Brain Research, 576 (1992) 49–58

BRES 17555

Melanocortins stimulate proliferation and induce morphological changes in cultured rat astrocytes by distinct transducing mechanisms

Muriel Zohar and Yoram Salomon

Department of Hormone Research, The Weizmann Institute of Science, Rehovot (Israel)

(Accepted 5 November 1991)

Key words: Melanocyte-stimulating hormone; Adrenocorticotropic hormone; Melanocortin; Cyclic adenosine 3′,5′-monophosphate; Astrocyte; Rat brain; Cell proliferation

Melanocyte stimulating hormone (MSH), adrenocorticotropic hormone (ACTH), and several peptides derived from pro-opiomelanocortin, are present in the dorsolateral hypothalamus and arcuate nucleus of several vertebrate species. These peptides affect central nervous system (CNS) functions including behavior, memory, and foetal brain development. In this study we investigated the effects of $ACTH_{1-24}$, $ACTH_{1-17}$, $ACTH_{4-10}$, α-MSH, β-MSH, and a potent analog (Nle^4,D-Phe^7)-α-MSH (melanocortins) on immunocytochemically defined astroglial cells prepared from primary cultures of 1-2-day-old rat brains. A cyclic adenosine 3′,5′-monophosphate (cAMP) response to the melanocortins was only detected in astrocytes and not in other cell types in the culture. The extent of the cAMP response was greatest on day 21, the latest time tested. On the other hand, (methyl^{3}H)-thymidine incorporation in astrocytes was significantly stimulated (1.5–2-fold) by melanocortins only in 7 and not in 14 and 21 day cultures. This mitogenic activity of melanocortins was not mimicked by other agents such as forskolin or isoproterenol which efficiently stimulate cAMP production in astrocytes. $ACTH_{1-17}$ as a melanocortin representative induced significant morphological changes in 7 and 14 day cultures which included rounding of the cell body and process extension. This response, however, resembled that induced by forskolin and hence appears to be cAMP mediated. These findings suggest that astrocytes in the CNS may serve as a target for melanocortins. These peptides appear to affect differentiation and proliferation of these cells during certain developmental periods. While the morphological effects of melanocortins seem to be cAMP mediated, induction of proliferation of the astrocytes by melanocortins appears to involve an alternative signal transduction pathway.

INTRODUCTION

The melanocortin family of peptides is processed from a common precursor glycoprotein, pro-opiomelanocortin (POMC) of 31 kDa[33,50] and includes adrenocorticotropin (ACTH) and the melanotropins, α-, β-, γ-melanocyte stimulating hormone (MSH). All melanocortins share the heptapeptide sequence 4–10 of ACTH. In the central nervous system (CNS) and peripheral nervous system (PNS) these peptides have been shown to affect diverse and complex networks (for review, see Eberle[13]). These include regulating behavior in test animals[11,64], affecting memory and learning[64], inducing lacrimal secretion in the rat[14,27,31], facilitating recovery of motor performance and nerve regeneration in the PNS[9,10,43,57] and CNS[61–63], causing transmitter release in motor nerve terminals[28] and having therapeutic importance in peripheral nerve disease[22].

Immunocytochemical studies have shown cell bodies containing melanocortins within the arcuate nucleus and in the dorsolateral hypothalamus which send axonal projections to many brain areas, including the limbic midbrain structures[42,59]. More defined sites of melanocortin action had not been identified until recently, when specific, high affinity binding sites for [^{125}I]$ACTH_{1-14}$ in brain membrane preparations[30] and receptors which recognize both ACTH and α-MSH by autoradiography in the adult rat brain have been described[53]. However, the specific cells or mechanisms by which melanocortins modulate their effects on the CNS are still unclear.

MSH and ACTH receptor pathways in melanogenic tissue and in adrenal cortical cells, respectively, are mediated by G-proteins, through adenylate cyclase (AC) and the cAMP cascade[19,32]. Uniquely, MSH[20,31,45] and ACTH[7] binding to their respective receptors in melanoma, lacrimal and adrenal cells is regulated by Ca^{2+} ions. Similar transduction mechanisms regulating different cell specific responses are expected to exist in melanocortin sensitive cells in the mammalian brain. Cyclic AMP stimulation in neonatal rat brain primary cell cultures[55] and in more purified astroglial cells[15] suggested the presence of a specific melanocortin receptor within the brain astroglial cell population. In the vertebrate adult and immature CNS, astrocytes are one of the ma-

Correspondence: Y. Salomon, Department of Hormone Research, The Weizmann Institute of Science, Rehovot, 76100, Israel. Fax: (972) (8) 344116.

jor cell types involved in regulating axonal growth or regeneration after injury (for review see Schwartz et al.[46]). Therefore the behavioral and developmental actions of 'melanocortins may in part be mediated by astroglial cells.

Cyclic nucleotides, in general, influence growth of various cell types. In many tissues cAMP appears to be a negative growth regulator. Long term culturing of M2R melanoma cells with phorbol esters increased proliferation which was blocked by the elevation of cAMP by forskolin or β-MSH[17]. In other cellular models. such as avian chondroprogenitor cells, cAMP has been reported to have a mitogenic effect[38].

Astrocytes in culture undergo marked morphological and biochemical differentiation. Two specific markers of astrocytes, glial fibrillary acidic protein (GFAP) and glutamine synthetase (GS), increase by several fold both in vivo and in vitro suggesting that maturation of this cell type can be achieved in vitro[25]. Astrocytes in culture appear to change their morphology to round, short processed type cells. Cyclic AMP derivatives (such as dibutyryl cyclic AMP), forskolin and neurotransmitters, which increase cAMP levels in astrocytes, accelerate both morphological and biochemical astroglial cell differentiation[29,39].

This study was aimed at identifying particular cell-specific responses elicited by melanocortins in well defined homogeneous primary astrocyte cultures. Employing a [methyl-^{3}H]thymidine incorporation assay and monitoring morphological changes[40], we report here that melanocortins influence astrocyte proliferation and differentiation by distinct transducing mechanisms which are differentially expressed throughout the 3 weeks of development in culture.

MATERIALS AND METHODS

[2-^{3}H]Adenine (9.77 Ci/mmol) was obtained from Kamag, Israel. [Methyl-^{3}H]thymidine (6.7 Ci/mmol) was from New England Nuclear, Boston, U.S.A. α-Melanocyte-stimulating hormone (α-MSH), atropine, carbachol, isoproterenol (ISO), EGTA, gentamicin sulfate, isobutylmethylxanthine (IBMX), poly-L-lysine, bovine pancreatic trypsin were from Sigma, St. Louis, MO and Forskolin (Fo) was from Calbiochem Corp., CA U.S.A. Synthetic $ACTH_{4-10}$ and $ACTH_{1-24}$ were kindly donated by Organon. The synthetic analog, Synchrodyn $ACTH_{1-17}$ was kindly donated by Hoechst, β-MSH and (Nle^4,D-Phe^7)-α-melanocyte-stimulating hormone (4,7-α-MSH) were a gift of A. Lerner, Yale University. All peptides and hormones were dissolved indouble distilled water unless otherwise stated.

Rabbit anti-mouse immunoglobulin conjugated with tetramethylrhodamine isothiocyanate isomer R was supplied by Dakopatts, Denmark. Monoclonal (mouse IgG_1) anti GFAP was purchased from BioMakor, Israel. Dulbecco's Modified Eagle Medium (DMEM) and L-15 medium were supplied by Gibco, England. Foetal Calf Serum (FCS) and L-glutamine were purchased from Biological Industries, Israel. Male Wistar neonatal rats (1–2 days old) were supplied by the departmental animal breeding center.

