Functional and neurophysiological evidence for the efficacy of trophic pharmacotherapy using an adrenocorticotrophic hormone 4-9 analog in experimental allergic encephalomyelitis, an animal model of multiple sclerosis

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FUNCTIONAL AND NEUROPHYSIOLOGICAL EVIDENCE OF THE EFFICACY OF TROPHIC PHARMACOTHERAPY USING AN ADRENOCORTICOTROPHIC HORMONE$_{4-9}$ ANALOG IN EXPERIMENTAL ALLERGIC ENCEPHALOMYELITIS, AN ANIMAL MODEL OF MULTIPLE SCLEROSIS

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Abstract—Chronic experimental allergic encephalomyelitis (CEAE) is a well-established animal model for the human syndrome, multiple sclerosis. CEAE has striking histological, electrophysiological and clinical analogies with multiple sclerosis and is a valuable animal model for the preclinical pharmacotherapeutical development of new putative therapeutic agents. In this paper, we describe a neurotrophic repair approach in Lewis rats suffering from CEAE. The neurotrophic peptide used is a degradation resistant adrenocorticotrophic hormone$_{4-9}$ analog. The development of CEAE was examined using a combination of clinical, functional and electrophysiological parameters including somatosensory and motor evoked potentials. The latencies and amplitudes of the various evoked potentials can provide quantitative, objective data regarding the involvement of different nerve tracts in CEAE and the effectiveness of the neurotrophic peptide.

Repeated subcutaneous injections of the neurotrophic peptide suppressed the development of CEAE-related clinical symptoms, markedly improved motor performance and reduced the reaction time upon thermal stimulation as compared to saline-treated CEAE animals during a 17 week follow-up study. Prolonged onset latencies of corticomotor evoked potentials and peak latencies of somatosensory evoked potentials due to the demyelination were normalized upon peptide treatment. In addition, peptide treatment substantially prevented total blocking of the corticomotor pathway in CEAE-animals and reduced the attenuation of sensory evoked potentials-related peak amplitudes as compared to saline-treated animals.

The functional and electrophysiological improvements observed in CEAE-animals treated with the adrenocorticotrophic hormone$_{4-9}$ analog, suggest that a neurotrophic repair approach could be of great value to promote the restoration of function in a disabling demyelinating disorder.

Key words: ACTH$_{4-9}$ analog, neuroprotection, melanocortins, rat, chronic experimental allergic encephalomyelitis, demyelinating disorder.

Multiple sclerosis (MS) is a severely disabling syndrome for which no effective pharmacotherapy exists.[49,53] This demyelinating disease affects predominantly young adults (incidence of 100 per 100,000);[62] and has both genetic and environmental preponderance.[30,31,52,67,68] The clinical manifestation of the syndrome is diverse and depends on the localization and extent of the demyelinating lesions in the CNS. Diagnosis is based on the finding of two or more neurological signs, involving different sites at different times. To date, the diagnosis can be established with great certainty by advanced electrophysiological, immunological and CNS imaging techniques e.g., magnetic resonance imaging.[9,45,48,55]

In contrast to the advances in the diagnosis of MS that were achieved in the last decade, an effective MS pharmacotherapy has so far not become available.[2,5] To date, most treatments for MS aim at the modulation of the deviant immune-system and range from non-specific corticosteroids to that of monoclonal antibodies directed against specific immune cells.[51,60] In addition to the immune-based therapies,

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Abbreviations: CEAE, chronic experimental allergic encephalomyelitis; CFA, complete Freund's adjuvant; CMEP, corticomotor evoked potential; dpi, days post-inoculation; EAN, experimental allergic neuritis; EEG, electroencephalogram; EMG, electromyogram; IFA, incomplete Freund's adjuvant; ITS, inter-toe spreading; MS, multiple sclerosis; PBS, phosphate-buffered saline; pi, post-inoculation; PL, print length; SL, step length; SSEP, somatosensory evoked potential; TS, toe spreading.

pharmacological attempts to stimulate regeneration in order to reduce the impairment of neuronal functions should be considered. These attempts include the transplantation of glial progenitor cells in experimentally induced lesions in order to restore (re)myelination[12,26] and the application of naturally occurring melanocortins or peptide analogs with well-established neurotrophic effects.[17]

We employed a commonly well characterized model for MS, i.e. chronic experimental allergic encephalomyelitis (CEAE) induced in Lewis rats.[39] In this model we tested the effectiveness of a synthetic, degradation resistant neurotrophic peptide (an adrenocorticotrophic hormone $[ACTH]_{4-9}$ analog, H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH). This peptide is devoid of corticotrophic and melanotrophic properties.[15] This peptide is able to exert a myelinoprotective effect in experimental allergic neuritis (EAN), an animal model for an auto-immune mediated peripheral demyelinating disease, the Guillain–Barré syndrome.[18,19] In this study, peptide treatment reduced neurological EAN related symptoms, normalized motor function and preserved the ultrastructure of myelinated fibres in the sciatic nerve.

In the present experiment, the functional integrity of ascending and descending tracts in the spinal cord was studied in CEAE using somatosensory and corticomotor evoked potentials. In addition, motor and sensory function were assessed. The combination of short latency somatosensory evoked potentials (SSEP; ascending tracts) and corticomotor evoked potentials (CMEP, descending tracts), as employed here, has proven to be a sensitive method of evaluating spinal cord dysfunction.[9,48] Here, we have elicited CMEPs by stimulating the primary motor cortex and recording the evoked electromyographic potential from the intrinsic small muscles of the contralateral hind paw. SSEPs were elicited by stimulating the sciatic nerve. The electroencephalogram (EEG) from the contralateral somatosensory cortex using permanent cortical electrodes was recorded and averaged. Both event-related potentials provide complementary information regarding the involvement of the afferent and efferent tracts in the spinal cord and at the subcortical level in animals suffering from CEAE. This technique allows the objective evaluation of the peptide effect on the functional integrity of CNS tracts.[16,63]

EXPERIMENTAL PROCEDURES

Animals

Sixty-five female Lewis rats of an inbred strain were used (initial weight about 200 g;[28,37] derived from the Central Department of Laboratory Animals (University of Limburg, Maastricht, NL). The animals were housed individually in Macrolon cages (RUCO, NL) on sawdust with free access to water and commercial rat chow. A 12–12 light–dark cycle was maintained with white lights on from 8.00 a.m. to 8.00 p.m. Whenever animals were severely affected by paresis or paralysis, the food pellets were supplemented with soaked rat chow in order to prevent decreased food and water intake.

Surgery

Five weeks preceding the inoculation, animals were anesthetized using a combination of Hypnorm® (Janssen Pharmaceutica, Tilburg, NL, containing 10 mg/ml fluanisone, 0.315 mg/ml fentanylcitrate, dose 0.8 ml/kg s.c.) and Diazepam® (Hoffman-La Roche, Mijdrecht, NL; 0.3 ml/kg i.m.). Subsequently, in 40 animals, under sterile conditions, a bipolar cortical electrode (MS303/2, Plastics One, U.S.A.) was inserted into the skull: one tip inserted over the right somatosensory cortex, (area 1; A 2.0, L 4.0) and one near the midline over area 4 (A 2.0, L 2.0; interpolar distance 2 mm). The electrodes were inserted 1 mm into the skull with the tip just above the pia mater. The electrodes were secured by two stainless steel screws and dental acrylic cement (Polyfast Selfcuring, U.K.). The wound was closed over the dental cement. Subsequently, the animals were allowed to recover for three weeks.

Sensitization of animals

Two hours prior to the inoculation, spinal cords of Duncan Guinea-pigs (500 g, CPB, Zeist, NL) were collected and homogenized in an equal volume of phosphate-buffered saline (0.1 M PBS, pH 7.3; 50% g/v). Subsequently, the homogenate was emulsified in equal parts of complete Freund's adjuvant [(CFA) 10 mg/ml M. Tuberculosis; Difco Laboratories, MI, U.S.A.]. The homogenate emulsion was kept on ice until use. Anesthetized animals (Hypnorm®, Janssen Pharmaceutica; 0.4 ml/kg body weight) were inoculated with 0.4 ml of CNS emulsion injected subcutaneously in the dorsum pedis of the two front limbs and the right hind limb.[40,71] Fifteen age-matched control animals were challenged with an identical volume of PBS in incomplete Freund's adjuvant (IFA). The day of inoculation was designated as day 0 dpi.

