Department of Pharmacology
Faculty of Medicine
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Melanocortins: regulators of behavioural and neurochemical processes

Academic Dissertation

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(née Opmane)

Riga
2004

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ACADEMIC DISERTATION

To be presented for public criticism at University of Latvia Promotion Council in the research branch of the biology and medicine, in Riga, O. Vācieša str. 4, on June 17th, 2004, at 14 o'clock noon.

Riga
2004

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To my family

CONTENTS

ABSTRACT 6
LIST OF ORIGINAL PUBLICATIONS 7
ABBBREVIATIONS 8

1. INTRODUCTION 9

2. REVIEW OF THE LITERATURE 11

2.1 Melanocortins 11
2.1.1 History of the discovery of MSH peptides and their nomenclature 11
2.1.2 Proopiomelanocortin and the formation of the MSH peptides 11
2.1.3 Primary structure of melanocortins 12
2.1.4 Specific structure features of melanocortins in different species 13
2.1.5 Expression of melanocortins in the CNS and peripheral tissues 13

2.2 Melanocortin receptors 14
2.2.1 Discovery of melanocortin receptors 14
2.2.2 Melanocortin receptor 1 (MC1R) 14
2.2.3 Melanocortin receptor 2 (MC2R) 14
2.2.4 Melanocortin receptor 3 (MC3R) 15
2.2.5 Melanocortin receptor 4 (MC4R) 15
2.2.6 Melanocortin receptor 5 (MC5R) 16
2.2.7 Structural characterization of the MCR and signal transduction pathways 17

2.3 The biological effects of MSH peptides 20
2.3.1 Pigmentation 20
2.3.2 Inflammation 20
2.3.3 Behavioural effects 21
2.3.3.1 Grooming behaviour 21
2.3.3.2 Sexual behaviour 22
2.3.3.3 Feeding behaviour 22
2.3.4 Cardiovascular effects 22
2.3.5 Other functions of melanocortins 23

2.4 Synthetic MCR agonists and antagonists 24
2.4.1 Agonists 24
2.4.2 Antagonists 26

3. AIM AND TASKS OF THE STUDY 27

4. MATERIAL AND METHODS 28

4.1 Animals 28

4.2 Drugs **28**

4.3 Behavioural studies (I, II, III) **29**
4.3.1 In rats 29
4.3.1.1 Intraventricular (ICV) and *ventral tegmental area* (VTA) cannulation and drug infusions (I, II) 29
4.3.1.2 Analysis of behavioural responses (I, II) 29
4.3.1.3 NMDA-toxicity test 30
4.3.2 In mice 30
4.3.2.1 Locomotor activity tests in mice (III) 30
4.3.2.2 Analgesia tail flick test (V) 30

4.4 Neurochemical experiments - brain microdialysis in anaesthetized rats (IV, VII) 31
4.4.1 Surgery procedure 31
4.4.2 Estimation of extracellular concentration of dopamine and DOPAC using high-performance liquid chromatography (HPLC) 32
4.4.3 Histological examination (IV, VII) 32

4.5 Statistical analysis **33**

5. RESULTS **34**

5.1 Behavioural effects of centrally administered melanocortins and their analogues in rats (I, II) **34**
5.1.1 Effects of α-MSH, γ_1-MSH and γ_2-MSH (II) 34
5.1.2 Effects of combined administration of γ_2-MSH and γ_1-MSH 35
5.1.3 Effects of MC4R antagonists HS964 and HS014 and their influence on α-MSH (I) 36

5.2 Influence of γ_1-MSH and γ_2-MSH on hyperlocomotion induced by psychoactivating drugs **37**
5.2.1 Phencyclidine (PCP) and amphetamine (AMP) hyperlocomotion tests in mice (III) 37
5.2.2 Influence of γ_2-MSH on the NMDA-induced behavior in rats 38

5.3 Examination of analgesic effects of melanocortins (V) **38**
5.3.1 Studies of α-, γ_1-, γ_2-MSH and HS014 38
5.3.2 Studies of the influence of the test-drug-pretreatments on γ_2-MSH-induced analgesia 40

5.4 Influence of melanocortins on extracellular dopamine and DOPAC concentrations in anaesthetized rat *nucleus accumbens* in microdialysis study (IV, VII) **42**
5.4.1 Effects of γ_1-MSH and γ_2-MSH (VII) 42
5.4.2 Effects of MC4R antagonist HS131 in microdialysis study (IV) 44

6. DISCUSSION **45**

6.1 Behavioral repertoire of MSH peptides **46**
6.2 Neurochemical basis of the dopaminergic component **48**

6.3 Glutamatergic component **49**
6.4 GABAergic component **49**

7. CONCLUSIONS **52**

ACKNOLEDGEMENTS **53**
REFERENCE LIST **54**
APPENDIX: ORIGINAL PAPERS I-VII

ABSTRACT

Knowledge of melanocortins (α-MSH, β-MSH, γ-MSH, ACTH) and of their functional role has increased tremendously over the last 10 years when five melanocortin receptor subtypes (MC1R-MC5R) were identified, cloned and characterized. Most studies are carried out to clarify action of MC1R, MC4R and α-MSH, while the role of MC3R and γ-MSH remains still unclear. However, attention may be payed to the fact that γ-MSH peptides can bind with high affinity to MC3R. Besides, this subtype is abundantly expressed in structures of the dopaminergic mesolimbic system, such as *ventral tegmental area* (VTA) and *nucleus accumbens* (NACC). Dopamine (DA) A10 cells which are located in the VTA, receive projections from both inhibitory (GABAergic) and excitatory (glutamatergic) interneurons. In this context, our research studies are devoted to γ-MSH peptides (γ_1- and γ_2-MSH) to elucidate their neuropharmacological activities and a putative role in the regulation of brain processes. Experiments were performed in laboratory animal models by use of behavioural and neurochemical tests, agonists and antagonists of different receptors.

Intra-VTA administration in rats demonstrated distinct behavioural responses caused by both γ-MSH: γ_1-MSH (likely to previously described α-MSH) induced excessive grooming and increased vertical activity that can be attributed to hyperactivation of the dopaminergic system. Unlike, γ_2-MSH caused psychodepressive state (catalepsy) indicating its anti-psychotic activity at the level of the dopaminergic mesolimbic system. Moreover, γ_2-MSH showed antagonizing potency by reducing the γ_1-MSH-induced behavioural responses. These results are in good line with the data obtained in our neurochemical (microdialysis technique) studies. Concentrations of the NACC extracellular DA and its metabolite DOPAC after γ_1-MSH (and also α-MSH) intra-VTA injection resulted in their considerable elevation, whereas γ_2-MSH caused a decrease in the concentrations of these monoamines. γ_2-MSH antagonized the γ_1-MSH effects. This phenomen indicates that these peptides are capable to modulate dopaminergic activity of the mesolimbic system in opposite manner. That was confirmed also by the data obtained in phencyclidine (PCP)-induced hyperlocomotion test (shizoprenia model) in mice: γ_1-MSH potentiated the PCP locomotion activity, whereas γ_2-MSH reduced it, and also antagonized the observed γ_1-MSH potentiating effect. γ_2-MSH fully reduced neurotoxic effects caused by intra-VTA (in rats) injected NMDA, a glutamate receptor ligand. Intriguingly, analgesia test showed that γ_2-MSH (but not γ_1-MSH) induced a stable and prolonged (90 min) non-opiate analgesia realized *via* $GABA_A$ receptor-mediated processes. Thus, muscimol (agonist of GABA site of the $GABA_A$ receptor) potentiated γ_2-MSH analgesic activity, and bicucculine (competitive antagonist of the GABA site) antagonized the γ_2-MSH analgesia. In turn, γ_1-MSH acted as antagonist of diazepam, a ligand of the $GABA_A$ receptor benzodiazepine site. Both peptides have distinct influence on analgesic effects caused by ethanol, a ligand of the $GABA_A$ receptor modulatory site. The data obtained firstly show a pleiotropic influence of γ-MSH peptides on brain processes by involving not only melanocortinergic mechanisms but also modulation of dopamin-, glutamat- and GABAergic processes. From our point of view, a distinct action of γ_1-MSH and γ_2-MSH and their mutual antagonism can be considered as the most important phenomenon, that gives us enough ground to postulate their endogenous regulatory role to maintain balanced psychoactivation/anti-psychotic (anti-schizophrenic?) and pain perception states. These data may open new vistas in understanding of psychopathologies and their correction possibilities by use of melanocortins and novel MCR ligands.

LIST OF ORIGINAL PAPERS

This dissertation is based on the following papers, herein referred to by their Roman numerals (I-VII). Additionally some unpublished data are also presented.

I. Klusa, V., Svirskis, S., Opmane, B., Muceniece, R. Skujins, A., Mutulis, F., Wikberg, J.E.S. and Schioth HB (1998) Evaluation of behavioural effects of neural melanocortin receptor antagonists injected ICV and in VTA in rats.
Neuropeptides 32, 573-580

II. Klusa, V., Svirskis, S., Opmane, B., Muceniece, R. and Wikberg , J.E.S. (1999) Behavioural responses of γ-MSH peptides administered into the rat ventral tegmental area.
Acta Physiol Scand 167, 99-104

III. Klusa, V., Svirskis, S., Germane, S., Opmane, B., Muceniece, R., Schiöth, H.B. and Wikberg, J.E.S. (1999) Melanocortins and their receptors: behaviour and neurochemistry. (Materials of the 57th Conference dedicated to the 80th Anniversary of the University of Latvia)
Acta Universitatis Latviensis 622, 155-163

IV. Lindblom, J., Opmane, B., Mutulis, M., Mutule, I., Petrovska, R., Klusa, V., Bergstrom, L., and Wikberg, J.E.S. (2001) The MC_4 receptor mediates α-MSH induced release of nucleus accumbens dopamine.
Neuroreport 12, 2155-2158

V. Klusa, V., Germane, S., Svirskis, S., Opmane, B. and Wikberg, J.E.S. (2001)
The γ_2-MSH peptide mediates a central analgesic effect via a GABA-ergic mechanism that is independent from activation of melanocortin receptors.
Neuropeptides 35, 50-57

VI. Klusa, V., Jansone, B., Svirskis, S., Rumaks, J. and Muceniece, R. (2003)
The fundamental roles of melanocortins in brain process (Review).
In: Chemical Probes in Biology, M.P.Schneider (eds), KLUWER Academic Publishers, Netherlands, pp 255-267

VII Jansone, B., Bergström, L., Svirskis, S., Lindblom, J., Klusa, V. and Wikberg, J.E.S. (2004) Opposite effects of γ_1- and γ_2-melanocyte stimulating hormone on regulation of the dopaminergic mesolimbic system in rats.
NeurosciLett 361, 68-71

ABBREVIATIONS

α-MSH	alpha melanocyte stimulating hormone (alpha melanocortin)
β-MSH	beta melanocyte stimulating hormone (beta melanocortin)
γ_1-MSH	$gamma_1$ melanocyte stimulating hormone ($gamma_1$ melanocortin)
γ_2-MSH	$gamma_2$ melanocyte stimulating hormone ($gamma_2$ melanocortin)
γ_3-MSH	$gamma_3$ melanocyte stimulating hormone ($gamma_3$ melanocortin)
AGRP	*agouti* related peptide
ACTH	adrenocorticotropic hormone
ASIP	*agouti* signaling protein
AMP	L-amphetamine
cAMP	adenosine 3'5'-cyclic monophosphate
DA	dopamine
DNA	deoxyribonucleic acid
CNS	central nervous system
DOPAC	3'4'-dihydroxyphenylacetic acid
CSF	cerebrospinal fluid
EDTA	ethylenediamine tetraacetic acid
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid $_A$ type receptor
hGH	human Growth Hormone
HPLC	high performance liquid chromatography
IUPAC-IUB	International Union of Pure and Applied Chemistry-International Union of Biochemistry and Molecular Biology
i.c.	intracisternal
i.p.	intraperitoneal
ICV	intracerebroventricular
IL	interleukin
IP_3	inositol 1'4'5'-triphosphate
K_i	dissociation constant (in competitive binding analysis)
LPH	lipotropins
LPS	lipopolysaccharide
MC1R	melanocortin receptor 1
MC2R	melanocortin receptor 2
MC3R	melanocortin receptor 3
MC4R	melanocortin receptor 4
MC5R	melanocortin receptor 5
MSH	melanocyte stimulating hormone (melanocortin)
NACC	*nucleus accumbens*
NF-kB	Nuclear Factor-Kappa B
NMDA	N-methyl-D-aspartate
NO	nitric oxide
PC	prohormone convertase
PCP	phencyclidine
PKA	protein kinase A
POMK	proopiomelanocortin
mRNA	messenger ribonucleic acid
TNF-α	tumor necrosis factor -α
VTA	*ventral tegmental area*

1. INTRODUCTION

Since the beginning of the neuropeptide era started in the 1970th, a lot of studies on peptide formation, amino acid sequence and the functional role of their receptors have been increased tremendously. The first scientific report about the biological activity of α-MSH peptide appeared in 1912, however until the end of the 20th century very little was known about the mechanisms underlying the central effects of melanocortin peptides. Only in the beginning of the 1990th, peptides (ACTH, α-, β-, γ_1- and γ_2-MSH) that belong to the 'melanocortin family' became as the most studied peptides. During 1992-1993 the genes encoded five melanocortin receptor subtypes (MC1R-MC5R) were identified, cloned and characterized in different types of cells. These discoveries have shed a light in understanding of molecular mechanisms underlying different melanocortin-induced effects, such as skin darkening, changes in behaviour, food intake, as well as anti-inflammatory and lipolytic actions, analgesia and others.

MC1R and MC4R have been studied more intensively than other types of the melanocortin receptors. The most clarified function of MC1R is the binding with α-MSH that leads to the stimulation of melanogenesis in melanocytes and thus induces the changes in the skin pigmentation. Over the last 5-7 years numerous studies have revealed the role of MC1R and α -MSH in melanoma tumorogenesis and in the regulation of anti-inflammatory processes. MC4R has important role in the control of feeding behavior. The latest investigations confirm that MC4R agonists are capable to reduced food intake thus exerting the anorexive effects. In contrast, MC4R antagonists induce overeating and weight gain. These findings stimulate an intensive drug design to obtain selective MC4R agonist that could be useful to treat obesity and antagonists in order to treat anorectic conditions.

In comparison to other melanocortins and MC receptors, less understandable is the functional role of both γ-MSHs and MC3R. γ_1-MSH and γ_2-MSH show high binding activity to MC3R that is widely expressed in many regions of the brain. Particularly abundant expression of the MC3R (also the MC4R) is found in the brain mesolimbic dopaminergic system. It is well known that the mesolimbic dopaminergic system is involved in regulation of the motivational processes, drug dependence, manifestation of schizophrenic hyperactivation and emotion. The mesolimbic dopaminergic system involves two important brain structures: the *ventral tegmental area* (VTA) and *nucleus accumbens* (NACC). VTA contains A10 dopaminergic cells that receive interneurons from both the inhibitory (e.g.GABAergic) and the excitatory (e.g. glutamatergic) neurons, hence dopamine (DA) release in the NACC can be modulated by these influences. Abnormal dopamine release in the NACC may lead to psychoses, paranoidal delutions in schizophrenic patients and drug addicted persons.

In Latvia, the functional role of γ-MSH peptides in central nervous system (CNS) have been investigated since 1995. The studies were started in the Laboratory of Pharmacology, Latvian Institute of Organic Synthesis and continued in the Department of Pharmacology, Faculty of Medicine, University of Latvia and supervised by Professor Vija Kluša. The collaboration between the Latvian scientists Swedish scientists group headed by Professor Jarl Wikberg (Uppsala University), as well as financial support from the Howard Hughes Medical Institute (USA, 1995-2000) have given good basis for detailed studies by rise of variety of experimental approaches to disclose the central effects of melanocortins.

The main goal of the present study was to investigate the pharmacological effects of melanocortins (particularly the γ-MSHs), and to clarify neurochemical mechanisms for MSH peptides. We have demonstrated that MSH peptides differ for each other by their behavioral repertoire in experimental animals, and these processes are provided not only *via* melanocortinergic mechanisms but also dopamine-, glutamate- and GABAergic pathways. The most intriguing fact that we have revealed was the opposite action of γ_1-MSH and γ_2-MSH, as well as the antagonizing phenomena between both γ-MSHs. The data obtained in our studies allow us to suggest that γ-MSH peptides may play a significant functional role in the regulation of brain processes, particularly the homeostasis of psychoactivation, pain perception (perhaps, also reward) processes.

The obtained data open new avenues in understanding the central effects of melanocortins and their putative endogenous roles. That also may initiate a design and synthesis for novel substances-prototypes anti-psychotic, analgesic and motivation-regulating drug based on the completely new mechanisms discovered in the present pharmacological studies.

2. REVIEW OF THE LITERATURE

2.1 Melanocortins

2.1.1 History of the discovery of MSH peptides and their nomenclature

According to the IUPAC-IUB convention the full name of the hormone that causes melanin dispersion is a melanocyte-stimulating hormone, abbreviated as MSH, and the trivial name is melanotropin. The term opiomelanocortins is used for any or all of the proopiomelanocortin (POMC) peptides, the term melanocortins relates only to ACTH and MSH-derived peptides [α-MSH, β-MSH, γ_1-MSH, γ_2-MSH and γ_3-MSH] (Eberle, 1988). The first scientific report on biological activity of melanocortins appeared in 1912 when it was demonstrated that pituitary extracts darken the skin of frogs (Fuchs, 1912). The isolation of the melanophore stimulants has lead to the identification of α-MSH, and it was one of the first peptide hormones discovered (Lerner and Lee, 1955). α-MSH appeared as a right peptide responsible for the skin darkening by melanocyte dispersion process. In the 1960th a lot of studies were devoted to clarification of the functional role of α-MSH and ACTH. In comparison to the quite well identified mechanisms of α-MSH, much less is known about the functional roles of γ-MSH peptides.