Purified cell cultures

Primary astroglial cell cultures were prepared from the cerebral cortex of 1–2-day-old rats as described by McCarthy and de Vellis[36] with modifications of Wolswijk and Noble[60]. The tissue was dissected to 1–2 mm^3 sections in L-15 medium, filtered through a nylon mesh of 220 μm and incubated in 2 ml Ca^{2+}-, Mg^{2+}-free Dulbecco's modified Eagle's medium (DMEM-CMF) containing 0.5 mM EDTA and trypsin (300 i.u. ml^{-1}) for 10 min at room temperature. Trypsinization was terminated by adding DMEM containing 10% FCS, and the cells were sedimented at 1000 *g* for 5 min. The pellet was washed to remove degris and resuspended in DMEM supplemented with 2 mM L-glutamine, 10% FCS and 15 μg/ml gentamicin (DMEM-FCS). Dissociated cells were plated at a density of 2 brains per 75 cm^2 NUNC tissue culture flask pre-coated with poly-L-lysine (20 mg/ml) and incubated at 37°C in a humidified incubator with 5% CO_2–95% air. Culture medium was replaced with fresh DMEM-FCS the following day and then 3 times each week until cells were utilized. Two days prior to the desired age of culture, i.e. on day 5, 12 and 19, for 7, 14 and 21 day experiments, respectively, the layer of nonadherent cells growing on top of the flat monolayer was removed by shaking the flask for 8 h at 37°C on a rotary platform (150 revs min^{-1}). The medium containing the loosely bound process bearing cells was removed and normally discarded except for experiments in Fig. 1. The remaining adherent astrocytes in the flask were allowed to recover for 1 day, shaken briefly, medium removed and then harvested (0.1% trypsin for 15 min at room temperature). After centrifugation, the pellet was resuspended in DMEM-FCS and seeded in the desired concentration to poly-L-lysine precoated 18 mm glass coverslips (10^5 cells/50 μl for immunofluorescence), 24-well Nunc plates (3×10^6 cells/well for [methyl-^{3}H]thymidine incorporation studies, $\approx 10^6$ cells/well for cAMP accumulation studies) or 35 mm petri dishes (for microscopy) as described in the individual experiments. Each culture was tested for GFAP immunoreactivity. Most cultures routinely tested >90% GFAP positive; the lowest degree of homogeneity permitted for experiments was 85% GFAP positive.

Immunocytochemical staining

The culture's cellular homogeneity was determined by indirect immunofluorescence labelling[40]. Cells, plated to glass coverslips in 35 mm culture dishes, were fixed with 5% glacial acetic acid in ethanol for 20 min at 4°C, followed by permeabilization with cold acetone (–20°C) for 5 min. The coverslips were then washed 3 times with Hank's Balanced Salt Solution (HBSS) containing 4% FCS (HBSS-FCS). Incubation with GFAP monoclonal antibodies (mouse IgG_1, 1:100 in HBBS-FCS) was carried out at room temperature for 30 min. Coverslips were washed 3 times with HBSS-FCS followed by a 30 min incubation at room temperature with rabbit anti-mouse IgGs, conjugated to tetramethylrhodamine isothiocyanate (RAM-TRITC, 1:50 in HBSS-FCS). The coverslips were subsequently washed 3 times in phosphate buffered saline solution (PBS), once in double distilled water (DDW), and fixed on a microscope slide in a mixture of 45% glycerol, 45% ethanol, and 10% PBS. Immunolabelled cells were photographed using a Zeiss Universal microscope equipped with phase contrast and fluoreascence optics. Purity of the culture is expressed in terms of GFAP positive cells per 100 cells counted.

Measurement of cAMP accumulation

Intracellular [^{3}H]cAMP accumulation in cell monolayers was measured using the [2-^{3}H]adenine prelabelling method described by Salomon[44]. Briefly, cells were incubated with [2-^{3}H]adenine (5–15 μCi/ml) in DMEM for 2 h at 37°C in a humidified 5% CO_2 atmosphere, and the wells were then washed with 1 ml of DMEM to remove excess [2-^{3}H]adenine. Cyclic AMP accumulation studies were conducted in 0.5 ml of DMEM containing 0.1 mM IBMX, 100 μg/ml BSA, and the appropriate stimulants at the concentrations indicated. Incubation at 37°C for the indicated time, was terminated by aspiration of the assay medium and addition of 1 ml 2.5% perchloric acid containing 0.1 mM cAMP. The clear perchlo-

ric acid extract was neutralized with KOH and subjected to double column (Dowex 50/alumina) chromatographic fractionation to determine the amount of [2-^{3}H]cAMP formed. Intracellular accumulation of [2-^{3}H]cAMP was calculated as a percentage of total [2-^{3}H]adenine uptake/well. Data was expressed as mean fold increase ± S.E.M. of 3 separate wells similary treated (triplicate determination) over nonstimulated controls.

Measurement of [methyl-^{3}H]thymidine incorporation

Incorporation of [methyl-^{3}H]thymidine into astrocyte DNA was measured as described by Pandiella et al.[37], with slight modifications. Medium of 7-, 14-, or 21-day-old cultures was changed to the same medium without serum but supplemented with 0.1% BSA (essentially fatty acid free) for 48 h. More than 90% of the cells survived serum-free conditions, as determined by trypan blue exclusion. The test substances were then added for 24 h, and 1 μCi/ml [methyl-^{3}H]thymidine was included for the last 6 h of the incubation at 37°C under an atmosphere of 5% CO_2/95% O_2. Cells were washed twice with cold PBS and nucleic acids and proteins were then precipitated with 7.5% trichloroacetic acid (TCA) for 30 min at 4°C. Acid insoluble material was washed twice with 0.5 ml ethanol, then solubilized in 0.3 ml of 1.0 N NaOH. Radioactivity was measured by beta-scintillation counting and the means from 3 separate wells similarly treated (triplicate determination) were calculated ± S.E.M.

Determination of morphological changes

The shape of the intermediate filament cytoskeleton was monitored by GFAP indirect immunofluorescence microscopy. Seven-, 14- or 21-day-old astrocyte cultures were incubated with or without test substances in DMEM-FCS. Cells were monitored by phase contrast microscopy during this time. After 24 h, cells were fixed in ethanol:acetic acid (95:5 v:v), for 20 min at 4°C, permeabilized with acetone at -20°C for 5 min and treated with anti-GFAP antibodies, as described above.

Statistical analysis

All statistical tests were carried out using the STATVIEW 512 program in a MacIntosh personal computer. Student's *t*-test or one way analysis of variance (ANOVA) followed by the Fisher's Least Significance Difference (LSD) test were used to determine significant ($P < 0.001$) differences over control means.

RESULTS

Regulation of cAMP levels by melanocortins

In order to examine whether the cAMP response to melanocortins, in primary brain cultures, is indeed confined to astrocytes we determined the melanocortin response in the nonadherent cells and in the purified astrocytes derived from the heterogeneous brain cell population (Fig. 1). The melanocortins induced a significant increase in cAMP in the purified 21-day-old astrocytes (Fig. 1A). $ACTH_{4-10}$ (0.1 mM), $ACTH_{1-17}$ (100 nM) and 4,7-α-MSH (100 nM) raised the cAMP levels by 14-, 9- and 11-fold, respectively, over nonstimulated controls. Isoproterenol (1 μM), used as a positive control in these experiments, stimulated cAMP accumulation in the purified astrocytes to a much higher extent (~40-fold). The nonadherent mixed population of cells (separated by shaking and usually discarded) were essentially nonresponsive to melanocortin stimulation, but exhibited a slight response to isoproterenol (Fig. 1B). In

