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Neurotrophic $ACTH_{4–9}$ analogue therapy normalizes electroencephalographic alterations in chronic experimental allergic encephalomyelitis

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Abstract

Chronic experimental allergic encephalomyelitis (CEAE) is an established experimental model for multiple sclerosis (MS). The demyelinating lesions in the white matter of the central nervous system observed in CEAE and in MS are accompanied by various neurophysiological alterations. Among the best defined electrophysiological abnormalities are the changes in event-related potentials, in particular evoked potentials involving the spinal cord, i.e. motor and sensory evoked potentials. Less familiar are the changes observed in the electroencephalogram of CEAE-affected animals, which are also encountered in the human equivalent, MS. In the present experiment we evaluated the therapeutic value of a neurotrophic peptide treatment [H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH, an $ACTH_{4–9}$ analogue] and its effect on the delayed flash visual evoked potentials (VEP) and power spectra of the electroencephalogram, during a 17-week follow-up of CEAE. CEAE animals treated with the neurotrophic peptide were protected against the development of neurological symptoms during the course of the demyelinating syndrome. VEPs of animals suffering from CEAE showed a delay of the latencies of the late components which was significantly counteracted by peptide treatment. The peak-to-peak amplitude of the VEP afterdischarge recorded from CEAE animals was significantly increased during the course of CEAE and correlated closely with the progression of the myelinopathy. Furthermore, CEAE animals showed an increase of electroencephalogram (EEG) beta activity of up to 500% as compared with the age-matched control group. This increase in beta power mainly consisted of a prevailing 20–21 Hz peak, a frequency that normally is not dominant in control EEG recordings of the rat during passive wakefulness. All these electrophysiological phenomena were absent in $ACTH_{4–9}$ analogue-treated animals. The present findings underscore the potential importance of a neurotrophic peptide treatment in the pharmacotherapy of central demyelinating syndromes, and possibly of MS.

Introduction

Multiple sclerosis (MS) is the most common of the central demyelinating syndromes and is characterized by recurrent inflammation and demyelinating, gliotic lesions scattered throughout a number of regions in the CNS (Lassman, 1983a; reviewed in McAlpine, 1991). This disease imposes a threat of progressive severe disability in, mostly, young adults. The aetiology of the syndrome is still elusive despite extensive research (Bernard & De Rosbo, 1992; Allen & Brankin, 1993; reviewed in McAlpine, 1991). However, susceptibility to the disease is determined by genetic and environmental factors (Tienari *et al*., 1992; Beall *et al*., 1993; Hillert, 1993; Oksenberg *et al*., 1993; Utz *et al*., 1993). Current treatment strategies focus on the modulation or suppression of the immune system (Oksenberg & Steinman, 1991). Their efficacy in MS is modest and at best these immune-based therapies can retard the progress of the disease or alleviate symptoms that occur shortly after the onset of the disease. However, while temporary effects on the progression of MS may be achieved by immune suppression, ultimate restoration of function will depend on the remyelinization of the demyelinated nerves. Treatment with neurotrophic peptides could support the compromised neuron by impeding demyelinization or enhancing myelin recovery.

Here the ameliorative effects of a neurotrophic pharmacotherapy were evaluated in a well-established animal model for MS, chronic

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experimental allergic encephalomyelitis (CEAE) (Paterson, 1980, 1983). CEAE is a cell-mediated autoimmune syndrome directed against myelin proteins of the central nervous system (CNS) (Zamvil *et al*., 1985, 1986; Sobel *et al*., 1990; Tuohy *et al*., 1992), which can be induced in genetically susceptible animals by sensitization with central nervous tissue emulsified in complete Freund's adjuvant (Freund *et al*., 1950). The neurotrophic peptide used in the present experiment was a degradation-resistant $ACTH_{4–9}$ analogue (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH) devoid of corticotrophic and melanotrophic activity (reviewed in De Wied & Jolles, 1982). The present experiment outlines the (long-term) CEAE-related changes of the electroencephalogram (EEG) and visual evoked potentials (VEPs). VEPs are claimed to be one of the most sensitive indices for detecting functional impairment of the CNS (McDonald & Halliday, 1977; Naweron, 1978; Kjaer, 1980; Chiappa, 1980; Purves *et al*., 1981; Rossini *et al*., 1989; Van Dijk *et al*., 1992) and allow an objective quantification of the severity of CEAE. As it is important to assess whether changes in VEPs are related to the ongoing EEG, the latter was also analysed and quantified by spectral analyses.

Using neurological examination and VEPs and EEGs recorded from permanent skull electrodes in awake animals, we demonstrate that a neurotrophic pharmacotherapy reduced the neurological symptoms induced by CEAE and reverses electrophysiological CEAE-related phenomena. In view of these beneficial effects in an animal model of MS, neurotrophic peptides could be a significant asset to the conventional immune-based therapies or the transplantation of progenitor myelin-forming cells (Groves *et al*., 1993).

Materials and methods

Animals

Forty-five male Lewis rats (Keith, 1978; Happ & Heber-Katz, 1988) of about 300 g body weight were used as subjects (Department for Laboratory Animals, University of Limburg, Maastricht, the Netherlands). The animals were housed individually under stress-free conditions in Plexiglas observation boxes (25 × 30 × 35 cm), which also served as recording boxes, and were maintained on a 12–12 light/dark cycle with white lights on from 21.00 to 9.00 h. The animals received commercial rat chow and water ad libitum. When necessary, CEAE-weakened animals were fed soaked food in order to support them during the initial stage of the disease and during exacerbations.

Operation procedures

Under Hypnorm® (containing fluanisone 10 mg/mL and fentanylcitrate 0.315 mg/mL, dose 0.8 mg/kg, s.c.; Janssen Pharmaceutica, Tilburg, the Netherlands) and diazepam® (0.3 mL/kg, i.m.; Hoffman-La Roche, Mijdrecht, the Netherlands) anaesthesia, tripolar cortical electrodes (MS333/2-A, Plastics One, Roanoke, VA, USA; stainless steel, diameter 0.25 mm, insulated except for the tips) were implanted permanently into the skull of 30 Lewis rats: one over the frontal region [reference point, (bregma 0–0); coordinates: A 2.0, L 2.0] and one over the visual cortex (A – 7.0, L 3.0). The ground electrode was placed over the cerebellum. A bipolar, silicone rubber-covered electrode (MS303/71, Plastics One; stainless steel, diameter 0.125 mm) was placed subcutaneously on the dorsal neck muscles to record the electromyogram (EMG). All electrodes were secured to the skull by four gilded screws and dental acrylic cement (Polyfast Selfcuring, London, UK). The skin was sutured over the dental cement. Subsequently, the animals were allowed to recover for 3 weeks.

TABLE 1. Clinical rating of CEAE-related neurological signs and symptoms

Rating	Description
0	No visible neurological symptoms
1	Loss of tip tail reflex, half limp tail
2	Completely flaccid tail
3	Moderate paresis, minor locomotion disturbances
4	Severe paresis accompanied by lordosis, severe disturbed locomotion and severe weight loss
5	One paralytic hind limb
6	Complete paralysis (both hind limbs)
7	Paralysis from diaphragm downwards
8	Tetraplegia, only head movements possible
9	Moribund state, death by CEAE

Induction of chronic experimental allergic encephalomyelitis

CEAE was induced by injections of an emulsion containing equal parts of freshly prepared guinea-pig spinal cord homogenate in 0.1 M phosphate-buffered saline (PBS) and complete Freund's adjuvant (CFA) as described by Lassmann (1983b) and Wisniewski & Keith (1977). The spinal cords were homogenized in an equal volume of PBS to a 50% g/v homogenate. CFA was prepared by adding 10 mg of *Mycobacterium tuberculosis* (H37RA, Difco) per mL of incomplete Freund's adjuvant (Difco Laboratories, USA).