2.1.2 Proopiomelanocortin and the formation of the MSH peptides

The melanocortins (α-MSH, β-MSH, γ_1-MSH, γ_2-MSH and γ_3-MSH) as well as several other biologically active fragments including β-endorphins, β-lipotropin and γ-lipotropin, are proteolytically cleaved from the common precursor, a glycosylated protein proopiomelanocortin (POMC) having 240 amino acid residues (Uhler and Herbert, 1983). The POMC sequences are identical in the pituitary, brain and peripheral tissues, however they are processed differentially at these sites that results in the release of active hormones (Smith and Funder, 1988). The prohormone convertases PC1 and PC2 participate in processing of the POMC. PC1 may generate the ACTH, whereas PC2 may lead to cleavage of the peptide bound after the first 13 amino acids of ACTH, yielding α-MSH. These PCs are soluble and primarily localized to large dense core vesicles in neurons and endocrine cells. Proteolytic cleavage occurs mostly at sites where two polar amino acids form a peptide bound, e.g., Arg-Arg (R-R), Lys-Lys (K-K), Arg-Lys (R-K), Lys-Arg (K-R). The prefix α (α-MSH) denotes the basic tridecapeptide derived from adrenocorticotropin (ACTH) and the prefix β (β-MSH) the acidic octadecapeptide derived from lipotropin (LPH), see Fig. 1 (Eberle, 1988).

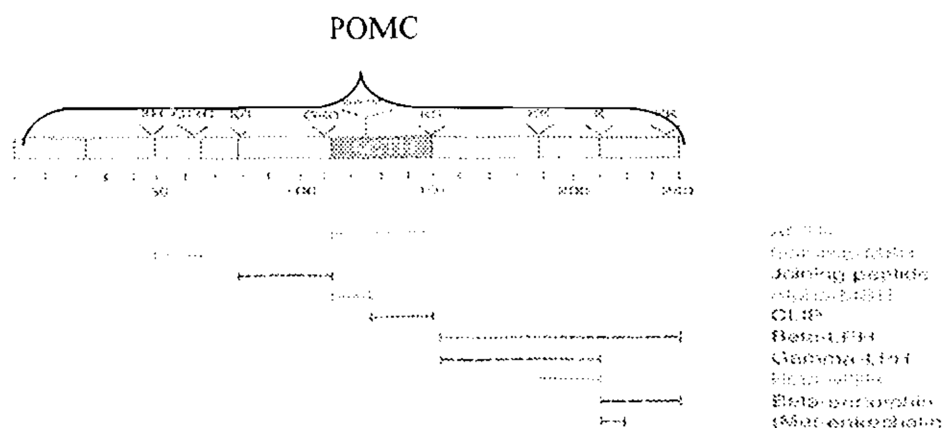

Fig. 1. Schematic presentation of the formation of POCM-derived peptides

In the central nervous system, POMC-immunoreactive neurons form essentially two neural systems: one originates in cell bodies localized in the posterior hypothalamus, and the other in cell bodies of the brain stem. These neurons project to distinct regions of the central nervous system including the *telencephalon*, *diencephalon*, *mesencephalon*, brain stem and the spinal cord (Low et al., 1994). The main source of circulating melanocortins is the hypophysis (pituitary). Melanotrophs of the pars intermedia and corticotrophs of the anterior hypophysis are typical cells that are known to process POMC. α-MSH and β-lipotropin are the main products from the intermediate lobe. α-MSH is also detectable in hypothalamus, stomach, kidney, intestine, testis, placenta, ovaries, pancreas, adrenal medulla, skin and immuno-competent cells. Presence of γ-MSHs was found in some regions of the CNS, neurons of intestines and adrenal medulla (Eberle, 1988). New discovery was done in 2003 when Delta-MSH has been detected in the POMC gene of a dogfish and a stingray (Dores et al., 2003).

2.1.3 Primary structure of melanocortins

Melanocortin peptides differ from each other by their amino acid sequences and length of the chain; however they all share a common pharmacophoric unit, a tetrapeptide His^6-Phe^7-Arg^8-Trp^9. α-MSH has an identical amino acid sequence to the 13 first amino acids in ACTH, however α-MSH has acetyl group in the N-terminus and amide group in the C-terminus (Fig. 2). There are three naturally occurring forms of α-MSH: desacetyl-, monoacetyl – and diacetyl- α-MSH. Desacetyl- α-MSH may be post-translationally processed into the α-MSH (Verburg-van Kemenade et al., 1987). The majority of α-MSH originates from pituitary melanotropic cells, whereas the hypothalamus releases the desacetylated form. The amino acid sequences of the γ-MSH peptides are almost identical. These peptides differ from each other only with extra C-terminal glycine residue in γ_2-MSH molecule (Fig. 2).

AKTH
H-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-Lys-Lys-Arg-Arg-
Pro-Val-Lys-Val-Tyr-Pro-Asn-Gly-Ala-Glu-Asp-Glu-Leu-Ala-Glu-Ala-Phe-
Pro-Leu-Glu-Phe-OH

α-MSH (acetylated)
Ac-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2

γ_1-MSH
H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2

γ_2-MSH
H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH

γ_3-MSH
H-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Arg-Arg-Asn-Gly-Ser-
Ser-Ser-Ser-Gly-Val-Gly-Gly-Ala-Ala-Gln-OH

Fig. 2. The primary structure of melanocortin peptides; the common pharmacophoric unit of the melanocortins is shown in the brown color, the homological amino acid sequence of γ_1-MSH and γ_2-MSH is framed.

2.1.4 Specific structure features of melanocortins in different species

Phylogenetically, α-MSH is an ancient molecule that has remained essentially unchanged over million years. The amino acid sequence of α-MSH is remarkably conserved across different species. In seven mammal species from which α-MSH has been isolated (pig, sheep, horse, monkey, camel, rat and ox), it showed identity in the structures. Birds' α-MSH (duck and turkey) shows a high degree of similarity with mammalian hormone. The γ-MSHs structures of different species have been deduced from the respective DNA sequences (Eberle, 1988).

2.1.5 Expression of melanocortins in the CNS and peripheral tissues

In the central nervous system, α-MSH immunoreactive perykaria are found in three regions: the *nucleus arcuatus* of the *hypothalamus*, the dorsolateral region of the hypothalamus and the *nucleus tractus solitarius* (Eberle, 1988). From these regions nerve fibres project throughout virtually the whole brain, including the *hypothalamus, thalamus*, midbrain, *amygdala, medulla*, spinal cord, *hippocampus* and cerebral cortex. Besides, α-MSH is widespread in peripheral tissue (Table 1.). γ-MSH immunoreactivity is present in POMC neurons of the *nucleus arcuatus* and *nucleus commissuralis* as well as in their projections (Bloom, 1980). In the peripheral tissues, γ-MSH is found in the adrenal cortex, heart, kidney and other ones (Table 1.). α-MSH- and γ-MSH are present in the nerves of baroreceptor areas in the brainstem, thus indicating possible melanocortin regulation of cardiovascular activity (Palkovits, 1987).

Table 1. Expression of MSH peptides in the hypophysal and peripheral tissues

PEPTIDES	TISSUES
	Hypophysis
α-MSH, γ-MSH	*Pars intermedia*
ACTH	*Pars anterior*
	Peripheral tissues
ACTH	Adrenal gland, stomach, small intestine, liver, colon, ovary, spleen, lung and kidney
α-MSH	Adrenal gland, duodenum, heart, small intestine, liver, lung, ovary, placenta, spleen, skeletal muscle, skin, spinal cord, testis and thyroid
γ-MSH	Adrenal cortex, heart, kidney, ovary, stomach and blood vessels

Various forms of stress induce a 2- to 8-fold increase in ACTH, α-MSH and γ-MSH concentrations in rat plasma. The basal γ_3-MSH levels are similar to those of α-MSH, they are low in the morning (8.2pM) while in the evening they are slightly elevated. Basal morning plasma α-MSH concentrations vary from 10-50pM with an average of about 28pM. The rate of α-MSH breakdown *in vitro* pooled blood or plasma samples from rat are the same as for endogenous or synthetic peptides. The breakdown is proximately exponential with a mean half life of 39 min for samples kept at 37°C, 54 min for sample kept on ice. The Phe^7-Arg^8 bond is particularly labile and thought to be the primary site for cleavage. Once this bond is cleaved, the fragments are degraded further to free amino acids by exopeptidases (Eberle, 1988).

2.2 Melanocortin receptors

2.2.1 Discovery of melanocortin receptors

Five different subtypes of melanocortin receptors (MC1-5R) were cloned during 1992 and 1993. In 1992 the genes encoding G protein coupled MSH receptor, termed MC1R, was cloned from cDNA libraries derived melanoma cell lines independently by two groups: Prof. Jarl Wikberg, Uppsala, Sweden, and Prof. Rogers Cone, Oregon, USA (Chhajlani and Wikberg, 1992; Montjoy et al., 1992). At the same time, a structurally similar receptor, now termed MC2R, was also cloned (Montjoy et al., 1992). In 1993, the genes encoding three larger MSH receptors, termed MC3, MC4 and MC5R, were identified, cloned and characterized (Chhajlani et al., 1993; Gantz et al., 1993).

2.2.2 Melanocortin receptor 1 (MC1R)

The MC1 receptors was firstly detected in melanoma cells and cells of solid melanoma tumours (Chhajlani and Wikberg, 1992) (Table 2.). However, the expression levels of the MC1R increase generally 10- to 20-fold upon transformation of the normal melanocytes into malignant melanoma (Loir et al., 1999). In normal adult human skin the immunoreactivity for the anti-MC1 receptor antibody was detected in hair follicle epithelia, sebocytes, secretory and ductal epithelia of sweat glands (Bohm et al., 1999). Now the MC1R has been cloned in a number of different mammalian and non-mammalian species such as rodents, cows and birds (Wikberg, 1999). The MC1R was also found in Leydig cells of the testis, lutein cells of the corpus luteum and trophoblastic cells of the placenta (Wikberg et al., 2000). The localization of MC1R to macrophages and monocytes (Hartmeyer et al., 1997), neutrophils (Catania et al., 1996), endothelial cells, fibroblasts (Boston and Cone, 1996), keratinocytes (Luger et al., 1997) and microglial (Wong et al., 1997) cell types have recently been much discussed in relation to anti-imflammatory actions of MSH peptides. In the CNS, MC1R is found to be located to only a few scattered neurons of the periaqueductal grey area in both rat and human brains and MC1R does not have a major role in the CNS (Xia Y et al., 1995). The MC1R contains substantial single nucleotide polymorphisms (SNP's) in its coding region and it results in phenotypic variations in hair and skin colour. Increased risk of melanoma is also linked to some mutations in the MC1R (Wikberg et al, 2000). MC1R binds α-, β- and γ-MSH peptides, however α-MSH-MC1R binding is considered as predominant for many physiological effects, for instance, skin darkening, melanoma formation.

2.2.3 Melanocortin receptor 2 (MC2R)

Melanocortin receptor subtype 2 proved to be the receptor for the corticotropin (ACTH) binding, but not for the MSHs (Schioth et al., 1996). The MC2R is highly expressed in the adrenal cortex, where most of the expression occurs in the cells of *zona glomerulosa* and *zona fasciculate* and in a few scattered cells in the medulla, however, both the hypophysis and the hypothalamus proved to be negative (Xia and Wikberg, 1996). In the adrenal cortex MC2R is responsible for the ACTH mediated control of glucocorticoid and mineralocorticoid production (Mountjoy et al., 1992). Natural mutations in the MC2R gene may be related to familial glucocortical deficiency (Weber et al., 1993). The MC2R has also been detected in mice (but not in human) adipocytes, where it may be responsible for stress-induced lipolysis mediated by ACTH released from the pituitary (Boston, 1999; Chhajlani, 1996). In the chicken, MC2R are expressed in both the spleen and the adrenal gland, indicating additional functions of the receptor in birds compared to mammals (Takcuchi et al., 1998).

The expression of the MC2R was recently identified in the skin, thus indicating a possible novel role for this receptor and corticotropins in the physiology of the skin (Slominski et al, 1996).

2.2.4 Melanocortin receptor 3 (MC3R)

The MC3R was originally found as an orphan G-protein coupled receptor and was characterized as an unknown receptor useful as a polymorphic marker linked to non-insulin-dependent *diabetes mellitus* on human chromosome 20q (Bell et al., 1991; Yamada et al., 1991). MC3R was firstly identified, cloned, expressed and localized in the brain, placental and gut tissues by RT-PCR in 1993 (Gantz et al., 1993). Expression of MC3R was also found in the heart of mammals, in the adrenal gland of chicken and in the thymus and peritoneal macrophages of mouse (Chhajlani, 1996; Takeuchi and Takahashi, 1999; Getting et al., 1999). MC3R is predominantly expressed in the CNS; *hippocampus, ventral tegmental area* (VTA), *cortex, thalamus, nucleus arcuatus, septum, corpus amygdaloideum, substantia grisea centralis mesencephali, nucleus raphes, nucleus accumbens* (NACC), *hypothalamus* (Gantz et al., 1993). Relatively high density of the MC3R is present in the NACC and VTA (Lindblom et al., 1998). The much wider distribution of the MC3R protein compared to its mRNA and expression of the MC3R on the POMC neurons of the *arcuate nucleus*, indicates the possibility that the central nervous system MC3R is a largely presynaptically located receptor. The MC3R are expressed on the cell bodies of POMC neurons of the posterior *hypothalamus* that form major projections to large parts of the CNS. So, the MC3R may function as an autoreceptor, regulating the release of MSH peptides from POMC neurons. The melanocortins receptor expression during the development of the fetus shows that the rat MC3R expression appears only after birth (Xia and Wikberg, 1997). Recently it was showed that anabolic androgenic steroids cause a strong unregulation of MC3R at sites of the *hypothalamus*, where MSH peptides can also stimulate sexual behaviors (Wikberg et al., 2000). Nevertheless, the physiological functions of the MC3R are at present poorly understood. γ-MSH peptides have a relatively high affinity to the MC3R (Table 4.).

2.2.5 Melanocortin receptor 4 (MC4R)

The ontogenesis of the mammalian MC4R expression showed that it is predominant during the whole fetal period (Kistler-Heer et al., 1998). The MC4R is widely distributed in the central nervous system and is represented in almost every brain region, including the *cortex, thalamus, hypothalamus*, brain stem, spinal cord, *septum lateralis, tuberculum olfactorium* and *ventral tegmental area* (Mountjoy et al., 1994). In a study covering 20 human peripheral organs, use of RT-PCR failed to detect the MC4R in any of the organs studied (Chhajlani, 1996). Compared to mammals, the MC4R was expressed in many peripheral tissues of the chicken (Takeuchi and Takahashi, 1998). However, there is evidence that the MC4R may be present also in human adipose tissue (Chagnon et al., 1997). This is in line of the reported role of the MC4R in control of body weight. A number of mutations have been found in the coding region of the MC4R gene which link with dominant autosomally inherited forms of severe obesity. The MC4R of the *hypothalamus* and brain stem are believed to play an important role in the control of feeding behaviour. Direct injections of α-MSH or ACTH-(1-24) into *hypothalamus* or intracerebroventricularly (icv) caused a marked inhibition of food intake (Poggioli et al., 1986). A relationship between feeding control and melanocortin receptors was confirmed in 1994, when it was shown that endogenous *agouti* peptide acted as MC4R antagonist (Lu et al., 1994). Further it was strengthened by the discovery that MC4R knockout mice developed morbid obesity (Huszar et al., 1997). The selective MC4R antagonist HS014 induces overeating and severe obesity (Kask et al., 1998).

Many studies indicate that an MC4R agonist could be useful to treat obesity, while an MC4R antagonist might be of use to treat anorectic conditions. Chronic morphine and cocaine administration causes down-regulation of the MC4R expression in areas of the brain regions involved in opiate addiction, suggesting about MC4R role in drug addiction (Alvaro et al., 1996). Some studies show the MC4R involvement in the regulation of the cardiovascular system, since increase in mean arterial pressure by α-MSH or ACTH was blocked by icv administration of the natural MC4R antagonist *agouti* signaling peptide (Dunbar and Lu, 1999). There is also evidence that neurotrophic effect of α-MSH may be mediated by MC4R (Van Der Kraan et al., 1999). Activation of central MC4R may play a central role in the regulation of luteinising hormone and prolactin secretion (Wikberg et al., 2000). The main difference between the MC4R and the other melanocortin receptors is its particularly low affinity for the γ-MSH peptides, and slightly higher affinity for β-MSH than α-MSH and ACTH, see Table 4 (Schioth, 2000).

2.2.6 Melanocortin receptor 5 (MC5R)

The MC5R is ubiquitously expressed in many peripheral tissues (e.g., adrenal glands, fat cells, liver, kidneys, lung, lymph nodes, bone marrow, thymus, mammary glands, testis, ovary, pituitary, uterus, oesophagus, stomach, skin, skeletal muscle) and it is also present in the brain and spinal cord (Chhajlani et al., 1993; Labbe et al., 1994). The receptor is richly expressed in the exocrine gland tissues (lacrimal, prostatic, seminal, pancreatic, preputial and Harderian) where it is present in the gland's secretory epithelia (Wikberg et al., 2000). There is evidence suggesting that MC5R plays a role in regulation of functions of exocrine glands. MC5R in mice is involved in production of sebum from sebaceous glands resulted in water repulsion of their furs and thermoregulation (Wikberg, 2001). Lipolysis in adipocytes may be stimulated by α-MSH peptides by activating MC5R. However, there are species differences in the stimulation of lipolysis in adipocytes, showing the rabbit and guinea pig as being considerably more sensitive to α-MSH than rodents and primates (Boston, 1999). α-MSH peptide has been shown to stimulate aldosterone secretion in the adrenal gland, and it is likely that the effect produced by α-MSH is mediated *via* the MC5R. Interestingly, α-MSH stimulates inositol-trisposphate and protein kinase C (PKC) activity in glomerulosa cells, indicating that the MC5R might be involved in signaling *via* the inositol-triphosphate pathway in the adrenal gland (Kapas et al., 1996). The function of the MC5R expressed in the various tissues remains to be elucidated. Major sites of expression for different melanocortin receptor subtypes are summarized in Table 2.

