Peptides, Vol. 18, No. 7, pp. 1015–1021, 1997

0196-9781/97 $17.00 + .00

ELSEVIER

PII S0196-9781(97)00043-0

Trophic Effects of Melanotropin-Potentiating Factor (MPF) on Cultures of Cells of the Central Nervous System

D. B. OWEN,* J. S. MORLEY,‡[1] D. M. ENSOR,* Y. S. ALLEN† AND J. B. MILES‡

*Department of Evolutionary & Environmental Biology, and †Department of Human Anatomy & Cell Biology, University of Liverpool, PO Box 147, Liverpool L69 3BX, UK
‡University Department of Neurological Science, % Pain Research Institute, Rice Lane, Liverpool L9 1AE, UK

Received 27 November 1996; Accepted 12 February 1997

OWEN, D. B., J. S. MORLEY, D. M. ENSOR, Y. S. ALLEN AND J. B. MILES. *Trophic effects of melanotropin-potentiating factor (MPF) on cultures of cells of the central nervous system.* PEPTIDES **18**(7) 1015–1021, 1997.—MPF is a tetrapeptide (structure Lys-Lys-Gly-Glu) that elicits a variety of neurotrophic effects in vivo consistent with a role in neuronal regeneration. In support of this role, we now show that MPF stimulates the proliferation of cultured astrocytes and neurite outgrowth from cultures of neocortical cholinergic and mesenchephalic dopaminergic neurons. The dose–response relationships are biphasic (''bell shaped''), maximal responses being obtained with 10^{-6} *M* concentrations of MPF. MPF and nerve growth factor seem to act on different receptors, because their effects on cholinergic neurons are synergistic. © 1997 Elsevier Science Inc.

Melanotropin-potentiating factor (MPF) NGF Neurotrophic effects Astrocytes Neurons

MELANOTROPIN-POTENTIATING Factor (MPF) is the *C*-terminal tetrapeptide of human β-lipotropin, of structure Lys-Lys-Gly-Glu (3). It initiates limb regeneration in hypophysectomized newts (10) and increases dopamine output from cultures of rat adrenal medullary strips (4). A metabolically stabilized analogue of MPF, of structure Ac-Lys-D-Lys-Sar-Glu (11), has profound long-term neurotrophic effects in rats with unilateral lesions of the nigrostriatal pathway; starting 6 weeks after injection of the analogue, the characteristic turning behavior of such rats is reduced (5), and there is a reversal of the mitochondrial and other cytological damage to substantia nigra neurons (9), which is mainly of dopaminergic neurons (2).

The present investigation was undertaken to ascertain if these neurotrophic effects are also seen in cultures of isolated rat brain neurons, and if the effect of MPF is mediated by astrocytes or by a direct action on neurons. Cholinergic neuron-enriched cells from the basal forebrain region, dopaminergic neuron-enriched cells from the ventral mesencephalon region of the fetuses of 14-day pregnant rats, and astrocytes from the ventral neocortical region of rat neonates were employed. The effects of 10^{-8} to 10^{-4} *M* concentrations of MPF on these cells were examined, and possible interactions of the effects of MPF and nerve growth factor (NGF) on cholinergic neurons were investigated.

METHOD

MPF and the analogue D-Lys-Lys-Gly-Glu were prepared as previously described (11) and were assessed as >95% pure by high performance liquid chromatography and amino acid analysis of enzymic digests. Recombinant human NGF was obtained from Genentech, Inc. All experiments took place within the confines of a laminar flow sterile cabinet with usual sterilization precautions. Sterile multiwell dishes (Nunclon), plastic tissue culture vessels (Nunclon), or glass coverslips were coated with either collagen (type 1 from rat tail, Sigma) or poly-L-lysine (MW 70–150,000, Sigma). Trypsin solution (0.05%) was prepared immediately prior to use by dilution of a 0.5% solution (trypsin EDTA, Gibco) (1 ml) with Basal Medium Eagle (BME) (9 ml) containing 25 m*M* HEPES W/Earles salts (Flow Labs). Trypsin inhibitor solutions (0.05% and 0.1%) were prepared immediately prior to use by appropriate dilution of a 0.5% solution [dehydrated form of the trypsin inhibitor (Sigma), in sterile water] with BME containing 25 m*M* HEPES W/Earles salts and 0.04 mg/ml of deoxyribonuclease (Sigma). Astrocyte culture medium was Dulbecco's Modified Eagles Medium (DMEM) (Gibco) containing 10% fetal calf serum and 1% penicillin-streptomycin solution (Sigma). Chemically Defined Medium (CDM) was a mixture of DMEM (75 ml) and Ham's

[1] Requests for reprints should be addressed to Dr. J. S. Morley, University Department of Neuroscience, % Pain Research Institute, Rice Lane, Liverpool L9 1AE, UK.