Lewis rats, anaesthetized with Hypnorm® (Janssen Pharmaceutica, 0.4 mL/kg body weight), were challenged with 0.3 mL of the CNS emulsion injected subcutaneously in the dorsum pedis of the hind limbs. Age-matched control animals ($n = 9$) received an equal volume of vehicle containing CFA in PBS. The animals were inoculated on two consecutive days as VEP and EEG recording also required 2 days per week. Animals that had been sensitized on the first day, were subsequently measured on the first day of each recording week, whereas the remainder of the animals were measured 1 day later. The day of inoculation is designated as day 0 post inoculation (p.i.).

Peptide administration

The peptide used in the present study was a degradation-resistant $ACTH_{4–9}$ derivative (Org 2766; H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH; Organon International, Oss, The Netherlands), which lacks corticotrophic and melanotrophic action (reviewed in De Wied & Jolles, 1982). The neurotrophic properties of the peptide have been demonstrated in a number of peripheral neuropathies in rats with optimal efficacy when given at a dose of 7.5–75 μg/kg per 48 h (Gispen, 1990; Van der Zee *et al*., 1991).

Animals were treated with either 75 μg/kg body weight $ACTH_{4–9}$ analogue every 48 h in 0.5 mL saline injected subcutaneously in the neck or with saline injections (0.5 mL). Control animals were also treated with saline injections. Treatment was initiated on the day of inoculation and proceeded until the experiment was terminated (week 17 p.i.). As treatment was given every 48 h, the animals received the saline/peptide injections alternately 24 h and 48 h before registration of the EEG. Previous studies indicate that this should not affect the EEG data (reviewed in De Wied & Jolles, 1982). This was confirmed in a control experiment (see Results: 'Control experiment: Direct effect of the $ACTH_{4–9}$ analogue and CFA on EEG and VEP parameters').

Clinical condition of the animals

The clinical status of the animals was scored every week following inoculation on a scale from 0 to 9. The clinical scores are summarized in Table 1. Accurate grading was verified by scoring the animals on several occasions by two different investigators blind to the treatment

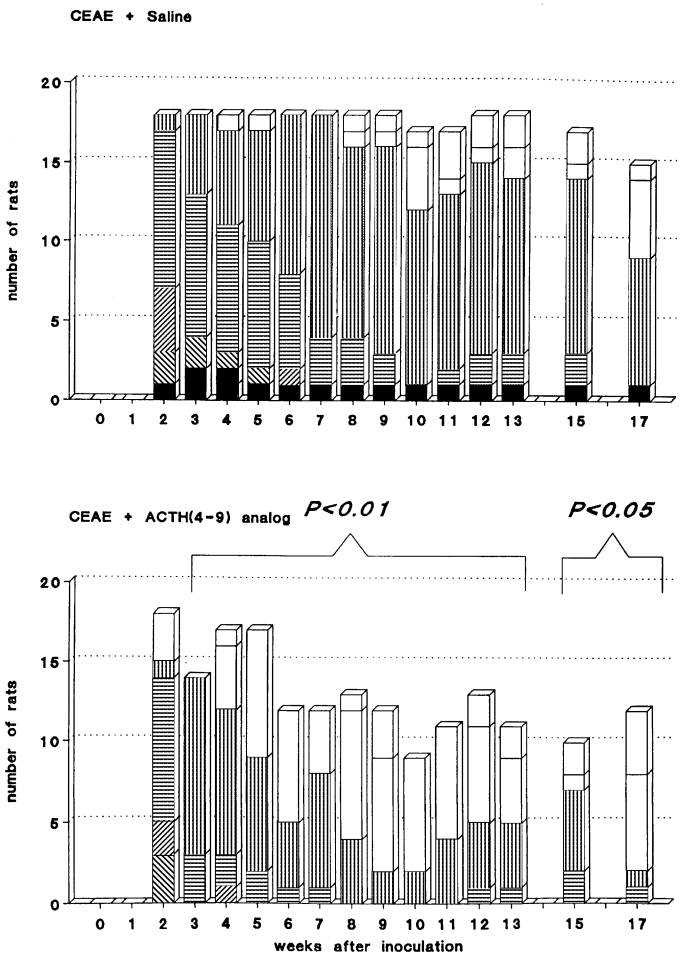

FIG. 1. Analysis of the effect of the $ACTH_{4–9}$ analogue on the clinical score of CEAE-rats. The severity of CEAE in rats was graded weekly on a scale of 0–9 every day (see Table 1). One animal died (CEAE-animal saline-treated) due to very severe CEAE. This animal was scored 9 throughout the rest of the experiment. Age-matched control animals were not affected by CEAE and are therefore not shown. For visualization purposes, animals with minor clinical symptoms, i.e. grade 1 (half limp tail) and grade 2 (limp tail) were combined into one group and animals with severe deficits were grouped together, i.e. grade 5 (paralysis) through 9 (death). The bars depict the number of animals with a given grade, as follows: white bars, limp tail (grades 1–2); light grey bars, moderate paraparesis (grade 3); dark grey bars, severe paraparesis (grade 4); black bars, paralysis, tetraparesis and death of the animal (grades 5–9). Animals not showing clinical symptoms are not indicated; total number of animals was 18 throughout the whole experimental period. Upper graph: Clinical score distribution of the group of rats with CEAE treated with 0.5 mL saline injections every 48 h ($n = 18$). Lower graph: Clinical score distribution of the group of rats with CEAE treated with 75 μg $ACTH_{4–9}$ analogue/kg body weight in 0.5 mL saline ($n = 18$). Clinical scores were compared using non-parametric Wilcoxon rank sum tests: $P < 0.05$ or $P < 0.01$ when comparing saline-treated vs. peptide-treated CEAE animals.

given to the animals. The intraobserver correlation of 0.9 indicates the reproducibility of the clinical evaluation. For the visualization in Fig. 1, animals with minor clinical symptoms, i.e. grade 1 (half limp tail) and grade 2 (limp tail) were combined into one group and animals with severe deficits were also grouped together, i.e. grade 5 (paralysis) through 9 (death). The graph illustrates the clinical score composition in the saline and $ACTH_{4–9}$ analogue treatment groups throughout time.

Registration of electroencephalogram and flash evoked visual potential

EEG and VEPs were recorded at a weekly interval on 2 consecutive days. An animal in a Plexiglas observation box was placed in a sound-attenuating electrically shielded chamber with mirrors on the walls and floor. The electrical signals were transmitted via an impedance transformer and cables attached to the head of the animal. The EMG and EEG signals were registered using a polygraph (Mingograph 34, Elema, Schönander, Sweden) with bandwidth 0.5–70 Hz for the EEG, and 27–700 Hz for the EMG. The latter was thereafter rectified and smoothed before being sampled. The output signals from the preamplifiers were digitized using a Tektronix computer (2630 Fourier Analyser with anti-aliasing filter, Tektronix, Campbell, OR, USA) with a sampling rate of 2 ms. Samples of EEG records of each animal, with the corresponding EMG signals and trigger markers of photic stimulation, were stored on optical disk for later off-line analysis. During the recording sessions, a paper registration of EEG and VEP recordings of each animal was made on the Mingograph 34 along with the EMG trace, calibration pulse and stimulus pulse (Fig. 3; paper speed 10 mm/s). Prior to each recording session, the amplifiers were calibrated using a sine-wave generator (Hewlett Packard).

