European Journal of Pharmacology, 147 (1988) 351–357
Elsevier

EJP 50136

α-MSH and Org.2766 in peripheral nerve regeneration: different routes of delivery

Catharina E.E.M. Van der Zee, Jan H. Brakkee and Willem Hendrik Gispen *

Division of Molecular Neurobiology, Rudolf Magnus Institute for Pharmacology and Institute of Molecular Biology and Medical Biotechnology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands

Received 24 September 1987, revised MS received 4 November 1987, accepted 24 November 1987

The efficacy of melanocortins (α-MSH and an ACTH-(4-9) analog, Org.2766) in accelerating functional recovery from sciatic nerve damage following various types of subcutaneous and oral administration was assessed in the rat. Furthermore, the effectiveness of the local delivery of melanocortins to the site of injury was examined. An accelerated recovery was evident following subcutaneous constant delivery of Org.2766 from an osmotic mini-pump and from biodegradable polymere microspheres, and was as effective as repeated subcutaneous injections of α-MSH or Org.2766. Oral administration of Org.2766 was ineffective. Local application of Org.2766, achieved by wrapping a peptide-impregnated biodegradable gelatine foam matrix around the site of injury, facilitated recovery as well. The biodegradable microspheres and gelatine foam matrix may be of importance in eventual clinical use as effective vehicles for administration of melanocortins in the treatment of peripheral nerve damage.

Peripheral nerve regeneration; α-MSH; Org.2766; (Osmotic mini-pump, Biodegradable microspheres, Biodegradable gelatine foam matrix, Rat, Subcutaneous injections)

1. Introduction

ACTH- and α-MSH-like peptides and Org.2766, an ACTH-(4-9) analog, accelerate axonal regeneration following crushing of rat sciatic nerve as demonstrated by an increase in the number of outgrowing sprouts and by the facilitation of the return of sensorimotor function (Strand and Kung, 1980; Bijlsma et al., 1981; 1984; Edwards et al., 1984; Verhaagen et al., 1986; De Koning et al., 1986). Previous studies, in which melanocortins were administered systematically, indicated that a dose of 1 or 10 μg per rat, administered every 48 h, was required for the accelerated return of sensorimotor function. Doses of 0.1 or 30 μg were ineffective (Bijlsma et al., 1981; De Koning et al., 1986). The administration of peptide from immediately after the crush lesion until post-operation day 8 was as effective as treatment throughout the experimental period, indicating a critical period for α-MSH to facilitate functional recovery (Edwards et al., 1984). The most common route of administration of these peptides is by repeated subcutaneous (s.c.) injection into the neck. Recently, Dekker et al. (in press) reported that the delivery of Org.2766 from s.c. implanted osmotic mini-pumps and from biodegradable microspheres also accelerated functional recovery following peripheral nerve damage.

We now compare the effectiveness of melanocortins on the functional recovery from sciatic nerve damage in the rat following various types of s.c. and oral delivery. Furthermore, the effectiveness of local delivery at the site of injury was examined.

* To whom all correspondence should be addressed.

2. Materials and methods

2.1. Animals

Female rats of an inbred Wistar strain (TNO, Zeist, The Netherlands) weighing approximately 120-140 g were used. The animals were housed in Makrolon cages (5 rats/cage) and maintained on a 12 : 12 h light : dark cycle, with food and water ad libitum. The rats were randomized over experimental groups prior to surgery.

2.2. Surgery

A crush lesion was made in the right sciatic nerve, under Hypnorm anaesthesia (Duphar, Weesp, The Netherlands; 0.8 ml/kg body weight), with hemostatic forceps with a waffle-shaped inner side of the mouth (Crile, diamant, 15 cm) as described in detail by De Koning et al. (1986).

2.3. Peptides

Synthetic α-MSH, Org.2766 (the ACTH-(4-9) analog H-Met(O_2)-Glu-His-Phe-D-Lys-Phe-OH) and ([^{3}H]Phe^7)-Org.2766 (specific activity 17.2 mCi/μmol) were a gift from Organon International B.V. (Oss, The Netherlands).