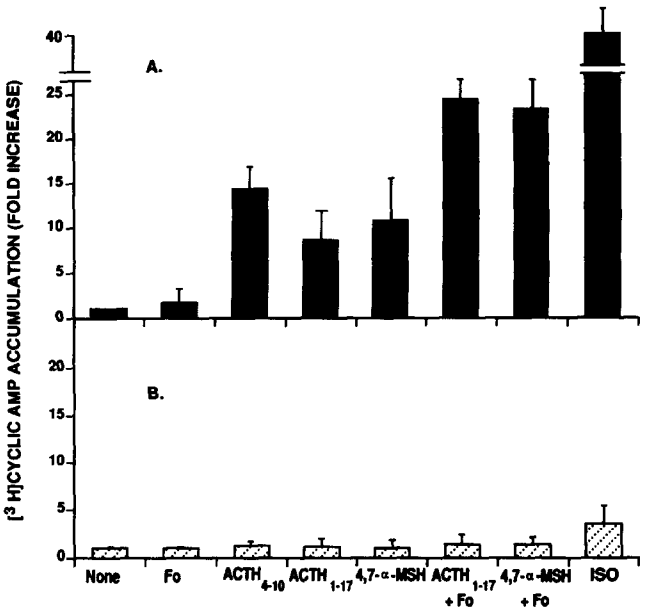

Fig. 1. The differential response to melanocortins of purified rat astrocytes and of other non-adherent heterogeneous brain cells. Cell monolayers were stimulated and [2-^{3}H]cyclicadenosine 3',5'-monophosphate (cAMP) accumulation determined under standard conditions in the presence of adrenocorticotropine hormone ($ACTH_{1-14}$ (0.1 mM), $ACTH_{1-17}$ (100 nM), 4,7-α-MSH (100 nM) and isoproterenol (ISO) (1 μM). Incubation time was 30 min. All other details were as described under Materials and Methods. A: purified 21-day-old astroglial cultures. (% conversion to [2-^{3}H]cAMP in absence of stimulants (None) = 0.069) B: heterogenous cell population of non-adherent layer removed from 19-day-old culture, replated and tested at 21 days. (% conversion to [2-^{3}H]cAMP in absence of stimulants (None) = 0.175). The experiment is representative of 3 experiments with comparable results. Each value represents the mean ratio (stimulated/nonstimulated) ± S.E.M. of triplicate determinations.

order to examine the melanocortin response in the two cell populations at higher sensitivity we also measured the response to melanocortins in the presence of forskolin. We tested the effect of forskolin (100 nM) alone and in combination with melanocortics on the 21-day-old astrocyte cultures and observed a synergistic response (Fig. 1A). However, in spite of the presence of forskolin, the melanocortins failed to stimulate cAMP accumulation in the nonadherent mixed cells (Fig. 1B). These results indicate that even under more sensitive assay conditions a cAMP response to melanocortins in the nonadherent cells in undetectable. The same synergistic effect of forskolin and melanocortins was also observed in 14-day-old astrocytes (Fig. 2) and in 7-day-old astrocytes (not shown). When culturing several regions of the rat brain, it appeared that melanocortins evoked the highest cAMP response from the cerebral cortex which included the astrocytes of the limbic system (data not shown).

Since astrocytes undergo spontaneous maturation in culture, we next examined whether the responsivenss to melanocortins varied over a period of 21 days. The

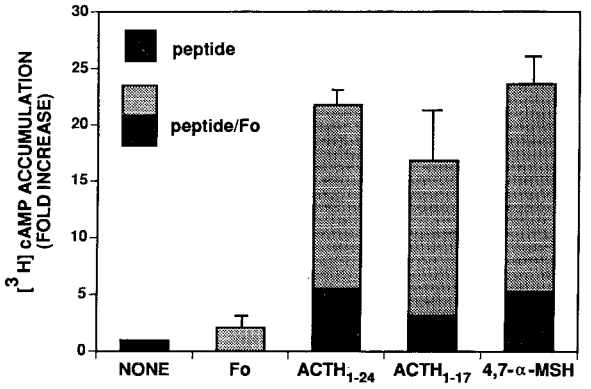

Fig. 2. The synergistic effect of forskolin on melanocortin stimulated cAMP accumulation in 14-day-old cultured cortical astrocytes. Cells were incubated with melanocortins (100 nM), in the absence or presence of forskolin (FO) (100 nM) for 10 min and [2-^{3}H]cAMP accumulation determined under standard conditions. (% conversion to [2-^{3}H]cAMP in absence of stimulants (None = 0.055). All other details were as described under Materials and Methods. Values are means ± S.E.M. of triplicate determinations. Data are representative of 4 similar experiments with comparable results.

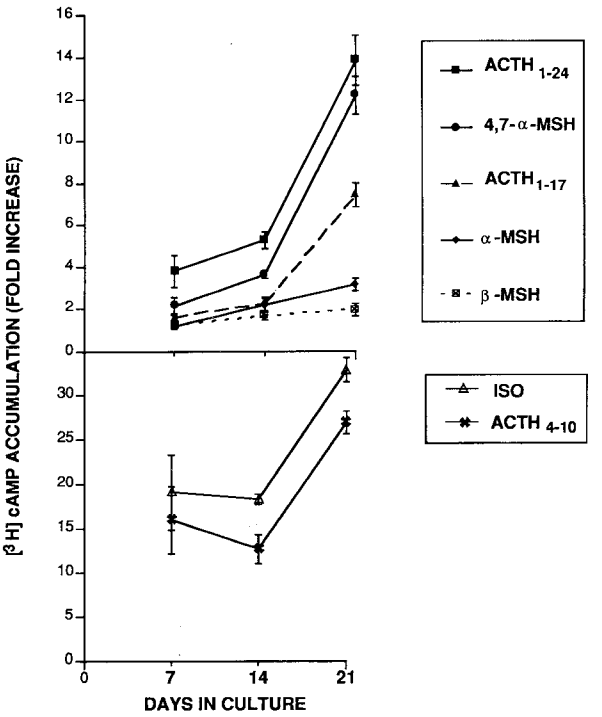

Fig. 3. Stimulation of cAMP accumulation by melanocortin in purified cortical astrocytes as a function of the age of the culture. Cells at the indicated age in culture were stimulated with the various melanocortins (1 μM), $ACTH_{4-10}$ (0.1 mM), and isoproterenol (ISO)((1 μM) for 10 min and [^{3}H]cAMP accumulation was determined under standard conditions. (% conversion to [2-^{3}H]cAMP in absence of stimulants was 0.125 (7 days), 0.191 (14 days), 0.198 (21 days)). Values represent the means of ratios (stimulated/non-stimulated) ± S.E.M. for 6–9 determinations. Data is a compilation of 6 separate experiments; two of which included 7 and 14 day cultures, another two which included 14 and 21 day cultures, and two in which all three 7, 14 and 21 day cultures were tested. Experiments employed the same protocols and before combining results, data were tested for any interaction due to the day of the experiment (two way-ANOVA). All other details were as described under Materials and Methods.

cAMP response to melanocortins and isoproterenol gradually increased with the age of the culture and varied for the different melanocortins used (Fig. 3). All tested peptides (except for $ACTH_{4-10}$) were used at a final concentration of 1 μM to elicit maximal cAMP response. Stimulation of the cells was seen as early as 7 days in culture, with β-MSH, α-MSH, $ACTH_{1-17}$, and 4,7-α-MSH eliciting a cAMP increase over nonstimulated controls of 1.2-, 1.2-, 1.6- and 2.2-fold, respectively. $ACTH_{1-24}$ induced a significantly higher response at 7 days (3.8-fold increase) and remained a most potent agonist at all ages tested (Fig. 3, upper panel). The cAMP response to alltested peptides was greatest at 21 days in culture, the longest period tested in these experiments (Fig. 3). By 21 days, it appears that $ACTH_{1-24}$ and 4,7-α-MSH are similar in their ability to increase cAMP levels, followed (in decreasing order) by $ACTH_{1-17}$ and α- and β-MSH. The behaviorally active heptapeptide fragment, $ACTH_{4-10}$ at 0.1 mM[11,55], also showed the highest degree of stimulation at 21 days, and the extent of stimulation was about twice that seen with the other melanocortins. Even at 7 days, $ACTH_{4-10}$ was formidable in elevating the cAMP in these cells to levels comparable to those induced by isoproterenol.