During recordings the animals could move freely in the observation box placed in the sound attenuated chamber. The animals were observed through a one-way mirror, in order to ensure that the recordings were always obtained during quiet wakefulness. The animals were made familiar with this environment in three recording sessions that took place prior to the immunological challenge. After a 20–30 min habituation period, 200 samples of 1 s baseline EEG were recorded during the quiet wakefulness state of the animal. Light flashes were delivered by a Grass PS22 stroboscope (duration 10 μs, intensity at the source 7.5×10^5 lux/m^2) at a frequency of 0.3 Hz. The flash light was positioned in front of the recording chamber (at $\approx$ 30 cm distance from the rat). The noise accompanying the triggering of the stroboscope was masked by a background noise generator placed inside the recording chamber. After a habituation period, 200 VEPs were recorded during the quiet behavioural states, and stored. EEG epochs starting 100 ms before and ending 900 ms following the flash stimulus were recorded for analysis.

For each rat, baseline EEGs were analysed off-line with an IBM computer by calculating the power frequency spectra using fast Fourier transformation (FFT, Hanning taper; Cooley & Tukey, 1965). EEG epochs with prevailing EMG activity (i.e. large and changing EMG, movement artefacts), sleep spindles or slow wave sleep were omitted from averaging. At least 50 EEG epochs of 1 s duration per animal were transformed by FFT and subsequently ensemble averaged. The frequency resolution thus obtained was 1 Hz. Furthermore, the frequency distribution was partitioned in frequency bands: 1–4 Hz, 7–10 Hz, 11–15 Hz, 16–30 Hz and 31–70 Hz for statistical analysis. The mean power of 10 animals was calculated per band.

EEG epochs following flash were analysed in an identical manner. At least 50 of these EEG epochs were averaged. The components of the obtained VEP were designated as P1, N1, P2, N2, P3, N3 and P4 (Creel *et al*., 1974; Inoue *et al*., 1992). In this experiment, amplitudes of the VEP peaks were defined as the difference between the peak of a component and the baseline. The point of onset of the P1 component of the VEP was defined as the baseline. The afterdischarge of the VEP was measured as the total excursion (TE) of the EP late components; this was defined as the transient, starting at 200 ms and lasting up to 700 ms following the flash (Fleming *et al*., 1973; Shearer *et al*., 1976). Latencies of the peak components

and the TE of the VEP afterdischarge were measured using especially designed computer software (KUN, Nijmegen, the Netherlands). Amplitudes of the VEP peaks were derived from each hard copy of the VEP and expressed in microvolts.

Design of the study

Effect of chronic experimental allergic encephalomyelitis and the $ACTH_{4–9}$ analogue on central neurophysiological parameters

Forty-five male Lewis rats were used in the experiment. Seven weeks prior to the experiment, 30 animals selected at random were provided with permanent cortical electrodes and were allowed to recover for 3 weeks. Baseline VEPs and EEGs were determined on three occasions prior to the induction of the myelinopathy to obtain clear and reproducible basal values for VEPs and EEG power spectra. Subsequently, the animals were assigned at random to three treatment groups. At the beginning of the experiment, all treatment groups had identical average basal values for latencies and amplitudes of the VEP components, total excursion of VEP afterdischarge and body weight, and exhibited similar EEG power spectra distributions.

CEAE was induced in two treatment groups by injections of CNS–CFA emulsion. Each group consisted of 18 animals: 12 animals with EEG/EMG electrodes and six animals without electrodes. VEPs and EEG were recorded in 10 animals per treatment group. The third group was inoculated using an emulsion containing PBS in CFA and served as an age-matched control group for CEAE animals ($n = 9$: seven animals with EEG/EMG electrodes and two animals without electrodes). In all animals, a weekly neurological examination was carried out using a clinical scoring method. Baseline EEGs and flash VEPs were recorded for up to 4 months following sensitization ($n = 10/10/6$).

Acute effect of the $ACTH_{4–9}$ analogue and complete Freund's adjuvant on electroencephalogram and visual evoked potential parameters

In a second experiment, the effect of CFA and the direct effect of the $ACTH_{4–9}$ analogue on EEG power spectra and VEP characteristics were evaluated. Fifteen male Lewis rats were provided with cortical EEG and EMG electrodes and allowed to recover from the operation procedure for 3 weeks. Baseline EEGs and VEPs were recorded in two independent recording sessions and subsequently the animals were assigned at random to one of the three treatment groups ($n = 5/5/5$).

CEAE was induced in one group ($n = 5$) by CFA-guinea-pig spinal cord homogenate injections. A second group was challenged with CFA–PBS emulsion injections, while the third experimental group ($n = 5$) was inoculated with PBS. At day 50 p.i., basal EEGs and VEPs were recorded. Subsequently, the CEAE animals received an $ACTH_{4–9}$ analogue injection (75 μg/kg in 0.5 mL saline, s.c., in the neck). The short-term effects of the $ACTH_{4–9}$ peptide on VEP and EEG changes were evaluated 24 h following administration.

Statistics

The experiments were carried out in a blind fashion: all treatments given to the animals were coded. The recording of the EEGs, analysis of the EEG recordings and the application of the treatment to the animals were all performed by different investigators unaware of which treatment any given rat had received. The code of the experiment was broken after analysis of the data had been completed. Data were statistically evaluated using an analysis of variance for repeated measurements (MANOVA), supplemented with Student's *t*-tests (two-sided). Clinical ratings were tested for putative differences using a non-parametric Wilcoxon rank sum test.

Results

Clinical progression of chronic experimental allergic encephalomyelitis

In the present experiment, the first manifestations of CEAE became evident 2 weeks p.i., ranging from limp tail to complete paralysis with faecal and urinary incontinence. Subsequently, animals suffering from CEAE began to recover gradually. Nevertheless, even after 17 weeks, the CEAE animals that received a placebo still displayed neurological symptoms (Fig. 1). One saline-treated CEAE animal died during the experiment (week 4 p.i.). This animal was graded 9 throughout the rest of the experiment. As illustrated in Fig. 1, neurotrophic peptide therapy significantly suppressed the development of CEAE-related signs (including limp tail, paraparesis and paralysis) during the chronic phase of the syndrome. One-third of the peptide-treated animals did not show any neurological symptoms ($n \geqslant 6$ of 18, from week 6 through week 17 p.i.). In contrast, in the saline-treated group, all animals exhibited clinical symptoms. As shown in Fig. 1, the $ACTH_{4–9}$ analogue significantly reduced the number of animals suffering from a limp tail by 37.7% ± 4.2 (mean over period 3 weeks p.i. until 17 weeks p.i.). CEAE-afflicted animals did not show any prevailing behavioural changes, including myoclonia, epileptiform seizures or myokymia (of facial musculature).

Body weight changes

Animals suffering from CEAE typically lost weight at the onset of the exacerbation. CEAE animals treated with saline lost up to 35.1% of their initial weight (96.4 g, week 3 p.i.). $ACTH_{4–9}$ analogue-treated animals showed a comparable weight loss (31.7%, 88.5 g, week 3 p.i.) but recovered more rapidly and more completely. Six weeks p.i., peptide-treated animals differed only 7.2 g from the age-matched control animals, whereas saline-treated animals differed 25.8 g from control values. However, this difference was not statistically different (using a MANOVA). CFA inoculation in age-matched control animals resulted also in a transient weight loss of 10.8% as compared with their initial weight (35 g, week 3 p.i.; data not shown).

