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Evidence that the Neurotrophic Actions of α-MSH may Derive from its Ability to Mimick the Actions of a Peptide formed in Degenerating Nerve Stumps

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SUMMARY

The ability of α-MSH to facilitate the recovery of sensorimotor nerve function following crush lesion is restricted to a critical period following such a lesion. This period coincided with the initiation of sprouting and the disappearance of the 150 kD neurofilament protein from the degenerating distal stump of the nerve.

Degenerating nerve contains a factor that is active in a bioassay system for MSH. This factor could not be detected in control nerves.

The hypothesis is forwarded that a neurotrophic factor known to be present in degenerating nerve stumps is an α-MSH-like peptide formed by the breakdown of the 150 kD neurofilament protein.

Key words: *α-MSH – Nerve regeneration – Neurofilament protein – Trophic factors*

INTRODUCTION

Following a lesion to a nerve fibre, there is a switch in the function of that nerve from a non-growing state to one of active growth and sprout formation. The critical

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signals initiating this activity are still unknown in spite of many years of active research efforts. Studies on the problem have followed two basic approaches. Firstly, the ability of a considerable number of chemically-defined agents to modulate nerve outgrowth has been described (reviewed by Bijlsma et al. 1984). Secondly, the role of various biological components, such as denervated target tissues and degenerating nerves have also been indicated (reviewed by Willard and Skene 1982). Apart from some evidence for the role of NGF in synaptic nerve regeneration, it has been impossible to bridge the gap between these two approaches and connect a chemically-defined agent with a biological process resulting from the nerve injury.

The trophic properties of peptides related to ACTH and MSH and the ability of these peptides to facilitate the regeneration of peripheral nerve has been reported by this laboratory and others (Swaab et al. 1976; Strand and Kung 1980; Strand and Smith 1980; Bijlsma et al. 1981). The effect on regeneration is apparent in an accelerated functional recovery and an increase in the number of regenerating nerve fibres at early times after the lesion (Bijlsma et al. 1983a,b,c). Recently, this neurotrophic activity has been shown to be more closely linked to the melanotrophic than to the corticotrophic activity of these peptides; α-MSH is the most potent peptide that has been found to date (Bijlsma et al. 1983c). In this paper, we report that there is a critical sensitive period for the effectiveness of α-MSH treatment following the crush lesion.

The results of the sensitive period study led us to investigate whether there was a connection between the ability of α-MSH and that of degenerating nerve tissue (Politis and Spencer 1983) to stimulate sprouting. This possibility seemed all the more compelling in view of the recent report (Dräger et al. 1983) that neurofilament protein contains an immunologically-recognised α-MSH-like portion. This protein has been shown to break down in the early stages of the degenerative process (Bignami et al. 1981; Soifer et al. 1981). We therefore investigated the possible presence of an α-MSH-like principle in the degenerating nerves during the period of 150 kD neurofilament protein breakdown and α-MSH sensitivity.

MATERIALS AND METHODS

Chemicals

Synthetic α-MSH was a gift from Organon Int. BV (Oss, NL). All other chemicals were of analytical grade.

Animals and treatments

Female Wistar rats 120–160 g body weight were used throughout. Crush lesion of the sciatic nerve and marking of the position of the crush was performed as described by Bijlsma et al. (1983b). For the measurement of functional recovery, a unilateral lesion was performed, for all other experiments, the lesion was bilateral. Sham operations were performed on control rats taking care to avoid any trauma to the sciatic nerve. α-MSH (10 μg/rat/0.5 ml saline) was injected subcutaneously on alternate days.

Measurement of functional recovery

Return of sensorimotor function was judged by the return of reflex withdrawal of the hind paw from a hot-air stimulus as described by Bijlsma et al. (1981). Tests were performed on alternate days, prior to dosing with peptide, by an investigator who did not know which treatment the rat had received. Data were analysed by analysis of variance, followed by a supplemental *t*-test.

