottom panel) after treatment. The effects on T_{br} are expressed as absolute differences between baseline and postinjection days. Asterisks indicate significant difference between baseline and postinjection conditions (paired t-test). On day 7, statistical analysis was not performed for the T_{br} data ($n = 3$).

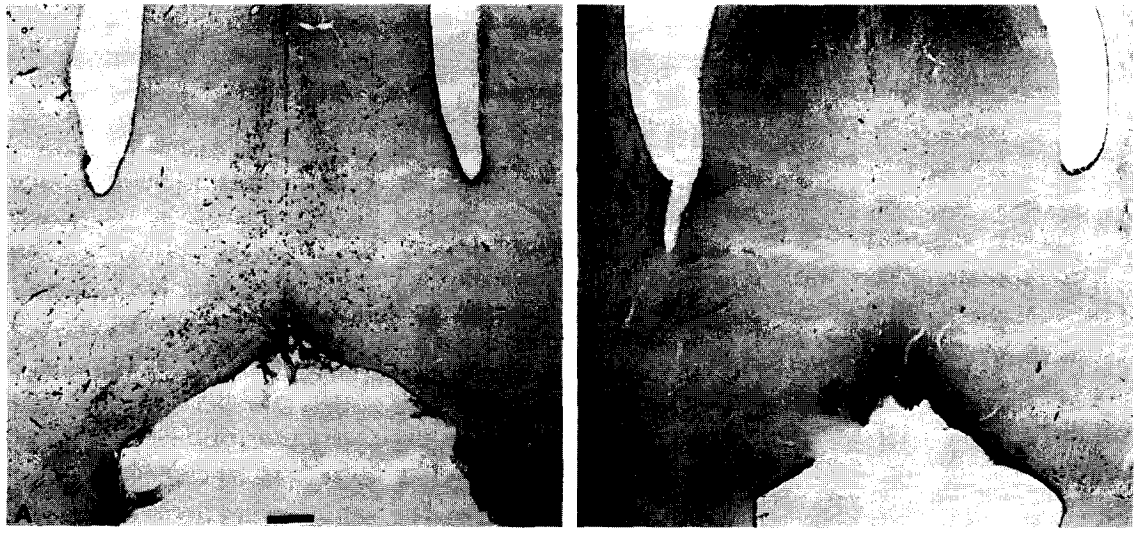

Fig. 3. Brightfield photomicrographs of coronal sections of rat forebrain treated with anti-Choline acetyltransferase (ChAT) immunohistochemistry. A: control rat demonstrating the typical distribution of ChAT (+) somata in medial septal and diagonal band nuclei. Magnification bar = 300 μm. B: comparable section taken at the same magnification from an experimental rat treated with IgG 192-saporin. ChAT (+) somata are almost completely absent.

tal day were −1.4 and −0.6°C ($P < 0.05$ for both) and on day 7, −0.9 and −0.3°C during the dark and light periods, respectively.

3.4. Histology

Immunohistochemistry verified the loss of cholinergic neurons. Of the seven animals, four had a complete lack of ChAT-positive neurons in the basal forebrain (Fig. 3); one animal had a greatly diminished number of ChAT-positive cells (but still a few) in all of the areas of the CBF; two animals showed a moderately decreased number of ChAT-positive neurons.

4. Discussion

After icv injection of 192 IgG-saporin, the number of cholinergic cells is greatly reduced in the medial septal nucleus, diagonal band of Broca, and nucleus basalis Meynert [4,10,35]. Acetylcholinesterase staining decreases in the corresponding projection areas in the cortex, olfactory bulb, and hippocampus. The loss of NGF receptor-positivity is complete as early as day 2 after toxin injection [10], but the disappearance of ChAT immunoreactivity from the CBF or regions innervated by the CBF is not complete before days 5–7 postinjection [10,33]. The loss of CBF neurons was verified after sleep recording in our experiments. In addition to the actual neuronal degeneration, it is likely that the functional impairment of NGF-responsive cells precedes the actual cell loss. In fact, 192 IgG itself may also influence cellular activity without causing degeneration. For example, 192 IgG acts as an antagonist of NGF in neurite regeneration [7]. The alterations in sleep and EEG power in our experiments occurred already on the first day after the injection. Since icv injection of nonspecific IgG does not cause hypothermia, EEG changes, or alterations in the diurnal rhythm of sleep-wake activity [25], the effects of 192 IgG-saporin are likely to result from the functional impairment of NGF-sensitive cells.