Peptide treatment

The neurotrophic peptide used in the study was a modified $ACTH_{4-9}$ peptide fragment (H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH). This $ACTH_{4-9}$ analog is devoid of corticotrophic or melanotrophic activity[15] and has well-established neuroprotective and neurotrophic properties exemplified *in vivo* and *in vitro* models.[3,25] As indicated in more detail in the outline of this study, myelin-challenged animals were treated either with 0.5 ml saline containing 75 μg/kg body weight $ACTH_{4-9}$ analog every 48 h injected subcutaneously in the neck ($n = 25$) or with saline injections ($n = 25$) from the day of inoculation until cessation of the experiment (123 dpi). This dose and route of administration have been reported to be optimally effective in *in vivo* models.[70] Age-matched control animals ($n = 15$) were treated with an identical volume of saline injected s.c. in the neck.

Clinical status of chronic experimental allergic encephalomyelitis animals

In order to substantiate the functional relevance of repeated neurotrophic peptide therapy, the neurological signs and symptoms were scored on a 0 to 9 scale as follows: grade 0, no visible neurological symptoms; grade 1, loss of tip tail reflex; grade 2, complete flaccid tail; grade 3, moderate paraparesis with minor locomotion disturbances; grade 4, severe paraparesis accompanied by lordosis, severe disturbed locomotion; grade 5, one paralytic hind limb; grade 6, both hind limbs paralysed; grade 7, paralysis from diaphragma downwards; grade 8, tetraplegia, only head movements possible; grade 9, moribund state or death. Animals which died during the experiment were subsequently scored 9 throughout the remnant of the experiment. The classification of the animals was verified at

random occasions by a second investigator. For practical reasons, the clinical scores in Fig. 1 are regrouped into three categories: 'minor' symptoms: grade 1, 2 and 3; 'moderate' neurological symptoms: grade 4; 'severe' symptoms: grade 5 through 9. Graph 1 illustrates the clinical score composition in the saline and $ACTH_{4-9}$ analog treatment groups throughout time.

Functional tests

Walking pattern analysis. Walking requires the proper function of the central program of locomotion with adequate afferent peripheral input resulting in a co-ordinated use of different muscle groups with a distinct temporal pattern of activation.[7] Therefore, the integrity of the afferent and efferent tracts, delivering the necessary inputs and outputs to the musculature, is essential for normal walking. Thus, analysis of walking in the rat reflects the integrity of these tracts in models of central or peripheral nerve damage.[7,14] The unrestrained walking pattern of the animals was analysed weekly according to the method originally described by De Medinacelli *et al.*[14] with minor modifications according to De Koning and Gispen.[13] In short, the hind paws of the animals were dipped in photodeveloper solution. After excessive fluid was removed, the animal was placed in a 50 cm long, confined corridor (inclination 10°) with a blinded, dark box at the end. On the floor of the corridor, photographic paper was placed that stains at contact with the photodeveloper fluid on the paws of the animals. The obtained foot prints were analysed by an independent investigator not involved in the present study. From the obtained walking tracks several parameters can be measured including step length (SL); print length (PL); toe spreading (TS) and inter toe spreading (ITS; see Fig. 2A). Left and right foot print readings of a given rat were averaged. From the TS and ITS distances, a toe spreading index was calculated according to De Koning and Gispen;[13] see Fig. 2A). Upon paralysis, animals were assigned a toe spreading and inter toe spreading distance of 6 mm, while print length was set at 60 mm.[14]

Ascension latency. A simple and rapid method of testing muscle weakness in affected animals is to determine the time that the animals require to climb upon a platform while clinging to it with their front limbs. Ascension performance was assessed weekly in the treatment groups. Prior to the experiment, animals were trained to perform the task in three training sessions. As the animal was supported at the trunk, front limbs were placed at the edge of a rough surface platform. Subsequently, the animal was released (t = 0). The time, the animals needed to climb upon the platform was recorded. Animals which failed to clamber upon the platform fell in a tray filled with sawdust, placed below the platform. A maximal latency of 6 s was allowed. Animals which fell were also assigned a latency period of 6 s. Each animal was tested three times per session. Performance of an animal on a given day was the average of these three latencies. The number of animals which fell per treatment group, the latency to climb upon the platform and the latency between releasing the animal and possible falling was determined.

Nociception. The initial pain associated with intense heat (higher than 45°C) is mediated by heat nociceptors (Aδ) whereas the later burning sensation is mediated by the unmyelinated C fibres.[34] In the present study, we assessed the performance of CEAE-animals in a hot plate reaction procedure according to Woolfe and McDonald[72] at week 0, 10 and 18 pi. A hollow aluminium plate was maintained at 54°C using a water bath. The rat was placed (t = 0), within a perspex cylinder, on the heated plate. The actual time the rats took to respond to the noxious thermal stimulation was measured. These reactions ranged from licking the fore or hind limbs to jerking, kicking or hind limb lifting. A maximal response latency of 30 s was maintained to prevent injury of the animals.

Electrophysiology

Corticomotor and somatosensory evoked potentials. Electrophysiological examinations using event related potentials was performed weekly. Animals were anesthetized with ketamine hydrochloride (1 ml/kg i.p.; Ketalar®, Parke-Davis, Amsterdam, NL, containing 50 mg ketamine and 0.1 mg phemerol chloride per ml). Prior to the SSEP registration, anesthesia was supplemented with an additional dose of xylazin (0.5 ml/kg, s.c.; Rompun®, 20 mg/kg, Bayer). The rectal body temperature was monitored during the recording sessions and maintained at 36°C.

CMEPs were elicited by electrical stimulation of the (right) cerebral cortex with permanent cortical scalp electrodes which evoke electromyographic potentials in the contralateral limb muscles. The electromyograph (EMG) response was recorded by surface electrodes from the small muscles of the contralateral foot. The recording patch was attached to the plantar side, while the reference electrode was attached to the dorsum pedis of the paw. A ground electrode was placed between the stimulation and recording site e.g., a 4 mm wide copper wire around the ankle of the left paw. Constant current, anodal impulses of 200 μs duration and a frequency of 0.1 Hz were delivered to the pial surface of the motor cortex by a SEP Neurotrac stimulator (Interspec Medical, Moberg Inc., U.S.A.) triggered by the sync pulse from the IBM computer. The recorded traces were led through a pre-amplifier (8 K, Neurolog NL104, Digitimer Ltd, U.K.), digitized (sample frequency 20 kHz, 1024 Hz points, epoch sweep 50 ms) and visualized on a 486 IBM microcomputer. Filters were set at 5 Hz (high pass) to 5 kHz (low pass) with an additional 50 Hz notch filter (Neurolog, NL125, Digitimer Ltd, U.K.).

The conduction time necessary to evoke the EMG response in the limb muscles is compatible with the conduction by the large myelinated fast fibres in the efferent descending tracts of the spinal cord.[4,11,23,27,47,61,74] CMEPs were easy to elicit and were highly reproducible. A typical example of CMEP trace is illustrated in Fig. 3A. In most animals, CMEP consisted of a biphasic wave at a latency approximately 6.3 ms following stimulus, however, more complex wave forms were also observed. The onset latency of the first (negative) deflection was designated as the conduction time of CMEP. Per animal, three CMEPs and hence three onset latencies were measured and averaged. These latencies varied little between successive stimulations (about 0.5%). In order to check whether the impairment of the CMEP (i.e. blocking) was due to changes proximal or distal to the sciatic notch, the sciatic nerve was stimulated at the notch to elicit muscle action potentials in the foot muscles (M-response by direct stimulation of α-motor fibres and the Hoffmann-response by monosynaptic projection of Ia afferents onto the alpha motoneurons), in order to determine peripheral nervous system contribution to the blocking.