Visual evoked responses in chronic experimental allergic encephalomyelitis

Latencies and amplitudes of VEPs recorded from age-matched control animals were comparable with values available in the literature (Creel *et al*., 1974; Inoue *et al*., 1992). A typical VEP signal is shown in Fig. 2A. The difference in latencies of the responses of a given control animal varied by no more than 10% (average all peaks from week 1 until week 17 p.i.: 6.1%). Table 2 shows the latency data of the VEP components. The N2 and P3 VEP components were omitted because of the inconsistency with which they occurred. In animals suffering from CEAE a latency delay of all VEP components was first noticed in week 4 p.i. and was most prominent in the late VEP components (week 4 p.i.: mean latency delay P2: 8.4 ms, N3: 18.3 ms, P4: 28.5 ms; Table 2). The observed latency shifts did not coincide with the clinical progression of the disease as maximum clinical scores preceded the course of VEP aberrations. $ACTH_{4–9}$ analogue therapy counteracted the delay of all VEP components. However, only the latency of the late P4 VEP components of peptide-treated CEAE animals was significantly different from that of the placebo-treated CEAE animals at weeks 4 and 7 p.i. [Table 2; MANOVA P4

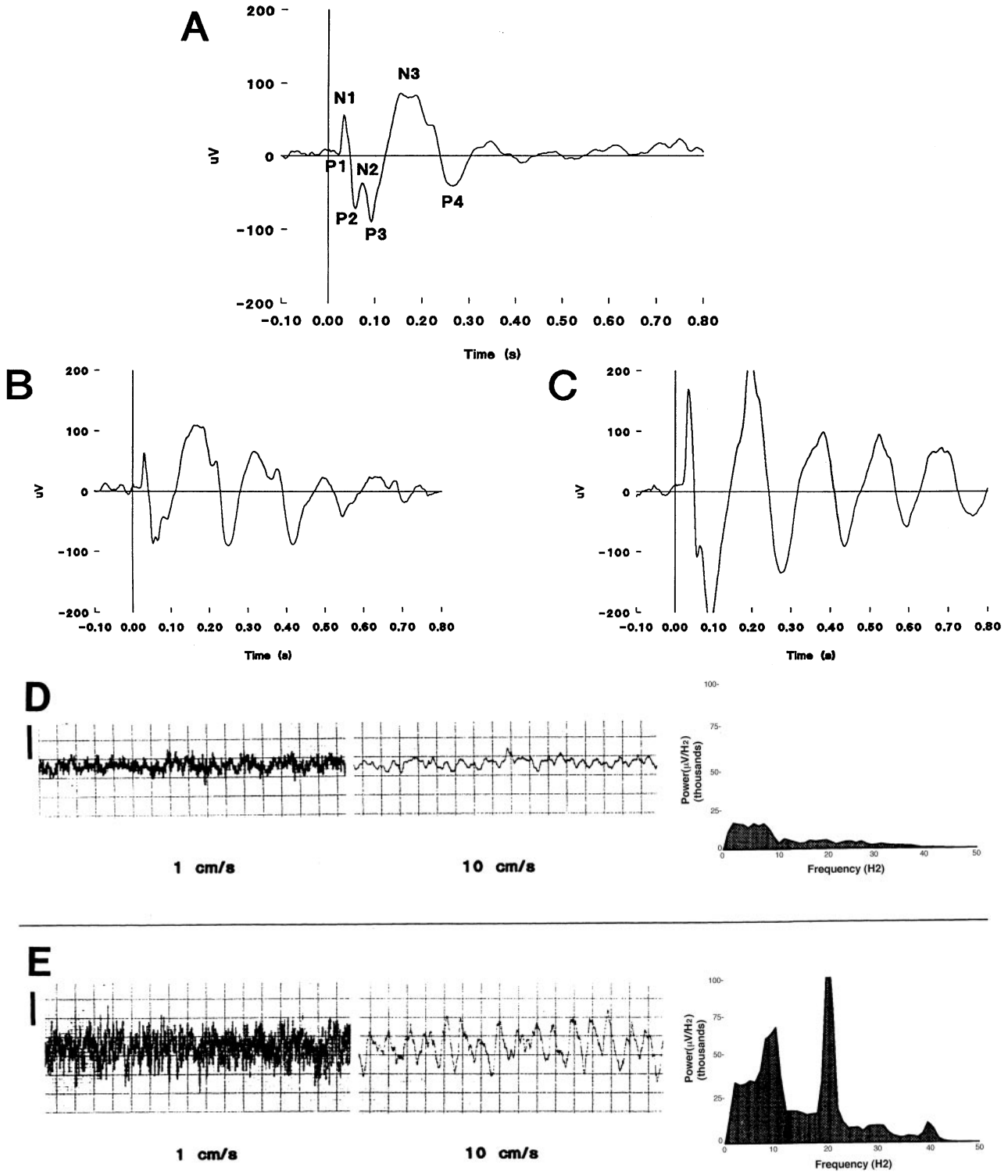

FIG. 2. Typical VEP registrations and baseline EEG during the course of CEAE. Negativity is deflected upwards. (A) Normal VEP registration recorded in a control animal at 6 weeks p.i. (B) Abnormal VEP signal recorded in an animal suffering from CEAE (week 6 p.i.). The animal was treated with saline during the experiment and suffered from a severe paraparesis. (C) Example of a severely disturbed VEP signal recorded in a saline-treated CEAE animal 6 weeks following inoculation. (D) Normal base-line EEG, with the corresponding power spectrum at the right-hand side of a control animal during passive wakefulness (week 8 p.i.). (E) Typical disturbed baseline EEG (and power spectra) of a CEAE animal (week 8 p.i.; saline-treated) during passive wakefulness. At this time-point, the animal was moderately paretic and did not present any convulsions or behavioural abnormalities. Vertical bars = 200 μV.

TABLE 2. Latencies and amplitudes of VEP specific components in CEAE under $ACTH_{4–9}$ analogue treatment. N2/P3 peaks were inconsistently present in averaged VEP registrations and were difficult to designate. Therefore, they have been omitted from analysis. Latency and amplitude values of week 0 p.i. are calculated from three independent recording sessions. At week 4 p.i. maximal differences were observed in latencies of VEP peaks; at week 7 p.i. maximal amplitude changes were observed