F12 (Flow Labs) (25 ml) containing 100 μg/ml transferrin (Sigma), 60 μM putrescine (Sigma), 5 μg/ml insulin (Sigma), 20 nM progesterone (Sigma), 30 nM sodium selenite (Sigma), 1 mM sodium pyruvate (Sigma), 5% fetal calf serum (Gibco), and penicillin-streptomycin (1 ml of Sigma solution per 100 ml). Cells were removed from culture substrates with 0.1% trypsin solution [prepared by diluting a 0.5% solution with pH 7.4–7.6 Hanks Balanced Salt Solution (Ca^{2+} and Mg^{2+} free, Sigma)].

Astrocyte Cultures

Neonatal rats were sacrificed by decapitation and the ventral neocortical region of the brain was removed and placed in BME. Sections of the neocortex were then incubated for 20 min at 37°C with freshly filtered (millipore) 0.05% trpsin solution (1 ml). The supernatant was decanted off, 0.1% trypsin inhibitor solution (1 ml) was added, and the mixture was triturated 20 times, and then centrifuged for 3 min at 1000 rpm. The trypsin inhibitor treatment, trituration, and centrifugation were repeated, and then residual cells were suspended in astrocyte culture medium (2–3 ml). The cell suspension was then either (i) plated down (50 μl per coverslip) on coated coverslips with 500 μl culture medium in a 24-well multidish, or (ii) placed (1 ml) in 25 or 100 ml Nunclon tissue/cell culture flasks containing, respectively, 5 or 12 ml of culture medium. The cultures were then incubated (5% CO_2) at 37°C for 1–2 h). Cells were removed from the substrate and repeatedly subcultured until predominantly astrocytes could be seen in microscopic examination. Optical nerve astrocyte cultures were prepared similarly from optical nerve astrocytes retrieved from neonatal rats.

Dopaminergic Neuron-Enriched Cultures

Fourteen-day pregnant rats were sacrificed by IP injection of pentobarbitone sodium BP, and the embryonic sac was exposed via a vertical slit through the abdominal wall. The sac was opened and the crown rump length of the embryo was measured to determine the exact age. After removal of the amniotic sac and the placenta, the fetuses were collected and placed in BME. After peeling off skin and the cranium, the fetal brain was positioned to provide a good view of the ventral mesencephalon, and all meningeal tissue was carefully removed. The ventral mesencephalon tissue was dissected out, placed in BME, repeatedly triturated for 5-min periods at 37°C, then centrifuged for 3 min at 1000 rpm. The residual cells were suspended in CDM, plated down (50 μl per coverslip) on collagen-coated coverslips with 500 μl CDM in a 24-well multidish, then incubated (10% CO_2) at 37°C for 1–2 h.

Cholinergic Neuron-Enriched Cultures

Fetal brain was collected, as described above, from 14-day pregnant rats, and basal forebrain (E15) tissue was dissected out and processed by the procedure described for preparing astrocyte cultures, except that after the trypsin inhibitor treatment the residual cells were suspended in CDM, and CDM was used in the subsequent incubations.

Astrocyte-Free Neuron-Enriched Cultures

After the appropriate neuron-enriched culture had been growing for 48 h in CDM, the medium was removed, and replaced with one containing 10^{-5} M cytosine arabinoside [Ara-C][3]. The incubation was continued for a further 24 h, then the [Ara-C][3]-containing medium was removed and replaced by the appropriate [Ara-C][3]-free medium. The absence of astrocytes and other glial cells was confirmed by microscopic examination.

Effect of MPF on Astrocyte Cultures

Pure astrocytes were allowed to grow to confluence in polylysine- or collagen-coated 200 ml Nunclon flasks (the time required was usually 2 weeks). The cells were removed from the substrate using trypsin, suspended in astrocyte culture medium, and incubated (10% CO_2) at 37°C until growth recommenced (usually 1–3 days). The appropriate concentration of MPF was added, the mixture was incubated for 24 h, then [^{3}H]thymidine (to provide 1 μCi thymidine/100 μl) was added and the incubation was continued for a further 24 h. The cells were removed by trypsinization, spun down at 1000 rpm for 5 min, then, after removal of the supernatant, they were treated with Scintran tissue solubilizer, left to lyse for 2–3 h, and then transfered to scintillation vials. The thymidine content of both control and treated cells was then measured using a Packard β-scintillation counter. In some experiments the incubations were continued for 14 days, the medium being replaced on days 3, 7, and 10 either with fresh medium (control) or with fresh medium containing the appropriate concentration of MPF or other agent; in these cases, the [^{3}H]thymidine was added on day 14.