Subsequently, anesthesia was supplemented with an additional dose of xylazin (Rompun®, 20 mg/kg, Bayer, 0.5 ml/kg, s.c.). In order to evoke SSEPs, the left sciatic nerve was electrically stimulated at the sciatic notch. The SSEPs were recorded bipolarly from cortical electrodes overlying the contralateral somatosensory cortex and placed at an interelectrode distance of 2 mm. Monopolar needle electrodes (insulated except for the (blunt) tip; Dantec Electronics, Denmark) were used to stimulate the sciatic nerve. One stimulation electrode was placed at the sciatic notch while the second was placed subcutaneously, approximately 5 mm distal to the other. Grounding of the animals by a copper band placed around the ankle contralateral to

the stimulation site was found to yield the best result. Three series of 128 rectangular, anodal stimuli (50 ms sweep duration, 1 Hz) were delivered to the sciatic nerve using a SEP Neurotrac stimulator (Moberg Inc., USA) triggered by an IBM microcomputer. The stimulus intensity was two to three times stronger than the stimulus required to produce a visible twitch of the paw (approximately 5 mA, pulse duration 200 μs.[54,58] The EEG traces recorded from the cortical electrodes were amplified (using a Neurolog N104) with a bandwidth of 1–50 Hz (Neurolog NL125). The output signals from the preamplifier were digitized, averaged (128 traces) and visualized on an IBM microcomputer (sample frequency 20 kHz, 2048 points, epoch sweep 100 ms).

The SSEPs were very reproducible. Characteristically, a first negative deflection was observed, designated as N1 at a mean peak latency of 11.6 ms (see Fig. 3A, negativity is deflected upwards), followed by respectively a positive deflection, P1 (at 15.6 ms), and a negative deflection, N2 (at 21.2 ms; see Fig. 3A). Peak latencies and peak-to-peak amplitudes (N1–P1; P1–N2) were measured using appropriate computer software. Amplitudes and latencies of three readings per animal (each 128 traces) were averaged.

Experimental design

Sixty-five Lewis rats were used. Five weeks prior to the inoculation, forty animals, selected according to a random table, were provided with permanent bipolar cortical electrodes and allowed to recover for three weeks. Subsequently, the basal values for walking pattern, placing performance, hot plate response and the CMEPs and SSEPs were determined three times to obtain reliable values. At day 0 pi, animals were assigned at random to three groups: CEAE was induced by injections of CNS emulsion both in the saline and in the neuropeptide treatment group each containing 25 animals (15 animals with electrodes, 10 animals without electrodes). Following the inoculation, the CEAE-animals were treated either with the $ACTH_{4-9}$ analog (75 μg/kg every 48 h, s.c.) or with saline injections during the 4.5 month follow-up. A third group of age-matched control animals (total of 15 animals: 10 with electrodes; 5 without electrodes) were inoculated with an inert inoculum (IFA-saline) and treated with saline injections until cessation of the experiment.

The animals were weighed and subjected to a daily neurological examination in order to classify neurological signs. Each week, walking tracks were recorded and placing performance was evaluated ($n = 25$ vs 25 vs 15). Animals were subjected to the hot plate test at week 0, 10 and 18 pi. In addition, CMEPs and SSEPs were recorded on a weekly basis in animals with chronically indwelling electrodes ($n = 15$ vs 15 vs 10), up to 17 weeks following CNS emulsion challenge. The peak-to-peak amplitudes (SSEP), onset (CMEP) and peak latencies (SSEP) of these evoked potentials were determined, as well as the distance between stimulation site and recording site.

Statistical procedure

Treatments were all assigned to animals according to a random table. Furthermore, the experiments were carried out with coded treatment solutions blind to the investigators evaluating the animals. Only after completion of group analyses, the code was broken. Denoted differences were statistically tested using an analysis of variance for repeated measurements (MANOVA), supplemented with Student's t-tests (two-sided) to delineate the effects. Data are presented in the graphs as means $\pm$ S.E.M. Clinical scores and CMEP failures, however, were analysed using a non-parametric Mann–Whitney Rank Sum test and a Chi-square test, respectively.

RESULTS

Chronic experimental allergic encephalomyelitis-related signs and symptoms

Animals challenged with myelin developed the characteristic limp tail, muscle weakness and paralysis complicated with urinary or, less frequently, faecal incontinence, within 10–11 days following inoculation. CEAE-animals deteriorated rapidly within days followed by an incomplete recovery at day 16–17 pi (Fig. 1A). A second exacerbation, further designated as the chronic phase, was denoted following day 19 pi. Deterioration of neurological signs and symptoms during the second exacerbation was more gradual and reached a more severe level than during the acute phase. In the present experiment, the animals suffered from a severe form of CEAE, as indicated by the fact that placebo-treated CEAE-animals presented half of the time (i.e. nine weeks) severe neurological deficits (grade 4 and higher; Table 1). Two animals died prior to the inoculation with the CNS emulsion due to anesthesia and were therefore omitted from the experiment. Two additional animals died during the experiment after inoculation, respectively at day 25 and 80 pi. These two animals were given a clinical score of 9 throughout the remainder of the experiment. Placebo-treated CEAE-animals reached a plateau of maximal scores between four and seven weeks pi. Subsequently the animals gradually recovered, however, within the time period of the experiment (4.5 months), complete recovery was not accomplished. Age-matched control animals challenged with an inert inoculum, did not present any neurological deficits. Peptide treatment did not alleviate the clinical manifestation during the acute phase of the CEAE, but did suppress significantly the CEAE-related signs during the chronic phase of the demyelinating syndrome (three to 15 weeks pi, reduction of mean score by 31%; see Fig. 1). As illustrated in Table 1, peptide treatment resulted in an approximately two-fold longer period free of symptoms (grade 0), when compared to the corresponding period in saline-treated animals ($P = 0.043$), whereas the average period with paralysis (designated grade 5) was reduced ($P = 0.036$). Moreover, throughout the course of CEAE, six out of 25 peptide-treated animals (24%) did not display any neurological symptom, whereas in the placebo-treated group, all animals showed neurological signs (week 4 until week 14 pi).

CEAE typically resulted in a severe weight loss starting in the acute phase and proceeding throughout the second attack (Fig. 1B). Weight loss was most prominent in the second and third week of CEAE in which animals lost about 40 g in six days. Subsequently the animals gradually recovered. Application of the $ACTH_{4-9}$ analog did not alter the weight loss in CEAE-animals (data not shown).