Table 2. Tissue distribution of the MC receptor subtypes (Wikberg, 2001)

MCR subtype	Tissues reported with MCR subtypes
MC1R	melanocytes, melanoma cells, macrophages, brain, adipose tissue and testis
MC2R	adrenal gland and adipose tissue
MC3R	brain, placenta, duodenum, pancreas, stomach and heart
MC4R	different regions of the brain
MC5R	brain, adrenal gland, skin, spleen, thymus, testis, ovary, muscle, lung, adipose tissue, liver, uterus, stomach, bone marrow, leucocytes, pineal, mammary and thyroid gland

2.2.7 Structural characterization of the MCR and signal transduction pathways

The melanocortin receptors belong to the class of G protein coupled 7-transmembrane (TM) region or heptahelix receptors. G-protein coupled receptors belong to the largest family of receptors with approximately 1000 members in mammals. The MC receptor family has the shortest amino acid sequence among the super family of G protein coupled receptors. The human MC1R, MC2R, MC3R, MC4R and MC5R are 317, 297, 361, 333 and 325 amino acids long, respectively. They are characterized by having short N-terminal (25-39 amino acids) and C-terminal regions (17-21 amino acids) as well as very small second extracellular loop (9 amino acids). All the MCRs ave several potential N-glycosylidation sites in their N-terminal domains. The MCRs also have conserved cysteins in their C-termini, which may serve as sites for fatty acid acylation anchoring the C-terminus to the plasma membrane (Tatro, 1996). Insights into the structural organization of the MSH peptides binding to MCRs and their ligand pockets have been studied at the late 1990th. By using site-directed mutagenesis, there was initially identified two amino acid residues, Asp^{117} (D117) in the third TM segment, or His^{260} (H260) in the sixth TM segment of the human MC1R, that were involved in the binding of α-MSH (Frändberg et al., 1994). All natural MSH peptides tested (which have an L-Phe in position 7) lost affinity for both mutants, whereas the D-Phe^7 containing NDP-MSH was not affected. Further studies indicated that actually these two amino acids rather participate in the maintenance of the over all structure of the MC1R and this conformational changes in the receptor is not linked to any specific amino acids in the MSH peptides (Schioth et al. 1997a). The linear MSH peptides are too flexible to allow accurate ligand locking. Therefore a novel series of small rigid cyclic MSH core peptides (for example [Cys^4, Cys^{10}]α-MSH(4-10) was synthesized to study the MC1R binding affinity, for the illustration see Fig.3).

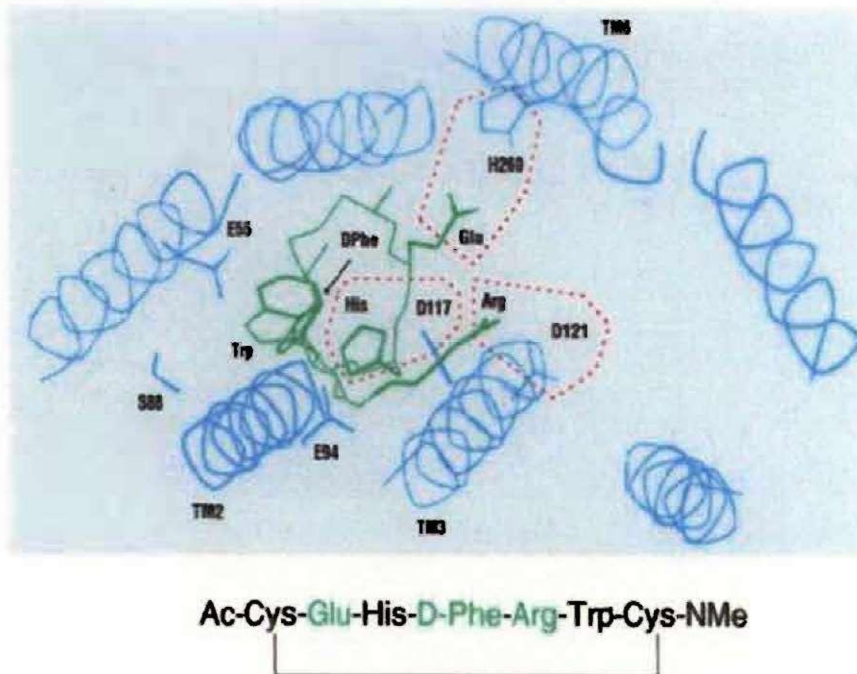

Fig. 3. Molecular 3 dimensional model represents interaction of the cyclic [Cys^4, Cys^{10}]α-MSH(4-10) wit MC1R (Prusis et. al., 1997).

This model presented in Fig.3 indicates that the receptor binding site forms a binding pocket by involving most TM domains with exception of TM4 and TM5. Obviously all domains are necessary to arrange optimal protein conformation to provide high binding activity.

The five melanocortin receptors exhibit sequence homologies ranging between 40% and 60%. The identity is lowest between the MC1R and the MC2R (38% identity) and highest between MC4R and the MC5R (60% identity), see Table 3.

Table 3. Amino acid identity (in %) between the five cloned human MC receptor subtypes (Schiöth et al., 1995; Schiöth et al., 1997)

	MC1R	MC2R	MC3R	MC4R	MC5R
MC1R	100	38	45	47	44
MC2R		100	42	46	44
MC3R			100	42	57
MC4R				100	60
MC5R					100

It is interesting that the MC2R has a similar degree of homology to the MC3, MC4 and MC5 receptors, as to the MC1R, despite the dissimilarity among themselves. Generally, the MC receptor subtypes show lowest homology in the intra- and extracellular loops and in TM4 and TM5, and highest homology in TM1, TM3 and TM7.

The ability of ACTH and different MSH peptides to bind to specific melanocortin receptor subtypes is showed in Table 4.The MC2R binds only ACTH and its fragments but not α, β, and γ-MSH peptides. Moreover, although the other MC receptors bind ACTH and larger ACTH fragments, ACTH may be subjected to breakdown to shorter MSH peptides that generally show higher activity for the MSH-binding MC receptors (Schioth et al, 1995). Diacetylation lowers the affinity of α-MSH for all of the melanocortin receptors. While the natural desacetylated form of ACTH and α-MSH favours the melanocortin 3 receptor even though the affinity between acetylated and desacetylated peptides are not very high. The MC1R and MC5R have more than 30-fold and 2-fold higher affinity for α-MSH, respectively, compared to that of γ_1-MSH, whereas the MC3R has 2-3-fold higher affinity for γ_1-MSH than α-MSH. The MC3R shows a relative preference for γ-MSH peptides.

Table 4. Ligand preferences of the five mammalian MC receptor subtypes (Schiöth et al., 1995; Schiöth et al., 1996; Schiöth et al., 1997)

Melanocortin receptor subtype	Potency order of melanocortins to melanocortin receptors
MC1R	NDP-MSH>α-MSH>β-MSH>γ_1-MSH
MC2R	AKTH
MC3R	NDP-MSH>γ_1-MSH>γ_3-MSH>β-MSH>γ_2-MSH>α-MSH>AKTH
MC4R	NDP-MSH>>β-MSH>α-MSH>AKTH>γ_1-MSH
MC5R	NDP-MSH>>α-MSH>β-MSH>AKTH>γ_1-MSH

The γ_2-MSH has similar relative MCR affinity profile as γ_1-MSH, although it is somewhat less potent. The main difference between the MC4R and the other receptors is its particularly low affinity for the γ-MSH peptides. So, the MC1R shows preference for α-MSH, the MC3R for γ-MSH and the MC4R for β-MSH (Schiöth et al., 1995; Schiöth et al., 1996; Schiöth et al., 1997).

All five melanocortin receptors subtypes are positively coupled in a stimulatory fashion, leading to the increase in intracellular cAMP after ligand activation (Gantz et al., 1993). The effect on cAMP production is rapid, reaching a maximum within 15 minutes. There are reports showing that other signal pathways also may be involved, namely the phosphoinositol pathway for the MC3R (Konda et al, 1994), and the MC5R may additionally be linked to the Jak/STAT signaling pathway (Buggy, 1998). Pretreatment with a cAMP antagonist, abolished the decrease in IP_3 production at higher concentrations of α-MSH or ACTH and resulted in an increase in intracellular calcium levels, therefore indicating that MC3R may be coupled to two second messenger systems: PKA/cAMP and IP_3/Ca^{2+} (see Fig. 4). The latter probably is under control of PKA. The melanocortin receptors have consensus recognition sites for protein kinase C, and in some case also for protein kinase A, indicating that they may be subjected to regulation by phosphorylation (Wikberg et al., 2000).

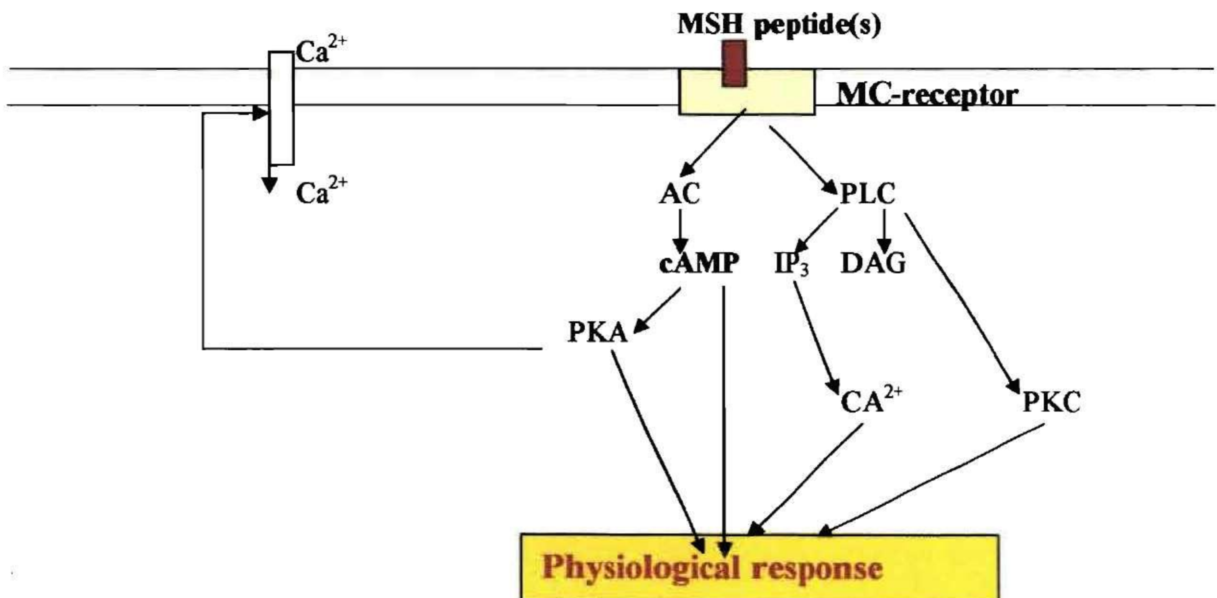

Fig. 4. Overview of the possible signalling pathway induced by melanocortins through MCRs transduction. AC, adenylate cyclase; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP_3, inositol 1,4,5-triphosphate; MC receptor, melanocortin receptor; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (Frändberg, 1998).

2.3 The biological effects of MSH peptides

2.3.1 Pigmentation

Early observation in the 1960th showed that α-MSH increased skin darkening in humans (Eberle, 1988). α-MSH and ACTH are both synthesized in human epidermis in response to ultraviolet radiation. Shortly after the cloning of the MC1R it was shown that the extension gene locus that controls coat color in mammals encodes the gene for the MC1R. Population studies have revealed more than 20 allelic variants of the MC1R gene. The activation of the MC1R results in stimulation of cAMP formation, induction of pigmentation enzymes, including tyrosinase and leads to melanin synthesis. The human MC1R thus plays a central role in regulation of eumelanin (black/brown) and phaeomelanin (red/yellow) synthesis within the mammalian melanocyte (Schioth et al., 1999).

α-MSH serves as a growth stimulator for normal melanocytes, when other synergistic mitogens are present, thus indicating that the MSH peptides and other factors are of importance for melanocyte cell proliferation *in vivo* (Halaban, 2000). Malignant transformation of melanocytes into melanoma leads to growth autonomy and is associated with the production of multiple growth factors and receptors by the tumour cells. High levels of α-MSH immunoreactivity were detected in melanoma tumours and melanoma cell lines express up to 20-fold higher levels of MC1R mRNA compared to normal melanocytes (Loir et al., 1997). So, the most clarified function of MC1R is binding with α-MSH that leads to skin darkening and melanoma tumorogenesis.

2.3.2 Inflammation

The POMC can express melanocortins not only in pituitary gland but also in immune cells, indicating the role of MSH peptides in the regulation of the CNS, immune and endocrine system. The POMC-derived peptides may be produced in immunocompetent cells when they receive non-cognitive (bacterial, viral) stimuli. The peptides then can be released to initiate glucocorticoid synthesis along the immuno-adrenal axis, hence induce immunosupression and anti-inflammatory action (Fig.5).

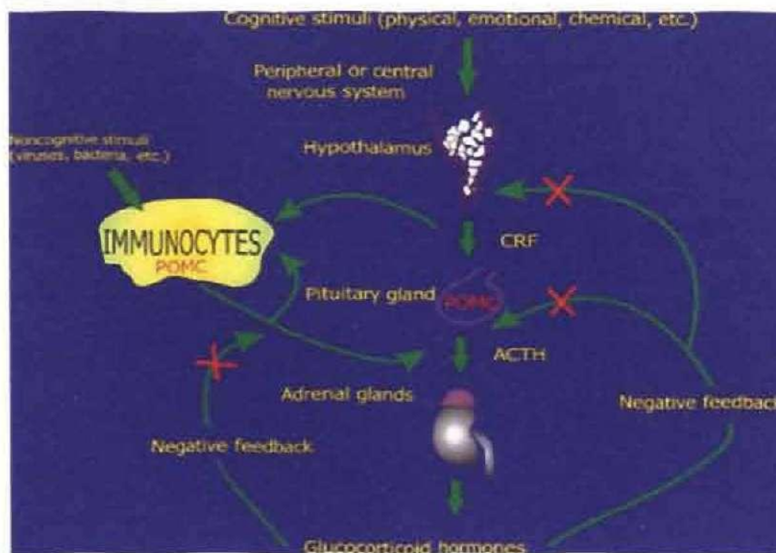

Fig. 5. The POMC-derived peptides take part in the transduction of the information between CNS and immune system (Blalock et al., 1985).

It was noted in the early 1980th that administration of α-MSH and ACTH(1-24) caused hypothermia and reduced fever induced by a leukocytic pyrogen (Glyn and Lipton, 1981). α-MSH seems to act both directly on inflammatory cells in the periphery and through central mechanisms. Many inflammatory processes, such as neurodegenerative diseases, stroke, closed head injury, encephalitis, and bacterial endotoxin-induced inflammation within the brain, are marked by a local increase in cytokines, particularly TNF-α. The effects of α-MSH include inhibition of production and activity of immunoregulatory and proinflammatory cytokines such as interleukin (IL)-1α and IL-1β, IL-2, interferon-γ, and inflammatory cytokine tumor necrosis factor (TNF)-α (Luger et al., 1997, 1998; Lipton and Catania, 1998). α-MSH is also found to produce by macrophages, monocytes, and mast cells. Elevated levels of α-MSH were also reported to be present in plasma from HIV infected patients and patients with sepsis (Airaghi et al., 1999; Catania et al., 2000). Melanocortin peptides significantly inhibit the release of both NO and TNF-α into cell-free supernatants from microglia cells. Melanocytes, keratinocytes, several other cutaneous cell and inflammatory cell types express the MC1R. The MC1R has received the most attention as the mediator of the effect of α-MSH on inflammatory processes. Recently it was found that not only α-MSH but also its shorter fragments (α-MSH 1-10 and α-MSH 11-13) might considerably suppress NF-kB production in macrophage cell line expressing MC1R, when they are exposed to inflammatory agents such as LPS and interferon-γ (Mandrika et al., 2001). γ-MSH immunoreactive material was demonstrated to be present in human neutrophil granulocytes (Johansson et al., 1991).

2.3.3 Behavioural effects

Melanocortin peptides induce a large array of behavioural effects which include improvement of attention, motor effects, facilitating actions of learning, modification of sexual and feeding behaviours (De Wied and Jolles, 1982). There is also evidence that the α-MSH and γ-MSH peptides have quite disparate and sometimes even opposing actions on the central nervous system. In comparison to the quite well studied effects of α-MSH much less is known about the functional roles of the γ-MSH peptides in CNS and peripheral tissues. Studies on the influence of γ-MSH peptides on behaviour are one of the main tasks of the present PhD thesis.