The Effect of MPF and Other Agents on Neuron Cultures

Unless otherwise stated, the appropriate culture in CDM was grown for 11 days, alone (control) or in the presence of the appropriate concentration of MPF, NGF, mixtures of NPF + NGF, or D-Lys-Lys-Gly-Glu, replacing the medium with fresh CDM every 3 days. In some experiments (described in the text as ''multiple dosing'') the cultures were grown for 14 days, and the medium was replaced on days 4, 7, and 10 by fresh CDM (control) or with CDM containing the same concentration of MPF or other agent (treated). Neurons (usually 60) were then selected randomly and viewed on a video screen linked with a microscope. The total neurite outgrowth from each selected neuron was then carefully traced onto a transparent sheet. Using a digitizing pen, the total neurite outgrowth in micrometers (μm) for each neuron was determined, and the mean total neurite outgrowth per control or treatment was calculated.

Statistical Analysis of Results

Unless otherwise stated, results were compared using the unpaired Student's t-test.

RESULTS

Astrocytes

Using 1–3-day cultures of neocortical astrocytes grown on DMEM in collagen-coated vessels, single doses of MPF at 10^{-4}, 10^{-6}, and 10^{-8} M concentrations caused increases, compared with controls (no MPF), in [^{3}H]thymidine uptake, measured as disintegrations per minute (dpm) after correction for quenching (Table 1, Fig. 1). The effect was maximal with 10^{-6} M MPF, so this concentration was used in subsequent experiments. In 16 experiments, highly significant, but variable, increases were seen, the mean increase being 271% ($p < 0.4 \times 10^{-7}$) (Table 1). The mean increase in 14 experiments where the culturing was extended to 12 days, and the culture medium was removed and replaced by fresh medium containing the same concentration (10^{-6} M) of MPF on days 1, 3, 7, and 10 of the culturing, was 197% ($p < 0.0001$) (Table 1). Significant increases were also observed when neonatal opical nerve astrocytes were used, or when the astrocytes were grown in CDM (Table 2).

TABLE 1

THE EFFECT OF MPF ON THE GROWTH OF NEOCORTICAL ASTROCYTES GROWN ON DMEM IN COLLAGEN-COATED VESSELS

MPF Conc. (*M*)	Control (dpm ± SE)	Treated (dpm ± SE)	Increase (dpm ± SE)	*p* (Sign Test)
10^{-6}	23860 ± 8351*	57307 ± 20057*	33447 ± 1170* (271%)	$<0.4 \times 10^{-7}$
10^{-6}†	13148 ± 3353‡	29173 ± 7439‡	16025 ± 4086‡ (197%)	<0.0001
10^{-4}	24572	28754	4182 (17%)	
10^{-6}	24572	39029	14457 (59%)	
10^{-8}	24572	34696	10124 (41%)	

Cell growth is represented by [^{3}H]thymidine levels given as disintegrations per minute (dpm) after correction for quenching.

* Mean of 16 experiments.

† In these experiments the culture medium was removed and replaced by fresh medium containing MPF at the same concentration (10^{-6} *M*) on days 3, 7, and 10 (''multiple dosing'').

‡ Mean of 14 experiments. DMEM = Dulbeccos Modified Eagles Medium containing 10% foetal calf serum and 1% penicillin-streptomycin solution. The final three entries give the results of a single experiment in which three different molarities (M) of MPF were compared.

Cholinergic Neurons

The effect of three different concentrations of MPF on cholinergic-enriched neurons that had been freed of astrocytes by treatment with [Ara-C]^3 is shown in Table 3. At an MPF concentration of 10^{-6} *M*, there was a 69% increase in neurite outgrowth of the cultured neurons; the increase was less (53%) at a concentration of 10^{-8} M, and only 7% at a concentration of 10^{-4} *M*. In six repeat experiments using 10^{-6} *M* MPF, increases in neurite outgrowth were always observed but the increases were variable (mean 30.3%) (Table 3, Fig. 1).

The effect of four different concentrations of MPF on cholinergic-enriched neurons that had not been treated with [Ara-C]^3 is shown in Table 4. In two sets of experiments, the effect of MPF was again maximal (increase in neurite outgrowth, 58% in one experiment, and 51% in the other) at a concentration of 10^{-6} *M*, and reduced to 25% or 21% at 10^{-5} *M*, 32% or 16% at 10^{-7} *M*, and 14% or −2% at 10^{-8} *M*. In five repeat experiments using 10^{-6} *M* MPF, increases in neurite outgrowth were always observed, but the increases were variable (mean 40.4%) (Table 4). No significant increase in the effect was seen when multiple doses of MPF (see the Method section for definition) were given.