PAGE separation of proteins in nerves

The sciatic nerve and the posterior tibial branch were dissected out free from connective tissue and placed straight on graph paper, taking care to avoid stretching. The nerve and paper were then frozen in liquid nitrogen and stored at $-70\,^{\circ}C$ until further processing. The nerves were cut into 5-mm lengths and each piece was homogenised in 50 μl ice-cold buffer (Tris/HCl, 50 mM; KCl, 25 mM; $MgCl_2$, 2 mM; pH 7.6) in a Potter-type homogeniser using 15 up-and-down strokes and a 0.10 mm clearance. The protein content of the homogenates was measured according to Lowry et al. (1951). Subsequent electrophoretic and staining procedures were carried out as described by Zwiers et al. (1980) except that the samples were heated for 10 min at $60\,^{\circ}C$ in the presence of SDS prior to application to the 11% polyacrylamide–SDS gels. Densitometric scanning of photographic prints of stained gels was performed using a Zeiss M4-QII spectrophotometer set at 550 nm.

Xenopus laevis test for MSH-like activity

A portion of the sciatic nerve and its branches was dissected from the distal limit of the crush lesion (or the equivalent place in sham-operated animals) for a distance of approximately 2 cm distally. Left and right nerves from each rat were combined and minced in 1.5 ml ice-cold buffer (Leibovitz's medium L15 diluted 1 : 1 with water containing bovine serum albumin, 0.1%; $CaCl_2$, 2 mM; penicillin, 100 units/ml; streptomycin, 100 μg/ml; amphotericin B, 125 μg/ml; adjusted to 200 mOsm with NaCl; pH 7.6). The tissue and buffer were then transferred to a Potter-type homogeniser and the nerve homogenised by 30 up-and-down strokes. The homogenate was centrifuged at $1000 \times g$ for 10 min in a Sorvall SS34 rotor and the resultant supernate (S1) centrifuged for 10 min at $10000 \times g$ in a Janetzki centrifuge to obtain the $10000 \times g$ supernate (S10). All homogenisation and centrifugation procedures were carried out at 0–4 °C. The assay for melanotropic activity was carried out using the in vitro assay on *Xenopus laevis* tail-fin pieces as described by De Graan et al. (1983), applying the melanophore index method for quantification of the response. The assay was carried out by an investigator who did not know the nature of the samples tested.

RESULTS

Critical period for the ability of α-MSH to facilitate function recovery from crush lesion

Continuous treatment of rats with α-MSH (10 μg/rat, on alternate days) accelerated the return of sensorimotor function following crush lesion (Fig. 1). Administration of the peptide from immediately after the crush lesion until Day 8 was equally effective

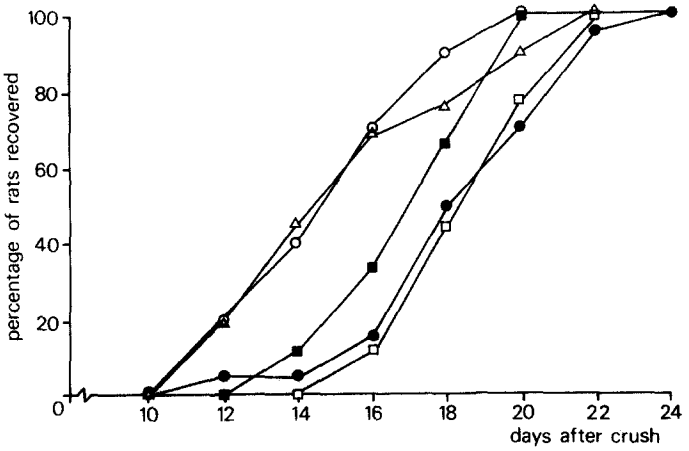

Fig. 1. The effect of α-MSH on the recovery of sensorimotor function following crush lesion of the sciatic nerve was measured as described in the Methods. Rats were injected every 48 h subcutaneously with either saline or α-MSH (10 μg/rat). Analysis of variance indicated that there was a significant difference ($F = 7.7$, $_{4/67}$; $P < 0.05$, supplemental t-test, two-tailed) between the group treated continuously with saline (●, n = 20) and the groups treated with α-MSH continuously (○, n = 10), on Days 0–8 (Δ, n = 20) or on Days 0–6 (■, n = 9), but not the group treated on Days 10–18 (□, n = 9). There is also no significant difference between the groups treated with α-MSH on Days 0–8 and continuously.

as continuous treatment. In both cases, the time taken for recovery of 50% of the rats was shortened from 18 to 15 days. Treatment from immediately after the lesion until Day 6 also significantly accelerated the repair process. However, if the commencement of the 8-day treatment regime was delayed until 10 days surgery, α-MSH was totally ineffective in reducing the time required for functional recovery (Fig. 1).