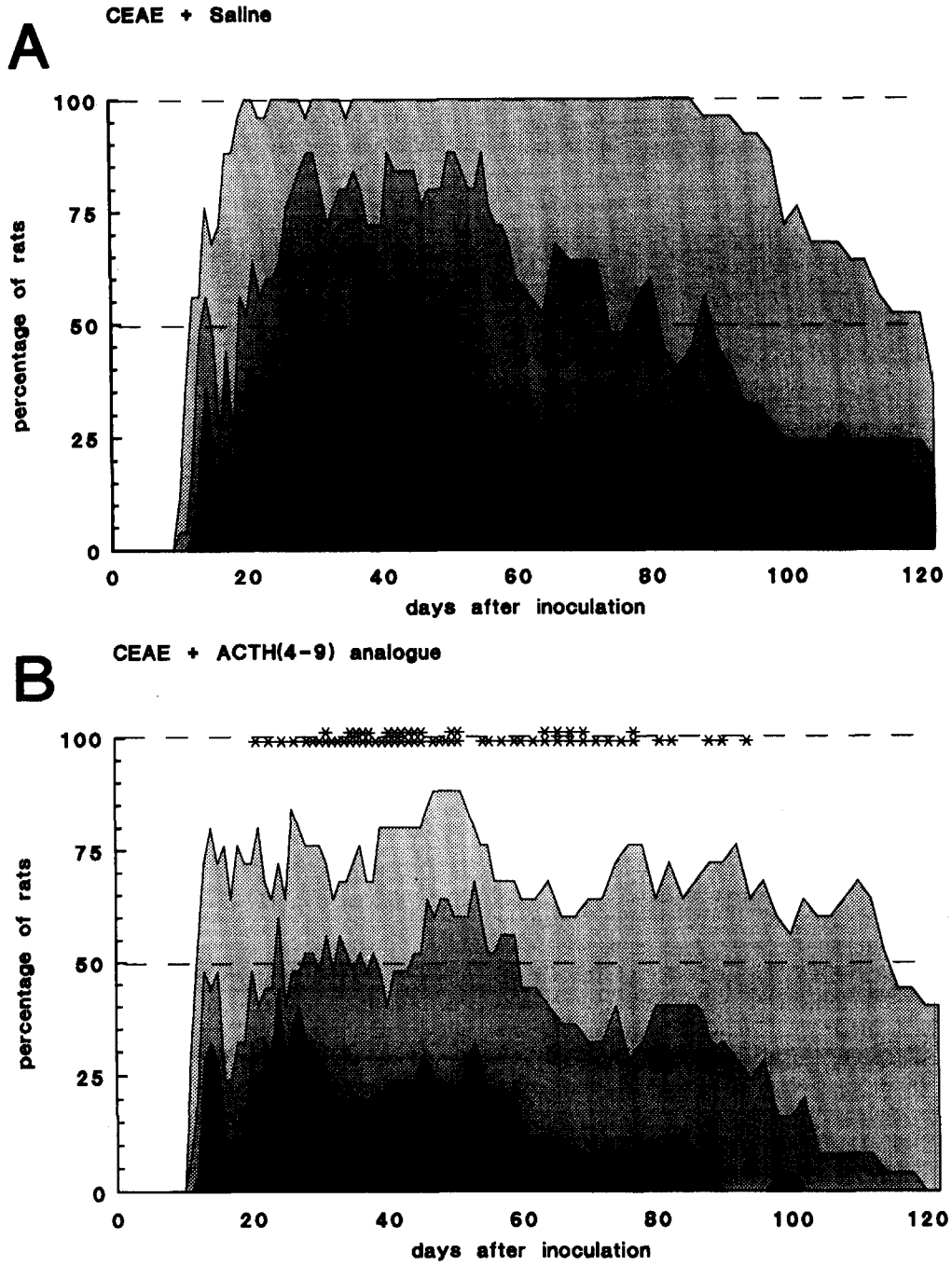


Fig. 1. Development of CEAE-related neurological symptoms in different treatment groups. CEAE was induced by subcutaneous injections of guinea-pig spinal cord homogenate (1:1 g/v homogenate in PBS) in CFA. The severity of CEAE in rats was graded on a scale of 0 to 9 every day (during 0 to 60 dpi) and every other day (during 62 to 123 dpi; see Experimental Procedures), however for practical reasons scores were regrouped in minor moderate and severe symptoms as follows: 1 (light grey): limp tail with mild paraparesis; 2 (dark grey): severe paraparesis with severely afflicted locomotion; 3 (black): paralysis (uni- or bilateral), tetraplegia or death. Data are shown using stacked areas depicting the number of rats with a score of 1 to 3 at a particular day following inoculation. Two animals died during the experiment due to the induced CEAE (both saline-treated CEAE-animals; at 25 and 80 dpi) and were subsequently scored 3 throughout the rest of the experiment. Age-matched control animals did not develop neurological symptoms of CEAE. (A) Clinical score distribution of the group of rats with CEAE treated with 0.5 ml saline injections every 48 h ($n = 25$). (B) Clinical score distribution of the group of rats with CEAE treated with 75 μg ACTH(4–9) analog/kg body weight in 0.5 ml saline ($n = 25$). Clinical scores were compared using non-parametric Wilcoxon Rank Sum tests. $^{*}P < 0.05$; $^{**}P < 0.01$ comparing saline-treated vs peptide-treated CEAE animals.

Table 1. Data represents the mean composition of the chronic experimental allergic encephalomyelitis progression in 25 animals of each treatment group following the inoculation with myelin

	Mean number of days with a given grade	
Clinical score	Saline-treated CEAE-animals	$ACTH_{4-9}$-treated CEAE-animals
0	20.0 ± 2.2	38.0 ± 6.1*
1	2.3 ± 0.9	4.5 ± 1.1
2	1.9 ± 0.5	4.7 ± 1.7
3	39.6 ± 4.6	36.3 ± 3.5
4	27.1 ± 3.4	26.0 ± 3.9
5	23.5 ± 5.2	10.2 ± 2.7*
6	2.3 ± 0.7	2.2 ± 1.0
7	0.6 ± 0.2	1.1 ± 0.4
8	0.04 ± 0.04	0.0 ± 0.0
9	5.7 ± 4.2	0.0 ± 0.0
Total	123 dpi ($n = 25$)	123 dpi ($n = 25$)

Age-matched control animals did not exhibit any symptoms and are therefore not shown in the table. Data are presented as mean ± S.E.M. and tested for potential differences using a non-parametric Mann–Whitney *U* Rank Sum test. *$P < 0.05$ comparing saline-treated vs peptide-treated group.

Functional parameters in chronic experimental allergic encephalomyelitis

Walking pattern analysis. Motor performance of age-matched control animals did not show any impairment. Hence, the walking track parameters of controls were used as reference to values derived from walking pattern analysis of CEAE-animals. Mean toe spreading index (TSI) values, derived from TS and ITS, of placebo-treated CEAE-animals began to deteriorate rapidly by week 2 pi, in parallel with the evolving clinical manifestation of the CEAE (Fig. 2B). Impairment of motor performance was maximal at week 2 pi, followed by an incomplete recovery. A second moderate deterioration was observed following week 6. TSI values did not return to normal values by week 17 pi.

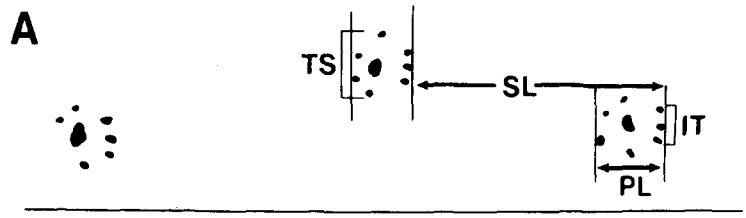
The $ACTH_{4-9}$ analog did not delay or shorten the acute deterioration of TSI. However, recovery upon the decline of motor scores at week 2 pi was more pronounced in peptide-treated animals, resulting in an advantage over placebo-treated animals throughout the observation period. Peptide treatment significantly ameliorated motor performance of CEAE-animals throughout the course of CEAE, as indicated by the reduction of the mean TSI impairment by 49% (Fig. 2B, average over week 3 to 17 pi; MANOVA (peptide vs saline-treated CEAE-animals) week 3 to 17 pi; $F(45,1) = 5.90$, $P = 0.019$).

In addition to the TSI, walking pattern analysis provided two additional parameters: print length (PL) and step length (SL). SL declined within the first five weeks and remained low until week 8 pi after which SL returned to normal values by week 15 pi (data not shown). SL was not affected upon peptide treatment.

Furthermore, mean PL was prolonged in saline-treated CEAE-animals concomitantly with the changes in TSI e.g., a rapid deterioration following week 1 pi, that reached maximal impairment during the period two to four weeks pi. After an incomplete recovery, a second regression was found, as in TSI (week 8 to week 17 pi). During this second deterioration phase, a clear tendency to normalize the value of PL was detected in $ACTH_{4-9}$ analog-treated animals (week 10 pi: mean PL controls: 22 ± 0.7 mm; CEAE-saline: 28 ± 2.5 mm; CEAE-$ACTH_{4-9}$ analog: 24 ± 0.4 mm). This amelioration of PL however, was not statistically different from saline-treated CEAE-animals (data not shown).

Ascension latency. In addition to the track analysis, we investigated an additional aspect of the animal's motor performance, namely a climbing task. Age-matched control animals were able to consistently perform the ascension task in less than 2 s. As illustrated in Fig. 2C, performance of placebo-treated CEAE-animals decreased substantially within the first two weeks. Subsequently, no recovery of impaired climbing performance was observed. Seventeen weeks following sensitization, 60% of

Fig. 2. Evaluation of the functional performance of CEAE-animals. Line with open circles represents the mean functional performance of CEAE-animals (○, $n = 25$) treated with 0.5 ml saline every 48 h. Line with closed circles represents the mean functional performance of the CEAE-animals (●, $n = 25$) treated with 75 μg/kg every 48 h $ACTH_{4-9}$ analog in 0.5 ml saline injected s.c. in the neck. Line with crosses represents mean motor performance of age-matched control animals (+, $n = 15$) treated with 0.5 ml saline injections every 48 h. (A) Walking pattern analysis. From walking tracks several distances, representing innervation of different muscle groups, can be measured, including: step length (SL), print length (PL), toe spreading (TS) and inter toe spreading (ITS). ITS and TS were converted into the toe spreading index[13] as shown in the figure.[14] PL of paralytical animals were omitted from analysis. (B) Effect of $ACTH_{4-9}$ analog treatment on mean toe spreading index in CEAE-animals. Analysis of variance for repeated measurements (all three treatment groups over week 2 to 17 pi): $F(57,2) = 9.45$, $P < 0.0005$. (C) Effect of $ACTH_{4-9}$ analog on median ascension performance in CEAE-animals. Peptide treatment did not differ from placebo-treated animals on any of the time points using a Mann–Whitney *U* Rank Sum tests. (D) Nocisensoric function in CEAE-animals under $ACTH_{4-9}$ analog treatment using a hot plate test. Lines represent the mean response latency to the thermal stimulus (maintained at 54°C). MANOVA (all three treatment groups over week 0 to 18 pi: $F(58,2) = 8.84$, $P < 0.0005$) followed by two-paired Student's *t*-tests (saline-treated vs peptide-treated CEAE-groups) week 10 pi: $P = 0.002$; week 18 pi: $P = 0.006$. *$P < 0.05$; **$P < 0.01$ comparing saline-treated vs peptide-treated CEAE animals.