When a biologically inactive analogue of MPF of structure D-Lys-Lys-Gly-Glu (11) was used in these experiments at a concentration of 10^{-6} *M*, the results were not significantly different from those of controls. Thus, using [Ara-C]^3-treated neurons, the mean neurite length was 201.2 ± 22 μm and that in the control was 199.0 ± 36 μm; and using neurons that had not been treated with [Ara-C]^3, the mean neurite length was 181.2 ± 39 μm and that in the control was 185.9 ± 41 μm.

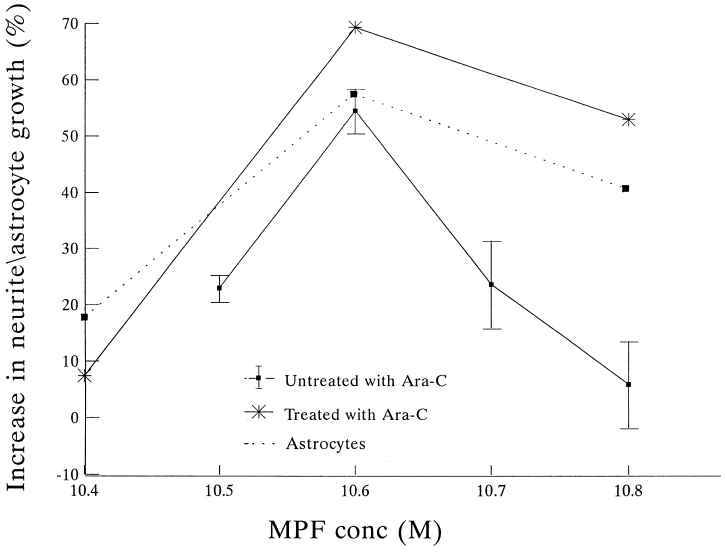

FIG. 1. Biphasic response of basal forebrain cholinergic neurons (treated or untreated with [Ara-C]^3) and astrocytes to varying concentrations of MPF. Data from Tables 1, 3, and 4. Note that the results with astrocytes and neurons treated with [Ara-C]^3 are from single experiments.

TABLE 2

THE INFLUENCE OF MEDIA AND ASTROCYTE SOURCE ON THE EFFECT OF MPF (10^{-6} *M*) ON ASTROCYTE PROLIFERATION

Astrocyte Source	Medium	Control (dpm ± SE)	MPF-Treated (dpm ± SE)	Increase (dpm ± SE)	*p* (vs. control)
Neocortical	CDM	8930 ± 1696	10807 ± 2053	1878 ± 357 (21.0%)	<0.0005
	DMEM	8295 ± 2157	8834 ± 2297	539 ± 140 (6.5%)	NS
Optical nerve	CDM	4163 ± 1166	4673 ± 1308	510 ± 143 (12.2%)	<0.01
	DMEM	3367 ± 1010	3932 ± 1180	565 ± 169 (16.8%)	<0.01

Cell growth is represented by [^{3}H]thymidine levels given as disintegrations per minute (dpm) after correction for quenching (all means of five experiments). CDM, chemically defined medium; DMEM, Dulbecco's Modified Eagles Medium; NS, not significant. See the Method section for full descriptions of media. The optical nerve astrocytes were retrieved from neonatal rats and processed as described in the Method section for neocortical astrocytes. Neocortical CDM vs. DMEM, $p < 0.0005$; optical CDM vs. DMEM, NS.

The effect of astrocytes or lymphocytes on these effects of MPF is shown in Table 5. In these experiments, [Ara-C]^3-treated cholinergic-enriched neurons were grown either on CDM, astrocyte beds, or lymphocyte medium, alone or in the presence of 10^{-6} *M* MPF. Neurite outgrowth was less in astrocyte beds or lymphocyte medium than in CDM. MPF caused significantly increased outgrowth of 28% or 48% in two experiments when it was added to the astrocyte medium, but no significant effect when it was added to the lymphocyte medium.

Table 6 shows the effects of MPF, NGF, and mixtures of MPF + NGF on cholinergic-enriched neurons that had previously been untreated or treated with [Ara-C]^3. Respectively, 10^{-6} *M* MPF caused 41% or 47% increases in neurite outgrowth, whereas 10^{-9} *M* NGF caused 83% or 54% increases; however, a mixture of 10^{-6} *M* MPF and 10^{-9} *M* NGF caused 105% or 77% increases.

Dopaminergic Neurons

The effect of MPF on the growth of dopaminergic-enriched neurons grown on collagen that had previously been treated with [Ara-C]^3 is given in Table 7. In three experiments, 10^{-6} *M* MPF caused significant 28–62% (mean 41.5%) increases in neurite outgrowth.