Decrease in 150 kD protein content of nerves undergoing degeneration

The profile of proteins present in segments of the sciatic nerve proximal and distal to the crush lesion, 4 days after the injury, is shown in Fig. 2. The quantity of protein present in several bands is altered in the degenerating fragment. Decreases in the major band at approximately 32 kD and the protein bands at approximately 150 and 200 kD [identified by Soifer et al. (1983) as the peripheral nerve myelin protein, P_0, and the high-molecular weight protein components of neurofilaments, respectively] were observed. Densitometric scanning of the protein band at 150 kD indicated that, at 4 days following the crush lesion, approximately 70% of the protein had disappeared. By 8 days (data not shown), the 150 kD protein band was reduced by 90% and remained at this low level during the subsequent 17 days. The protein profile in all segments of nerves from sham-operated rats was indistinguishable from that in the proximal portion of the lesioned nerves.

Presence of α-MSH-like activity in degenerating nerve stumps

The results described above indicated that if a functionally-significant, α-MSH-like factor is formed by the breakdown of neurofilament protein in degenerating nerves,

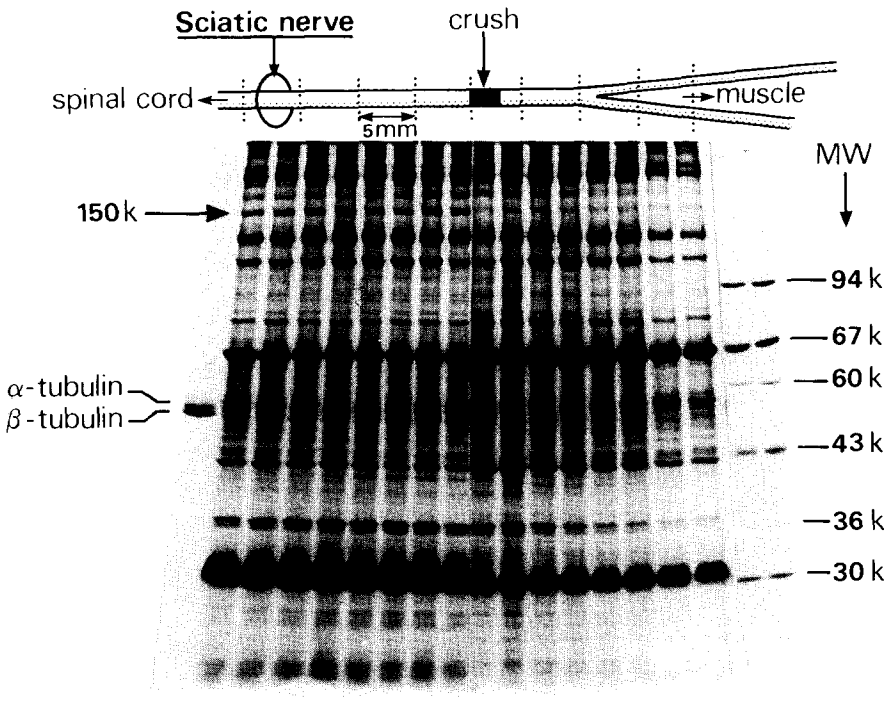

Fig. 2. Profiles of proteins present in 5-mm segments of sciatic nerve 4 days after crush lesion were obtained by SDS–PAGE as described in the Methods. Duplicate samples, containing 25 μg total protein, are shown below the corresponding segments of the sciatic nerve to indicate the position relative to the lesion. The location in the gel of purified tubulin and of a mixture of standard proteins of the molecular weights indicated, are also shown. The arrow marks the position of the band that is presumed to be the 150 kD neurofilament protein.

it should be detectable in degenerating nerves between 4 and 8 days after the nerve injury. The melanotropic activity present in extracts of degenerating nerves was therefore assayed in 6 days following the crush lesion. Both S1 and S10 supernatant fractions from degenerating nerves contained melanotropic activity in the in vitro bioassay system (Fig. 3). No activity could be detected in S1 or S10 extracts from control nerves (data for S1 only shown in Fig. 3). The activity in the nerve extracts from degenerating nerve homogenates was equivalent to between 0.5 and 1.0 ng/ml of α-MSH.