Induction of astrocyte proliferation by melanocortins

It was hypothesized that the cAMP response to melanocortins in astrocytes might mediate a cell specific response such as growth. We therefore examined the possible mitogenic effect of these peptides using [methyl-^{3}H]thymidine incorporation into DNA as an index of cell proliferation. Following serum deprivation for 48 h, 7, 14, and 21 day astrocyte cultures were exposed to the various melanocortins (Fig. 4). Triplicate wells containing 10% FCS were included with each experiment as a positive growth control. Melanocortins enhanced DNA synthesis only in the 7-day-old cultures. $ACTH_{1-24}$ (100 nM), 4,7-α-MSH (100 nM) and $ACTH_{4-10}$ (0.1 mM) elicited a 1.7-, 1.9-, and 1.8-fold increase in [^{3}H]thymidine uptake over the nonstimulated control, respectively. In contrast, none of the peptides tested induced proliferation in 14 and 21 day cultures. FCS (10%) elicited a 5–8-fold increase and carbachol (1 mM) a 2-fold increase in [methyl-^{3}H]thymidine incorporation irrespective of the culture age, i.e. at 7-, 14- and 21-day-old cultures (Fig. 4). The proliferative response to carbachol, first reported by Ashkenazi et al.[1] was abolished by the addition of 10 μM atropine, suggesting muscarinic acetylcholine receptor involvement. These results indicate that cultured cortical astrocytes were capable of proliferation under a mi-

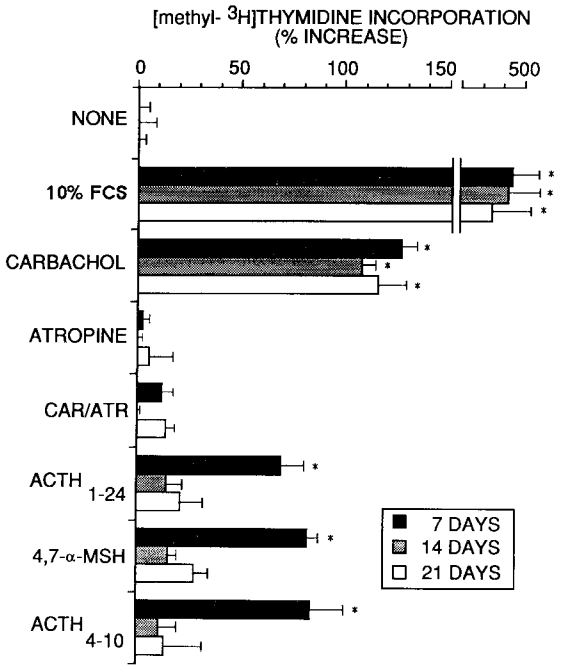

Fig. 4. Differential effect of melanocortins on [methyl-^{3}H]thymidine incorporation in 7-, 14- and 21-day-old cortical astrocyte cultures. Cells were incubated either without (None) or with 100 nM $ACTH_{1-24}$, 100 nM 4,7-α-MSH, 0.1 mM $ACTH_{4-10}$, 10% FCS, 1 mM carbachol, 10 μM atropine, and carbachol plus atropine (car/atr) for 24 h and [^{3}H]thymidine incorporation was then determined. All other details were as described under Materials and Methods. Values are the mean percent increase of stimulated over NONE ± S.E.M. of 3–6 individual determinations. Nonstimulated controls (NONE) incorporated 8490 CPM (7 days), 8440 CPM (14 days) and 8904 CPM (21 days). Data are representative of 5 experiments with comparable results. *$P < 0.001$, compared with nonstimulated values (ANOVA and Fisher LSD).

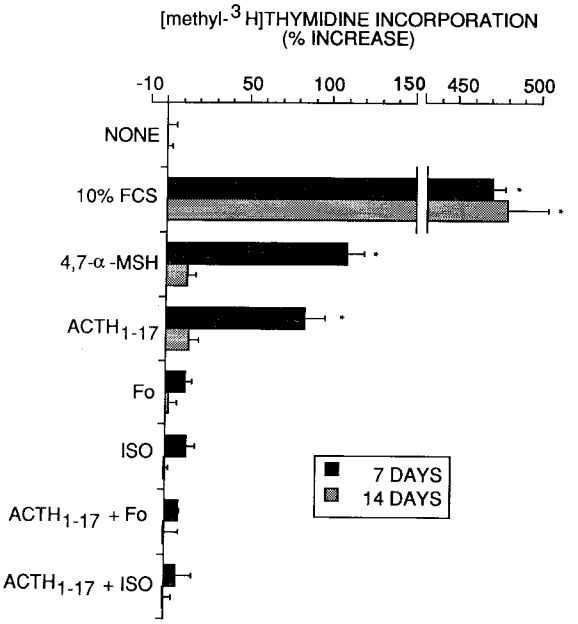

Fig. 5. Effect of cAMP elevating agents on [methyl-^{3}H]thymidine incorporation in cortical astrocyte cultures. Cells were stimulated with 100 nM $ACTH_{1o17}$, 100 nM 4,7-α-MSH, 10% FCS, 100 nM forskolin (Fo), 100 nM isoproterenol, (ISO) or $ACTH_{1-17}$ in combination with either forskoling or isoproterenol for 24 h and [^{3}H]thymidine incorporation was then measured under standard conditions. All other details were as described under Materials and Methods. Nonstimulated controls (NONE) incorporated 7732 CPM (7 days), 6914 CPM (14 days). Values are means ± S.E.M. of 3 individual determinations. Data are representative of 3 experiments with comparable results. *$P < 0.001$, compared with nonstimulated values (one way analysis of variance and Fisher (LSD).

togenic signal at all ages tested, but selectively responded to melanocortin stimulation only at the defined age of 7 days.

While the mechanism mediating the growth response

TABLE I

Effects of melanocortins on DNA synthesis and cAMP accumulation in 7 and 14 day cortical astrocyte cultures

Stimulants	*7 Days*			*14 Days*		
	[Methyl-^{3}H]thymidine incorporation			*[Methyl-^{3}H]thymidine incorporation*		
	(c.p.m./well ± S.E.M.)	*(fold)*	*cAMP (fold)*	*(c.p.m./well ± S.E.M.)*	*(fold)*	*cAMP (fold)*
None	11382 ± 302	1.0	1.0	5266 ± 185	1.0	1.0
10% FCS	72084 ± 2040*	6.3	NT	31069 ± 1365*	5.9	NT
$ACTH_{1-24}$ (100 nM)	22791 ± 972*	2.0	3.8	6252 ± 245	1.2	5.2
$ACTH_{1-17}$ (100 nM)	21610 ± 526*	1.9	1.6	6891 ± 592	1.3	2.2
$ACTH_{4-10}$ (0.1 mM)	20997 ± 289*	1.8	13.9	5831 ± 429	1.1	12.7
4,7-α-MSH (100 nM)	21374 ± 985*	1.9	2.2	5955 ± 460	1.1	3.6
α-MSH (100 nM)	22525 ± 659*	2.0	1.2	5737 ± 483	1.1	2.2
β-MSH (100 nM)	22206 ± 300*	1.9	1.2	5426 ± 158	1.0	1.7
ISO (1 μm)	9381 ± 548	0.8	19.1	4859 ± 416	0.9	18.2

Data for thymidine incorporation is compiled from 4 experiments. Values for [methyl-^{3}H]thymidine incorporation are means ± S.E.M. for 3–6 individual determinations. cAMP was determined as described in Materials and Methods.
*$P < 0.001$ over basal determinations (one way analysis of variance and Fisher LSD).