2.4. S.c. peptide administration

S.c. injections of α-MSH or Org.2766, dissolved in 0.5 ml saline (0.9% NaCl), were placed in the neck region. The doses tested were 0.75, 7.5 and 75 μg/kg body weight (approximately 0.1, 1 and 10 μg/rat). The first injection was given immediately after the sciatic nerve was crushed and was repeated every 48 h. Control rats received 0.5 ml saline/48 h.

Osmotic ALZET 2002 mini-pumps (length 15 mm, volume 250 μl, pumping rate 18 μl/day; ALZA Corp., London, England) were implanted s.c. in the neck region immediately following the crush procedure. The pumps were filled with 70 μg α-MSH or 70 μg Org.2766 in 250 μl saline and gave a constant release of 5 μg/day. In another group the pump contained 1680 μg Org.2766 in 250 μl saline and gave a constant release of 5 μg/h. Control pumps were filled with saline. The pumps were removed under anaesthesia two weeks after implantation.

Biodegradable microspheres, consisting of a 1 : 1 copolymere of lactic and glycolic acid containing 3% (w/w) Org.2766, were obtained from Organon International B.V. (Oss, The Netherlands). Forty milligram of microspheres (peptide release was 40 μg/day for 14 days in vitro), were suspended in 0.5 ml of an isotonic mannitol solution containing 0.05 μg/ml benzalkonium chloride, and were injected s.c. immediately following surgery. Control rats received microspheres loaded with vehicle.

2.5. Oral administration

Org.2766 was dissolved in drinking water. Rats received either 5 or 25 mg Org.2766 in 25 ml water/rat per day. A fresh solution was made every day. Control rats received normal drinking water. Rats received 2 mg Org.2766/day in 1 ml tap water, administered orally via a stomach tube in another experiment. Treatment in both cases began immediately after the surgery and lasted throughout the experimental period.

2.6. Local application

Peptide was applied locally by implanting a biodegradable matrix around the site of the crush lesion. The matrix (Willospon® 10 × 10 × 1 mm, gelatine foam) was impregnated with peptide by soaking the matrix in 100 μl saline containing 20 μg Org.2766 at room temperature, for 5 min. The Org.2766 content of the matrix was monitored in another matrix that was impregnated at the same time by measuring the amount of ([^{3}H]Phe^7)-Org.2766 (specific activity 0.55 μCi/20 μg) that was absorbed in the matrix and the amount that remained in the impregnation fluid. Radioactivity was determined by liquid scintillation counting. The matrix used for implantation contained a total of 14 μg Org.2766. We attempted to remove the matrix at different times following implantation to determine the remaining amount of radioactive peptide. However, this was only possible up to 1 h after implantation, thereafter the matrix is degraded and cannot be quantitatively removed.

The peptide content was reduced to 50% after 5 min and after 1 h the peptide content was only 10% of the original amount.

2.7. Functional recovery

The return of sensorimotor function was measured by the reflex withdrawal from local noxious stimulation of the footsole. Testing was performed daily beginning 14 days after surgery. In the first experiments the test was performed as described by De Koning et al. (1986). In the last series of experiments the more sensitive version of this reflex withdrawal test as developed by De Koning and Gispen (1987) was used. The investigator performing the functional recovery tests was not aware of the treatment a given rat had received. The treatment code was partially broken at the end of the experiment to allow the analysis of data. The final treatment code was broken only after the analysis of the data was completed.

2.8. Data analysis

The data obtained with the procedure described by De Koning et al. (1986) were expressed as percentage of animals that showed full recovery on a given day, and group differences were analyzed with the Chi-square-test. The data obtained with the procedure described by De Koning and Gispen (1987) were expressed as the mean percentual recovery per day (±S.E.M.) and group differences were analyzed by an analysis of variance for repeated measures, followed by a supplemental t-test.

3. Results

3.1. Effect of repeated injections

Rats bearing a crush lesion in the sciatic nerve and receiving Org.2766 at a dose of 75 μg/kg body weight every other day (10 μg/rat per 48 h) showed an accelerated recovery of function as tested in the foot reflex withdrawal test (fig. 1A and B). In fig. 2A the effect of different doses of Org.2766 and α-MSH on the number of animals that were fully recovered on day 17 following surgery is shown. Both peptides were active at doses of 7.5 and 75 μg/kg body weight whereas 0.75 μg/kg α-MSH was ineffective in this treatment regime.