2.3.3.1 Grooming behaviour

Grooming behaviour of mammals, such as rats, rabbits or cats, is a pattern of characteristic movements with readily definable components consisting of activities directed to the animal's body surface, such as face washing, body grooming, licking, scratching and genital grooming. Novel environment is well known to induce excessive grooming behaviour in small mammals and birds. MSH and ACTH peptides can induce excessive grooming in the rat. Excessive grooming could be elicited by icv injections of α-MSH, synthetic MCR agonists Melanotan-II and NDP-MSH (Adan et al., 1999) and also by γ_1-MSH (see results). The grooming responses of α-MSH and ACTH(1-24) were blocked by melanocortin receptor blockers HS014 and SHU9119 (Schioth, 2000). There is evidence that the effects are mediated by the activation of MC4R subtype (Vergoni et al., 1998). α-MSH and ACTH injected directly into the VTA, paraventricular nucleus of the *hypothalamus*, *dorsomedial nucleus* of the *hypothalamus* and *anterior hypothalamus* induce grooming behavior (Argiolas et al., 2000). MCR blockers can attenuate the effects of the melanocortic peptides.

2.3.3.2 Sexual behaviour

ACTH and MSH peptides are known to effect sexual behaviour in both male and female rats. In male rats, rabbits and cats icv injections of ACTH(1-24) or α-MSH may elicit elements of copulatory behaviour such as erection, ejaculation, sexual postering and genital licking, even in the absence of a female partner (De Wied and Jolles, 1982). Both ACTH(1-24) and α-MSH induced similar increases in erection frequencies when injected directly into paraventricular nucleus of the *hypothalamus* (Argiolas et al., 2000). The three major kinds of neural autonomic control of erection are stimulatory adrenergic, inhibitory cholinergic and inhibitory mediated by nitric oxide (Argiolas, 1999). Peripheral administration of α-MSH or ACTH does not seem to induce erection. The synthetic MCR agonist Melanotan-II induced a marked increase in erection frequency in double-blind study in men with erectile dysfunction (Wessells et al., 1998). MC4R antagonist HS014 only marginally blocked the frequency of penile erections induced by α-MSH (Vergoni et al., 1998). It is thus apparent that the molecular mechanisms eliciting the changes in sexual behaviour by MSH and ACTH peptides are distinctly different from those eliciting the grooming behaviour. Today, it is not clear which receptor mediates the effects of melanocortins on erection, nor is it well established how this effect interacts with other central regulators (such as dopamine, serotonin, acetylcholine, nitric oxide, oxytocin and opioid peptides).

2.3.3.3 Feeding behaviour

Complex machinery is known to regulate feeding behaviour. Peripheral input to the central feeding systems is mediated by leptin, a hormone produced in the adipocytes. Inside the brain leptin may upregulate the α-MSH and downregulate the neuropeptide Y/*agouti* related peptide (AGRP). Within the central nervous system various regulatory factors are involved, which include neuropeptide Y, corticotropin-releasing hormone, galanin, orexin, catecholamine-regulated transcript, melanin-concentrating hormone and monoamines. A role for melanocortins in the central control of appetite was described as early as the 1980th (Vergoni et al., 1986). The central melanocortin system effects on feeding seem to be mediated by the actions of α-MSH and AGRP on MC4R in hypothalamic areas and other regions of the CNS, such as in the brainstem. The reduction of food intake was still seen after 4 weeks of administration of α-MSH (Lim et al., 2000). Inactivation of the MC4R gene in mice results in marked increase in body weight and length (Huszar et al., 1997). The central administration of α-MSH, ACTH(1-24) and Melanotan-II induces anorexia, while the non-selective MC4 receptor antagonist SHU9119, and the MC4 receptor-selective antagonist HS014 induced marked orexigenic effects (Kask et al., 1998). The anorectic effect of Melanotan-II is blocked by SHU9119, as well as the anorectic effect of α-MSH is blocked by HS014 (Vergoni et al., 1998). The relatively MC4-receptor-selective agonist β-MSH inhibits dose-dependently fasting-induced food intake, whereas the relatively MC3R selective agonist γ_1-MSH fails this effect. (Kask et al., 2000). Thus, there exists very strong support for the role of the MC4 receptor in regulation of feeding behaviour.

2.3.4 Cardiovascular effects

Melanocortins have been described as having a variety of cardiovascular effects. α-MSH and ACTH are effective in prohibiting death in anesthetized rats in a volume-controlled model of hemorrhagic shock and during conditions of prolonged respiratory arrest (Ludbrook and Ventura, 1995). Studies on the mechanism of this anti-shock effect suggest that melanocortins inhibit the overproduction of TNF-α and nitric oxide seen in shock (Guarini et al., 1997).

HS014, a selective MC4R antagonist is able to block the reversal to shock induced by ACTH(1-24) after both icv and iv administration in rats (Guarini et al., 1999).
Moreover, γ-MSH peptides, which have a preference for the MC3R, produced no effect. Injection of α-MSH into the dorsal vagal complex of the *medulla oblongata* of the rat elicited hypotensive and bradycardic effects that were blocked by the MCR antagonist SHU9119 (Li et al., 1996). However, the icv administration of α-MSH or ACTH leads to the increase in mean arterial pressure, concomitant with increase in the lumbar sympathetic nerve activity. The effect of α-MSH or ACTH was blocked by icv administration of the natural MC4R antagonist *agouti* signaling protein (ASIP) (Dunbar and LU, 1999). Intracerebroventricular injection of γ-MSH produced prolonged pressor effects that could not be blocked by melanocortin receptor antagonist SHU9119. γ_1-MSH and γ_2-MSH peptides induce a dose-dependent, short-lasting increase in blood pressure, heart rate, and pulse amplitude following iv administration in normotensive and normovolemic rats. ACTH (1-24), α-MSH and γ_3-MSH that are lacking Arg-Phe sequence in the C-terminal, were to be found either inactive or much less active (Van Bergen et al., 1995, 1997). In generally, the regulation of cardiovascular system by melanocortic peptides is mediated by both melanocortin receptor and non-melanocortin receptor mediated mechanisms.

2.3.5 Other functions of melanocortins

Melanocortin peptides are reported to antagonize opiate dependence and tolerance and to induce opioid withdrawal effects (Szekely et al., 1979; Contreras et al., 1984). It was speculated that the antagonism of opiate self-administration, analgesic tolerance, and physical dependence caused by melanocortins could be mediated through the MC4R (Alvaro et al., 1997). Melanocortins (α-MSH) are known to improve axonal regeneration following peripheral nerve injury and stimulate neurite outgrowth from CNS neurons both *in vitro* and *in vivo*.
Rat spinal cord, dorsal root ganglia, sciatic nerve, and soleus muscle were analyzed for the expression of the MC3R, MC4R, MC5R and POMC. The only MCR found by RNAse protection assays was the MC4R in the lumbar spinal cord, indicating a possible role in neuroregeneration for the MC4R (Van der Kraan et al., 1999). Administration of α-MSH either centrally or peripherally inhibits fever induced by endotoxins (lipopolysaccharides), IL-1, IL-6 or TNF-α (Lipton and Catania, 1998). α-MSH also decreases ischemia/reperfusion injury after ischemia and inhibits both neutrophil accumulation and nitric oxide production. α-MSH decreases renal injury when neutrophil effects are minimal or absent, indicating that α-MSH inhibits neutrophil-independent pathways of renal injury (Chiao et al., 1998). There is evidence that α-MSH increases release of luteinizing hormone (LH) in female but not in male rats (Limone, 1997). The physiological effects of the melanocortins are summarized in the Table 5.

Table 5. Effects of the melanotropins on the pituitary gland and various peripheral organs (Eberle, 1988)

Peptide	Target organ	Effect
α-MSH	skin	stimulates melanogenesis
α-MSH	adenohypophysis	inhibits prolactin release modulates LH release stimulates hGH release
α-MSH, β-LPH	sebaceous glands	stimulate sebum secretion
α-MSH, β-MSH	adipocytes (rabbit)	stimulate lipolysis
α-MSH, β-MSH γ_3-MSH	adrenal cortex	stimulate aldosterone secretion modulate ACTH effects
α-MSH	immune system	inhibits IL-1 induced host-defence reactions (anti-inflammatory properties)
α-MSH, γ-MSH	heart vessels	increase heart rate increase blood pressure
α-MSH, γ_2-MSH	kidneys	increase natriuresis
α-MSH	eye	stimulate permeability changes

2.4 Synthetic MCR agonists and antagonists

The important issues for the clarification of the functional roles of melanocortins in CNS and periphery will be the development of the selective agonists and antagonists on the five melanocortin receptors and evaluate the pharmacology, pharmacokinetics and toxicology of these compounds. Already in the past, quite large number of linear or cyclic MSH peptide analogues were designed and screened. Early seeking of new ligands was based mainly on their ability to cause the effects on skin darkening in amphibians or lizards. Now most of these peptides have only historical interest however a few are still useful for studying the pharmacology of the five melanocortin receptor subtypes. There is intensive search for the new highly active MCR ligands.

2.4.1 Agonists

Linear peptide NDP-MSH ([Nle^4-D-Phe^7]α-MSH; Melanotan-I) is one the most useful synthetic agonists from the early studies (Table 6.). NDP-MSH shows high binding affinity for the all of MC receptors (Table 7.). Therefore NDP-MSH has been widely used in its radio-iodinated form for radioligand binding studies (Schiöth et al., 1995).

Table 6. Alignments of structures of useful ligands for melanocortin receptors (Schioth, 2001; Wikberg, 2001).

Peptide	Structures of ligands
NDP-MSH	Ac-Ser-Tyr-Ser-Nle-Glu-His-D-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2
MTII	Ac-Nle-Asp-His-D-Phe-Arg-Trp-Lys-NH_2
MS05	Ser-Ser-Ile-Ile-Ser-His- L-Phe-Arg- L-Trp-Gly-Lys-Pro-Val- NH_2
MS09	Ser-Ser-Ile-Ile-Ser-His- D-Phe-Arg- L-Trp-Gly-Lys-Pro-Val- NH_2
RO27-3325	Buturyl-His-Phe-Arg-Trp-N-methyl glycine- NH_2
HS131	(cyclo (S-S)-Ac-L-Cys^5-Gly^6-D-Nal^7-L-Cys-$NH_2{}^{10}$) α-$MSH^{5\text{-}10}$ trifluoroacetate
HS014	Ac-Cys-Glu-His-D-Nal-Arg-L-Trp-Gly-Cys-Pro-Pro-Lys-Asp- NH_2
SHU9119	Ac-Nle-Asp-His-D-Nal-Arg-Trp-Lys-NH_2
HS024	Ac-Cys-Nle-Arg-His-D-Nal-Arg-Trp-Gly-Cys-NH_2
HS028	Ac- Cys-Glu-His-dCl-D-Phe-Arg-Trp-Gly-Cys-Pro-Pro-Lys-Asp-NH_2

Note. The amino acid residues which make up the ring closure in the cyclic compounds are shown underlined. Nle, norleucine; D-Nal, β-(-2-naphthyl)-D-alanine; dCl-D-Phe, 3,4-dichloro-D-phenylalanine; D-Phe, D-phenylalanine; L-Phe, L- phenylalanine.

In 90th of the last century a series of 23 cyclic lactam peptides was claimed, all of which were designed around a variant of the critical four amino acid pharmacophore (His-Phe-Arg-Trp) in MSH in which the L-Phe has been replaces by D-Phe. One of this peptide that has been applied quite widely in assessment of the physiological effects of the melanocortins is the metabolically stable MSH lactam analogue Melanotan-II (Hadley et al., 1989). The binding profile of Melanotan-II indicates that it is a fairly non-selective agonistic peptide at all the MSH binding MCR (Wikberg et al., 2000). Melanotan-II was used for stimulation of sexual responses and melanin synthesis in mammals. Central administration of Melanotan-II in rats inhibits food intake, this effect has believed to be exerted by stimulation of MC4R (Fan et al., 1997). Clinically, Melanotan-II has been evaluated for treatment of psychogenic erectile dysfunction (Wikberg et al, 2000). More recently a highly active and full selective MC1R agonist MS05 was synthesized. Another close analogue to MS05 is MS09 that is a more potent agonist than MS05 on the MC1R, although it is less selective compared to MS05 (see Table 6). Both MS05 and MS09 have shown to inhibit the production of the inflammatory mediator nitric oxide in macrophage cells (Szardenings et al., 2000). The use of the MS05 and MS09 may be considered as potential anti-inflammatory agents. Recently, were synthesized an interesting new MC4R agonistic peptide RO27-3325 (Benoit et al., 2000). However, the RO27-3325 showed low affinity for the human MC4R (Wikberg et al., 2000). The synthetic MCR agonist and antagonist used in different tests are shown in Table 7.

Table 7. Binding affinity (K_i) of synthetic peptides for human melanocortin MC1, MC3, MC4 and MC5 receptors (Wikberg et al., 2000)

	MC1R	MC3R	MC4R	MC5R
Agonists				
NDP-MSH	0.085	0.40	3.8	5.1
Melanotan-II	0.67	34	6.6	46
MS05	0.87	1100	>100 000	>100 000
MS09	0.16	6.5	46	270
RO27-3325	33 000	340 000	250 000	>300 000
Antagonists				
SHU9119	0.71	1.2	0.36	1.1
HS014	110	54	3.2	690
HS024	19	5.5	0.29	3.3

2.4.2 Antagonists

Various melanocortin receptor antagonistic peptides have also been developed. One of the first antagonists synthesized was a cyclic peptide, SHU9119, a full antagonist for the MC3R and MC4R and a partial agonist for the MC1R and MC5R. However, SHU9119 is not selective for any of these receptors and is likely to have a pronounced effect on all four MCR when injected into experimental animals. Thus the usefulness of SHU9119 as an experimental tool for MC-receptor subtype classification seems to be limited. Further development of cyclic peptides leads to the discovery of the first selective MC4R antagonist HS014 (Schiöth et al., 1998b). However HS014 similarly to SHU9119 is a partial agonist of MC1R and MC5R, it shows also antagonistic properties towards MC3R. Both, SHU9119 and HS014 have been found to increase food intake and body weight on their central administration to rats (Kask et al., 1999). Also MC4R blocker HS014 is capable of completely attenuating the loss of body weight induced by leptin in animals. Other cyclic MSH peptide analogue HS024 shows about 10-fold more potent antagonism on the MC4R compared to HS014 (see Table 6). Unfortunately, HS024 appears to be antagonistic on all the MSH-binding MCRs. The HS024 causes a dose dependent increase in food intake in free feeding rats after its icv administration (Kask et al., 1998). Recently efforts have been made in search for non-peptide MCR agonist and antagonists. Intriguingly, a 1,4-dihydropiridine compound cerebrocrast showed highly selective affinity to MC4R (Jansone et al., in press) indicating that structure of this compound may conform MC4R optimally.
Newly synthesized MCR ligands mostly serve for experimental studies as model-analogues of MSH peptides. In 2002-2003 have been designed and synthesized several highly selective agonists and antagonists for the MC3R and MC4R. Unfortunately, these compounds are not yet commercially available. Highly melanocortin-receptor-subtype-selective agonists and antagonists are needed to for better understand the mechanisms underlying the various effects of the melanocortins and the roles of the melanocortin receptors in the mediation of these effects.

3. AIM AND TASKS OF THE STUDY

To study behavioural and neurochemical repertoire of natural melanocortins γ_1-MSH and γ_2-MSH, and at least in part to clarify their functional role in the central nervous system.

TASKS

1. To perform studies of γ-MSH peptides in comparison to reference peptide α-MSH in behavioural tests (rats and mice) and microdialysis experiments (rats) by peptide intra-VTA, ICV and intracisternal administration.

2. To study influence of melanocortin receptor subtype 4 (MC4R) antagonists HS014, HS964 and HS131 on MSH-peptide induced central effects.

3. To examine central mechanisms of α-MSH, γ_1-MSH and γ_2-MSH induced effects by use of different neurotransmitter receptor (dopamine, GABA, glutamate, opiate) agonists and antagonists as test-drugs.

4. MATERIAL AND METHODS

The methods used have been described in details in the respective papers (I-VII).

4.1 Animals

Adult male Wistar rats (Breeding Facility of the Joint Stock Company GRINDEX, Riga, Latvia) and male Sprague-Dawley rats (Beco, Sweden) weighing 270-350g were used in the experiments (papers I, II and IV,VII). In analgesia experiments BALB/c male mice 20±2g were obtained from the Institute of Microbiology and Virusology, University of Latvia, Riga, Latvia (paper V). In the study of assessment of locomotor activity, BALB/c male mice 19±1g came from the Breeding Facility of the Joint Stock Company GRINDEX, Riga, Latvia (paper III). The animals were housed in groups of 4 (rats) and 8 (mice) under standard laboratory conditions: temperature 21±1°C, relative humidity of 55±10% and artificial 12h dark-light cycle (lights on 07.00-19.00). Free access to standard rat or mice diet and tap water was available at all times. Experimental sessions were conducted between 09.00-14.00. Each experimental group consisted of 4-10 rats and 7-9 mice. The experimental set-ups were approved by the local Ethics Committees on Laboratory Animal Use of Latvia and/or Sweden.

4.2 Drugs

γ_2-MSH peptide was purchased from BACHEM, Switzerland. The HS964, HS131, HS014 (structures for synthetic peptides see in chapter 2.10.), α-MSH and γ_1-MSH were synthesized in the Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden. Synthesis of the peptides was done by using the solid phase approach applying a Fmoc-based Pioneer peptide synthesis system (PerSeptive Biosystems) and purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. The peptides were dissolved in water and stored frozen in aliquots until used. Prior to the experiments aliquots with peptides were unfrozen and dissolved until appropriate dose in sterile artificial cerebrospinal fluid (CSF) or saline. α-MSH was used as a reference peptide.
Dopamine (DA) and 3,4-dihydroxyphenylacetic acid (DOPAC) were obtained from Sigma Chemicals (St Louise, USA). Stock solutions were made in sterile water and stored until use. Before experiments, the stock solutions were unfrozen and diluted until needed concentrations with artificial CSF, of the following composition: 8.65g/l NaCl; 201.31mg/l KCl; 176.42mg/l $CaCl_2$; 172.81mg/l $MgCl_2$ in sterile water (Apoteket, Produktion&Laboratorium, Umeå, Sweden). Phencyclidine (PCP), L-amphetamine (AMP), naloxone hydrochloride, bicuculline were purchased from Sigma Chemicals (St Louise, USA). Haloperidol (0.5% solution) and diazepam (5% solution) were from Gedeon Richter, Hungary. Muscimol was obtained from Fluka AG, Switzerland.