$$TSI = ((TS_{experimental} - TS_{control}) / TS_{control}) + (IT_{experimental} - IT_{control}) / IT_{control})) * 100$$

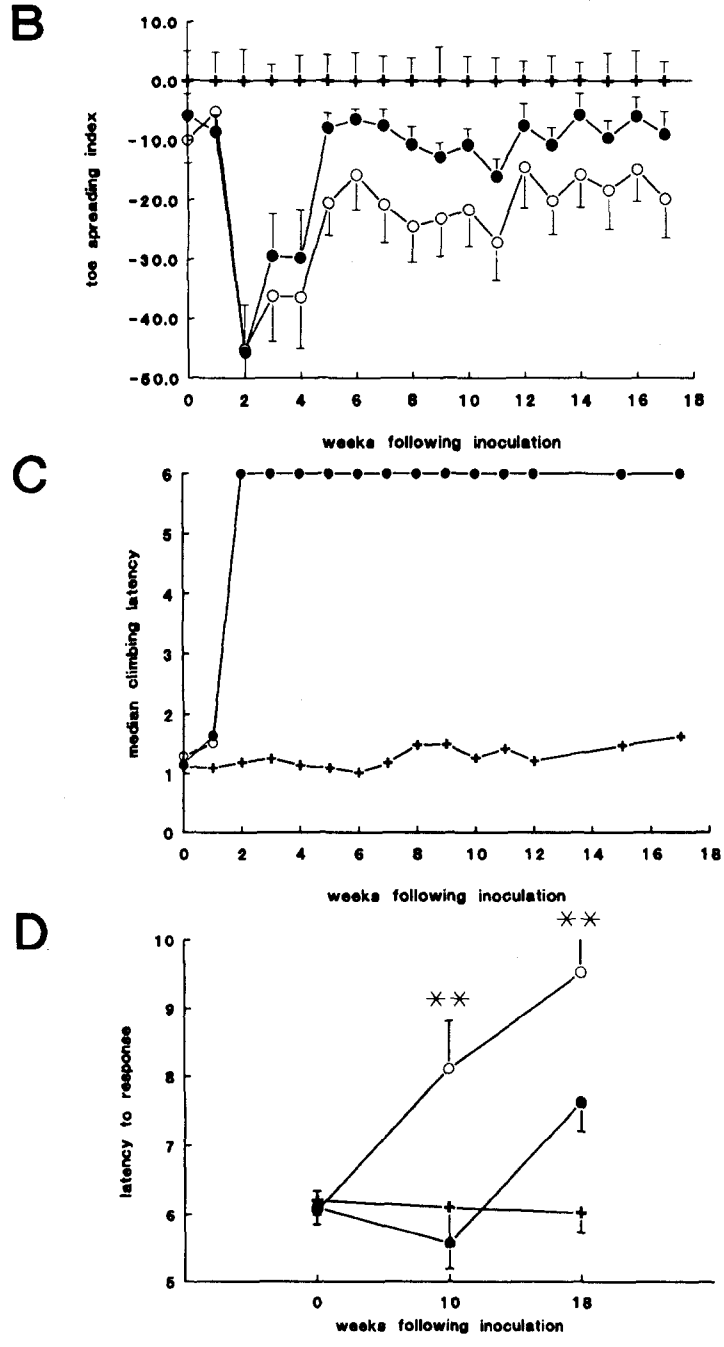


Fig. 2.

CEAE-animals still invariably fell from the platform, in contrast to the spontaneous recovery as demonstrated by clinical and other functional tests. Hence, no difference was detected between saline- and peptide-treated animals.

Hot plate response latency. Animals were subjected to a hot plate test at week 0 (prior to myelin challenge), 10 and 18 weeks pi. Hot plate performance led to consistent results with little variability. Control animals responded to the thermal stimulation consistently in 6.1 s (± 0.26) throughout the experiment. The induced demyelinating syndrome significantly prolonged the reaction time upon the noxious stimulus by 23 (10 weeks pi) and 58% (18 weeks pi; Fig. 2D).

Repeated $ACTH_{4-9}$ analog treatment substantially and significantly shortened the response latency of animals suffering from CEAE in the hot plate test (Fig. 2D; week 10 pi: $P = 0.002$; week 18 pi: $P = 0.006$).

Electrophysiological parameters

Corticomotor evoked potentials. A typical MEP recorded in a control animal is depicted in Fig. 3A. In the present experiment, the deterioration of CMEP due to the experimental demyelination, displayed two exacerbations characterized by prolonged onset latencies, temporal dispersion and in some cases there was an absence of response upon cortical stimulation.

Figure 3B illustrates the number of animals failing to produce an EMG response upon stimulation. This phenomenon was mainly confined to week 2 to 6 pi, corresponding to the primary attack as identified on motor performance. In case of a failure to elicit a CMEP response, we stimulated the sciatic nerve at the sciatic notch. In all cases, normal M- and H-reflexes (e.g., normal amplitude and onset latencies) could be registered from the small foot muscles upon stimulation. Therefore, the observed demyelination-induced total block appeared not to reside at the peripheral nerve nor in the structures involved in the segmental reflex. Furthermore, the CMEP failure correlated closely with motor disability, as quantified by the walking pattern analysis: on 28 occasions CEAE-animals were totally paralytic on the day of electrophysiology (saline- as well as peptide-treated animals). In 27 out of these 28 occasions, the paralytic rats failed to elicit a CMEP. Whereas, in 32 out of 38 cases of CMEP failure, the corresponding motor function was absent (paralysis) or severely impaired (i.e. TSI lower than -30). The afore mentioned two exacerbations in CMEP deterioration are clearly illustrated in Fig. 3C depicting the CMEP latency prolongation throughout the course of CEAE. Whereas the primary exacerbation was characterized by CMEP blocking and conduction slowing (Fig. 3B), the second exacerbation demonstrated mainly a delay in the mean onset latency. The second period of prolonged CMEP latencies commenced in week 7 pi and progressed gradually until cessation of the experiment at week 17 pi. Mean CMEP onset latency of age-matched control animals persisted at 6.30 ± 0.03 ms (mean $\pm$ S.E.M. over week 0 to week 17 pi) throughout the experiment.

Neurotrophic peptide treatment reduced the number of CEAE-animals failing to give a CMEP by 81% (Fig. 3B; Chi-square test (Area under the curve) placebo vs peptide-treated CEAE-animals, week 0 to 17 pi: $P < 0.0005$). Furthermore, the $ACTH_{4-9}$ analog substantially prevented the CEAE-associated delay of CMEP onset latencies (Fig. 3C). This amelioration was most explicit in the second deterioration of CMEP latencies e.g., week 7 to 17 pi (MANOVA (saline- vs peptide-treated EAE-animals; week 7 to 15 pi: $P = 0.023$).

Somatosensory-evoked potentials. SSEP traces characteristically showed three reproducible peaks (see Fig. 3A). In age-matched control animals, the cortical recorded SSEP presented a small negative deflection (N1) at 11.6 ± 0.1 ms (mean over week 0 to 17 pi). The subsequent positive (P2) and negative deflections in controls followed at 15.7 ± 0.2 ms and 22.8 ± 0.4 ms respectively. Control peak-to-peak N1–P1 and P1–N2 amplitudes remained respectively at 51 ± 1 and $85 \pm 1\ \mu V$ throughout the 17 weeks observation period.

As in the analysis of CMEP onset latencies, two exacerbations characterized by delayed SSEP peak latencies were encountered in the course of CEAE. Total loss of SSEPs, however, was not encountered in CEAE-animals. As illustrated in Fig. 4A, the first N1 peak latency delay commenced at week 3 pi, reaching maximal mean latencies at week 5 to 7 pi (N1 delay 35% at week 6 pi). Subsequently, the mean latency returned to normal values. The second phase of SSEP latency deterioration was found following week 9 pi. This prolongation of SSEP latencies was more severe than the primary phase and reached maximal values at week 13 to 15 pi (N1 delay 48 percent at week 13 pi). P1 and N2 SSEP peaks showed comparable prolongation of latencies (Fig. 4B and C).