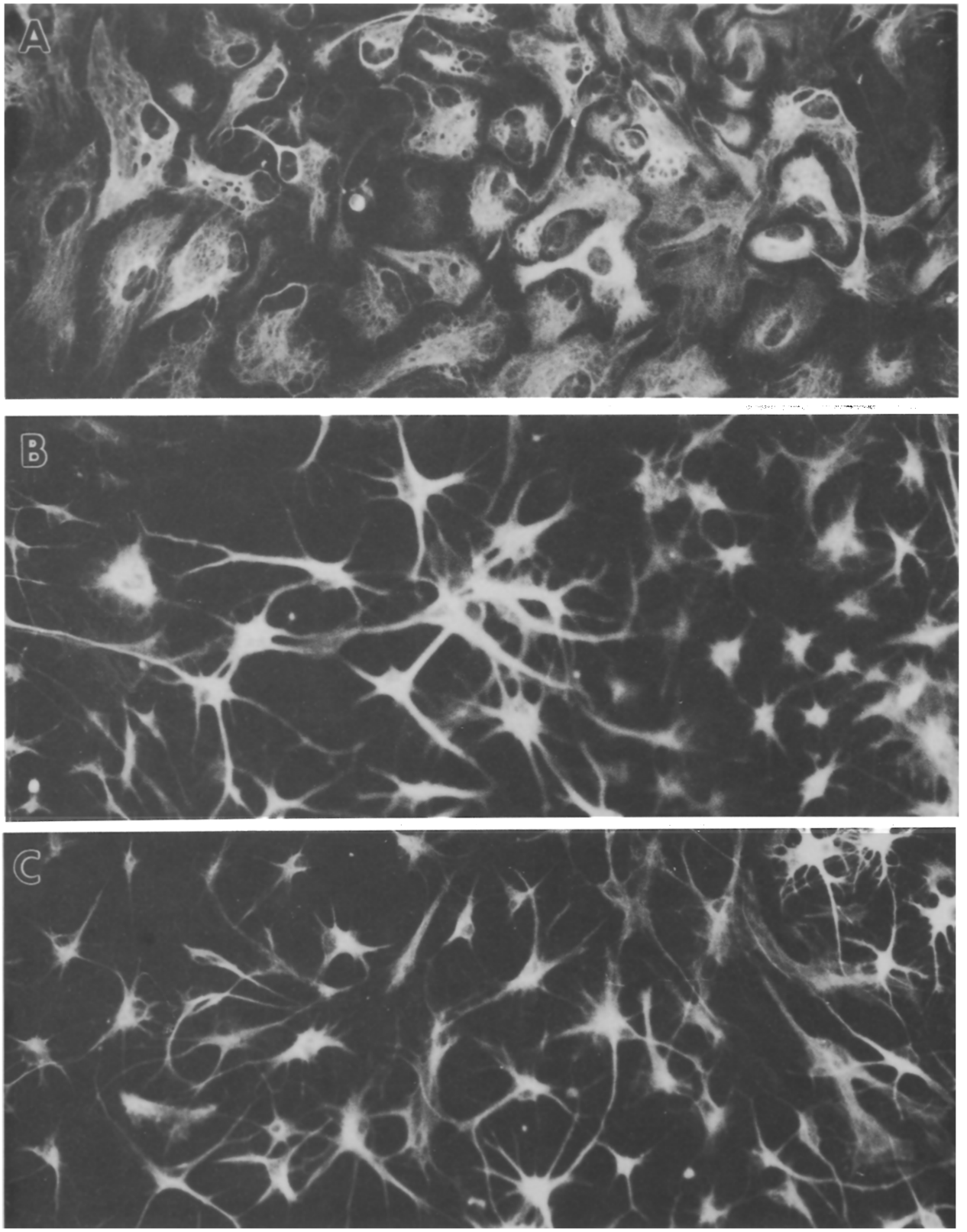

Fig. 6. Morphological changes induced in 14-day-old primary rat astrocytes by $ACTH_{1-17}$. Cells were cultured for 14 days in Dulbecco's modified Eagle's medium (DMEM-FCS), and incubated for 24 h in the presence of $ACTH_{1-17}$ (100 nM) or forskolin (100 nM). Incubation was terminated by washing with phosphate buffered saline (PBS) and cellular fixation followed by immunofluorescent labeling with anti-glial fibrillary acidic protein (GFAP) antiserum as described in Materials and Methods. A: nonstimulated controlcells, flat, polygonal morphology; B: incubated in the presence of $ACTH_{1-17}$, cells develop a process-bearing morphology; C: incubated in the presence of forskolin, cells develop similar process-bearing morphology.

to FCS is unknown, the proliferative effect of carbachol in astrocytes was reported to correlate well with the degree of activation of phosphoinositide hydrolysis[1]. To evaluate whether the proliferative effect of melanocortins in these cultures is related to their ability to stimulate cAMP accumulation, we incubated the cells with other stimulators of cAMP under similar experimental conditions. Isoproterenol (100 nM) and forskolin (100 nM) failed to elicit an increase in [methyl-^{3}H]thymidine incorporation in either 7 or 14 day cultures when included in the incubation medium (Fig. 5). In addition, the positive growth response to $ACTH_{1-17}$ (100 nM) in 7-day-old cultures was abolished by simultaneous incubation with either forskolin (100 nM) or isoproterenol (100 nM). In agreement with the results (Fig. 4), only the 7-day-old cells responded to 4,7-α-MSH (100 nM) and $ACTH_{1-17}$ (100 nM) by increasing [^{3}H]thymidine incorporation 2.1- and 1.8-fold over nonstimulated controls, respectively.

All of the melanocortins tested increased [methyl-^{3}H]thymidine incorporation in 7 day astroglial cells (Table I). There appeared to be no significant difference in the extent of proliferation induced by the various melanocortins tested irrespective of their ability to stimulate significantly different cAMP levels. In particular, β-MSH and α-MSH, which are weak stimulants of cAMP production in 7 day cultures, were as effective as $ACTH_{1-24}$ in enhancing [methyl^3-H]thymidine incorporation. The melanocortin peptides, in general, increased [methyl-^{3}H]thymidine incorporation with a pharmacological profile which did not parallel that observed for their ability to stimulate cAMP accumulation.In14-day-old cultures, no effect on DNA synthesis was observed although melanocortins elevated cAMP levels to a significantly higher extent.

Morphological changes in astrocytes as induced by melanocortins

Studies by others have described morphological differentiation incultured astrocytes upon administration of cAMP elevating agents or cAMP derivatives[29,39]. Further studies were therefore conducted to examine the influence of melanocortins via adenylate cyclase on astrocyte morphology using GFAP as a marker for changes of the intermediate filament organization. Astrocytes (14 days) were incubated in the presence of $ACTH_{1-17}$ (100 nM), forskolin (100 nM), isoproterenol (100 nM) or vasopressin (1 μM). Morphological changes were observed by light microscopy as soon as 2 h after commencing incubation with isoproterenol or forskolin. $ACTH_{1-17}$ as a representative of melanocortins induced morphological-changes after 20–24 h. In the presence of $ACTH_{1-17}$, the 14-day-old astroglia converted from polygonal (Fig. 6A) to process bearing cells (Fig. 6B) that resemble forskolin (Fig. 6C) and isoproterenol (data not shown) treated astrocytes. Similar morphological changes were observed in the 7- and 21-day-old astrocyte cultures (data not shown). Vasopressin did not elicit any observable morphological response (data not shown). This peptide served as a control as it was shown to be ineffective in elevating cAMP levels in these cells[15]. These data indicate that the morphological differentiation of cultured astrocytes can be influenced by melanocortins throughout the culture period and that this effect appears to be mediated by cAMP.