DISCUSSION

In this experiment, we have shown that three phenomena occur during the first 8 days following a crush of the sciatic nerve: (i) α-MSH is able to alter the time course of the recovery process, measured by an earlier return of sensorimotor function many days later; (ii) a protein with an apparent molecular weight of 150 kD, similar to the neurofilament protein identified by Soifer et al. (1983) disappears from the degenerating portion of the nerve, and (iii) a melanotropic factor appears in the degenerating portion of the nerve.

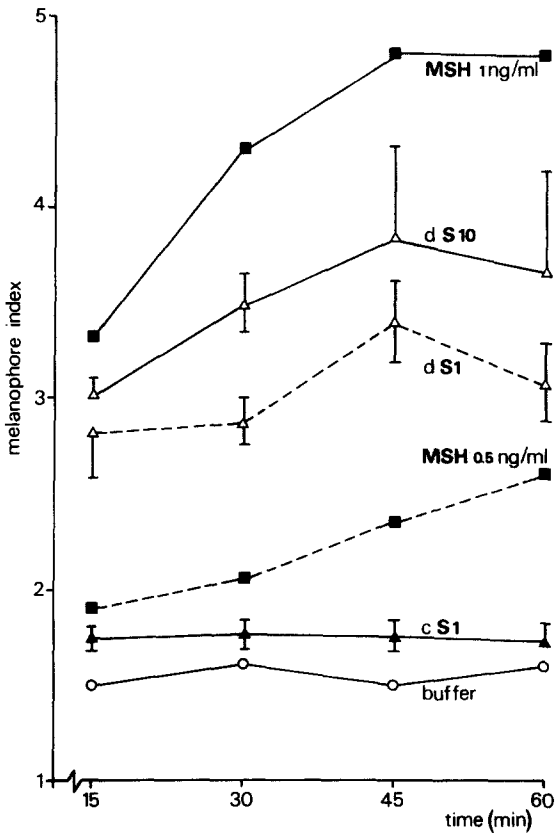

Fig. 3. Melanotropic activity was assayed on pieces of *Xenopus* tadpole tail-fin pieces as described in the Methods. The mean index for pieces obtained from 3 separate tadpoles during 1 h incubation with test substances was taken for each assay. The values plotted for buffer and α-MSH are the mean values obtained from two assays, carried out at the same time as tissue samples. The values plotted for control nerve (cS1) and for degenerating nerve S1 (dS1) and S10 (dS10) are the mean ± SEM of assays on nerves from 5 rats taken 6 days after sham operation or crush lesion, respectively.

The precise location of the critical period of sensitivity of functional recovery of α-MSH is under further investigation, taking into account the possible influence of the total dose given as well as the dosing regime. The data presented in this paper indicate that α-MSH effect is entirely and exclusively mediated during the initial 8 days after crush. This is the period during which the 150 kD protein disappears from degenerating nerve tissue. Clearly other proteins also change during the degenerating process, as has also been shown by other workers using different experimental procedures (Soifer et al. 1983). However, we believe the 150 kD protein to be of particular significance because the relative mobility in the gel indicates a similarity to the neurofilament protein that contains α-MSH-immunoreactivity (Dräger et al. 1983).

Taken together, these findings led us to the hypothesis that there may be a link between the beneficial effect of pharmacological treatment with α-MSH and the occurrence of a natural trophic substance formed in degenerating nerves (Politis and Spencer

1983). We propose that this link could be the formation of an α-MSH-like substance by biochemical processes, such as breakdown of the 150 kD neurofilament protein, occuring in degenerating nerve tissue. Indeed, we have shown that MSH-like activity, as measured by the ability to stimulate dispersion of melanosomes in *Xenopus laevis* tail-fins, is detectable in extracts of degenerating but not intact nerve extracts. Agents other than MSH are capable of causing melanosome dispersion in the *Xenopus* tail-fin and experiments are now in progress to demonstrate whether the observed activity is indeed the result of MSH receptor activation.

This putative connection between the therapeutic effects of α-MSH treatment and the neurotrophic actions of degenerating nerve tissue (Politis and Spencer 1983) may provide the first link between the pharmacological and the physiological approaches to the problem of the regulation of nerve regeneration.

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