	Week 0 p.i.			Week 4 p.i.		
Latency	Age-matched controls	CEAE + saline	CEAE + $ACTH_{4-9}$ der.	Age-matched controls	CEAE + saline	CEAE + $ACTH_{4-9}$ der.
P1	21.7 ± 0.8	22.7 ± 0.6	22.4 ± 0.4	23.7 ± 1.0	26.5 ± 1.4	23.8 ± 0.5
N1	30.7 ± 1.0	30.2 ± 0.3	30.3 ± 0.2	33.0 ± 0.7	34.6 ± 1.7	3 1.9 ± 0.4
P2	53.1 ± 0.5	52.8 ± 0.4	52.7 ± 0.2	55.0 ± 0.6	63.4 ± 4.2	54.8 ± 0.6
N3	162.1 ± 5.9	163.2 ± 2.4	164.2 ± 4.7	159.8 ± 5.8	178.0 ± 7.4	161.6 ± 4.2
P4	235.9 ± 2.7	236.8 ± 2.9	231.9 ± 4.1	235.2 ± 7.7	263.7 ± 8.4*	239.5 ± 3.1
	Week 0 p.i.			Week 4 p.i.		
Amplitude	Age-matched controls	CEAE + saline	CEAE + $ACTH_{4-9}$ der.	Age-matched controls	CEAE + saline	CEAE + $ACTH_{4-9}$ der.
N1	74.2 ± 13.9	71.1 ± 11.7	67.5 ± 12.3	57.5 ± 12.4	59.2 ± 13.6	47.3 ± 10.2
P2	91.0 ± 14.1	87.2 ± 9.1	85.3 ± 10.9	61.4 ± 8.4	82.4 ± 6.2	62.4 ± 2.4
N3	87.9 ± 7.5	108.3 ± 10.3	95.0 ± 11.5	86.3 ± 7.2	113.9 ± 12.2*	82.0 ± 6.9
P4	38.3 ± 8.0	43.4 ± 5.1	39.0 ± 4.2	35.7 ± 5.2	93.1 ± 10.5*	41.4 ± 5.4

*$P < 0.05$ using a Student's *t*-test comparing saline-treated vs. peptide-treated CEAE animals.

latencies (all treatment groups) over weeks 4–10 p.i.: $P = 0.050$ followed by *t*-tests].

The amplitudes during the 4 month follow-up varied considerably among the animals and time points. The P4 amplitude gradually increased during the course of CEAE, reaching maximum values in weeks 6 and 7 p.i. (Table 2). By week 13 p.i. the P4 amplitude had returned to normal. Owing to CEAE, the N3 peak of the VEP was also significantly increased 6, 7 and 11 weeks following inoculation. Peptide treatment normalized the observed amplitude changes of the P4 VEP component at weeks 7 and 12 p.i., and N3 amplitude changes at 6, 7 and 11 weeks p.i. [Table 2; MANOVA (all treatment groups): N3 amplitude: 5–12 weeks p.i.: $F_{17,2} = 3.57$, $P = 0.050$; P4 amplitude: 6–12 weeks p.i.: $F_{20,2} = 3.87$, $P = 0.038$, followed by *t*-tests]. The amplitude values of these animals treated with the $ACTH_{4–9}$ analogue were comparable with amplitudes of VEPs recorded in age-matched controls on that given day (Table 2).

Clear changes of the VEP afterdischarge became apparent by comparing VEPs of animals suffering from severe CEAE (B and C in Fig. 2) with a VEP of a control animal (Fig. 2A). As illustrated in Fig. 3, the immunological challenge induced a marked increase of the TE of the VEP after discharge [MANOVA (CEAE-saline vs. control animals) week 3 p.i. to week 18 p.i.: $F_{13,1} = 5.33$, $P = 0.038$]. The increase of the TE following the VEP was gradual, reaching maximum values at 6 weeks p.i. (104% increase as compared with the control group). In the chronic phase of the disease, the increase of the VEP TE followed the course of the clinical status of the animals closely. The relapse of the animals as observed by clinical scoring following the 10th week p.i. can also be found in the TE graph (Fig. 3).

The neurotrophic peptide significantly suppressed the CEAE-related increase in TE [Fig. 3; MANOVA (CEAE-peptide vs. CEAE-saline) weeks 3–18 p.i.: $F_{18,1} = 8.00$, $P = 0.011$]. Furthermore, TE values of peptide-treated animals did not differ from age-matched control animals throughout the experiment [MANOVA (CEAE-peptide vs. controls) weeks 3–18 p.i.: $F_{13,1} = 0.16$, $P = 0.698$].

Spectral analysis of baseline electroencephalogram during quiet normal vigilance

Two weeks after sensitization, a marked change in the EEG was observed, manifested by an increase of amplitude and fast activity. An example of the alterations observed in baseline EEG is shown in Fig. 2D. The abnormalities in EEG recordings were robust and reproducible in the same animals, but they varied considerably between individual animals. Power spectra analysis of the baseline EEG revealed an increase of all frequencies from 5 Hz onwards with prominent peaks at 8–9 Hz and 20–21 Hz (Fig. 4A). Power spectra of 50 s epochs are plotted three-dimensionally in Fig. 4B. The 8–9 Hz peak reached maximum values at 8 weeks p.i. [3.2-fold increase of 5–12 Hz power as compared with absolute spectra of controls, peak width 7 Hz (5–12)] while the 20–21 Hz peak reached maximum values at 5–8 weeks p.i. [10.3-fold increase of 18–23 Hz power as compared with control spectra values, peak width 4 Hz (18–23 Hz)]. A harmonic of the 20–21 Hz peak was also detected (41–42 Hz). As illustrated in Fig. 4C, the increase of high voltage fast activity was first noticed 2 weeks p.i. with an increase in both high and low frequencies. Three weeks following the immunological challenge, a distinct 20–21 Hz peak was detected in the power spectra that evolved to its nadir in 2 weeks (weeks 5–8 p.i.). The 8–9 Hz increased at week 5 and slowly progressed to a maximum at 8 weeks p.i. EEG abnormalities tended to return to normal by week 13 p.i. Power spectra distributions were divided in specific frequency domains. Statistical analysis of the power within the different frequency domains indicated that the beta 1 band (16–30 Hz) of CEAE-saline-treated animals was significantly increased throughout week 4 until week 9 p.i. as compared with control values [MANOVA weeks 4–8 p.i. (saline-CEAE vs. controls): $P = 0.035$] as well as the beta 2 band (31–70 c/s) throughout week 5 until week 8 p.i. [MANOVA weeks 5–8 p.i. (saline-CEAE vs. controls): $P = 0.012$]. Interestingly, animals with the most pronounced EEG abnormalities in the saline-treated CEAE group were also the most severely affected animals in terms of neurological symptoms.

As illustrated in Fig. 4D, the $ACTH_{4–9}$ analogue significantly counteracted the transient high frequency brain wave oscillations found in CEAE animals [including beta 1: MANOVA period weeks 4–8 p.i. (saline-CEAE vs. peptide-CEAE): $P = 0.020$ followed by supplemental *t*-tests]. The power spectra of EEG records derived from peptide-treated animals did not differ from control animals. Although the 20–21 Hz peak was also apparent in the power spectra of peptide-treated animals, its amplitude was much less pronounced