DISCUSSION

Our results show that MPF, a simple tetrapeptide is a potent stimulator of the proliferation of pure astrocytes from two distinct regions (the cortex and optical nerve) of the rat brain (Tables 1 and 2). The maximum effect was seen with an MPF concentration of 10^{-6} *M*, the effects of weaker and stronger concentrations of MPF being less (Table 1). In all replicate experiments, 10^{-6} *M* MPF caused statistically significant increases (mean 271%) in the growth of neocortical astrocytes.

The astrocyte cultures we used underwent many passages to exclude other neurone populations, and microscopic examinations suggested the presence of only pure astrocytes. Nevertheless, there was wide variation in their responses to MPF. We suggest that the most likely explanation of the variable results is (a) that the starting number of cells in different replicates differed appreciably (supported in a few cases by haemocytometer work), and/or (b) that, in different replicates, the astrocytes were in different stages of cell division, and that MPF acts as a trigger of growth only at certain stages of division. Multiple applications of MPF in these experiments had little effect on the degree of proliferation (Table 1).

MPF also caused highly significant increases in neurite out-

TABLE 3

EFFECT OF MPF ON NEURITE OUTGROWTH OF CHOLINERGIC ENRICHED NEURONS CULTURED (CDM) ON COLLAGEN, AND TREATED WITH [ARA-C]^3

MPF Conc. (*M*)	*N*	Mean Length (μm)	Control (μm)	*p* (vs. Control)	Increase (μm)
10^{-6}	63	158.1 ± 43	93.3 ± 47	<0.0005	64.8 (69%)
	60	26.4 ± .8	21.8 ± 6	<0.0005	4.6 (21%)
	120	293.6 ± 72	233.2 ± 58	<0.0005	60.4 (26%)
	60	364.3 ± 83	247.6 ± 47	<0.0005	116.7 (47%)
	30	243.1 ± 64	210.4 ± 59	<0.025	32.7 (16%)
	30	226.8 ± 68	201.3 ± 39	<0.05	25.5 (13%)
	120	317.6 ± 72	264.7 ± 66	<0.0005	52.9 (20%)* mean (30.3%)
10^{-4}	65	100.8 ± 43	93.3 ± 47	NS	7.5 (7%)
10^{-6}	63	158.1 ± 43	93.3 ± 47	<0.0005	64.8 (69%)
10^{-8}	62	142.8 ± 36	93.3 ± 47	<0.0005	49.5 (53%)

N = total number of neurites selected in determination of mean length; CDM, chemically defined medium. The final three entries give the results in one experiment in which three different molarities (*M*) of MPF were used.

* Multiple dosing.

TABLE 4

EFFECT OF MPF ON NEURITE OUTGROWTH OF CHOLINERGIC NEURONS CULTURED (CDM) ON COLLAGEN AND UNTREATED WITH [ARA-C]^3

MPF Conc. (*M*)	*N*	Mean Length (μm)	Control (μm)	*p* (vs. Control)	Increase (μm)
10^{-6}	60	265.1 ± 74	170.3 ± 37	<0.0005	94.8 (56%)*
	60	254.0 ± 76	164.8 ± 39	<0.0005	89.2 (54%)*
	30	229.1 ± 56	144.6 ± 33	<0.0005	58.5 (58%)
	60	228.1 ± 73	151.4 ± 49	<0.0005	76.7 (51%)
	60	273.9 ± 86	194.8 ± 67	<0.0005	79.1 (41%)
	60	336.3 ± 101	307.3 ± 88	<0.1	29.0 (9%)
	31	243.1 ± 64	213.5 ± 58	<0.05	29.6 (14%)
					mean 40.4%
10^{-5}	30	181.4 ± 34	144.6 ± 33	<0.0005	36.8 (25%)
	60	182.6 ± 41	151.4 ± 49	<0.001	31.2 (21%)
10^{-6}	30	229.1 ± 56	144.6 ± 33	<0.0005	58.5 (58%)
	60	228.1 ± 73	151.4 ± 49	<0.0005	76.7 (51%)
10^{-7}	30	190.2 ± 37	144.6 ± 33	<0.0005	45.6 (32%)
	60	175.4 ± 46	151.4 ± 49	<0.025	24.0 (16%)
10^{-8}	30	164.3 ± 35	144.6 ± 33	<0.025	19.7 (14%)
	60	148.2 ± 32	151.4 ± 49	NS	−2.8 (−2%)

N = total number of neurites selected in determination of mean length; CDM, chemically defined medium. The bottom entries give the results of two experiments when different molarities of MPF were used; significant statistical differences were found (sign test) in each experiment when the results at 10^{-6} *M* were compared with those at 10^{-5}, M, 10^{-7}, and 10^{-8} *M* ($p < 0.0005$), and the results with 10^{-5} and 10^{-7}, M were compared with that at 10^{-8} *M* ($p < 0.001$).