DISCUSSION

Melanocortins appear to specifically affect primary cultures of purified rat astrocytes and the magnitude of the response in terms of cAMP production seems to increase with the age of the cells in culture. Our findings support the hypothesis that putative melanocortin receptors reside on the astrocyte population of rat brain cells in culture[5,55], possibly in situ as well. In addition, our results suggest that melanocortins are regulating two cell specific responses in astrocytes via different signal transduction pathways: proliferation and morphological differentiation, both of which do not develop simultaneously.

The melanocortins failed to elicit a cAMP response in the heterogeneous nonadherent layer of cells derived from whole brain primary cultures, which consist of putative *O*-2A progenitor cells, fibroblasts, type-2 astrocytes, and oligodendrocytes. These cells were not typed with specific antigenic markers and therefore, their exact identity was only evaluated on the basis of morphology. Even under more sensitive assay conditions by inclusion of forskolin, which potentiates hormone stimulated cAMP accumulation in various cell types[47] and has been shown by us to increase the sensitivity to MSH by a factor of 40 and to increase maximal response level by 3-fold in mouse M2R melanoma cells[19], a cAMP response to melanocortins in the nonadherent cells is undetectable. The response to isoproterenol, however, indicates the viability of these cells which are known to express β-adrenergic receptors[36,56]. However, since the cells in this study were selected on the basis of their cAMP response, we cannot exclude the possibility that melanocortins may stimulate other brain cell types via alternative signal transduction mechanisms.

The melanocortins have been shown to affect complex behavioral functions, as well as fetal brain development. Various studies attempted to correlate specific brain regions with the induced behavioral effect (for review see De Wied and Jolles[11]). In our studies, the cerebral cor-

tex exhibited the highest responsiveness in terms of cAMP production confirming a previous study[8]. Included in this area when removed for culturing are structures associated with the limbic system, known to be involved in processes related to emotion and arousal[34]. According to De Wied and Jolles[11] the melanocortins might promote an arousal state and in this way modulate the significance of environmental cues.

The positive mitogenic effect of melanocortins in astrocytes does not correlate with their ability to increase intracellular cAMP levels. First, forskolin and isoproterenol were completely ineffective in stimulating [methyl-^{3}H]thymidine incorporation (Fig. 5) although they elevate cAMP levels in astrocytes at all ages tested. Second, melanocortins failed to induce proliferation in 14- and 21-day-old cultures when their ability to stimulate cAMP levels was highest (Figs. 3, 4 and Table I). Third, although the various melanocortins stimulate cAMP levels to varying degrees, their mitogenic capabilities in 7-day-old cultures were equipotent (Table I). Finally, elevation of cAMP levels by simultaneous treatment with melanocortins and forskolin or isoproterenol in 7-day-old cultures, abolished the positive trophic response to melanocortins in these cells, suggesting the counteraction of cAMP on proliferation in astrocytes (Fig. 5).

The trophic effects of melanocortins may therefore be mediated by a second separate signal transducing pathway. Brennemen et al.[3] have also suggested another effector, other than adenylate cyclase, for the observed mitogenic response to vasoactive intestinal peptide in astrocytes. Activation of two separate signal transduction pathways has been reported in classical melanocortin sensitive cells. Using ^{31}P nuclear magnetic resonance (NMR) spectroscopy measurements, in M2R melanoma cells MSH was recently shown to stimulate phosphonoester accumulation by what appears to be a cAMP independent mechanism[12]. Likewise, in cultured adrenal cortical cells, factors other than cAMP appear to mediate cell specific responses to ACTH[23]. Ashkenazi et al.[1] related the proliferative effect of carbamylcholine in astrocytes to the ability of the muscarinic acetylcholine receptor to regulate phosphatidyl inositol (PIP_2) phospholipase C (PLC) activity. We confirmed the mitogenic effects of carbamylcholine in astrocytes in our experiments (Fig. 4). Therefore, it is possible that the melanocortin receptor which mediates the proliferative effects in these cells may act via the same effector system. The possibility that melanocortin receptors may afford their pleiotropic effects in cells by effector enzymes other than adenylate cyclase has been considered[5,13,26,49]. However, to the best of our knowledge, no direct demonstration has been reported concerning the activation of inositol lipid specific PLC by melanocortins.

The results presented are compatible with the presence of two melanocortin receptor subtypes which may employ separate signal transducing pathways and a differential pattern of receptor expression associated with the development of the astrocytes in vitro. In the young cultures (7 days), both types might be present as indicated by both the proliferative and cAMP responses. As the cells mature, the relative ratio between the two types of receptors might change, leading to higher cAMP levels, and loss of the proliferative effect. The pharmacology of these putative melanocortin receptor subtypes is certainly different (Fig. 3 and Table I). While it appears that the melanocortins are equipotent in stimulating the effector system controlling proliferation, their relative potencies in regulating cAMP accumulation are significantly different. The efficacies of the different melanocortins on these putative receptor subtypes and consequently the resulting cellular responses may be complex. For example, $ACTH_{4-10}$ can elevate cAMP levels to nearly those attained with isoproterenol, yet unlike isoproterenol, can induce astrocyte proliferation in 7-day-old cultures. This may be explained by a difference in the type of receptor activated or the peptide fragment's relative potency on the putative melanocortin receptor subtypes. Melanocortins may also be working indirectly, stimulating the production of an additional substance responsible for the mitogenic effects observed. In support of this receptor subtype hypothesis it has been shown, using crosslinking techniques, that two putative MSH receptor proteins appear to reside on M2R melanoma cells[18,52]. Multiple receptors regulating parallel transductory pathways have also been suggested for ACTH[41] and other hormones such as glucagon[58].

Our results suggest that the control of adenylate cyclase by melanocortins may play a role in the regulation of astrocyte morphology. Since changes in astrocyte morphology are observed in brain trauma[35], occur during development[48] and may also regulate interactions between neurons as well as neuron survival[21], it is possible that these melanocortin induced changes may play an important role in astrocyte function. Phorbol esters were shown to induce significant morphological changes in these cells as well[24]. The cAMP induced morphological differentiation described here might however represent only a general nonspecific effect rather than a reflection on a real physiological process. It may be helpful to characterize the response of astrocytes to melanocortins in terms of biochemical markers, such as glutamine synthetase activity[6,54], or changes in protein synthesis[4] to determine if the morphological changes are being accompanied by changes in astrocyte function.

In addition, it was interesting to observe that both the

morphological changes, which we speculate to be cAMP mediated, and the proliferative effect could be induced by melanocortins inthe 7-day-old cultures. The data presented clearly demonstrate that melanocortins are stimulating astrocyte growth under serum free conditions but cannot exclude the possibility that they might exert a different effect in the presence of serum. The morphological assay, however, was conducted in the presence of 10% fetal calf serum and under these conditions, ACTH induced the morphological changes.

The influence of melanocortins on glial cells may be related to their more complex effects on the CNS. The temporal influence of melanocortins on immature, astrocyte proliferation might be intrinsically supportive to promote neuron survival and growth and synaptic plasticity during brain development. It has been demonstrated that the presence of glial cells is beneficial for the development and differentiation of neuronal cells in culture[2,51], as well as essential for neuronal migration[16].

In summary, this study shows that astrocytes in vitro respond to melanocortins. These findings are consistent with the possibility that astrocytes are targets for melanocortins in the CNS. Furthermore, two signal transduction mechanisms have been implicated in the regulation of the astrocyte specific responses. While it appears that proliferation is being mediated by a cyclic AMP independent mechanism that has still to be identified, the morphological changes appear to be regulated by a cyclic AMP dependent process.