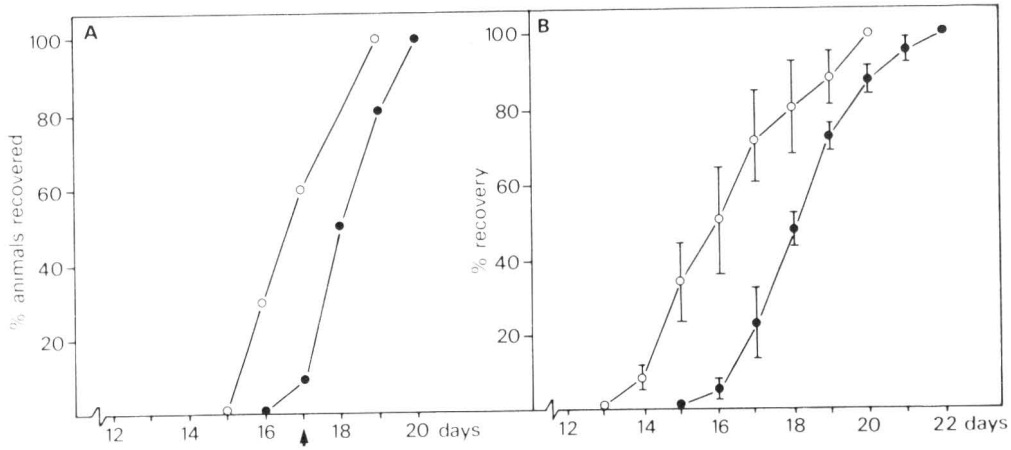

Fig. 1. The effect of the peptide Org.2766 (75 μg/kg per 48 h s.c.) on functional recovery following crush lesions of the sciatic nerve. ○ peptide (n = 10); ● saline (n = 10). (A) The percentage of animals that recovered was determined by measuring the reflex withdrawal reaction after local footsole stimulation of 0.1 mA. At day 17 (↑) a higher percentage peptide-treated animals had recovered, compared to saline-treated animals ($P < 0.025$, Chi-square-test). (B) The percentage recovery was determined by the reflex withdrawal reaction after local footsole stimulation with six different intensities ranging from 0.1-0.6 mA. The mean percentage recovery ± S.E.M. is plotted. Rats receiving peptide showed an enhanced recovery compared to rats receiving saline ($F(1,18) = 11.13$; $P < 0.005$).

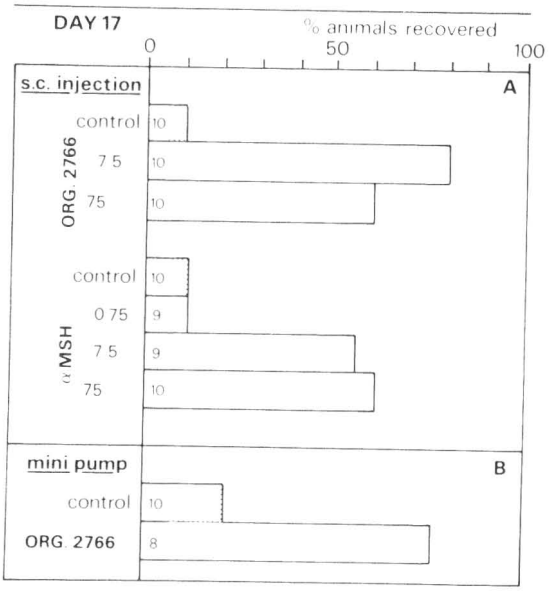

Fig. 2. (A) The effect of s.c. delivery of the peptides Org.2766 and α-MSH. Percentage of animals that recovered by day 17 following a crush lesion of the sciatic nerve, was determined by measuring the reflex withdrawal reaction after a local footsole stimulation of 0.1 mA. The number of animals per group is indicated in the bar. S.c. injection with Org.2766 (7.5 or 75 μg/kg per 48 h) showed a significant higher percentage of recovered animals compared to saline injection ($P < 0.005$ and $P < 0.025$ resp., Chi-square-test). The effects of a s.c. injection of α-MSH (7.5 or 75 μg/kg per 48 h) differed significantly from those following the s.c. injection of saline ($P < 0.01$ and $P < 0.025$ respectively, Chi-square-test). S.c. injection of 0.75 α-MSH μg/kg per 48 h did not have any effect. (B) The constant release of Org.2766 (120 μg/day = 5 μg/h) from a s.c. implanted osmotic mini-pump resulted in a higher percentage of recovery in the peptide-treated group, compared to the saline-treated group ($P < 0.005$, Chi-square-test).