4.3 Behavioural studies (I, II, III)

4.3.1 In rats

4.3.1.1 Intraventricular (ICV) and *ventral tegmental area* (VTA) cannulation and drug infusions (I, II)

Guide cannulas made from stainless steel syringes (15mm long, 0.56mm OD) were slowly implanted stereotaxically under Nembutal (CEVA, Sanofi, France) anesthesia (60mg/kg i.p.) into the left VTA or left lateral ventricle (ICV) at the following co-ordinates: -5.2mm caudal to *bregma,* 0.8mm lateral from midline, and –8.0mm ventral from *dura mater* or –1.5mm, 1.0mm, and –3.6mm, respectively (Paxinos and Watson, 1982). The guide cannula was implanted 2mm over the VTA for later insertion of a microinjection needle and kept in place with dental cement (De Trey, Sevriton, Germany) and covered with duracryl (SPOFA, Dental, Czech Republic). To prevent clogging of the guide cannulas, a bent stylet of stainless steel was inserted into them, and removed only when the injections took place. After implantation of intra-VTA or ICV cannulas animals were kept in individual cages for the following seven days in a recovery room. α-MSH, HS964, HS014, γ_1-MSH or γ_2-MSH was given at the doses of 0.3 and 3 nmol in volume of 0.5μl per rat. The control animal group received CSF in volume of 0.5μl per rat. For the combined treatments, HS964, HS014, γ_2-MSH or saline (control group) were administrated 15min prior to the α-MSH or γ_1-MSH. Drugs were injected manually into VTA or ICV *via* the guide cannula using a 75RN Hamilton Microliter syringe (Hamilton-Bonadaz AG, Schwitzerland) and a very fine polyethylene tube (PE-10, Clay Adams, Sweden) attached to a 30-gauge microinjection needle that extended 1.2mm deeper than the guide cannula. The total volume injected for each substance was 0.5 or 1μl and the speed of injection was 0.25μl/min. The microinjection needle was left in place after injection for 5 min to limit drug efflux up the cannula shaft.

4.3.1.2 Analysis of behavioural responses (I, II)

The day before behavioural experiment, male Wistar rats (300-350g) and male Sprague-Dawley rats (270-340g) were transported to an observation room for 1 day of handling and habituating. On the next day when experiments started, each rat was placed for 30 min into a Plexiglas observation cage (60x40x15cm^2) for adaptation to the novel environment. Registration of behavioural responses started on the 5th min after the injection of MSH peptide into VTA or IVC and continues for an hour for time-response studies by using a Psion Workabout microcomputer (Noldus, the Netherlands). Grooming behavior was expressed in seconds as the sum of the separate grooming reactions, i.e. total grooming (face washing, body licking, scratching, ano-genital grooming, head shakes and wet-dog shakes). The data were expressed in figures as four observation periods, like 0-15min, 16-30min, 31-45 min and 46-60min periods (paper II) or summed as 0-15min, 0-30min, 0-45min and 0-60min periods (paper I). The following non-grooming behaviour events were registered: vertical activity (VA), horizontal activity (HA) and catalepsy VA and HA were scored as incidences during the total observation period. Catalepsy was evaluated at the beginning and at the end of experiment in three consecutive tests for maximum 10s each: (1) by placing the rat with its forepaws on a 7cm high bar, (2) placing the rat with the hind legs on the same bar, and by (3) placing the rat across two 7cm high bars distanced at 15 cm (Kobayashi et al., 1997).

Each of the catalepsy tests were given a weighting coefficient, respectively, 0.2, 0.3 and 0.5 and the total catalepsy was scored as total time the animal spent in the separate tests multiplied by its corresponding coefficient (Sanberg et al., 1988).

4.3.1.3 NMDA-toxicity test (unpublished data)

Male Wistar rats (270-350g) received γ_2-MSH (at the dose of 0.3 nmol/0.5µl; intra-VTA) 5 min prior the injection of NMDA (10µg/0.5µl; intra-VTA). The dose of NMDA was appropriate for inducing tonic seizures. Rats of control group received intra-VTA injections of saline and NMDA. Following behaviour responses were observed: horizontal activity, vertical activity, ipsilateral and contralateral rotations.

4.3.2 In mice

4.3.2.1 Locomotor activity tests in mice (III)

Locomotor activity tests were performed in BALB/c male mice (19-21g) after intracisternal (i.c.) injection of γ_1-MSH, γ_2-MSH or MC4 receptor antagonist HS014 at the dose of 0.3 nmol/mouse in a volume of 10µl. Peptides were dissolved in saline. Phencyclidine (PCP) 5mg/kg intraperitoneally and L-amphetamine (AMP) 5mg/kg subcutaneously were administered 5min prior to peptide injections. Mice of control groups received saline i.c. in the volume of 10µl/mouse. For the assessment of the locomotor activity, the mice were placed in the Activity Cage (Ugo Basile, Cat.7400, Italy). Locomotor activity was registered from the 30th to 60th min after PCP or AMP administration.

4.3.2.2 Analgesia tail flick test (V)

α-MSH, γ_1-MSH, γ_2-MSH and HS014 (all at the doses 0.3, 1 and 3 nmol/ 10µl) were injected i.c. into the *cisterna magna* in conscious BALB/c male mice via a J-shape needle connected to a Hamilton syringe, as previously described by Takagi et al. (1979). Naloxone hydrochloride (2 mg/kg), bicuculline (0.5 mg/kg), haloperidol (0.5 un 1 mg/kg; 0.5% solution), diazepam (10 mg/kg; 5% solution), muscimol (1 mg/kg) and ethanol (10% ethanol in water) were used as test-drugs and injected intraperitonelly in mice prior to i.c. injections of melanocortins. The tail flick test was a slightly modified version of that previously described by Dewey (1981).
The tail of the mouse was placed on the photoelement window of a MODEL DS20 SOCREL tail flick apparatus (Ugo Basile, Italy) and an infrared beam was focused on the tail area 2cm from its basis. The latency for the mouse to react to the pain stimuli was recorded. Maximal exposure to the pain stimuli was to 15s. The tail flick latency test was performed at 30min, 60min, 90min, 90min, 2h, 3h and 24h after the following injections of melanocortins or test-drugs.

4.4 Neurochemical experiments - brain microdialysis in anaesthetized rats (IV, VII)

4.4.1 Surgery procedure

Microdialysis is an *in vivo* sampling technique, in which a hollow semi-permeable membrane is inserted into a particular brain area in the experimental animal and perfused with saline. For acute experiments, the rats were anaesthetized with inactin (80mg/kg, i.p.) and positioned in a Kopf stereotaxic frame (David Kopf Instruments, CA, USA). The body temperature of the rats was kept at 37C° using a body HB 101/2 temperature control unit (Letica, Scientific Instruments, Barcelona, Spain). A microdialysis probe (MAB, Agn Tho's AB, Lidingo, Sweden) was slowly implanted in the left *nucleus accumbens* (coordinates from *bregma*: B+2.2, L-1.5, V-7.1; Paxinos and Watson, 1982) and used to collect extracellular dopamine (DA) and its metabolite DOPAC. The outer diameter of the microdialysis probe was 0.6mm, the length 2mm and it had a 15 000 Daltons's cutoff PES (Polyethen Sulphone) membrane. The probe was constantly perfused with artificial cerebrospinal fluid (CSF; Apoteket, Produktion & Laboratorium, Umeå, Sweden) at a flow rate 2µl/min (infusion pump, Univentor 684 Syringe pump, Bulebel Industrial Estate, Malta). Two hours after the implantation of the probe, dialysates were collected every 20 min from the outlet line in the Microsampler (Univentor 810 Microsampler, Bulebel Industrial Estate, Malta) into polyethylene microcentrifuge tubes and analyzed immediately by high-performance liquid chromatography with electrochemical detection (see below). Three basal samples were collected with less than 15% variation of the DA and DOPAC levels before drug administration started. Figure 6. illustrates the experimental set-up of the brain microdialysis.

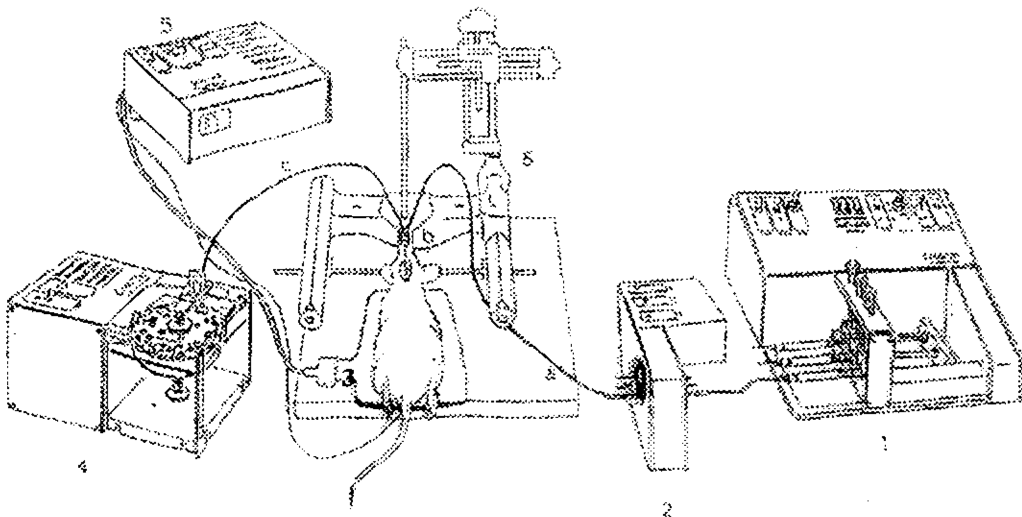

Fig.6. Brain microdialysis: schematic illustration of the experimental set-up. (1) Microdialysis pump; (2) syringe selector; (3) body temperature support; (4) microdialysis collector; (5) temperature controller (6) stereotaxic holder with a microdialysis probe implanted into a rat brain: (a) inlet tubing; (b) microdialysis holder (c) outlet tubing.

The guide cannula was implanted into the left VTA (coordinates from *bregma*: B-5.0mm, L-0.9mm, V-7.2mm; Paxinos and Watson, 1982) as was described above in section 4.3.1 (VTA cannulation) for later administration of α-MSH, γ_1-MSH, γ_2-MSH and HS131 at the doses of 10, 3, 3 and 1 nmol/0.5µl per rat, respectively. For the combined treatments, γ_2-MSH or HS131 were administered 40min prior to the injections of γ_1-MSH or α-MSH, respectively. Control animals receive CSF into the VTA.

4.4.2 Estimation of extracellular concentration of dopamine and DOPAC using high-performance liquid chromatography (HPLC)

Neurotransmitter DA and its metabolite DOPAC in collected brain *nucleus accumbens* dialysate were determined using HPLC technique with electrochemical detection (Gamache et al., 1993). For the separation of biogenic amines each 40µl sample of dialysate was injected onto a reversed-phase column (ReproSil-Pur C-18-AQ 150x3mm, particle size 5µm) using a Rheodyne injection valve equipped with a 100ml loop. Coulometric-electrochemical detection system (ESA Inc, Chelmsford, MA, USA) utilizing two electrodes were used to oxidize the amines. Preinjection part guard electrode voltage was +0.4V (ESA, Guard Cell Model 5020) and the working electrode voltage was +0.34V (ESA, Analytical cell Model 5011). The recycled mobile phase consisted of a mixture of 2g/l $CH_3COONa{\bullet}H_2O$, 38.75mg 1-octanesulfonic acid, 3.7mg EDTA and 100ml/l methanol at pH 4. The flow rate of the HPLC pump (LKB 2150 HPLC pump, Bromma, Sweden) was 0.6ml/min. Chromatograms were recorded using a MEGA series integrator (Carlo ERBA, Strumentazione, USA). The limit of detection was 0.4nM for both DA and DOPAC.

4.4.3 Histological examination (IV, VII)

The histological examination was performed at the end of microdialysis experiments to convince the correct positioning of the microdialysis probe in the *nucleus accumbens* and guide cannula directly above the VTA, Rats were decapitated and the brains were removed, frozen in cold (between –20° and –30°) 2-methylbutane. The frozen brains were mounted on a cryostat microtome (MICROM HM 500 OMV, Laborgerate GmbH Walldorf, Germany) and sectioned (35µm).
The sections were mounted on gelatin coated glass slides and stained with Mayer hematoxylline (Histolab Products AB, Sweden). The stained sections were digitized in a video camera (CCD-72, Dage-MTI, Michigan City, IN, USA) and the placement of microdialysis probe and the guide cannula were verified using NIH-Image software (NIH Image 1.54, NIMH, Bethesda, MD) and the brain atlas of Paxinos and Watson (Fig.7). Only animals with a correctly implanted probe and cannula were further included in the statistic analysis.

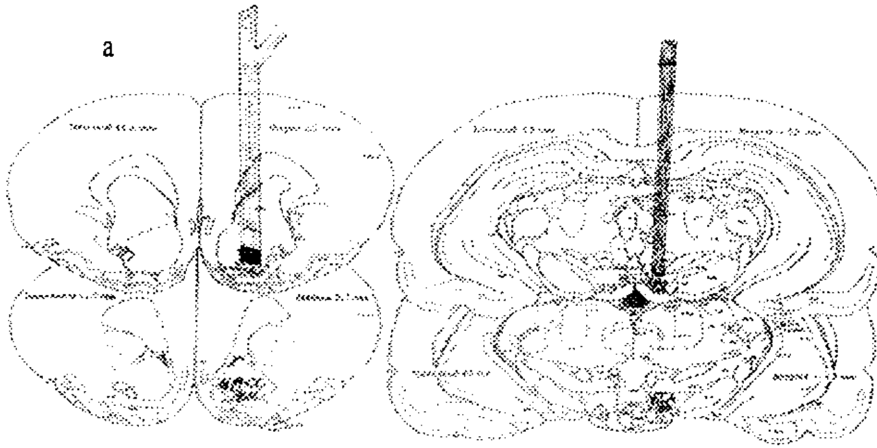

Fig.7. The rat brain sections showing the position of the tips of the microdialysis probe in the *nucleus accumbens* [a] and the guide cannula into the VTA [b] (the atlas of Paxinos and Watson, 1982).

4.5 Statistical analysis

Data for the behavioral and analgesia studies (I, II, V) were analyzed by one-way ANOVA followed by the Bonferroni's multiple comparison or Newman-Keuls tests as a *post hoc*. Results are expressed as the mean ±SEM. Statistics of the locomotor activity data (III) was calculated as mean values ±SEM and significance was evaluated by ANOVA followed by Student's t test. In the microdialysis studies (IV, VII) the average concentration of three baseline samples was considered as control values and expressed as 100%. The statistical evaluation of microdialysis data (VII) was carried out using one-way ANOVA followed by the Newman-Keuls multiple comparison test and paired *t* test. Data were analyzed using the statistical program Prism 3.0 (graph Pad). Inter-group comparisons (VI) were made with a factorial ANOVA test followed by Fisher's PLSD test where appropriate. Then statistics were performed using the StatView 4.51 software for Macintosh. P-values equal and lower than 0.05 were considered to be significant.

5. RESULTS

5.1 Behavioural effects of centrally administered melanocortins and their analogues in rats (I, II)

5.1.1 Effects of α-MSH, γ_1-MSH and γ_2-MSH (II)

Peptides were administered intra-VTA and/or intracerebroventricularly (ICV) and tested in the open field test in rats. First of all we found the optimal dose of peptides. α-MSH, γ_1-MSH, γ_2-MSH were administrated into the VTA at the doses 0.3 and 3 nmol. Total grooming and vertical activity were estimated for 15 minutes in the open field. At the smaller dose only α-MSH showed significant grooming activity in rats. So, the higher dose showed the more pronounced effects than 0.3 nmol. Thus, for studies of time-dependent effects the dose of 3nmol was chosen and total grooming (Fig.8) and vertical activity (Fig.9) were observed. Data with a dose of 0.3nmol are not presented in figures. Figures 8, 9 and 10 represent the data four consecutive 15 min observation intervals, 0-15min, 16-30min, 31-45min and 46-60min.
Both α-MSH and γ_1-MSH at the dose of 3 nmol caused significant and marked increase in total grooming at the first 15 minutes and it lasted throughout the 1-h observation period (Fig.8).
By contrast, neither 0.3 nor 3 nmol of γ_2-MSH affected the total grooming behavior in rats compared with the saline control (Fig.8).

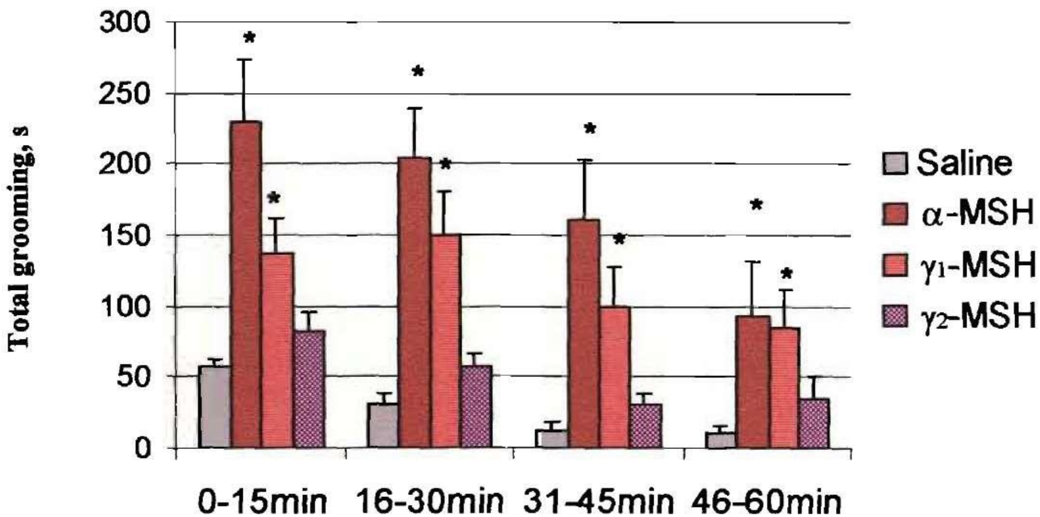

Fig.8. The influence of α-MSH, γ_1-MSH and γ_2-MSH (each peptide of 3 nmol/0.5μl into the rat left VTA) on grooming responses during 1 h after injections. n=8.
*$p< 0.05$ vs saline control.