Acknowledgements. The authors wish to acknowledge the devoted secretarial assistance of Malka Kopelowitz, and thank Israel Hanukoglu for helpful discussions. M.Z. completed this work in partial fulfillment of her M.Sc. thesis at the Feinberg Graduate School of the Weizmann Institute of Science. Supported in part by the Center of Brain Research, the Weizmann Institute of Science. Y.S. is the Tillie and Charles Lubin Professor of Hormone Research.

REFERENCES

1 Ashkenazi, A., Ramachandran, J. and Capon, D.J., Acetylcholine analogue stimulates DNA synthesis in brain-derived cells via specific muscarinic receptor subtypes, *Nature,* 340 (1989) 146–150.
2 Banker, G.A., Trophic interactions between astroglial cells and hippocampal neurons in culture, *Science,* 209 (1980) 809–810.
3 Brenneman, D.E., Nicol, T., Warren, D. and Bowers, L.M., Vasoactive intestinal peptide: a neurotrphic releasing agent and an astroglial mitogen, *J. Neurosci. Res.,* 25 (1990) 386–394.
4 Bridoux, A., Fages, C., Couchle, D., Nunez, J. and Tardy, M., Protein synthesis in astrocytes: 'spontaneous' and cyclic AMP-induced differentiation, *Dev. Neurosci.,* 8 (1986) 31–43.
5 Charkraborty, A., Orlow, S. and Pawelek, J.M., Stimulation of the receptor for melanocyte-stimulating hormone by retinoic acid, *FEBS Lett.,* 276 (1990) 205–208.
6 Charriere-Bertrand, C., Prince, G., Boucher, C., Fages, C., Tardy, M. and Nunez, J., Expression of tubulin, GFAP, and of their encoding mRNAs during the proliferation and differentiation of cultured astrocytes, *Neurochem. Int.,* 15 (1989) 215–222.
7 Cheitlin, R., Buckley, D.I. and Ramachandran, J., The role of extracellular calcium in corticotropin steroidogenesis, *J. Biol. Chem.,* 260 (1985) 5323–5327.
8 Cholewinski, A.J. ad Wilkin, G.P., Astrocytes from forebrain, cerebellum, and spinal cord differ in their responses to vasoactive intestinal peptide, *J. Neurochem.,* 51 (1988) 1626–1633.
9 Crescitelli, L.A., Strand, F.L. and Keim, K.L., Melanocortins and fast axonal transport in intact and regenerating sciatic nerve, *Peptides,* 10 (1989) 883–886.
10 De Koning, P. and Gispen, W.H., A rationale for the use of melanocortins in the treatment of nervous tissues damage. In D.G. Stein and B. Sabel (Eds.), *Pharmacological Approaches to the Treatment of Brain and Spinal Cord Injuries,* Plenum, New York, 1988, pp. 233–258.
11 De Wied, D. and Jolles, J., Neuropeptides derived from proopiocortin: behavioral, physiological, and neurochemical effects, *Physiol. Rev.,* 62 (1982) 976–1059.
12 Degani, H., DeJordy, J.O. and Salomon, Y., Stimulation of cyclic AMP and phosphomonoester levels by melanotropin in melanoma cells: ^{31}P-NMR studies, *Proc. Natl. Acad. Sci. U.S.A.,* 88 (1991) 1506–1510.
13 Eberle, A.N., *The Melanotropins: Chemistry, Physiology and Mechanisms of Action,* Karger, Basel, 1988, 556 pp.
14 Entwistle, M.L., Hann, L.E., Sullivan, D.A. and Tatro, J.B., Characterization of functional melanotropin receptors in lacrimal glands of the rat, *Peptides,* 11 (1990) 477–483.
15 Evans, T., McCarthy, K.D. and Harden, T.K., Regulation of cyclic AMP accumulation by peptide hormone receptors in immunocytochemically defined astroglial cells, *J. Neurochem.,* 43 (1984) 131–138.
16 Gasser, U.E. and Hatten, M.E., Neuron-glia interactions of rat hippocampal cells in vitro: glial-guided neuronal migration and neuronal regulation of glial differentiation, *J. Neurosci.,* 10 91990) 1276–1285.
17 Gerst, J.,Benezra, M., Schimmer, A. and Salomon, Y., Phorbol ester imparis melanotropin receptor function and stimulates growth of cultured M2R melanoma cells, *Eur. J. Pharmacol.,* 172 (1989) 29–39.
18 Gerst, J.E., Sole, J., Hazum, E. and Salomon, Y., Identification and characterization of melanotropin binding proteins from M2R melanoma cells by covalent photoaffinity labelin, *Endocrinology,* 123 (1988) 1792–1797.
19 Gerst, J.E., Sole, J., Mather, J.P. and Salomon, Y., Regulation of adenylate cyclase by β-melanotropin in the M2R melanoma cell line, *Mol. Cell. Endocrinol.,* 46 (1986) 137–147.
20 Gerst, J.E., Sole, J. and Salomon, Y., Dual regulation of β-melanotropin receptor function and adenylate cyclase by calcium and guanosine nucleotides in the melanoma cell line, *Mol. Pharmacol.,* 31 (1978) 81–88.
21 Gilad, G., Shanker, G., Dahl, D. and Gilad, V., Dibutyryl cyclic AMP-induced changes in neuron-astroglia interactions and fibronectin immunocytochemistry in dissociated rat cerebellar cultures, *Brain Res.,* 508 (1990) 215–224.
22 Gispen, W.H., Therapeutic potential for melanocortins in peripheral nerve disease, *Trends Pharmacol. Sci.,* 11 (1990) 2212–222.
23 Hanukoglu, I., Feuchtwanger, R. and Hanukoglu, A., Mechanism of corticotropin and cAMP induction of mitochondrial cytochrome P450 system enzymes in adrenal cortex cells, *J. Biol. Chem.,* 265 (1990) 20602–20608.
24 Harrison, B.C. and Mobley, P.L., Phorbol-ester induced change in astrocyte morphology: correlation with protein kinase C activation and protein phosphorylation, *J. Neurosci. Res.,* 25 (1990) 71–80.

25 Hertz, L.,Juurlink, B.J., Forsmark, H. and Schousboe, A., Astrocytes in primary cultures. In S.M. Pfeiffer (Ed.), *Neuroscience Approached through Cell Culture,* CRC, Florida, 1982-3, pp. 176–186.

26 Hill, S.E., Buffey, J., Thody, A., Oliver, I., Bleehen, S. and MacNeil, S., Investigation of the regulation of pigmentation in α-melanocyte stimulating hormone responsive and unresponsive cultured B16 melanoma cells, *Pigment Cell Res.,* 2 (1989) 161–166.

27 Jahn, R., Padel,U., Porsch, P. and Soling, H., Adrenocorticotropic hormone and α-melanocyte-stimulating hormone induce secretion and protein phosphorylation in the rat lacrimal gland by activation of a cAMP-dependent pathway, *Eur. J. Biochem.,* 126 (1982) 623–629.

28 Johnston, M.F., Kravitz, E.A., Meiri, H. and Rahamimoff, R., Adrenocorticotropic hormone causes long-lasting potentiation of transmitter release from frog motor nerve terminals, *Science,* 220 (1983) 1071–1072.

29 Kempski, O., Wroblewska, B. and Spatz, M., Effects of forskolin on growth and morphology of cultured glial and cerebrovascular endothelial and smooth muscle cells, *Int. J.dev. Neurosci.,* 5 (1987) 435–445.

30 Li, G., Queen, G. and LaBella, F.S., Adrenocorticotropin, vasoactive intestinal peptide, growth hormone-releasing factor, and dynorphin compete for common receptors in brain and adrenal, *Endocrinology,* 126 (1990) 1327–1333.