3.2. *Effect of mini-pump delivery*

Rats bearing a crush lesion in the sciatic nerve and receiving peptides from an osmotic mini-pump were tested for foot reflex withdrawal. No effect on the return of sensorimotor function was observed at a peptide (α-MSH or Org.2766) release of 5 μg/rat per day (data not shown). However, a significant acceleration of functional recovery following a crush lesion was demonstrated at a (higher) dose level of 5 μg Org.2766/rat per h (fig. 2B). Extrapolation of this dose level to a daily amount of peptide per kg body weight yielded an approximate dose of 900 μg/kg body weight per day.

3.3. *Effect of microspheres injections*

A single s.c. injection with biodegradable microspheres, causing a constant release of 40 μg Org.2766/day, resulted in an accelerated recovery of sensorimotor function following a crush lesion of the sciatic nerve (fig. 3). Extrapolation of the dose to the amount of peptide per kg rat/day yielded an approximate dose of 300 μg/kg body weight per day.

3.4. *Effect of oral administration*

Oral administration of Org.2766 via the drinking water which rats consumed ad libitum up to 25 ml per day did not facilitate the functional recovery following sciatic nerve damage. The doses tested were 5 or 25 mg Org.2766/rat per day or approximately 40 or 200 mg/kg per day (data not shown). Likewise, the forced consumption of 2 mg Org.2766 (15 mg/kg body weight) in 1 ml via

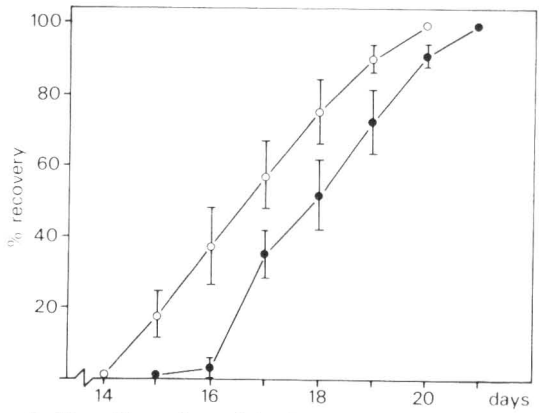

Fig. 3. The effect of s.c. injection of biodegradable microspheres containing Org.2766. The mean percentage recovery ± S.E.M. of rats is plotted against the number of days after the crush lesion of the sciatic nerve. The percentage recovery was determined by the reflex withdrawal reaction after local footsole stimulations of 0.1-0.6 mA. Rats receiving one injection of peptide-loaded biodegradable microspheres, resulting in a release of Org.2766 40 μg/day per rat (○, n = 10), showed an accelerated recovery compared to rats injected with vehicle-loaded microspheres (●, n = 9) ($F(1,17) = 8.68$; $P < 0.01$).

a stomach tube had no detectable effect on the return of function of the damaged sciatic nerve (data not shown).

3.5. *Effect of local administration*

Three groups of rats were used, all bearing a crush lesion at the right sciatic nerve. The biodegradable matrix (containing approximately 10-14 μg Org.2766) was wrapped around the site of the crush lesion in one group. In the second group the site of injury was covered with a saline impregnated matrix. In the third group no matrix was placed around the site of injury, but a peptide impregnated matrix (containing 10-14 μg Org. 2766) was wrapped around the contralateral uncrushed sciatic nerve at approximately the same level as the crush lesion in the right sciatic nerve. The local delivery of Org.2766 at the site of injury facilitated the return of function of the damaged sciatic nerve (fig. 4). Placement of a peptide impregnated matrix on the contralateral nerve was ineffective.