With respect to the vertical activity the MSH peptides show the same considerable difference: γ_1-MSH (0.3 and 3 nmol) and α-MSH (3 nmol) increased the vertical activity, but only during the first 15 minutes period. However none of doses of γ_2-MSH influenced vertical activity at any of the time periods studied (Fig.9).

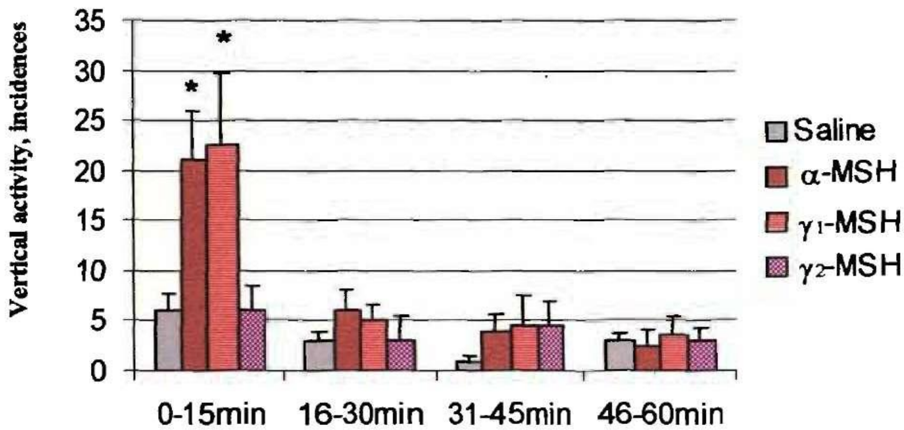

Fig.9. The influence of α-MSH, γ_1-MSH and γ_2-MSH (each peptide of 3 nmol/0.5µl into the rat left VTA) on vertical activity during 1 h after injections. n=8.
* $p < 0.05$ vs saline control.

5.1.2 Effects of combined administration of γ_2-MSH and γ_1-MSH

In order to assess the behavioural effect of combined administration of γ_1-MSH and γ_2-MSH, the rats were pre-treated with either saline or γ_2-MSH at the dose of 3 nmol 15 min prior to intra-VTA administration of γ_1-MSH at the dose of 3 nmol. The γ_1-MSH given to the saline-pretreated rats caused a significant increase in vertical activity and total grooming in rats during 1h observation. Pretreatment with γ_2-MSH significantly attenuated the γ_1-MSH induced grooming behaviour (Fig.10) and vertical activity in rats (not shown in figure) during 1h observation.

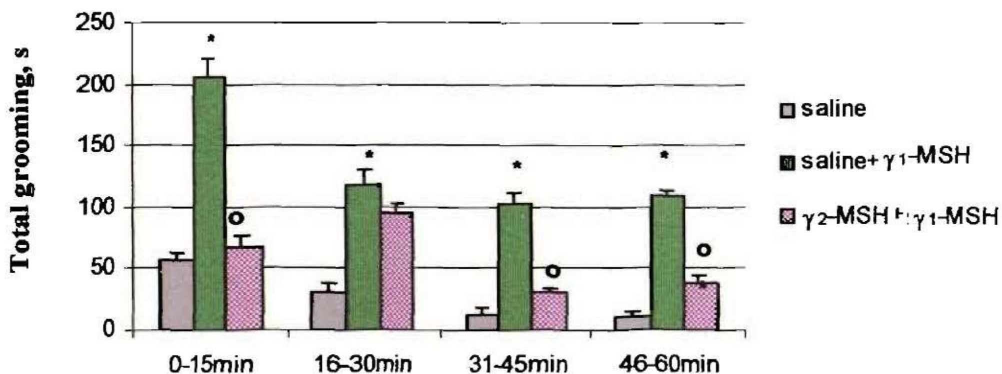

Fig.10. Effect of pre-treatment with γ_2-MSH or saline on the effect of γ_1-MSH (both peptides of 3 nmol/0.5µl into the rat left VTA) on grooming responses during 1 h after injections. n=8.
*$p < 0.05$ vs saline control, $^0p < 0.05$ vs saline+γ_1-MSH.

After 15 min and 1h following injection the animals were tested for catalepsy. In these tests it was revealed that only γ_2-MSH at both doses (0.3 and 3 nmol) caused catalepsy, whereas α-MSH and γ_1-MSH lacked this effect. After 15min following γ_2-MSH administration the catalepsy was 4-5 scores from the maximum 10 and after an hour - 4.9 scores. The animals that received the combined treatment (γ_2-MSH+γ_1-MSH) showed 3.1 score catalepsy.

5.1.3 Effects of MC4R antagonists HS964 and HS014 and their influence on α-MSH (I)

The aim of the study was to obtain behavioural effects (grooming, horizontal and vertical activity) induced by MC4R antagonists HS964 and HS014 administered either intra-VTA or ICV in rats. α-MSH was used as a reference substance. The results show that α-MSH caused considerable and significant (vs saline control) grooming activity already after 15 min following both intra-VTA and ICV injections (Fig. 11). Grooming activity was sustained for 60 min in a slightly gradually increasing manner.

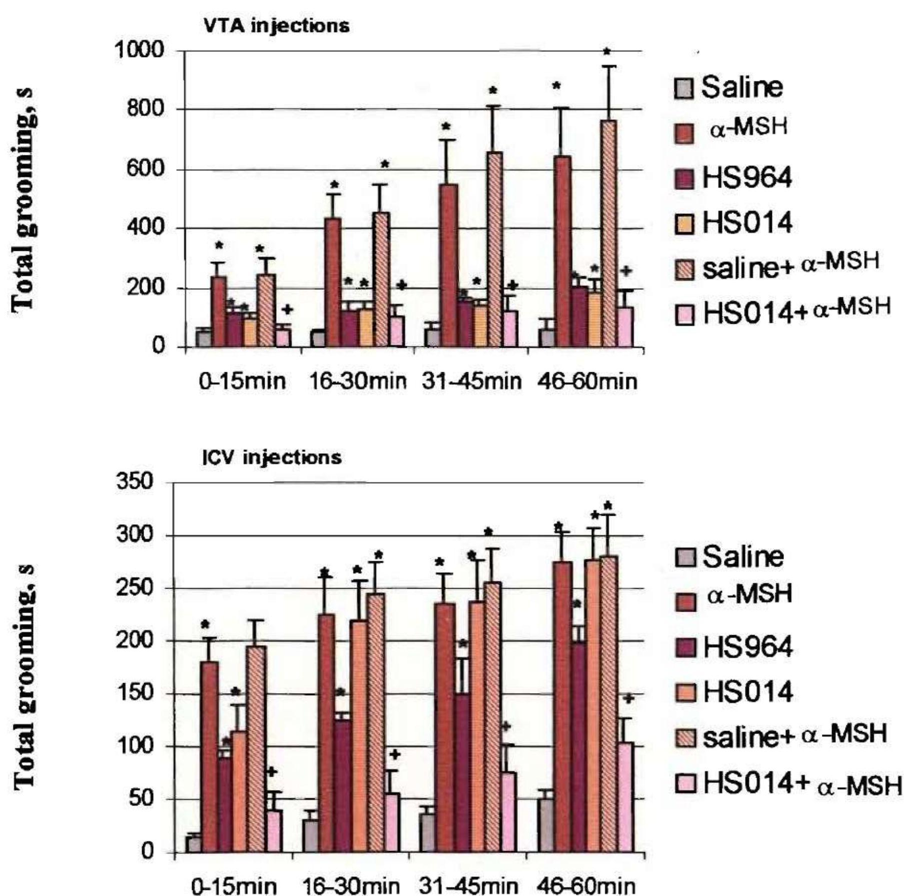

Fig.11. Effect of α-MSH, HS964, HS014 or pre-treatment with HS014 or saline on the effect of α-MSH (all peptides at the dose of 3 nmol/0.5µl) administered into the VTA or ICV on grooming responses in male rats. n=6-10.
*$p < 0.05$ vs saline control, $^{+}p < 0.05$ vs saline+α-MSH.

Intriguingly, that also HS964 and HS014 caused an increase in grooming activity after both ICV and intra-VTA administration. Both HS964 and HS014 administered into the VTA induced small increase of the grooming activity that contrasted with intensive grooming behaviour caused by the injection of α-MSH. Intracerebroventricular injection of HS014 but not HS964 induced more pronounced grooming behaviour in rats than its intra-VTA administration. This high grooming activity was similar to degree caused by the ICV injection of α-MSH. HS014 injection both ICV and intra-VTA 15 min prior to the administration of α-MSH blocked grooming behavior in rats (Fig.11). This blocking effect is more pronounced after the intra-VTA injection. HS964 did also block the α-MSH induced grooming after intra-VTA administration at 15 min time period (data not shown in fig.). α-MSH showed the increase in vertical activity only after intra-VTA administration, but not after ICV injections (not shown in figure). Intra-VTA administration of HS014 elevated the vertical activity in manner comparable to that of α-MSH. Horizontal activity was not influenced by α-MSH or HS964. Horizontal activity was slightly increased only after ICV injections of HS014 (locomotor activity data not shown in figure).

5.2 Influence of γ_1-MSH and γ_2-MSH on hyperlocomotion induced by psychoactivating drugs

5.2.1 Phencyclidine (PCP) and amphetamine (AMP) hyperlocomotion tests in mice (III)

PCP dose of 5mg/kg intraperitoneally caused a hyperlocomotion in mice. MSH peptides administered intracisternally acted differentially: γ_1-MSH potentiated the PCP-induced effects, whereas γ_2-MSH antagonized the PCP effects (Fig.12). Moreover, pre-treatment with γ_2-MSH reduced the γ_1-MSH-induced potentiating influence on PCP. Similar antagonizing effect against γ_1-MSH+PCP was observed in HS014-pre-treated mice (Fig.12).

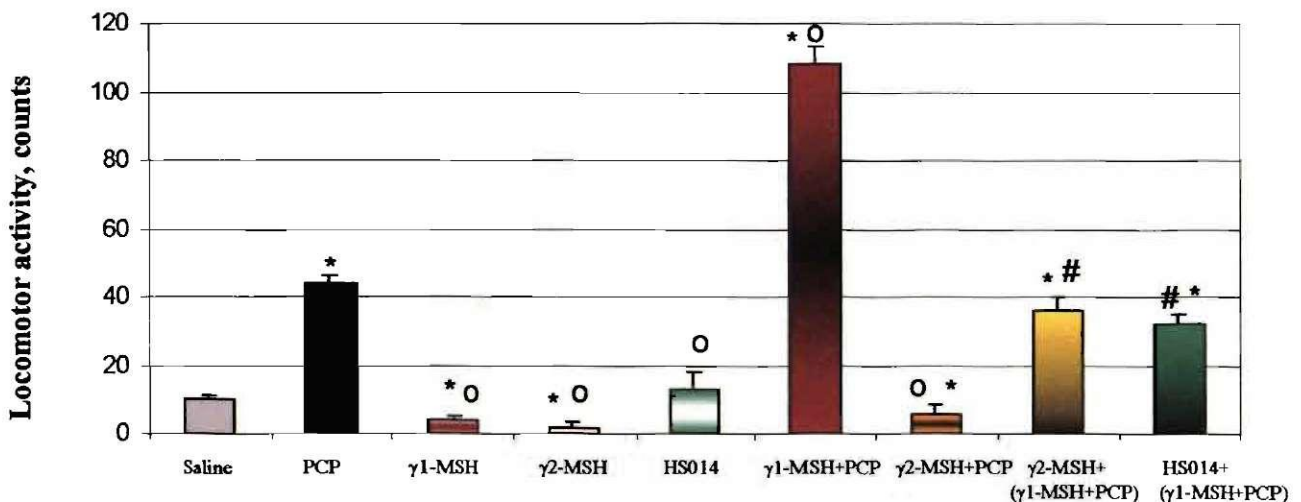

Fig.12. Influence of γ_1-MSH and γ_2-MSH (each of 0.3 nmol/10μl, i.c.) on the PCP- induced hyperlocomotion in BALB/c mice. PCP (5 mg/kg) was administrated intraperitoneally 5 min prior to peptide injections. Locomotor activity was registered from 30th to 60th min after PCP administration. n=9.
* $p < 0.05$ vs saline, $^{0}p < 0.05$ vs PCP, $^{\#}p < 0.05$ vs γ_1-MSH+ PCP.

Both γ_1-MSH and γ_2-MSH injected intracisternally in BALB/c male mice reduced AMP-induced (5mg/kg, subcutaneously, 5min prior to peptide injections) hyperlocomotion (data not shown in figure).

5.2.2 Influence of γ_2-MSH on the NMDA-induced behavior in rats (unpublished data)

Administration of NMDA (10 μg) into the *ventral tegmental area* in rats caused dramatic changes in behavior: increased locomotor activity and induced rotations (Fig.13). Intra-VTA injection of γ_2-MSH 5 min prior the NMDA completely antagonized the NMDA-caused neurotoxic behaviour responses in rats (Fig.13).

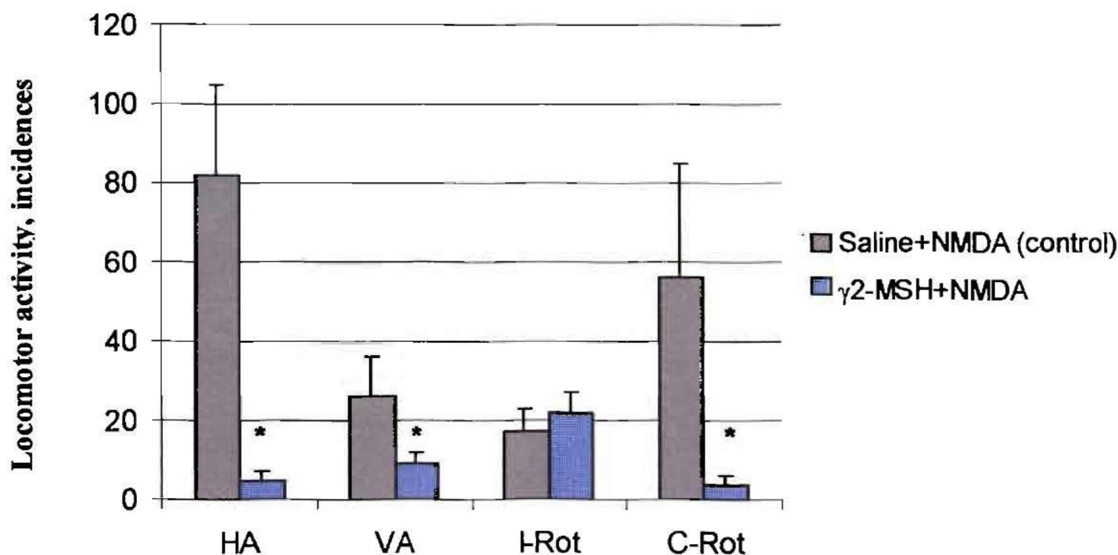

Fig. 13. Effects of the γ_2-MSH (0.3 nmol/0.5 μl, intra-VTA) pre-treatment (5 min prior the injection of NMDA) on NMDA (10 μg/0.5 μl, intra-VTA) induced locomotor activity. n=4.
HA, horizontal activity; VA, vertical activity; I-Rot, ipsilateral rotations; C-Rot, contralateral rotations.
*$p < 0.05$ vs saline+NMDA (control group).

5.3 Examination of analgesic effects of melanocortins (V)

5.3.1 Studies of α-, γ_1-, γ_2-MSH and HS014

In these studies α-MSH, γ_1-MSH, γ_2-MSH and MC4R antagonist HS014 were injected intracisternally in mice. Besides, the effects of different test-drugs (haloperidol, naloxone, bicuculline, muscimol, ethanol and diazepam) were used for the determination of mechanisms of analgesic action. Test-drugs were injected peripherally. Analgesic effects were estimated as tail-flick responses. The results obtained shows that i.c. (intracisternal) injection of γ_2-MSH at the all three doses (0.3, 1 and 3 nmol) significantly increased the tail-flick latencies in mice. Analgesic effect was stable and lasted until 90min following the injections (Fig.14).

The peak response for γ_2-MSH was at 60 min with an approximately 70% increase compared to the baseline (Fig. 14).