31 Lieba, H., Garty, N., Schmidt-Sole, J., Pitterman,l O., Azrad, A. and Salomon, Y., The melanocortin receptor in the rat lacrimal gland: a model system for the study of MSH (melanocyte stimulating hormone) as a potential neurotransmitter, *Eur. J. Pharmacol.,* 181 (1990) 71–82.

32 Londos, C., Salomon, Y., Lin, M.C., Harwood, J.P., Schramm, M., Wolff, J. and Rodbell, M., 5'-Guanylyl imidodiphosphate, a potent activator of adenylate cyclase systems in eucaryotic cells, *Proc. Natl. Acad. Sci. U.S.A.,* 71 (1974) 3087–3080.

33 Mains, R.E., Eipper, B.A. and Ling, N., Common precursor to corticotropins and endorphins, *Proc. Natl. Acad. Sci. U.S.A.,* 74 (1977) 3014–3018.

34 Martin, J.H., *Neuroanatomy: Text and Atlas,* Elsevier, New York, 1989, 376–380 pp.

35 Mathewson, A.J. and Berry, M., Observations on the astrocyte response to a cerebral stab wound in adult rats, *Brain Research,* 327 (1985) 61–69.

36 McCarthy, K.D. and de Vellis, J., Preparation of separate astroglial and oligodendroglial cell cultures from rat cerebral tissue, *J. Cell Biol.,* 85 (1980) 890–902.

37 Pandiella, A., Beguinot, L., Velu, T.J. and Meldolesi, J., Transmembrane signalling at epidermal growth factor receptors overexpressed in NIH 3T3 cells, *Biochem. J.,* 254 (1988) 223–228.

38 Pines, M. and Hurwitz, S., The effect of parathyroid hormone and atrial natriuretic peptide on cyclic nucleotides production and proliferation of avian epiphyseal growth plate chrondroprogenitor cells, *Endocrinology,* 123 (1988) 360–365.

39 Pollenz, R.S. and McCarthy, K.D., Analysis of cyclic-AMP-dependent changes in intermediate filament protein phosphorylation and cell morphology in cultured astroglia, *J. Neurochem.,* 47 (1986) 9–17.

40 Raff, M.C., Fields, K.L., Hakomori, S.I., Mirsky, R., Pruss, R. and Winter, J., Cell-type specific markers for distinguishing and studying neurons and the major classes of glial cells in culture, *Brain Res.,* 174 (1979) 283–308.

41 Ramachandran, J., Corticotropin receptors, cyclic AMP and steroidogenesis, *Endocrin. Res.,* 10 (1985) 347–363.

42 Ravid, R., Swaab, D.F., Van der Woude, P.T. and Boer, G.J., Immunocytochemically stained binding sites for oxytocin and α-melanocyte stimulating hormone in rat brain following ventricular administration, *Brain Research,* 379 (1986) 404–408.

43 Saint-Come, C., Acker, G. and Strand, F.L., Peptide influences on the development and regeneration of motor performance, *Peptides,* 3 (1982) 439–449.

44 Salomon, Y., Determination of cellular responsiveness to hormones and neurotransmitters by the conversion of [^{3}H]adenine to [^{3}H]cylic AMP in cell monolayers, cell suspensions and tissue slices, *Methods Enzymol.,* 195 (1990) 22–28.

45 Salomon, Y., Melanocortin receptors: targets for control by extracellular calcium, *Mol. Cell. Endocrinol.,* 70 (1990) 139–145.

46 Schwartz, M., Cohen, A., Stein-Iszak, C. and Belkin, M., Dichotomy of the glial cell response to axonal injury and regeneration, *FASEB J.,* 3 (1989) 2371–2378.

47 Seamon, K. and Daly, J.W., Activation of adenylate cyclase by the diterpene forskolin does not require the guanine nucleotide regulator protein, *J. Biol. Chem.,* 256 (1981) 9799–9801.

48 Skoff, R.P., Price, D.L. and Stocks, A., Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve, *J. Comp. Neurol.,* 169 (1976) 291–312.

49 Slominski, A., Moellmann, G. and Kuklinska, E., MSH inhibits growth in a line of amelanotic hamster melanoma cells and induces increases in cyclic AMP levels and tyrosinase activity without inducing melanogenesis, *J. Cell Sci.,* 92 (1989) 551–559.

50 Smith, A.I. and Funder, J.W., Proopiomelanocortin processing in the pituitary, central nervous system, and peripheral tissues, *Endocrin. Rev.,* 9 (1988) 159–179.

51 Smith, G.M., Rutishauer, U., Silver, J. and Miller, R.H., Maturation of astrocytes in vitro alters the extent and molecular basis of neurite outgrowth, *Dev. Biol.,* 138 (1990) 377–390.

52 Solca, F.F.A., Salomon, Y. and Eberle, A.N., Heterogeneity of the MSH receptor among B16 murine melanoma subclones, *J. Receptor Res.,* 11 (1991) 379–390.

53 Tatro, J., Melanotropin receptors in the brain are differentially distributed and recognize both corticotropin and α-melanocyte stimulating hormone, *Brain Research,* 536 (1990) 124–132.

54 Toru-Delbauffe, D., Baghdassarian-Chalaye, D., Gavaret, J.M., Courtin, F., Pomerance, M. and Pierre, M., Effects of transforming growth factor β1 on astroglial cells in culture, *J. Neurochem.,* 54 (1990) 1056–1061.

55 Van Calker, D., Loffler, F. and Hamprecht, B., Corticotropin peptides and melanotropins elevate the level of adenosine 3':5'-cyclic monophosphate in cultured murine brain cells, *J.Neurochem.,* 40 (1983) 418–427.

56 Van Calker, D., Muller, M. and Hamprecht, B., Adrenergic α- and β-receptors expressed by the same cell type in primary culture of perinatal mouse brain, *J. Neurochem.,* 30 (1978) 713–718.

57 Van der Neut, R., Bär, P.R., Sodaar, P. and Gispen, W.H., Trophic influences of alpha-MSH and $ACTH_{4-10}$ on neuronal outgrowth in vitro, *Peptides,* 9 (1988) 1015–1020.

58 Wakelam, M., Murphy, G., Hruby, V. and Houslay, M., Activation of two signal-transduction systems in hepatocytes by glucagon, *Nature,* 323 (1986) 68–70.

59 Watson, S.J., Richard, C.W. and Barchas, J.D., Adrenocorticotropin in rat brain: immunocytochemical localization in cells and axons, *Science,* 200 (1978) 1180–1182.

60 Wolswijk, G. and Noble, M., Identification of an adult-specific glial progenitor cell, *Development,* 105 (1989) 387–400.

61 Wolterink, G. and Van Ree, J.M., Functional recovery after destruction of dopamine systems in the nucleus accumbens of rats. III. Further analysis of the facilitating effect of the ACTH(4–9) analog ORG 2766, *Brain Res.,* 507 (1990) 109–114.

62 Wolterink, G., Van Zanten, E., Kamsteeg, H., Radhakishun, F.S. and Van Ree, J.M., Functional recovery after destruction of dopamine systems in the nucleus accumbens of rats. II. Facilitation by the ACTH(4-9) analog ORG 2766, *Brain Res.,* 507 (1990) 101–108.

63 Wolterink, G., Van Zanten, E. and Van Ree., J.M., Functional recovery after destruction of dopamine systems in the nucleus accumbens of rats. IV. Delay by intra-accumbal treatment with ORG 2766- or α-MSH antiserum, *Brain Res.,* 507 (1990) 115–120.

64 Zager, E.L. and Black, P.M., Neuropeptides in human memory and learning processes, *Neurosurgery,* 17 (1985) 355–369.