* Multiple dosing* v single dosing, NS.

growth from cultures of cholinergic enriched neurons (Tables 3 and 4). The method of preparation of the cultures only permits use of the term ''cholinergic-enriched neurons,'' but astrocytes were excluded in some of the experiments by [Ara-C]^3 treatment (Table 3). With both [Ara-C]^3-treated and untreated neurons, a maximum response was obtained with 10^{-6} *M* MPF, and the responses to weaker or stronger concentrations were significantly lower (Tables 3 and 4, Fig. 1). As with the effect on astrocytes, the dose–response relationship was therefore bell shaped. In replicate experiments, 10^{-6} *M* MPF caused consistent, but again variable, increases in neurite extension using astrocyte-free cultures (mean 30.3%) (Table 3), or astrocyte-contaminated (untreated with [Ara-C]^3) cultures (mean 40.4%) (Table 4). The specificity of this effect of MPF is illustrated by the results with D-Lys-Lys-Gly-Glu. Only the configuration of the *N*-terminal residue is changed in this analogue, yet at 10^{-6} *M* concentration it was without effect on the growth of cholinergic neurons.

To further explore the effect of astrocytes on MPF's action on neurons, [Ara-C]^3-treated cholinergic neurons were grown on either CDM or astrocyte beds, alone or in the presence of 10^{-6} *M* MPF. Neurite outgrowth was less in astrocyte medium than in CDM (the Controls in Table 5). MPF caused significantly increased outgrowth in both cases, but the increase was significantly higher when astrocyte medium was employed (Table 5). The suggestion—from the higher mean outgrowth in [Ara-C]^3 untreated, compared with treated neurons—that the effect of MPF is greater in the presence of astrocytes is thus supported. Similar

TABLE 5

EFFECTS OF ASTROCYTES OR LYMPHOCYTES ON NEURITE OUTGROWTH OF CHOLINERGIC ENRICHED NEURONS TREATED WITH ARA-C

MPF Conc. (*M*)	*N*	Medium	Mean Length (μm)	Control (μm)	Increase (μm)	Increase (%)
10^{-6}	60	astr	327.5 ± 98	255.4 ± 84	<0.0005	72.1 (28%)
	60	CDM	336.3 ± 101	307.3 ± 98	<0.1	29.0 (9%)
	100	astr	237.7 ± 53	160.6 ± 46	<0.0005	77.1 (48%)
	32	CDM	243.1 ± 64	210.4 ± 59	<0.025	32.7 (16%)
10^{-6}	42	lymph	190.2 ± 54	197.9 ± 64	NS	−7.7 (−4%)
	31	CDM	243.1 ± 64	213.5 ± 58	<0.05	29.6 (14%)
	30	lymph	187.7 ± 31	170.5 ± 49	<0.1	17.2 (10%)
	30	CDM	226.8 ± 69	201.3 ± 39	<0.05	25.5 (13%)

N = number of neurites measured in determination of mean length; CDM, chemically defined medium; astr, astrocyte; lymph, lymphocyte; NS, not significant. See the Method section for preparation of media. Differences ast vs. CDM, $p < 0.005$; lymph vs. CDM, NS.

TABLE 6

SYNERGISM IN THE EFFECTS OF MPF AND NGF ON CHOLINERGIC ENRICHED NEURONS GROWN IN CDM ON COLLAGEN

MPF (Conc.)	NGF (Conc.)*	*N*	Mean Length (μm)	Control (μm)	*p* (vs. Control)	Increase (μm)
Cultures previously untreated with [Ara-C][3]						
10^{-6} *M*	nil	60	273.9 ± 86	194.8 ± 67	<0.0005†	79.1 (41%)
nil	$\simeq 10^{-9}$*M*	59	356.7 ± 108	194.8 ± 67	<0.0005‡	161.9 (83%)
10^{-6} *M*	$\simeq 10^{-9}$*M*	60	404.2 ± 108	194.8 ± 67	<0.0005	209.4 (106%)
Cultures previously treated with [Ara-C][3]						
10^{-6} *M*	nil	60	364.3 ± 83	247.6 ± 47	<0.0005§	116.7 (47%)
nil	$\pm 10^{-9}$*M*	60	380.9 ± 87	247.6 ± 47	<0.0005	133.3 (54%)
10^{-6} *M*	$\pm 10^{-9}$*M*	60	438.3 ± 99	247.6 ± 47	<0.0005#	190.7 (77%)

CDM, chemically defined medium; *N*, number of neurites measured in determination of mean length.
* The conc was 20 ng/ml, which based on MW of NGF = 26,000 was calculated as 0.77×10^{-9} *M*.
† MPF vs. NGF, and MPF vs. MPF + NGF <0.0005.
‡ NGF vs. NGF + MPF <0.025; §MPF vs. NGF not significant; MPF vs. MPF + NGF <0.0005; #NGF vs. NGF + MPF <0.005.

experiments using lymphocyte medium (Table 5) suggest that the action of MPF is impeded by the presence of lymphocytes.