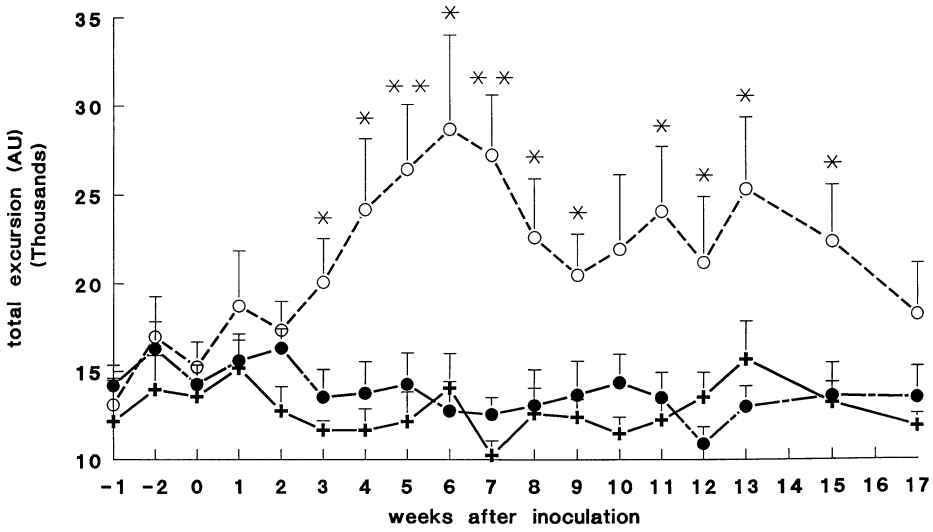

FIG. 3. Total excursion of the afterdischarge following VEP in animals suffering from CEAE. VEPs were recorded and averaged. The total excursion was calculated from the interval 200 ms to 700 ms post-stimulus by computer-specific software. The total excursion is expressed in arbitrary units (AU). Line with crosses represents mean TE of age-matched control animals ($n = 6$) treated with saline injections. Line with open circles represents mean TE of saline-treated animals suffering from CEAE ($n = 10$). Line with filled circles represent averaged TE of CEAE animals treated with the $ACTH_{4–9}$ analogue during the course of CEAE ($n = 10$; 75 µg $ACTH_{4–9}$ analogue/kg per 48 h). Statistics: Analysis of variance for repeated measurements (MANOVA) all treatment groups: weeks 3–18 p.i., $F_{22,2} = 6.13$, $P = 0.008$, supplemented with Student's *t*-tests (two-sided). * = $P < 0.05$ comparing saline-treated vs. peptide-treated CEAE animals.

[week 5 p.i.: increase 148% vs. 930% (18–23 Hz) power increase in placebo-treated CEAE group] as compared with control values. The 10 Hz peak was not conspicuous in the averaged power spectrum of $ACTH_{4–9}$ analogue-treated animals.

Control experiment: Direct effect of the $ACTH_{4–9}$ analogue and complete Freund's adjuvant on electroencephalogram and flash evoked visual potential parameters

At 50 days p.i., animals receiving either CFA, saline or CFA-myelin inoculations were examined for neurophysiological alterations. One CFA-inoculated animal lost its EEG electrodes and hence was omitted from the analysis. Latencies of late VEP components were delayed in CEAE animals as compared with saline-inoculated animals (N2, N3 and P4). The mean TE of the VEP afterdischarge in these CEAE animals was increased by 99.8%, whereas in the power spectrum of baseline EEG an increase of power in all frequency bands was found, with a prominent peak at 23 Hz (band width 21–25 Hz). Total power of the beta band increased by 298% as compared with pre-experimental values (Fig. 5).

CFA-inoculated animals manifested no changes of VEP latencies. In contrast, the total excursion of the VEP transient of these animals displayed a modest increase of 32% as compared with basal values (data not shown). In addition, EEG spectra showed some increase of the beta frequency range with a small peak at 21–23 Hz (Fig. 4B), which may suggest that these EEG phenomena are at least in part induced by CFA inoculation. Saline-inoculated animals manifested neither a delay of VEP latencies, nor an increase of VEP total excursion. Power spectra of baseline EEG were comparable with the pre-experimental power spectra of these control animals. No significant changes in power spectra, TE or VEP latencies of CEAE animals were observed 24 h following subcutaneous injection of the $ACTH_{4–9}$ analogue.

Discussion

In this investigation we found that in CEAE a delay of peak latencies of flash VEPs occurred, while the peak amplitudes of specific VEP components was attenuated. However, the changes of the latencies and the amplitudes of flash evoked VEP components were modest. In contrast, a marked increase of the VEP afterdischarge was observed. Apparently the damping of the transient VEPs mediated by regulatory neuronal systems of the visual cortex following a flash stimulus is impaired. This may be related to an altered excitation state of the visual cortex (Lopes da Silva, 1991) as is also reflected in the increase of the power in different frequency bands.

These findings are in line with the well-established fact that the visual pathways are commonly involved in MS patients (Rusell *et al*., 1991; Van Diemen *et al*., 1992; McDonald & Barnes, 1992; Moschos & Brouzas, 1992; Bilbool *et al*., 1993b). VEPs have been used extensively to diagnose demyelinating lesions in the visual pathways. This recording of VEPs has been advocated as one of the most sensitive indices of detecting functional impairment of the CNS in human demyelinating disorders (Naweron, 1978; Kjaer, 1980; Chiappa, 1980; Purves *et al*., 1981; Van Dijk *et al*., 1992). In EAE, several studies have reported the involvement of the visual system based on the study of histological, electrophysiological and biochemical parameters. The visual pathways have been singled out as one of the predilection sites of the primary demyelination in EAE (Bilbool *et al*., 1983a; Matsushima *et al*., 1986; Deguchi *et al*., 1992).

In the clinic, serial EEG analysis is valuable in following the course of neurodegenerative disorders (Kiloh *et al*., 1972; Kooi *et al*., 1978; Levic, 1978; Awerbuch & Verma, 1987), detecting the development of complications or relapses and the presence of putative residual brain damage. The initial studies of the EEG activity in EAE, as in MS, reported slowing of the background rhythms and occasional paroxysmal activity (Saragea *et al*., 1965; Baxter & Rosenthale, 1966; Feldman *et al*., 1969). However, only visual interpretation of the EEG was carried out in these studies. Quantitative analysis of EEG records in chronic EAE revealed a marked *non*-paroxysmal high voltage and fast activity using occipital–frontal leads. This change developed gradually in 3–4 weeks and returned to normal values ahead of the waning of symptoms. In other studies, this fast activity has been reported as a side phenomenon (Bilbool