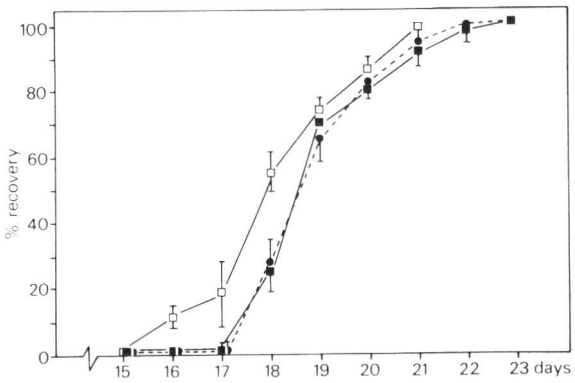

Fig. 4. The effect of Org.2766 delivered locally by a biodegradable gelatine foam matrix. The percentage recovery of rats following a crush lesion of the sciatic nerve was determined by measuring the reflex withdrawal reaction after local footsole stimulations of 0.1-0.6 mA. The values were averaged and the mean percentage recovery ± S.E.M. is plotted against the days following crush lesion. Rats of which the right sciatic crushed nerve was surrounded by a biodegradable matrix loaded with 14 μg Org.2766 (□, n = 17) showed an enhanced recovery compared to rats with a saline-loaded matrix. (●, n = 16), or compared to rats with a peptide-loaded matrix around the contralateral uncrushed sciatic nerve (■, n = 10). Analysis of variance showed significance at $P < 0.036$ ($F(1,31) = 2.70$ and $P < 0.006$ ($F(1,25) = 4.26$ respectively. There was no significant difference between rats with a saline-loaded matrix around the crushed nerve and rats with a peptide-loaded matrix around the contralateral uncrushed nerve.

4. Discussion

Melanocortins and some of their synthetic fragments and analogs facilitate axonal regeneration in the peripheral nervous system following mechanical damage (Strand and Kung, 1980; Bijlsma et al., 1981; 1983a, b; De Koning et al., 1986; Dekker, 1987; for a recent review see Gispen et al., 1987). The data collectively show that peptide treatment not only shortens the recovery period but also improves the quality of the recovered sciatic nerve function (De Koning and Gispen, 1987).

Despite the fact that the beneficial effect of these peptides on nerve repair has been demonstrated at the histological, neurophysiological and functional level, their precise mechanism of action is largely unknown. It would appear that the peptides do not facilitate axon elongation but rather enhance the initial sprouting response (De Koning et al., 1986; Verhaagen et al., 1987; Dekker, 1987). As the molecular events in post-lesion neural plasticity share many aspects of neural developmental processes (Gispen et al., 1987), it is of interest to note that melanocortins have been shown to enhance neuronal maturation, brain development and motor coordination in pre- and perinatal rodents (Van der Helm-Hylkema and De Wied, 1976; Swaab and Boer, 1978; Strand and Smith, 1986; Azmitia and De Kloet, 1987). Whether the melanocortin accelerates repair of damaged peripheral nerve because it mimics the action of a naturally occurring peptide factor, produced in degenerating peripheral nerve, is still under study (Edwards et al., 1984; Edwards and Gispen, 1985).

The present study confirms the effectiveness of repeated subcutaneous injections of relatively low doses of Org.2766 or α-MSH (fig. 1 and 2). We have also shown that other forms of s.c. administration were effective, i.e. the sustained delivery of peptide from osmotic mini-pumps or biodegradable microspheres. The latter form of peptide ad-

ministration could be of great importance for the eventual clinical use of the peptide in counteracting e.g. the neurotoxicity of cytostaticum treatment (De Koning et al., 1987), since only a few subcutaneous injections are needed and the microspheres containing the peptide are completely biodegraded (see also Dekker et al., in press).

Oral administration was effective in certain paradigms used to test CNS effects of Org.2766: for instance in the counteraction of a CO_2-induced amnesia of a conditioned passive avoidance response (Rigter et al., 1976) or in the facilitation of the behavioral recovery following lesions in the nucleus accumbens of rats (Wolterink and Van Ree, 1986). Our data do not show an effect of orally administered Org.2766 on the functional recovery from peripheral nerve damage as reported by Dekker et al. (in press). However, Dekker (1987) described a small effect of orally administered peptide on the time that the rats took to withdraw their paw from a noxious stimulus. We have used doses 1000 to 5000 times greater than the dose that is effective following s.c. administration as recommended and used by the cited authors who reported on the CNS activity of the peptide following its oral administration.