Inverted U-shaped or bell-shaped dose–response relationships, similar to those we found in the responses of astrocytes and neurons to MPF (Fig. 1), have frequently been reported in investigations of the neurotrophic effects of melanocortins (6) and the physiological effects of hormones (8). An explanation of this phenomenon (8) based on a multisubsite receptor model seems especially relevant in the case of MPF, because MPF's molecule is well-suited to participate in stabilized P4 states (see Fig. 2).

Our finding of synergism in the actions of MPF and NGF on cholinergic neurons is of particular interest. On a molecular basis, MPF was about 1000 times less potent than NGF in promoting neurite outgrowth, but our results (Table 6) show that the effect of mixtures of MPF and NGF was significantly higher than that of either given alone. This suggests that MPF and NGF act through different receptors.

Our finding that MPF also increases neurite outgrowth in cultures of mesencephalic dopaminergic neurons (Table 7) provides added support to our interpretation of in vivo experiments in which dopaminergic anatomy and function is restored by MPF (2,5,9,11). Based on these cumulative results, clinical investigation of the potential of MPF in the treatment of Parkinson's disease has been approved and is now in progress.

Our results further emphasize the interesting parallelism in the trophic actions of MPF and the hepatapeptide sequence 4–10 of ACTH. Thus, ACTH(4–10) has trophic effects on spinal neurons in vitro similar to those described for MPF (13). Of a large number of pituitary and other peptides examined, only MPF and α-MSH (which contains the ACTH sequence) stimulated limb regeneration in hypophysectomized newts (10) or dopamine output from cultures of adrenal medullary cells (4). In the form of metabolically stabilized analogues (Ac-Lys-D-Lys-Sar-Glu in the case of MPF; Org 2766 in the case of the ACTH sequence), both show effects in vivo suggestive of CNS regeneration (1,2,6,9,12). However, despite this broad parallelism, significant differences in the actions of the two peptides are emerging. MPF potentiates the pigmenting action of α-MSH on melanocytes (hence its name), but has itself no effect on melancocytes (3). The ACTH(4–10) analogue reverses the abnormal turning behavior of rats with unilateral lesions of their nigrostriatal pathways provided it is injected immediately after the lesioning, the effect being maximal 2 days after the

TABLE 7

EFFECT OF MPF ON NEURITE OUTGROWTH IN CULTURES OF DOPAMINERGIC ENRICHED NEURONS (ASTROCYTE FREE) GROWN IN CDM ON COLLAGEN

MPF Conc.	*N*	Mean Length	Control	*p* (vs. Control)	Increase (μm)
10^{-6} *M*	30	136.4 ± 37	106.5 ± 25	<0.0005	29.9 (28%)
	60	149.6 ± 39	111.1 ± 30	<0.0005	38.5 (35%)
	50	194.2 ± 37	120.0 ± 29	<0.0005	74.2 (62%)
					mean (30.3%)

N = number of neurites measured in determination of mean length. CDM, chemically defined medium.

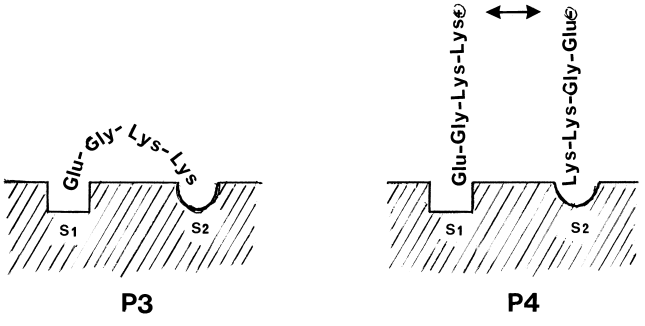

FIG. 2. Postulated mechanism (simplified), based on a multisubsite receptor model, to explain the biphasic (bell-shaped) dose–response relationships seen with MPF. MPF is represented as having binding sites located on its Glu and Lys residues, which interact with specific subsites (S1 and S2) of the receptor (shown shaded). In the activated state P3, one molecule of MPF interacts with the receptor via both of its binding sites, a conformational change occurs, and agonism results. It is postulated that another state P4, where two molecules of MPF interact with the receptor—one via its Glu site and the other via a Lys site—is stabilized in the case of MPF because of ionic interactions (represented by arrows) between the unbound Lys and Glu residues; consequently, formation of the active P3 state is hindered, and abnormal dose–response relationships are predicted. In extreme cases, when the P4 state is favored to the full exclusion of the P3 state, maximum responses will occur with 50% occupancy of the two subsites, and thereafter increasing concentrations of MPF will result in progressively decreased responses, the response being nil when there is 100% occupancy of the subsites.