The major findings of our experiments, however, are not the changes that occurred after 192 IgG-saporin injection but that 192 IgG-saporin failed to induce symptoms characteristic of basal forebrain lesions or administration of the muscarinic cholinergic antagonist atropine. Lesions in the basal forebrain that are not specific to cholinergic neurons cause severe insomnia [12,22–24]. Electrical stimulations in the area that includes the olfactory tubercle, preoptic region, and the diagonal band of Broca enhance sleep [3,30]. In contrast, lesioning the CBF neurons by 192 IgG-saporin was followed by only slight changes in sleep patterns, suggesting that CBF neurons are not involved in the mediation of the sleep effects of basal forebrain lesions. Our findings also support the idea that the basal forebrain neurons involved in generation of NREMS are not CBF cells but local interneurons [8] (see also [32] for a review). Injection of 192 IgG-saporin transiently altered the diurnal distribution of sleep. The suprachiasmatic nucleus (SCN) is important in the regulation of the circadian rhythm of sleep-wake activity. Many SCN neurons express NGF receptors [14,29]. Since the effects of 192 IgG-saporin on the survival of SCN neurons were not studied in the present experiments, it cannot be ruled out that the toxin also destroyed SCN cells. Furthermore, CBF neurons project to the SCN [11]. Administration of 192 IgG-saporin, therefore, may also indirectly alter the activity of the SCN. Basal forebrain lesions that spare the SCN may indeed

alter the diurnal rhythm of sleep-wake activity in the rat; e.g. radiofrequency coagulation of the medial preoptic area causes a decrease in NREMS during the day and a tendency toward increased NREMS at night [12]. The diurnal rhythm of sleep returned to normal on day 7 after the injection of 192 IgG-saporin in spite of the degeneration of CBF neurons, indicating that compensatory mechanisms corrected the deficit.

In a previous experiment, sleep-wake activity was recorded in rats for 3 h daily during the light period 8–17 d after the icv injection of 192 IgG-saporin [2]. Temporary suppressions in NREMS (days 10 and 12) and REMS (day 8) were observed. Thus, although the changes in sleep lasted longer in that experiment [2] than in our studies, the observations were similar to ours in that sleep decreased during the day, and this was a transient alteration.

Injection of a large dose of atropine is followed by an EEG-behavioral dissociation: the EEG is characterized by slow-wave activity while the animals are behaviorally active [21,26]. We did not observe similar phenomenon after the injection of 192 IgG-saporin, indicating that it is not the loss of the CBF input to the cortex that increases slow-wave activity after atropine treatment. In fact, EEG power decreased in each frequency range and in each vigilance state after 192 IgG-saporin. In the rat, atropine-sensitive and atropine-resistant low-voltage fast-wave activities are distinguished in the EEG [15]. The atropine-sensitive fast activity is characteristic of alert rats during behavioral immobility. Lesions in the basal forebrain by kainic acid are followed by a reduced acetylcholinesterase staining in the cortex, and slow waves appear in the EEG during behavioral immobility [31]. In our experiments, slow-wave EEG without body movements was regarded as NREMS; therefore, if quiet wakefulness was accompanied by EEG slow-wave activity after the injection of 192 IgG-saporin, then this was not recognized. This phenomenon, however, appears only for seconds in the rat [31], and it would not significantly influence the results of the evaluation of sleep-wake activity. Furthermore, 192 IgG-saporin does not change the EEG characteristics of behavioral immobility in rats and decreases the time they spend in the quiet wakeful state [2].

Changes in the EEG were not observed after microinjection of 192 IgG-saporin into the nucleus basalis, which resulted in 50% cell loss in the nucleus, leaving CBF neurons intact in other basal forebrain areas [34]. Intraseptal [19] or icv [2] administration of 192 IgG-saporin or colchicine [9] suppresses the hippocampal theta activity; the frequency of the theta activity is not affected, but the amplitude of the waves is decreased. A general decrease in EEG power in each frequency range was observed in the present experiments. This indicates that the suppression of theta activity in the hippocampus is part of a more generalized EEG suppression due to depressed neural activity at the various terminal fields of CBF projections: the reduced input to the hippocampus results in decreased theta activity, whereas the diminished cortical afferent activity causes a decreased EEG power in the other frequency bands. The changes in EEG after 192 IgG-saporin correspond to those observed after electrolytic coagulations in the substantia innominata [20]. After unilateral lesions in the substantia innominata, acetylcholine release from the ipsilateral cortex is greatly diminished and EEG activity is depressed in all frequency bands. In the present experiments, the EEG power did not recover by day 7 after injection of 192 IgG-saporin. Similarly, EEG theta activity is still suppressed 8 wk after 192 IgG-saporin injection [19], and the EEG activity is depressed 20 d after substantia innominata lesion [20]. These findings indicate that, unlike the disruption of the circadian organization of sleep, the suppressed EEG activity in CBF-lesioned rats is not rapidly compensated.

Injection of 192 IgG-saporin causes a gradually developing hypothermia. Large lesions in the hypothalamus impair defense against cold (reviewed [6]). In the present experiments, the ambient temperature was 21°C, i.e. below the thermoneutral zone. Thermoregulatory deficiency may, therefore, result in decreased body and brain temperature. It is unlikely, however, that a loss in cholinergic innervation of the preoptic area contributes to the hypothermic effects of 192 IgG-saporin since microinjection of atropine into that area results in increased body temperature [13].

In conclusion, the results suggests that NGF-receptive structures, CBF neurons, contribute to the regulation of the diurnal organization of sleep-wake activity, the maintenance of normal body temperature, and EEG activity. The role of NGF-receptive structures in the maintenance of normal EEG activity appears to be independent from their role in sleep regulation, and it might be related to the role that CBF neurons are postulated to play in cognitive functions [1].

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