After a period where peak-to-peak amplitudes varied in all treatment groups (Fig. 5A), by week 2 pi, stable amplitudes were reached in all treatment groups. In CEAE-animals, however, the amplitude decrease was more pronounced as comparable to age-matched controls. Mean N1–P1 amplitude of the control group was $51 \pm 1\ \mu V$, whereas the mean N1–P1 amplitude of placebo-treated CEAE-animals decreased to $25 \pm 2\ \mu V$ (mean over week 2 to 17 pi). No recovery of the low amplitudes in placebo-treated CEAE-rats was found during the 17 week observation period (Fig. 5A).

Upon $ACTH_{4-9}$ analog treatment, SSEP latencies were only slightly delayed during the first six weeks pi, and completely normalized in the remainder of the experiment (week 7 to 17 pi; Fig. 4A–C). The N1 latency of the peptide-treated group was significantly

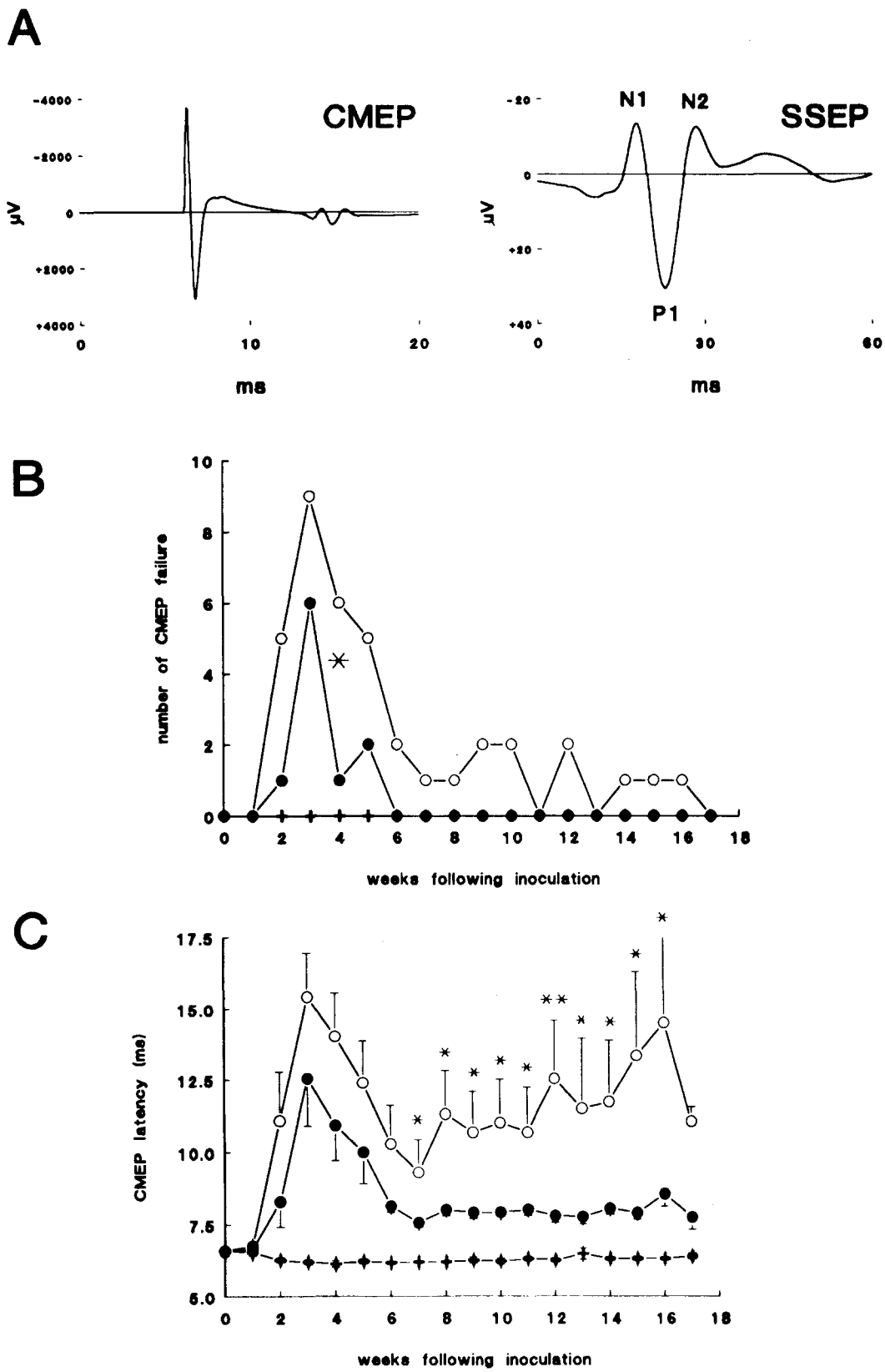


Fig. 3. Changes in CMEPs in CEAE-animals upon $ACTH_{4-9}$ peptide treatment. Line with open circles represents the saline-treated CEAE group (○, $n = 15$, 0.5 ml saline/48 h, s.c.). Line with closed circles represents the $ACTH_{4-9}$ analog-treated CEAE group (●, $n = 15$, 75 μg/kg every 48 h in 0.5 ml saline, s.c.). Line with crosses represents the age-matched control group (+, $n = 10$, 0.5 ml saline/48 h, s.c.). (A) Typical CMEP and SSEP signals recorded from respectively the small foot muscles by surface electrodes and the permanent electrode overlying the primary somatosensory cortex in an age-matched control animal (week 17 pi). (B) Absolute number of CEAE-animals failing to give a CMEP, i.e. total blocking of the corticomotoneuron pathways, in CEAE-animals. Chi-square test (total number of CMEP failures; week 0 to 17 pi; placebo-treated vs peptide-treated CEAE-animals): $P < 0.0005$; followed by supplemental Chi-square tests on the different time points. (C) Mean onset latency of CMEP in the different treatment groups. MANOVA (all three treatment groups; week 7 to 16 pi) $P = 0.006$; followed by supplemental t-tests (two-paired). $^{*}P < 0.05$; $^{**}P < 0.01$ comparing saline-treated vs peptide-treated CEAE-animals.