lesioning, and decreasing 5 days after the treatment (1); the MPF analogue has similar behavioral effects in this rat model even when it is injected 3 weeks after the lesioning, but in contrast to the ACTH analogue, its effects are first seen 6 weeks after the lesioning, develop in magnitude over the next 6 weeks, and are still evident 2 years after the lesioning (5). A compensatory mechanistic and neuronal protective role for ACTH(4–10) is indicated, whereas the results with MPF suggest that it may have a more direct role in the initiation of CNS regeneration.

Unpublished work in which we are applying an MPF assay (7) in the analysis of human tissue retrieved at autopsy leaves no doubt that MPF exists in the human brain, though its specific distribution is presently uncertain. The MPF sequence, like the ACTH sequence, is contained within the PMOC gene, but there is, as yet, no evidence that this gene is the genetic source of MPF.

The mechanism(s) by which MPF exerts its trophic effects remain unknown, but the results presented in this and our previous articles suggest a neurotrophic status for MPF akin to that postulated for the increasingly large number of small neurotrophic proteins for which receptors have been identified. In the therapeutic application of neurotrophic agents, there are clearly many possible advantages in the use of a small peptide as distinct from proteins.

ACKNOWLEDGEMENTS

We thank the SERC for the award of a Ph.D. studentship to D.B.O., and the Trustees of Remedi for other financial support.

REFERENCES

1. Antonawich, F. J.; Azmita, E. C.; Strand, F. L. Rapid neurotrophic actions of an ACTH/MSH (4–9) analogue after nigrostriatal 6-OHDA lesioning. Peptides 14:1317–1993; 1993.
2. Bazzaz, A. A.; Morley, J. S.; Miles, J. B. Neurotrophic peptide (MPF analogue) restores nigral dopaminergic neurons in a rat model of Parkinson's disease. J. Pathol. (submitted).
3. Carter, R. J.; Shuster, S.; Morley, J. S. Melanotropin is the *C*-terminal tetrapeptide of human β-lipotropin. Nature 279:74; 1979.
4. Ensor, D. M.; Morley, J. S.; Redfern, R. M.; Miles, J. B. Stimulation of dopamine output from adrenal medullary cells by β-endorphin and its *C*-terminal tetrapeptide (MPF). Brain Res. 490:196–199; 1989.
5. Ensor, D. M.; Morley, J. S.; Redfern, R. M.; Miles, J. B. The activity of an analogue of MPF (β-endorphin 28–31) in a rat model of Parkinson's disease. Brain Res. 610:166–168; 1993.
6. Gispen, W. H., et al. On the neurotrophic action of melanocortins. Prog. Brain Res. 72:319–331; 1987.
7. Lewis, J. R. E.; Morley, J. S.; Venn, R. F. Analysis of β-endorphin 28–31 (melanotropin potentiating factor) and analogues by high-performance liquid chromatography of their 9-fluorenylmethoxycsarbonyl derivatives. J. Chromatogr. 615:37–45; 1993.
8. Morley, J. S. The design of antagonists to gastrin. In: Miyoshi, A., Ed. Gut peptides and ulcer. Tokyo; Biomed. Res. Found.; 1983:1–14.
9. Morley, J. S.; Bazzaz, A. A.; Ensor, D. M.; Miles, J. B. Reversal of mitochondrial damage in a rat model of Parkinson's disease by a neurotrophic peptide (MPF analogue). Neuroreport 5:1877–1881; 1994.
10. Morley, J. S.; Ensor, D. S. Neurotrophic effects of β-endorphin *C*-terminal tetrapeptide (MPF). Neuropeptides 8:45–49; 1986.
11. Morley, J. S.; Hayward, C. F.; Carter, R. J.; Shuster, S. MPF analogue with stability to proteolysis. Neuropeptides 2:109–114; 1981.
12. Strand, F. L.; Rose, K. J.; Zuccarelli, L. A.; Kume, J.; Alves, S. E.; Antonawich, F. J.; Garrett, L. A. Neuropeptide hormones as neurotrophic factors. Physiol. Rev. 71:1017–1046; 1991.
13. Van der Neut, R.; Bar, P. R.; Sodaar, P.; Gispen, W. H. Trophic influences of alpha-MSH and ACTH 4–10 on neuronal outgrowth in vitro. Peptides 9:1015–1020; 1988.