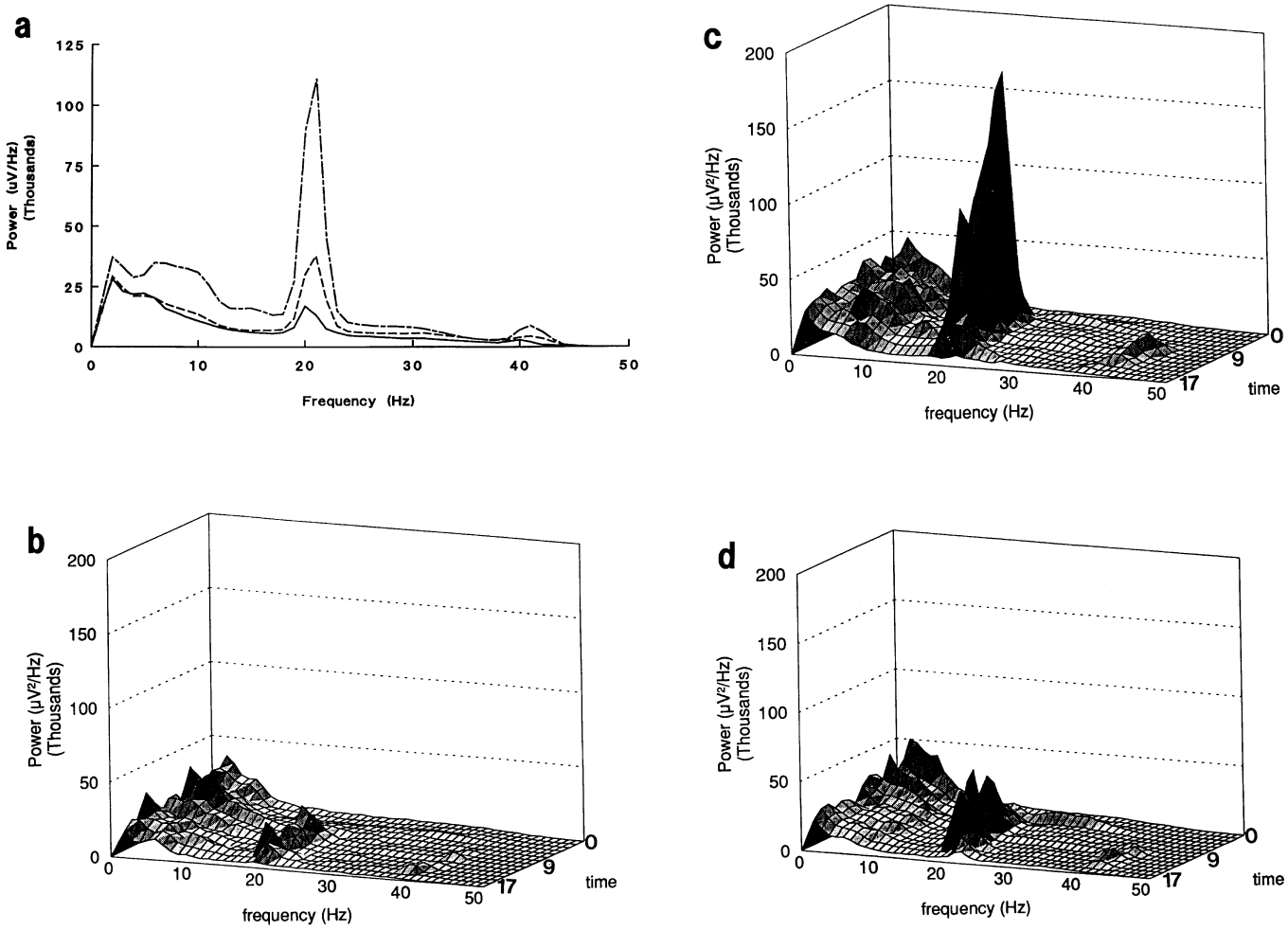

FIG. 4. Power spectra of baseline EEG during passive wakefulness throughout the course of CEAE. Baseline EEG was recorded during quiet stages of the animal. Two hundred second registrations were recorded (1 s epochs, sampling rate 512 Hz), digitized and analysed off-line. Power spectra were averaged per treatment group. The absolute power spectra of each treatment group are visualized in three-dimensional graphs depicting the frequency distributions of baseline EEG throughout the course of CEAE. The *x*-axis depicts the frequencies present in the EEG (Hz); the *y*-axis depicts the absolute power (μV^2/Hz) at a given frequency; the *z*-axis represents the time following inoculation of the animals (weeks p.i.). (a) Averaged power spectra of the different treatment groups (weeks 4–10 p.i.). Hatched-dotted line represents the mean power spectrum of rats suffering from CEAE treated with 0.5 mL saline ($n = 10$); Striped line represents the mean power spectrum of rats suffering from CEAE treated with 75 μg $ACTH_{4-9}$ analogue/kg per 48 h in 0.5 mL saline ($n = 10$); Solid line represents CFA-inoculated control rats which were treated with 0.5 mL saline ($n = 6$). (b) Power spectra of CFA-inoculated, control animals throughout time. These animals were treated with saline injections (0.5 mL/48 h, s.c.; $n = 10$) during the 17 weeks follow-up. (c) Power spectra of CEAE animals treated with saline injections every 48 h (0.5 mL/48 h; $n = 10$). (d) Power spectra of $ACTH_{4-9}$ analogue treated animals (75 μg $ACTH_{4-9}$ analogue/kg per 48 h in 0.5 mL saline; $n = 10$) suffering from CEAE.

et al., 1983a; Deguchi *et al.*, 1992). The main finding in this study was a diffuse increase of EEG power from 5 Hz onwards with an enhancement at 8–9 Hz (5–12 Hz) and a pronounced peak at 20–21 Hz (18–23). The increase of power in these two frequency bands was present consistently, appeared gradually, and diminished independently of one another during the course of CEAE. As the waxing and waning of these activities had different times of onset and optimum values during the development of CEAE, it is unlikely that they are expressions of the same underlying mechanism. This implies that CEAE obviously causes changes in the dynamics of neural networks, possibly by way of induced lesions in regions which regulate the oscillatory activities of the brain. In previous magnetic resonance studies, we showed that the CEAE lesions frequently develop in the thalamus and periventricular white matter (Duckers *et al.*, 1996), which may interfere with thalamic-control circuits.

The increase in the relatively fast oscillatory activity (18–23 Hz) was particularly conspicuous. Fast oscillations occur in the ongoing EEG due to depolarizing actions that depend on modulatory systems of thalamic and cortical neurons (Steriade *et al.*, 1996). Different systems may facilitate the occurrence of fast oscillations in thalamic and cortical networks, namely monoaminergic and cholinergic systems. It is possible that the disturbances induced in the thalamus by CEAE may change the balance between modulatory systems of thalamic-control circuits, leading to an increase of fast oscillations in the frequency range 18–23 Hz. How this may take place is an issue that needs further investigation. The EEG records were obtained from only two electrodes so that they can only give us a rough indication of the changes of such dynamics. However, this finding merits a more detailed analysis of the underlying mechanisms that cause the changes on electrogenesis. In addition, the observed EEG changes in CFA–PBS challenged animals (Figs 4B and 5) might also partially depend on the documented side-effects of

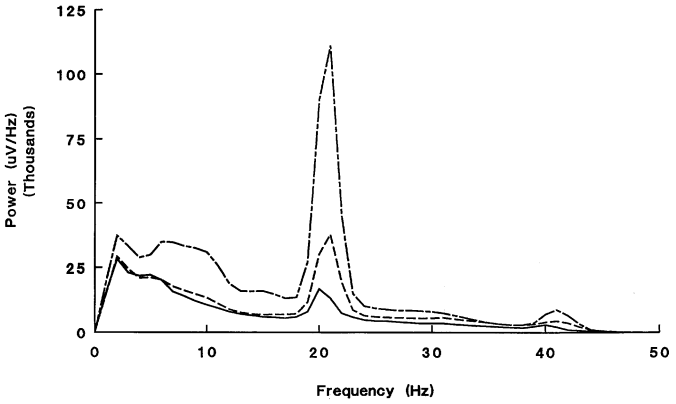

FIG. 5. Effect of CFA and CEAE on power spectra of baseline EEG. Lewis rats (325 g) were provided with permanent EEG and EMG electrodes and allowed to recover for 3 weeks. At 50 days p.i., the effect of the $ACTH_{4–9}$ analogue on baseline frequency distributions was assessed. Solid line represents the mean EEG power spectrum of rats with CEAE ($n = 5$) inoculated with CFA-myelin injections. Striped line represents the EEG frequency distribution of age-matched control animals inoculated with saline ($n = 5$). Dotted line represents the mean EEG power spectrum of CFA-only inoculated animals ($n = 4$).

the adjuvant (Aoyagi *et al*., 1983; Mizisin *et al*., 1987; Neidhart & Larson, 1990).

In the present experiment, $ACTH_{4–9}$ analogue treatment resulted in a marked suppression of neurological symptoms and CEAE-induced phenomena in the EEG. Neurotrophic $ACTH_{4–9}$ analogue treatment significantly reduced the beta power increase and the EEG power within the 8–9 Hz and the 20–21 Hz frequency bands. The mechanism by which the observed beneficial phenomena in EEG and VEP are elicited by the $ACTH_{4–9}$ analogue is unknown.