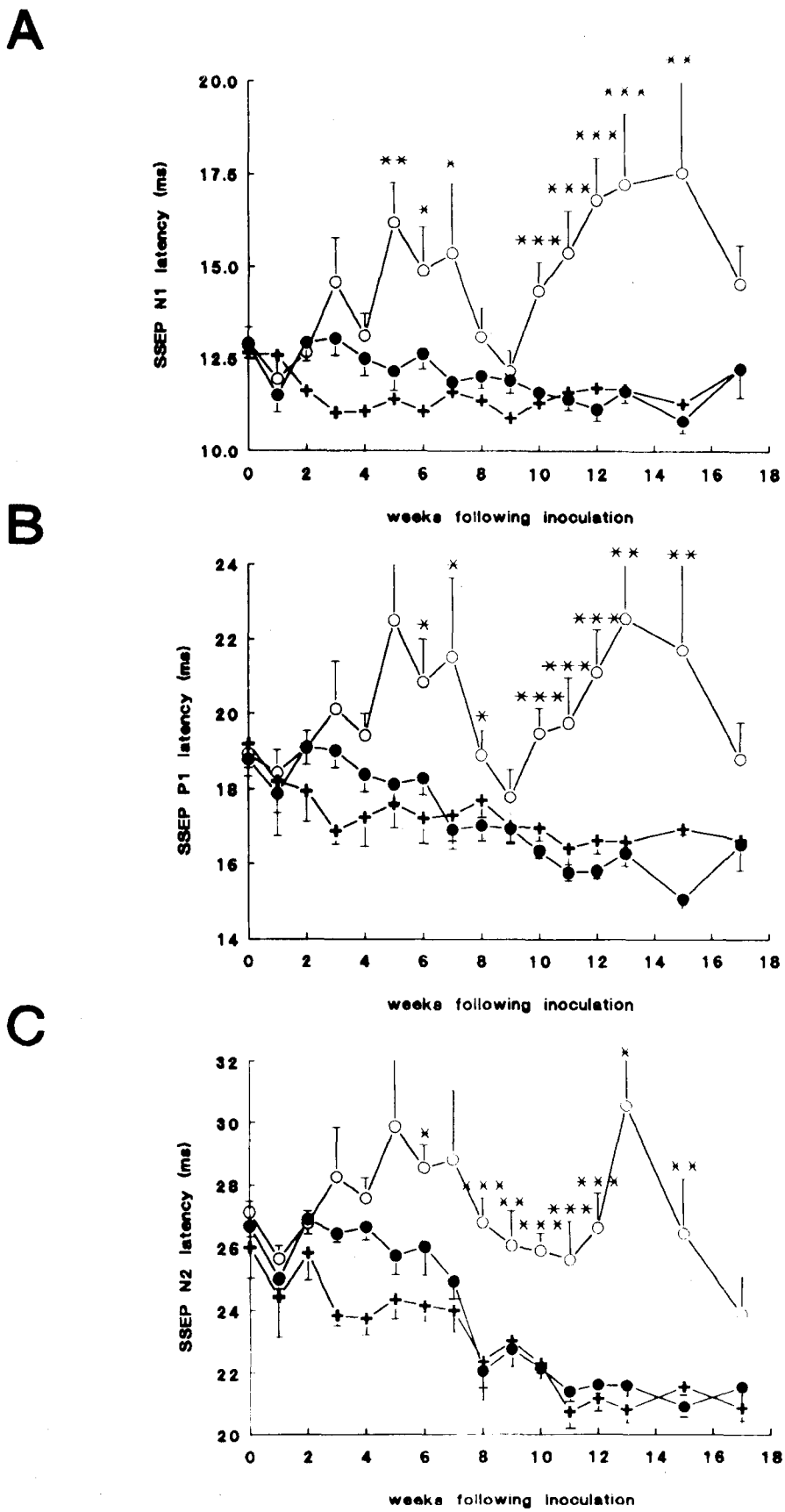


Fig. 4. Mean peak latency delay of averaged SSEP peak components (N1, P1 and N2) in CEAE-animals under neurotrophic peptide therapy. Line with open circles represents the saline-treated CEAE group (○, $n = 15$, 0.5 ml saline/48 h, s.c.). Line with closed circles represents the $ACTH_{4-9}$ analog-treated CEAE group (●, $n = 15$, 75 μg/kg every 48 h in 0.5 ml saline, s.c.). Line with crosses represents the age-matched, control group (+, $n = 10$, 0.5 ml saline/48 h, s.c.). The sciatic nerve was stimulated at the sciatic notch, while the subsequent SSEP was recorded from permanent cortical electrodes (contralateral to the stimulated site. (A) Mean N1 peak latency in the different treatment groups. (B) Mean P1 peak latency in CEAE and control groups. (C) Mean N2 peak latency in the different treatment groups. MANOVA (all three treatment groups; week 5 to 16 pi) N1: $P < 0.0005$; P1: $P < 0.0005$ and N2: $P = 0.003$; followed by supplemental t-tests (two-paired). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ comparing saline-treated vs peptide-treated animals.

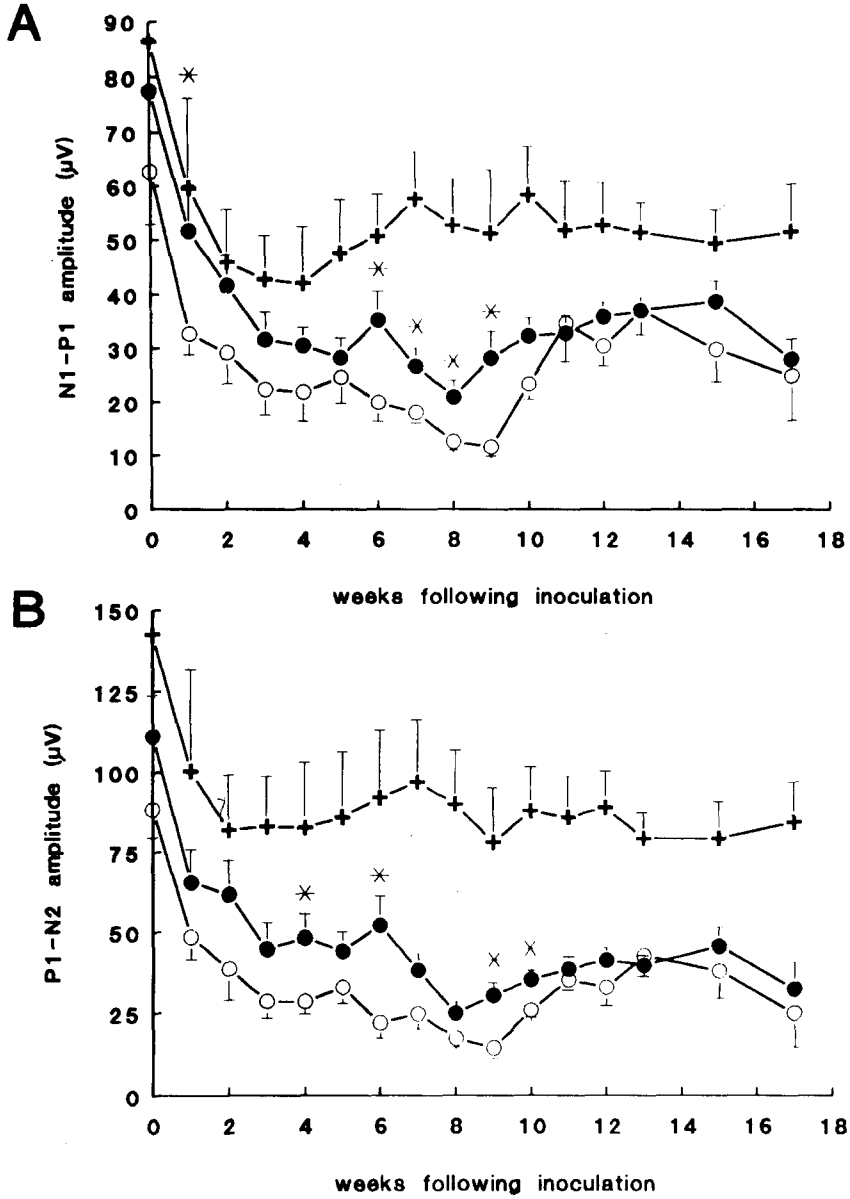


Fig. 5. Peak-to-peak amplitudes of the averaged SSEP trace in CEAE-animals. Line with open circles represents the saline-treated CEAE group (○, $n = 15$, 0.5 ml saline/48 h, s.c.). Line with closed circles represents the $ACTH_{4-9}$ analog-treated CEAE group (●, $n = 15$, 75 μg/kg every 48 h in 0.5 ml saline, s.c.). Line with crosses represents the age-matched, control group (+, $n = 10$, 0.5 ml saline/48 h, s.c.). (A) Mean N1–P1 peak-to-peak amplitude of averaged SSEP trace. (B) Mean P1–N2 peak-to-peak amplitude of averaged SSEP trace. MANOVA (all three treatment groups; week 3 to 16 pi) N1–P1 amplitude: $P = 0.044$ and P1–N2 amplitude: $P = 0.048$; followed by Students' t-tests (two-paired). $^*P < 0.05$ comparing saline-treated vs peptide-treated animals.

shorter during week 5 to 7 pi and week 10 to 15 pi from the saline-treated counterpart. Similarly, P1 latencies were significantly different from placebo-treated CEAE-animals during the interval week 5 to 15 pi (Fig. 4B), whereas N2 latencies were preserved during the period 6 to 15 weeks pi (Fig. 4C). In addition, peptide treatment significantly restored SSEP N1–P1 as well as P1–N2 peak amplitudes in CEAE (Fig. 5A and B; mean N1–P1 amplitude of peptide-treated animals week 2 to 17 pi: 33 μV ± 1; saline-treated animals 25 μV ± 2).