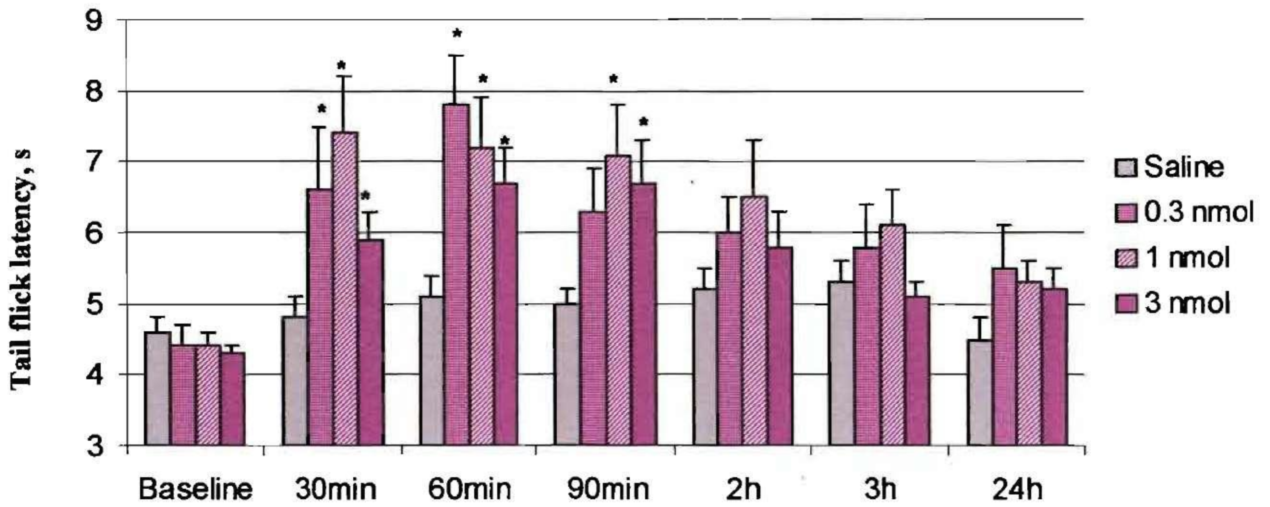

Fig. 14. Effects of the γ_2-MSH (0.3, 1 and 3 nmol, i.c.) on tail flick latencies in BALB/c mice. n=7-9.
*$p < 0.05$ vs saline.

γ_1-MSH gave a short increase in tail flick latencies at 60-90 min and only at the smaller, 0.3 nmol, dose (Fig. 15).

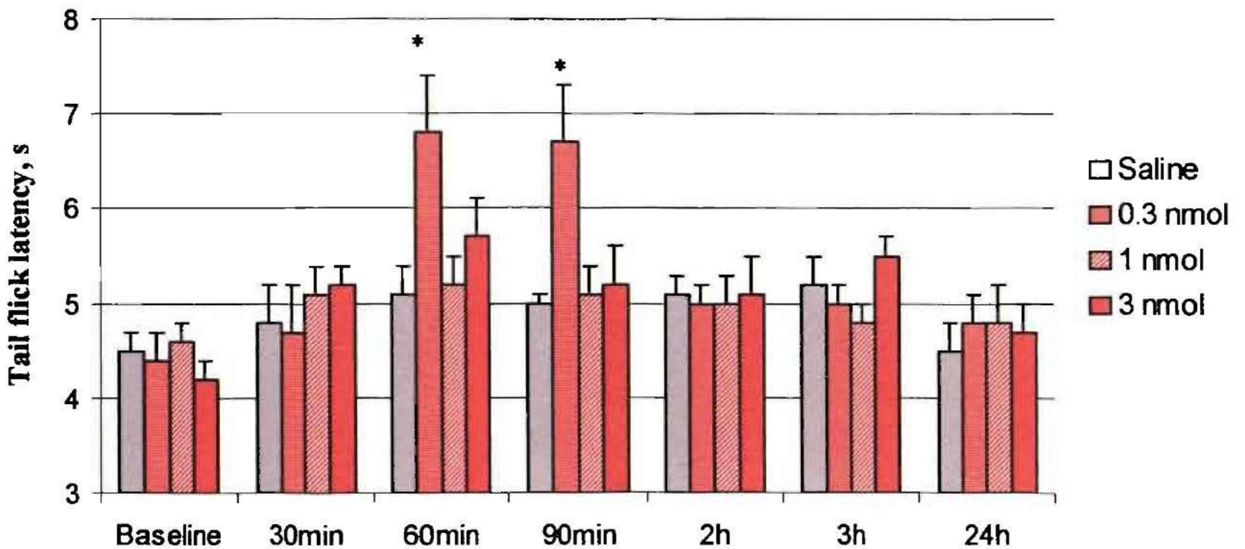

Fig. 15. Effects of the γ_1-MSH (0.3, 1 and 3 nmol, i.c.) on tail flick latencies in BALB/c mice. n=7-9.
*$p < 0.05$ vs saline.

Completely different picture was showed by the all three doses of α-MSH that caused at 30min hyperalgesia with maximum decrease being about 35% from the baseline level (Fig.16).

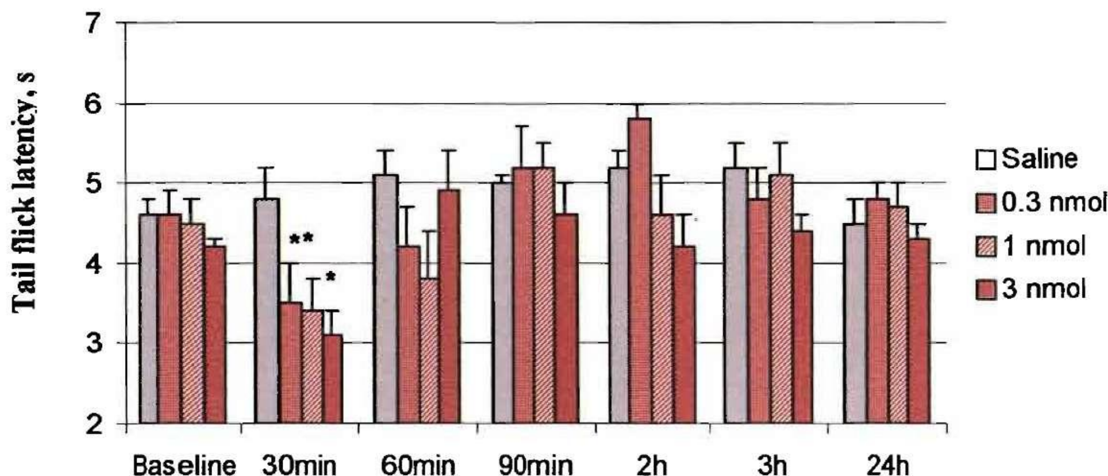

Fig.16. Effects of the α-MSH (0.3, 1 and 3 nmol, i.c.) on tail flick latencies in BALB/c mice. n=7-9.
*$p < 0.05$ vs saline.

The MC4R antagonist HS014 injected i.c. at the doses of 0.3, 1 and 3 nmol did not influence the tail-flick latencies in analgesia study (data not shown in figure).

5.3.2 Studies of the influence of the test-drug-pretreatments on γ_2-MSH-induced analgesia

Intracisternal administration of HS014 or γ_1-MSH (both at the dose of 1 nmol) 30 min prior to the injection of 1 nmol of γ_2-MSH have shown that neither HS014 nor γ_1-MSH pre-treatment affected tail-flick response induced by γ_2-MSH. The dopamine receptor antagonist haloperidol (0.5 and 1mg/kg; i.p.) *per se* induced a clear and significant increase in the tail-flick latency. There were no significant differences between the haloperidol pre-treated animals (haloperidol injected 30 min prior the i.c. injection of γ_2-MSH), γ_2-MSH treated and the animals receiving combined treatment (haloperidol+γ_2-MSH) on tail flick latency responses.
The opiate receptor antagonist naloxone (2 mg/kg i.p.) caused no effect on the tail-flick latencies. Similarly as haloperidol, naloxone administered 30 min prior to the i.c. administration of γ_2-MSH at the dose of 1 nmol did not alter the increase in tail-flick latency induced by the i.c. injection of γ_2-MSH.
Intraperitonal injection of ethanol (4 g/kg) *per se* caused significant increase in tail-flick latencies. For the combined treatment ethanol (4 g/kg; i.p.) were given 10 min prior to i.c. injections of α-, γ_1- or γ_2-MSH (all at the dose of 1nmol). The α-MSH did not significantly affect the ethanol response, whereas γ_1-MSH significantly attenuated the ethanol response. By contrast, γ_2-MSH significantly potentiated the ethanol-induced increase in tail-flick latencies (Fig.17).

The $GABA_A$ receptor antagonist bicuculline (0.5 mg/kg; i.p.) *per se* did not show any significant effects on the tail-flick latencies in mice. For the combined treatment bicuculline (0.5 mg/kg; i.p.) was injected 5 min prior to i.c. injections of α-, γ_1- or γ_2-MSH (each of 1nmol).
Pretreatment with bicuculline completely attenuated the increase in tail-flick latency induced by γ_2-MSH. However bicuculline did not affect response of α- or γ_1-MSH (Fig.17).

The GABA receptor agonist muscimol (1 mg/kg; i.p.) *per se* induced a significant increase in tail-flick latency at 30min observation time. For the combined treatment muscimol (1 mg/kg; i.p.) was administered 5 min prior to i.c. injections of α-, γ_1- or γ_2-MSH (all peptides at the dose of 1 nmol). Neither α-MSH nor γ_1-MSH gave any significant alterations of the tail flick latency response induced by muscimol. The muscimol significantly potentiated the γ_2-MSH induced analgesic effect. Figure 17 summarizes the data obtained with γ_1-MSH, γ_2-MSH and pretreated with test-drugs in analgesia test in mice.

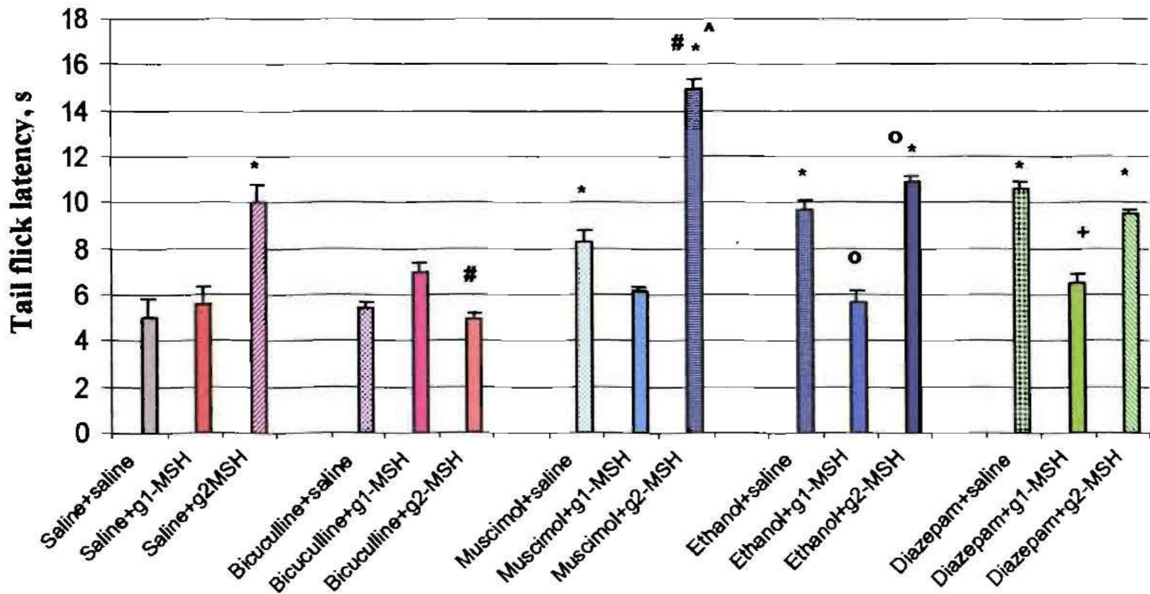

Fig. 17. Effects of γ_1-MSH and γ_2-MSH (both at the dose of 1 nmol, i.c.), bicuculline (0.5 mg/kg, i.p.), muscimol (1 mg/kg, i.p.), ethanol (4 g/kg, i.p.) and diazepam (10 mg/kg, i.p.) on tail flick latencies in BALB/c mice. n=7-9.
*$p < 0.05$ vs saline, #$p<0.05$ vs γ_2-MSH, ^$p<0.05$ vs muscimol+saline, $^0p<0.05$ vs ethanol+saline, + $p<0.05$ vs diazepam+saline.

$GABA_A$ receptor ligand at the benzodiazepines site, diazepam, at the dose of 10 mg/kg i.p. *per se* caused a significant analgesic effect. For the combined treatment diazepam was injected 5 min prior to i.c. injections of α-, γ_1- or γ_2-MSH (1 nmol each) in mice. Both the α-MSH and γ_1-MSH treatments significantly attenuated the increase in tail-flick latency caused by diazepam. However, the combined treatment of diazepam and γ_2-MSH did not affect significantly the tail-flick response, compared to any of these treatments given alone.

5.4 Influence of melanocortins on extracellular dopamine and DOPAC concentrations in anaesthetized rat *nucleus accumbens* in microdialysis study (IV, VII)

5.4.1 Effects of γ_1-MSH and γ_2-MSH (VII)

γ_1-MSH or γ_2-MSH (both at the dose of 3 nmol/0.5μl) were injected into the rat brain left VTA and extracellular dopamine and its metabolite DOPAC concentrations in the left NACC in anaesthetized rats were determined by brain microdialysis study. Injection of CSF served as control. For the combined administration, γ_2-MSH was injected 2 samples or 40min prior to the injection of γ_1-MSH. The dose of 3 nmol/0.5μl for MSH peptides was selected in accordance with the doses known to elicit maximal behavioral responses. Baseline dialysate levels of DA and DOPAC were 32.3±0.3 fmol/40μl and 24.6±1nmol/40μl, respectively. Injection of CSF did not induce any changes in the NACC extracellular DA and DOPAC level. In anaesthetized rats the intra-VTA injections of γ-MSH peptides had different effects on *nucleus accumbens* extracellular concentrations of DA and its metabolite DOPAC.
Administration of γ_1-MSH resulted in a significant increase in the extracellular DA release in the NACC. By contrast to the increase induced by γ_1-MSH, the intra-VTA injection of γ_2-MSH caused a marked decrease in the DA levels on the NACC microdialysates. Intra-VTA pre-treatment with γ_2-MSH abolished the influence of γ_1-MSH on dopamine concentration (Fig.18.)

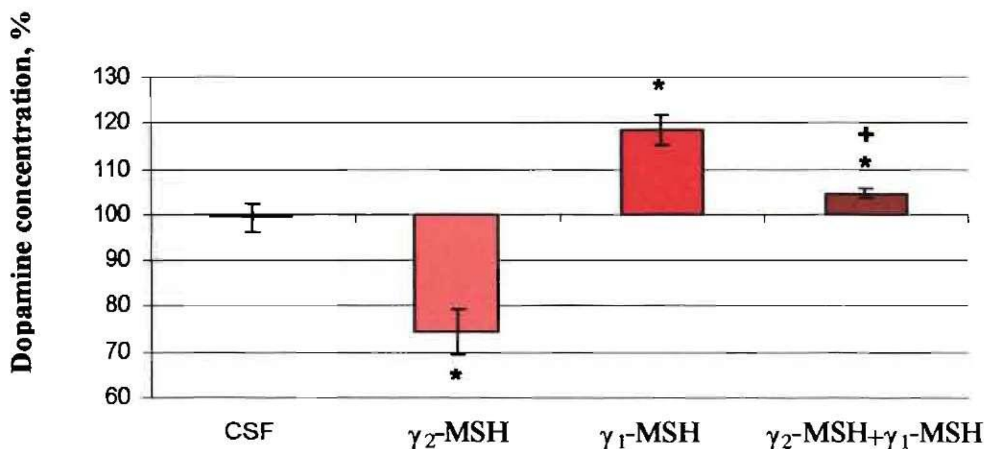

Fig. 18. Average levels of extracellular dopamine in the anaesthetized rats *nucleus accumbens* measured by microdialysis following intra-VTA administration of artificial CSF, γ_1-MSH, γ_2-MSH (each peptide in 3nmol), and influence of the pre-treatment of γ_2-MSH on the effect of γ_1-MSH. Changes are expressed as percentages (±SEM) from basal levels of dopamine (calculated as the mean of the three samples before the treatment of MSH peptides). n=7.
*$p<0.05$ vs CSF (control group), +$p<0.05$ vs γ_1-MSH.

Results from the measurements of DOPAC by brain microdialysis technique are shown in Fig. 19. Administration of γ_1-MSH into the left VTA gave a increase also in the extracellular DOPAC level. Moreover, the intensity of the increase of extracellular DOPAC induced by the intra-VTA injection of γ_1-MSH was markedly higher than that of the extracellular DA level in the NACC (Fig.18 and Fig. 19). γ_2-MSH caused a decrease in the DOPAC levels in NACC microdialysates. Pretreatment of γ_2-MSH (γ_2-MSH injected 40min prior γ_1-MSH) significantly attenuated γ_1-MSH-induced increase in DOPAC content (Fig.19).

So, *in vivo* microdialysis results similarly as in the behavioral studies results confirm that both γ-MSH peptides may act in an opposite manner.