Only 2-3% of the exogenous α-MSH administered by s.c. injection was absorbed ($T_{1/2} = 5.6 \pm 2.8$ min, Wright and Wilson, 1983). The plasma concentrations of Org.2766 after subcutaneous injection were 5% (peak plasma level) and 2.5-3% half an hour later. The elimination phase of Org.2766 was bi-exponential with an initial half-life for intact peptide of 4 min. Relatively high and stable plasma levels of intact Org.2766 were obtained shortly after s.c. injection reflecting metabolic stability (Verhoef and Witter, 1976).

It is extremely difficult to estimate the actual amount of intact peptide in the circulation following administration by the different routes used in this study. It is even more difficult to estimate the final amount that reached the site of action. These parameters will undoubtedly vary among the different manners of application. Nonetheless, the present data and those obtained earlier collectively suggest that a peak amount of peptide must be available in a short period following the crush. Higher doses are therefore required for the gradual release from a mini-pump or microspheres than those needed to be effective after a bolus injection.

Local application of the peptide has been shown to facilitate functional recovery following nerve damage (Edwards et al., 1986). However, α-MSH was absorbed to a matrix that was not biodegradable in that study. In the present paper we report that recovery was facilitated by wrapping an Org.2766-impregnated biodegradable gelatine foam matrix around the site of injury. Although biodegradation of the matrix might in principle also release the peptide into tissue fluid and ultimately into the circulation, the amount that is released with time is apparently so low that the contralateral placement of the matrix was ineffective. As the gelatine matrix is rapidly degraded and will certainly not result in a sustained release of peptide over weeks, the effect of this form of administration may at best be compared to the effect of a single injection. De Koning and Gispen (1987) reported that one s.c. injection of 10 μg Org.2766/rat (75 μg/kg) did not facilitate the return of function whereas Verhaagen et al. (1987) demonstrated that a single subcutaneous injection of 1 μg Org.2766/rat (7.5 μg/kg) increased the number of newly formed sprouts at the distal border of the crush. The effectiveness of the locally applied biodegradable matrix could be of use in the neurosurgical and/or plastic-surgical treatment of peripheral nerve damage.

Acknowledgements

The authors are grateful to Dr. H. De Nijs from Organon International B.V., Oss, The Netherlands, for his gift of the microsphere preparation. This study was supported in part by a grant from the Prinses Beatrix Fonds.

References

Azmitia, E.C. and E.R. De Kloet, 1987, ACTH neuropeptide stimulation of serotonergic neuronal maturation in tissue culture: modulation by hippocampal cells, in: Neuropeptides and brain function, eds. E.R. De Kloet, V. Wiegant and D. De Wied, Progr. Brain Res., Vol. 72, p. 311.

Bijlsma, W.A., F.G.I. Jennekens, P. Schotman and W.H. Gispen, 1981, Effects of corticotrophin (ACTH) on recovery of sensorimotor function in the rat: structure-activity study, European J. Pharmacol. 76, 73.

Bijlsma, W.A., F.G.I. Jennekens, P. Schotman and W.H. Gispen, 1983a, Stimulation by ACTH(4-10) of nerve fiber regeneration following sciatic nerve crush, Muscle Nerve 6, 104.

Bijlsma, W.A., F.G.I. Jennekens, P. Schotman and W.H. Gispen, 1984, Neurotrophic factors and regeneration in the peripheral nervous system, Psychoneuroendocrinology 9, 199.

Bijlsma, W.A., P. Schotman, F.G.I. Jennekens, W.H. Gispen and D. De Wied, 1983b, The enhanced recovery of sensorimotor function in rats is related to the melanotropic moiety of ACTH/MSH neuropeptides, European J. Pharmacol. 92, 231.

Dekker, A., 1987, Enhancement of peripheral nerve regeneration in the rat by a previous nerve injury and by treatment with neuropeptides, PhD Thesis, State University of Utrecht, The Netherlands.

Dekker, A.J.A.M., M.M. Princen, H. De Nijs, L.G.J. De Leede and C.L.E. Broekkamp, Acceleration of recovery from sciatic nerve damage by the ACTH(4-9) analog Org.2766: different routes of administration, Peptides (in press).