The normalizing effect of the peptide on the VEP is of particular significance as the visual system is clearly affected in most MS patients. It is hypothesized that the direct neuroprotective and neurotrophic properties of the $ACTH_{4–9}$ analogue could have prevented lesion formation in regulatory regions of the brain, resulting in a normalization of the CEAE-induced EEG and event-related potential phenomena. The effects of melanocortins [ACTH and α-melanocyte-stimulating hormone (MSH)-like peptides] on functional recovery following CNS damage has been well documented. These neurotrophic peptides facilitate functional recovery after central nervous tissue damage in the septal area (Isaacson & Polawsky, 1985), the nucleus accumbens (Wolterink & Van Ree, 1986; Vos *et al*., 1991), the parafascicular thalamic nucleus (Nyakas *et al*., 1985) and the fimbria fornix (Pitsikas *et al*., 1990; Spruijt *et al*., 1990), and diminish histomorphological and functional changes as a result of ageing of the brain (Landfield *et al*., 1986; Reul *et al*., 1988). Furthermore, in previous studies we have shown that the synthetic $ACTH_{4–9}$ analogue could also exert a myelinotrophic effect in CEAE (Duckers *et al*., 1993a,b, 1994, 1996). Prophylactic application of the peptide significantly reduced neurological CEAE-related symptoms, improved walking pattern performance of these CEAE animals and normalized delayed somatosensory (SSEP) and corticomotor evoked potential latencies. In addition, peptide treatment substantially prevented total blocking of the corticomotor pathway in CEAE animals and reduced the attenuation of SSEP-related peak amplitudes as compared with saline-treated CEAE animals. Magnetic resonance imaging studies indicated that treatment with the $ACTH_{4–9}$ analogue significantly reduced the development of cerebral lesions by 85% at 10 and 20 weeks p.i. The CEAE-related lesions were localized in particular in the hippocampus, thalamus and periventricular white matter of the CEAE animals. The present study substantiates these observations and provides further evidence that a neurotrophic pharmacotherapy results in functional improvement and a decrease of the CEAE-induced lesions in regulatory brain regions and the visual pathway.

Recently, several melanocortin receptors have been cloned (Mountjoy *et al*., 1992; Gantz *et al*., 1993a, 1993b) which interact with ACTH, αMSH and αMSH-derived peptide fragments. The neuronal melanocortin receptors, MC3, MC4 and MC5, are particularly interesting in view of the present beneficial effects of the $ACTH_{4–9}$ analogue on EEG as these receptors are expressed in the CNS, including the thalamus, hippocampus, and hypothalamus (Gantz *et al*., 1993b). These brain regions have been designated as regulatory structures of EEG and are affected by CEAE as shown by MRI studies. Current research is now focusing on the possible neuroprotection of the $ACTH_{4–9}$ analogue against CEAE by assuming a direct neurotrophic or myelinotropic effect mediated by a MSH receptor.

A direct myelinotropic effect by $ACTH_{4–9}$ analogue is further suggested by the induction cyclic adenosine monophosphate by melanocortin peptides in cultured astrocytes (Van Calker *et al*., 1983; Evans *et al*., 1984; Zohar & Salomon, 1992) resulting in differentiation and proliferation (Zohar & Salomon, 1992). Astrocytes are capable of ciliary neurotrophic factor (CNTF) secretion, which can protect oligodendrocytes against cytokine-induced cell death (Louis *et al*., 1993) and induce proliferation of glial progenitors. Binding sites for the $ACTH_{4–9}$ analogue have also been identified on glia cells. Dyer reported binding of biotin-labelled $ACTH_{4–9}$ analogue to cultured Schwann cells which could be displaced by excess unlabelled $ACTH_{4–9}$ analogue or αMSH (Dyer *et al*., 1993). Therefore, glia cells could be a target of $ACTH_{4–9}$ analogue.

Alternatively, besides a direct effect on the compromised neuron and glial cell, the peptide could also exert its beneficial effect by an anti-inflammatory mechanism (Murphy *et al*., 1983; Richards & Lipton, 1984; Hiltz & Lipton, 1989), by an effect on the permeability of the blood–brain barrier (BBB) (Goldman & Murphy, 1981; Goldman *et al*., 1982) or by an effect on the excitability of the neural network (reviewed in Spruijt, 1992). Breakdown of the BBB is essential in the pathogenesis of CEAE and MS. In some reports, the $ACTH_{4–9}$ analogue has been shown to influence the BBB (Goldman & Murphy, 1981; Goldman *et al*., 1982). The effect of the peptide on the decrease of BBB permeability, however, is rather small and it is unlikely that this peptide can counteract the induced CEAE-related BBB leakage. Alternatively, the neurotrophic peptide may enhance the excitability of cortical neurons mediated by a normalizing effect on the *N*-methyl-D-aspartate receptor binding (reviewed in Spruijt, 1992) or mineralocorticoid receptors (Reul *et al*., 1988; Joels & De Kloet, 1992) in pathological situations. The direct short-term effects of ACTH fragments on physiological VEPs have also been documented. Wolthuis and De Wied showed that the optic isomers 7-l-phe $ACTH_{4–10}$ and 7-d-phe $ACTH_{4–10}$ did not affect latencies and amplitudes of VEP components (Wolthuis & De Wied, 1976). However, the ACTH fragments diminished VEP afterdischarge within 1 h following systemic administration of the peptides. The observed EEG effects of the $ACTH_{4–10}$ fragments are, however, short-lived (Wolthuis & De Wied, 1976; Urban & De Wied, 1978; Sandman *et al*., 1985). Optimal EEG changes were seen within 2 h after peptide administration and returned to normal within 6 h following peptide injection. The observed transient changes could be mediated by an effect on the CNS vigilance regulatory system. In the present experiment, animals were injected with peptide alternately 24 h and 48 h before registration. Registration 24 h after injection of the

$ACTH_{4–9}$ analogue demonstrated that the putative short-term effects of the peptide did not interfere with VEP and EEG recordings 24–48 h after peptide administration. Furthermore, the short half-life time of the peptide in plasma ($t = 16$ min; Gispen *et al*., 1975; Van Nispen & Greven, 1982) also indicates that it is not likely that the peptide has a long-term effect on the CNS vigilance.

Based on the premise that MS is primarily an autoimmune disorder, many immunoregulatory therapies have been attempted to treat MS, ranging from total body irradiation to corticosteroids. Results with immunobased therapy are modest (Oksenberg & Steinman, 1991). This study demonstrates that neurotrophic peptides have a profound normalizing effect on VEP and EEG in CEAE and suggest that such peptides can be used supplementary to the conventional immune-based therapies, aiming at improving the function of the affected target tissue. Thus, the development of repair strategies in demyelinating syndromes can be aided by these neurotrophic peptides and this can potentially be of value in the human situation, i.e. in MS and the Guillain–Barré syndrome.

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Abbreviations

ACTH	adrenocorticotrophic hormone
BBB	blood–brain barrier
CEAE	chronic experimental allergic encephalomyelitis
CFA	complete Freund's adjuvant
CNS	central nervous system
CNTF	ciliary neurotrophic factor
p.i.	postinoculation
EEG	electroencephalogram
EMG	electromyogram
FFT	fast Fourier transformation
MANOVA	analysis of variance for repeated measurements
MS	multiple sclerosis
MSH	melanocyte-stimulating hormone
PBS	phosphate-buffered saline
s.c.	subcutaneous(ly)
TE	total excursion
VEP	flash evoked visual potential

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