DISCUSSION

Demyelinating lesions are the histopathological hallmark of CEAE and are present throughout the neuroaxis, with predilection sites in the spinal cord and brain stem.[32,41,42] In fact, twice as many lesions are detected in the spinal cord as compared to cortical or subcortical areas. The present data suggest that neurotrophic peptide therapy with the $ACTH_{4-9}$ analog in CEAE results in clinical, functional and electrophysiological benefits:[17] the development of

CEAE-related neurological signs was markedly reduced upon application of the $ACTH_{4-9}$ analog, whereas the recovery of sensorimotor function was significantly facilitated. CEAE causes a biphasic deterioration of the conduction in the corticomotoneuron pathway characterized by a marked delay, or even an absence of the motor response. A similar biphasic deterioration was found in the SSEP latency delay, indicating the involvement of the dorsal columns. Upon $ACTH_{4-9}$ analog treatment, motor tract as well as sensory tract latencies were preserved. Furthermore, total blocking in motor tracts was substantially prevented and SSEP peak-to-peak amplitude decrement was restored in peptide-treated CEAE-animals throughout the 17 weeks of observation.

Clinical manifestations of chronic experimental allergic encephalomyelitis and functional tests

In the present study, the four different functional tests employed in this study were differentially affected by the demyelinating disorder and the neurotrophic $ACTH_{4-9}$ analog. The clinical manifestation of CEAE was characterized by a brief, transient period with severe signs followed by a remission period and a second more gradual phase of exacerbation lasting up to several months. The acute, primary exacerbation is caused mainly by immunological responses resulting in damage to the blood–brain barrier accompanied by parenchymal inflammatory infiltration that are reflected in the impairment of nerve conduction delay. The symptoms and functional deficits encountered in the second, chronic deterioration phase, however, originate from tissue damage.[29,38,41,50] Although all functional parameters deteriorated rapidly following week 2 pi, but the subsequent remission and relapse were not encountered in all parameters. The differences encountered in the evolution of the clinical scores and functional tests probably reflect that the various tests reflect the integrity of different nerve tracts that are affected differently by the CEAE pathological processes. The locomotor function as quantified by walking pattern analysis reflects the integrity of both motor and sensory tracts.[7,14] The hot plate performance reflects the pain sensory function mediated via thinly myelinated Aδ fibres in the spinothalamic tract and cortical processing of the noxious stimulus.[44,56]

The CMEP profile provides numerical data on the physiological integrity of the myelinated corticomotoneuron projections,[47,61,74] whereas the SSEP have been extensively used to test the integrity of the myelinated afferent fibres in the dorsal column.[33,57] The slowing of central nerve conduction velocity and conduction blocking of SSEP and CMEP in CEAE, as documented extensively in the literature,[10,11,16,35,54,66] originates from focal primary demyelination with preservation of denuded axons.[41] Furthermore, temporal dispersion of nerve action potentials due to focal demyelination can result in altered evoked cortical responses. In the present study, a marked delay of latency was found in both event related responses, indicating the involvement of both the sensory and motor tracts in CEAE. The total conduction block of CMEP, however, did not appear to reside in the peripheral nervous system, since the ability to elicit electromyographic M- and H-responses with normal latencies and amplitudes upon stimulation of the sciatic nerve in all animals with total motor conduction block was unaffected. Here, we report a deterioration in CMEP latency simultaneous with the deterioration of locomotor function (as quantified by TSI). Total CMEP conduction block in the first exacerbation phase coincided with paralysis or severe paraparesis, whereas severe slowing of CMEP latency, detected in the chronic exacerbation phase, related to poor performance in the walking pattern analysis test (i.e. TSI). These observations further establishes the diagnostic potential of CMEPs in quantifying the neurological deficit and predicting the ability to walk.

Effects of melanocortins in neuroregeneration

Various studies have indicated the efficacy of melanocortins and some of their synthetic fragments to facilitate axonal regeneration and recovery of sensory and motor function in the peripheral nervous system.[25,46,55] The beneficial effects have been shown under different experimental conditions including lesioning,[13] metabolically-induced neuropathy[6] and neurotoxin-induced neuropathy,[24,64] Recently, a profound protection was demonstrated against the deterioration of motor function and histological damage associated with peripheral primary demyelination in EAN.[18,19] In addition, the $ACTH_{4-9}$ analog suppressed the neurological symptoms in animals suffering from CEAE, whereas analysis of T2-weighted magnetic resonance images indicated that peptide treatment reduced the development of lesions in the cortex of Lewis rats suffering from CEAE.[17]

The facilitation of the recovery of sensorimotor functions may be accomplished by an effect of the $ACTH_{4-9}$ analog in enhancing oligodendrocyte survival, protection and stimulation of central myelin production. Several lines of evidence suggest that the melanocortins may stimulate the proliferation and differentiation of cultured astrocytes.[22,36,69,75] Hence, activated astrocytes may trigger remyelination by secretion of platelet-derived growth factor (PDGF) and protect glial cells by secretion of ciliary neurotrophic factor (CNTF).[43] Furthermore, $ACTH_{4-9}$ analog binding sites on glia cells have been identified by Dyer *et al.*[20] These studies designate glial cells as a possible target for melanocortin induced facilitation of functional recovery in demyelinating disorders such as EAN and CEAE. The melanocortin receptors identified so far, however, do not appear to bind specifically to the $ACTH_{4-9}$ analog.[1,21] Southern blot analysis suggests that the melanocortin receptor

family contains additional members. Thus, the melanocortin receptor(s) involved in the neurotrophic and myelinotrophic effects may be identified in the near future. Albeit, the mitogenic activity on astrocytes and the binding sites identified on glia, implicate the glial cells as possible target cells of the myelinoprotective properties of melanocortins in CEAE and EAN.

Alternatively, the $ACTH_{4-9}$ analog may have prevented axonal degeneration, stimulated neuronal outgrowth upon damage, or attenuated the immunological responses that led up to the primary demyelination. The clinical, functional and electrophysiological beneficial effects of the peptide was restricted to the chronic phase of CEAE (four to 17 weeks pi, congruent to the development of lesions as reported in literature.[41] However, the $ACTH_{4-9}$ analog treatment did not alter CEAE symptoms and motor deficits in the period of acute exacerbation (one to four weeks pi), which is mainly determined by immunological responses rather than by morphological damage.[29,38,41,50] Finally, the $ACTH_{4-9}$ analog lacks the 10–13 amino acid sequence (lysine-proline-valine) which is regarded as being essential to the anti-pyretic properties of α-melanocyte stimulating hormone.[8,59] Therefore, it is unlikely that the observed benefits were mediated by a general immunosuppressive effect as seen in $ACTH_{1-39}$ therapy.

Our studies suggest a protective rather than a neurotrophic effect of the ACTH peptide in CEAE. Magnetic resonance imaging in CEAE-animals revealed a significant reduction of the development of CNS lesions due to the $ACTH_{4-9}$ analog.[17] Here, peptide treatment resulted in partial amelioration of the loss of peak-to-peak SSEP amplitudes in combination with normalized latencies. Thus, the $ACTH_{4-9}$ analog apparently partially prevented or restored conduction block present in peptide-treated animals. The normalized latencies in peptide-treated CEAE-animals, in contrast to the severely delayed latencies in saline-treated CEAE-animals, indicate that the remaining myelinated fibres in peptide-treated animals were unaffected as compared to the saline-treated ones. This suggests a myelinoprotective action of the $ACTH_{4-9}$ analog rather than a trophic action in the afferent pathways. Similar conclusions were drawn in the peripheral demyelinating model EAN, resembling the human Guillain–Barré syndrome. Morphological analysis of the sural nerves derived from peptide-treated EAN-animals demonstrated a complete preservation of the myelinated fibre diameter distribution identical to those observed in sural nerve sections of age-matched controls.[18] A neurotrophic response would have resulted in enhanced regeneration and hence more small and intermediate size myelinated fibres as compared to age-matched controls.

CONCLUSION

The present study shows that a neurotrophic pharmacotherapy using the $ACTH_{4-9}$ analog suppressed CEAE-related clinical symptoms, improved motor and (noci)sensory function, and preserved sensory and motor latencies. Moreover, peptide treatment prevented total motor conduction block of CMEPs throughout the experiment. The remaining fibres appeared to be unaffected by the demyelinating disorder as indicated by normal SSEP and CMEP latencies. Recently, comparable effects were reported for insulin-like growth factor-1.[73] The beneficial clinical, histological and biochemical effects of insulin-like growth factor-1 appear to be mediated through a direct receptor-mediated myelinotropic response, a direct effect on the endothelial cells of the blood–brain barrier as well as an immunomodulative effect. These trophic peptide pharmacotherapies may provide a MS repair strategy by (myelino)protection against further exacerbation, advancement of remissions or support to graft embryogenic cells.[26]

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