De Koning, P., J.H. Brakkee and W.H. Gispen, 1986, Methods for producing a reproducible crush in the sciatic and tibial nerve of the rat and rapid and precise testing of return of sensory function. Beneficial effects of melanocortins, J. Neurol. Sci. 74, 237.

De Koning, P. and W.H. Gispen, 1987, Org.2766 improves functional and electrophysiological aspects of regenerating sciatic nerve in the rat, Peptides 8, 415.

De Koning, P., J.P. Neijt, F.G.I. Jennekens and W.H. Gispen, 1987, Org.2766 protects from cisplatin-induced neurotoxicity in rats, Exp. Neurol. 97, 746.

Edwards, P.M. and W.H. Gispen, 1985, Melanocortin peptides and neural plasticity, in: Senile Dementia of the Altzheimer type, Springer Verlag, Berlin, eds. J. Traber and W.H. Gispen, p. 231.

Edwards, P.M., R.R.F. Kuiters, G.J. Boer and W.H. Gispen, 1986, Recovery from peripheral nerve transection is accelerated by local application of α-MSH by means of Accurel polypropylene tubes, J. Neurol. Sci. 74, 171.

Edwards, P.M., C.E.E.M. Van der Zee, J. Verhaagen, P. Schotman, F.G.I. Jennekens and W.H. Gispen, 1984, Evidence that the neurotrophic actions of α-MSH may derive from its ability to mimic the actions of a peptide formed in degenerating nerve stumps, J. Neurol. Sci. 64, 333.

Gispen, W.H., P. De Koning, R.R.F. Kuiters, C.E.E.M. Van der Zee and J. Verhaagen, 1987, On the neurotrophic properties of melanocortins, in: Neuropeptides and Brain Function, eds. E.R. De Kloet, V. Wiegant and D. De Wied, Progr. Brain Res., Vol. 72, p. 319.

Rigter, H., R. Janssens-Elbertse and H. Van Riezen, 1976, Reversal of amnesia by an orally active ACTH(4-9) analog Org.2766, Pharmacol. Biochem. Behav. 5(1), 53.

Strand, F.L. and T.T. Kung, 1980, ACTH accelerates recovery of neuromuscular function following crushing of peripheral nerve, Peptides 1, 135.

Strand, F.L. and C.M. Smith, 1986, LPH, ACTH, MSH and motor systems, in: Neuropeptides and Behavior, Vol. 1, eds. D. De Wied, W.H. Gispen and Tj.B. Van Wimersma Greidanus (Pergamon Press, Oxford) p. 245.

Swaab, D.F. and G.J. Boer, 1978, The fetal brain and intrauterine growth, Postgrad. Med. J., 54, 63.

Van der Helm-Hylkema, H. and D. De Wied, 1976, Effect of neonatally injected ACTH and ACTH analogs on eye-opening of the rat, Life Sci. 18, 1099.

Verhaagen, J., P.M. Edwards, F.G.I. Jennekens, P. Schotman and W.H. Gispen, 1986, α-Melanocyte-stimulating hormone stimulates the outgrowth of myelinated fibers after peripheral nerve crush, Exp. Neurol. 92, 451.

Verhaagen, J., P.M. Edwards, F.G.I. Jennekens, P. Schotman and W.H. Gispen, 1987, Early effect of an ACTH(4-9) analog (Org.2766) on regenerative sprouting demonstrated by the use of neurofilament-binding antibodies isolated from a serum raised by α-MSH immunization, Brain Res. 404, 142.

Verhoef, J. and A. Witter, 1976, In vivo fate of a behaviorally active ACTH(4-9) analog in rats after systemic administration, Pharm. Biochem. Behav. 4, 583.

Wolterink, G. and J.M. Van Ree, 1986, Org.2766 accelerates functional recovery of impaired motor activity due to lesions in the nucleus accumbens in rats. Abstract for meeting: 'Neuropeptides and brain function', May 28-30, Utrecht, The Netherlands.

Wright, A. and J.F. Wilson, 1983, Absorption of α-MSH from subcutaneous and intraperitoneal sites in the rat, Peptides 4, 5.