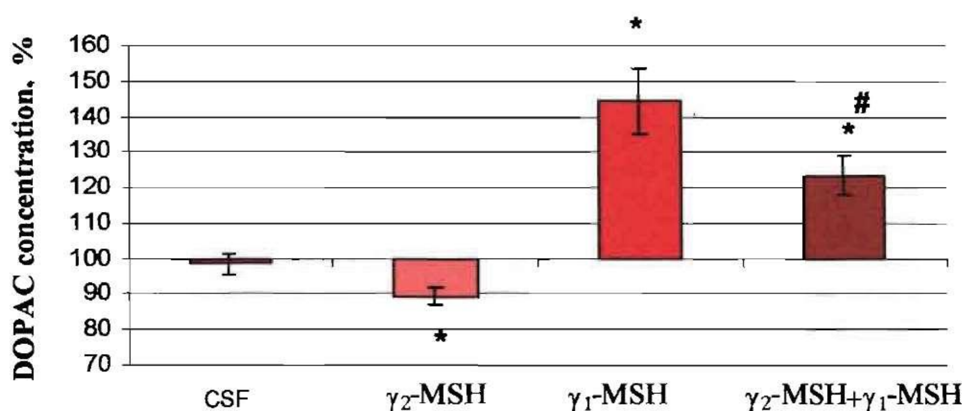

Fig. 19. Average levels of extracellular DOPAC in the anaesthetized rats *nucleus accumbens* measured by microdialysis following administration of artificial CSF, γ_1-MSH, γ_2-MSH (peptides each of 3nmol) and influence of the pre-treatment of γ_2-MSH on the effect of γ_1-MSH. Changes are expressed as percentages ($\pm$SEM) of basal pretreatment levels of DOPAC (calculated as the mean of the three samples before the treatment of MSH peptides). n=7. *$p<0.05$ vs CSF (control group), #$p<0.05$ vs γ_1-MSH.

5.4.2 Effects of MC4R antagonist HS131 in microdialysis study (IV)

The study reveals the effects induced by the intra-VTA administration of α-MSH (10 nmol/0.5μl) and the MC4R selective antagonist HS131 (1 nmol/0.5μl) on the level of extracellular DA and DOPAC in the anaesthetized rat *nucleus accumbens*. Injection of CSF served as control. Administration of MC4R antagonist HS131 into the VTA did not affected the extracellular level of both DA and DOPAC in NACC. Instead, injection of α-MSH into the left VTA caused a significant increase in the levels of both DA and DOPAC. This increase was blocked by pretreatment of HS131 injected 40min prior α-MSH (Fig.20).

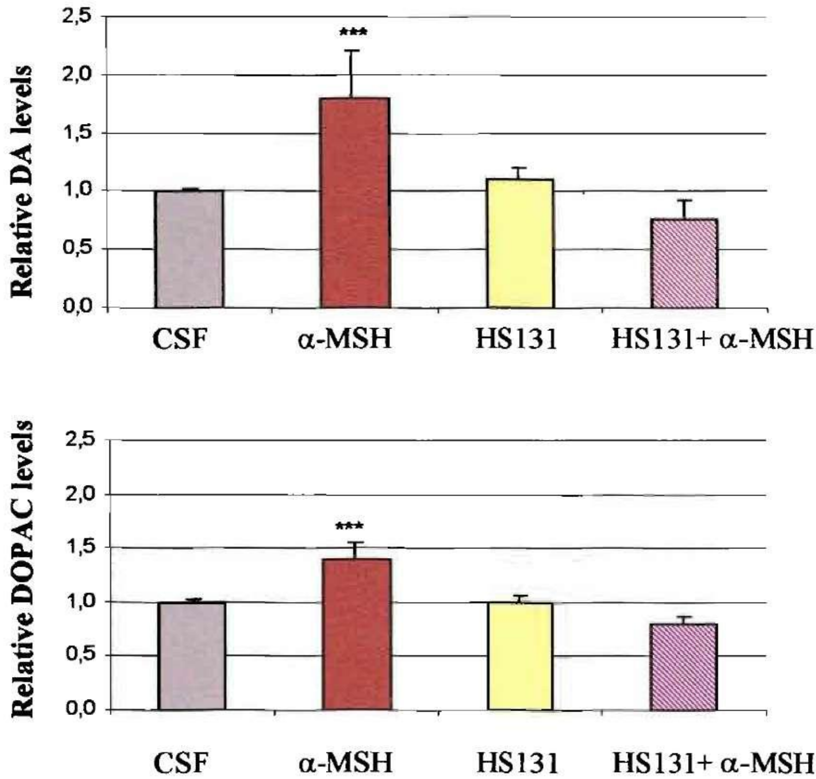

Fig. 20. Average levels of extracellular DA and DOPAC in the anaesthetized rats *nucleus accumbens* measured by microdialysis following intra-VTA administration of artificial CSF, 10nmol α-MSH, 1nmol HS131 or the influence of the pre-treatment of 1nmol HS131 on the effect of 10nmol α-MSH. The results are presented as mean ±SEM of DA and DOPAC 60 min after administration relative to basal levels. n=4-6.
***$p<0.05$ vs CSF (control group), HS131 and HS131+ α-MSH.

6. DISCUSSION

Melanocortins were the first peptide hormones among these discovered in the beginning of the last century (Eberle, 1988). Despite many years of investigation, mechanisms underlying the broad array of physiological effects of melanocortins in the brain and the whole body were poorly understood. In comparison to such melanocortin peptides, as ACTH and α-MSH, remarkably less is known about the functional roles of γ_1-MSH and γ_2-MSH peptides in the brain.
The last five-ten year studies have shed a light in clarifying the central role of melanocortin peptides. This substantial progress was made in the 1992-1993 when identifying, cloning and characterizing of five different types of melanocortin receptors, termed MC1R, MC2R, MC3R, MC4R and MC5R were performed separately in two scientific centers - the Uppsala University, Sweden (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993) and the Oregon University, USA (Mountjoy et al., 1992; Gantz et al., 1993). MCRs are widely expressed in different tissues of the body whereas in the brain mostly are present MC3R and MC4R. Resent autoradiographic studies also confirmed that MC3R protein dominates in many areas of the brain, such as the *nucleus accumbens* NACC, *ventral tegmental area* (VTA), medial pre-optic area and ventromedial nucleus of the *hypothalamus*. In the VTA area, not only MC3R expression but also MC4R has been determined (Lindblom et al., 1998). Since the discovery of MCRs, the melanocortinergic effects have been discussed in context of recently discovered neural melanocortin receptors. Melanocortins show specific affinities for each of the MCR subtype in the brain. Binding studies using recombinant human MCR indicate that γ_1-MSH has about 3-fold higher affinity for the MC3R than α-MSH, whereas α-MSH peptide shows 45-fold higher potency for the MC4R than γ_1-MSH (Schiöth et al., 1995, 1996). The γ_2-MSH has demonstrated a similar to γ_1-MSH affinity profile for human melanocortin receptors; however the binding affinities of γ_2-MSH for MCR are about 2- to 3-fold lower than these for the γ_1-MSH (Schiöth et al., 1996). Unfortunately, none of the natural melanocortins show selective binding activity for the MC3R and MC4R. Therefore, it might be of value to look for synthetic specific antagonists on the different MCR subtypes in order to clarify effects caused by natural melanocortins. Presently for the experimental use are only non-selective MC3R/MC4R antagonists, for instance HS014, HS964 and HS131 (Schiöth et al., 1998b). In 2002-2003 are synthesized selective antagonists for the MC3R, MC4R and MC5R antagonists (Grieco et al., 2002; Balse-Srinivasan et al., 2003), unfortunately they are not yet available commercially.

We have focused our interests on the effects induced by γ_1- and γ_2-MSH, taking into account that they show a high affinity for MC3R (Schiöth et al., 1995) on the one hand, and the abundant expression of the MC3R in the VTA, on the other hand. The VTA is the site of origin of mesolimbic dopaminergic A10 neurons that innervate the NACC, the major terminal area for these neurons. When the biologically active substances stimulate dopamine cells, the dopamine release in the NACC is increased, and that coincides with hyperlocomotion and stereotypical behavioral responses (e.g. exploring, grooming behaviour) in laboratory animals. Release of dopamine in the NACC is regarded as crucial factor in the mediation of cognitive, rewarding, motivational and affective functions (Spanagel et al., 1992). It is assumed that in schizophrenic patients or drug addicted persons, dopaminergic hyperactivity in the VTA-NACC pathways may lead to psychoses, paranoid delutions etc.
The cytoarchitectony of the VTA is very complicate showing that VTA dopamine neurons are influenced by many other systems, for instance, GABA, glutamate, opiate and cholinergic ones (Fig. 21). VTA A10 dopaminergic neurons receive a lot of interneurons that may modulate dopamine release in the NACC (Spanagel and Zieglgansberger, 1997), for instance, the inhibitory amino acids (e.g. GABA) acting on the $GABA_A$ receptors and the excitatory amino acids (e.g. glutamate) acting on the NMDA or non-NMDA receptors.

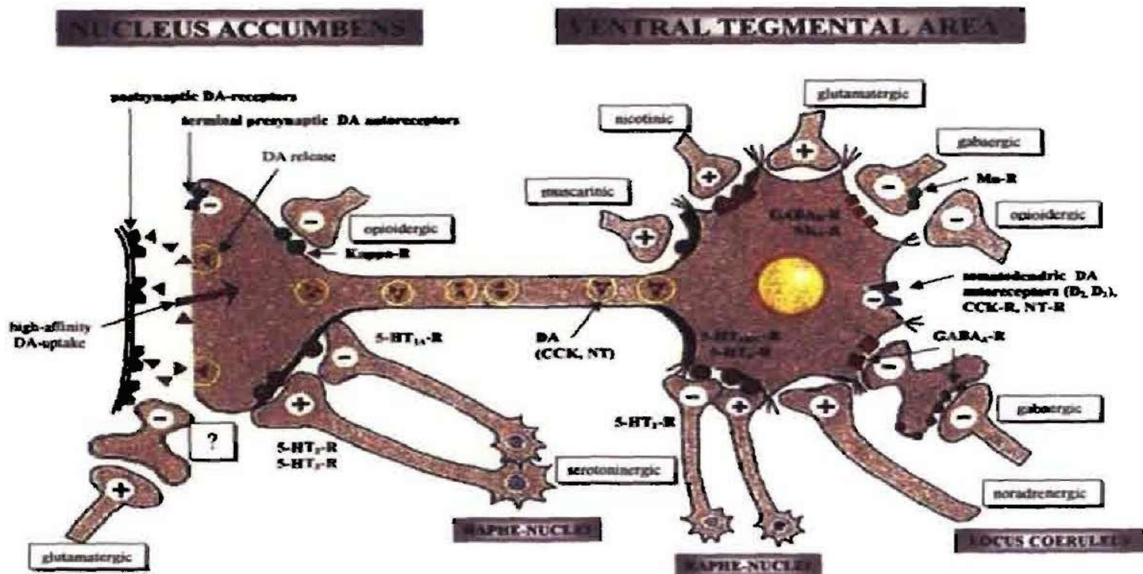

Fig. 21. The mesolimbic dopaminergic system: activity of VTA DA neuron and DA release in the NACC are modulated by different neurotransmitters, released from both inhibitory and excitatory interneurons (Rommelspacher H, 1997).

In our experiments, γ_1-MSH, γ_2-MSH as well as MC4R antagonists mostly were administered into the VTA to evaluate behavioral responses induced by these peptides. α-MSH served as reference peptide.

6.1 Behavioral repertoire of MSH peptides

Our results have shown that α-MSH injected into the VTA induced marked grooming responses in rats. Grooming is a pattern of behavioral condition which has been described as readily definable components consisting of activities directed to the animal's body surface, such as licking, face washing, body grooming and genital grooming. As it has been reported earlier, the ICV administration of α-MSH induced an excessive grooming activity in rodents (Gispen et al., 1975; Torre and Cellis, 1988). Our present data confirm that intra-VTA injection of α-MSH also showed a stable grooming effect. It is intriguing that grooming behaviour was increased to a much greater degree when α-MSH was injected into the VTA than after IVC administration. We have found that the intra-VTA administration of MC4R antagonist HS014 may cause a potent inhibition of the α-MSH induced grooming activity, indicating that α-MSH grooming effects may be provided *via* MC4R. Another support for this suggestion is that the MC4R antagonistic ACTH(4-10) analogues also are capable to block the grooming response induce by ICV administered α-MSH (Adan et al., 1997). That is in line with our data that shows the ability of MC4R antagonist HS014 to inhibit the α-MSH induced grooming response also following its IVC injection.
Surprisingly, that MC4R antagonists (HS014 and HS964) may also cause some increase in grooming behaviour, vertical and horizontal activity following their injections into the VTA or ICV. The grooming responses of MC4R antagonists HS014 and HS964 are more pronounced when MC4R antagonists are injected into ICV than after intra-VTA injection.

γ_1-MSH showed a slightly less potent grooming effect in comparison to α-MSH. Both α-MSH and γ_1-MSH increased not only grooming but also vertical activity. In contrast to α-MSH and γ_1-MSH, intra-VTA administration of γ_2-MSH showed strikingly different effect: the γ_2-MSH was practically ineffective in inducing grooming behavior and vertical activity in rats. Moreover, intra-VTA injection of γ_2-MSH peptide induced even a slight long-lasting catalepsy. Our data are in line with previously reported data indicating that γ_2-MSH is not capable to cause increase in grooming responses following intra-cerebroventricular administration (Van Ree et al., 1981). Furthermore, for the first time we have shown that in case of combined treatment, γ_2-MSH may attenuate the grooming response and the vertical activity induced by γ_1-MSH.

These data are very intriguing, since both γ_1-and γ_2-MSH structurally differs by the only an extra C-terminal glycine residue in the γ_2-MSH molecule. It is possible that this additional glycine residue, which is expected to be flexible as a 'moving tail', could change the peptide conformation quite different and thus may influence its pharmacological properties. One might speculate that therefore γ_2-MSH could become antagonistic, or it might even be capable of rapid bringing a MCR into a desensitized state.

Thus, our data confidentially show a distinct difference in the behavioral repertoire of intra-VTA administered MSH peptides and also the possible role of these peptides in the regulation of activity of the mesolimbic dopaminergic system: α-MSH and γ_1-MSH may act as activating peptide, whereas γ_2-MSH as inhibitory one. The γ-MSH peptides showed not only the opposite spectra of behavioral effects; moreover γ_2-MSH also may attenuate the grooming response and the vertical activity caused by the γ_1-MSH. Since γ_1-MSH acted similarly to some central stimulants (amphetamine), which are capable to induce hyperlocomotion stereotype, one may suggest that γ_1-MSH acts as psychoactivating peptide where as γ_2-MSH as anti-psychotic one. Their opposite action may serve for the balancing of psychoactivating state, possible via the mesolimbic system.

We can not yet explain for sure which of the MCRs exactly play the role in the behaviour effects caused by MSH peptides, either there is yet another unknown MCR or melanocortins are exerting their behavioral effects *via* another pathway distinct from the melanocortins receptors.

There are data describing that γ_1-, γ_2- and γ_3-MSH, but not the α-MSH are capable to prevent the binding of [^{3}H]-naloxone to opiate receptors of the brain (Oki et al., 1980). For the [^{3}H]-naloxone sites, the γ_2-MSH expresses the lower affinity than γ_1-MSH peptide (Oki et al., 1980). However, γ-MSH peptides have shown an about 1000-fold lower affinity to opiate receptors when compared with the affinities for an MC3R. So, it seems unlikely that opiate receptors are involved in the γ-MSH peptides induced behavior responses when administrated intra-VTA. Recently, the autoradiographic studies in the Biomedical Center of Uppsala (Lindblom et al., 1998) pointed out the intriguing fact: the peculiar binding properties of MSH peptides in the VTA seemingly indicating the presence of yet another MCR in this area.

The data obtained in the behavioral study indicated us to clarify the mechanisms of action of the different behavioral effects induced by melanocortins. Some answers we have obtained from further neurochemical studies.

6.2 Neurochemical basis of the dopaminergic component

To find the direct arguments for the MSH peptides influence on the mesolimbic dopaminergic system we used *in vivo* brain microdialysis method in anaesthetized rats. The concentrations of extracellular dopamine (DA) and DOPAC were measure in the rat *nucleus accumbens* (NACC) following the γ_1-MSH, γ_2-MSH, α-MSH and MC4R antagonist HS131 injections into the VTA. Intra-VTA injections of α-MSH and γ_1-MSH caused a considerable increase in the extracellular DA and DOPAC release in the NACC. However, α-MSH caused slightly higher influence on DA level than that on metabolite DOPAC. Interestingly that γ_1-MSH (which similarly to α-MSH is capable of inducing marked grooming) demonstrated a more pronounced ability to intensify the release of DOPAC than that of DA, indicating a strong and prolonged DA metabolism caused by this peptide. In contrast to both α-MSH and γ_1-MSH, the intra-VTA administration of γ_2-MSH caused a decrease in the extracellular DA and DOPAC contents in the rat NACC dialysates. The lowering of extracellular DA level was more pronounced than that of DOPAC. For the combined treatment, γ_2-MSH was injected prior to γ_1-MSH, and the same antagonizing phenomenon between both peptides which was obtained in the behavioral studies was noted: γ_2-MSH abolished the γ_1-MSH-induced increase of extracellular DA and DOPAC contents in the rat NACC. These data indicate that DAergic system directly or indirectly may be modulated by α-, γ_1- and γ_2-MSH peptides. The neurochemical data, showing that γ_1-MSH and α-MSH cause an increase in the extracellular DA and DOPAC release, are in good line with those demonstrated in behavioral experiments: α-MSH- and γ_1-MSH-induced hyperactivity (an excessive grooming and increase in vertical locomotor activity) can be attributed to intensified activity of the DAergic mesolimbic system. At the same time, depressed DAergic processes in the NACC may give some explanation for the cataleptic state caused by γ_2-MSH. The opposite action of γ_2-MSH and γ_1-MSH, a strong antagonism of γ_2-MSH towards the effects induced by the γ_1-MSH, suggest that the relationships between both γ-MSHs may be considered, perhaps, as functional antagonism based on their opposite influence at least on the mesolimbic dopaminergic system, particularly on their ability to influence DA metabolism.
The intra-VTA injection of MC4R selective antagonist HS131 did not cause any changes the DA and DOPAC contents in the rat NACC; however the pre-treatment with HS131 completely abolished the effect of α-MSH. One may suggest that α-MSH caused effect on the increase of NACC dopamine and DOPAC levels is mediated by activating melanocortin receptors, particularly; it might be MC4R subtype.
For the further dopaminergic system involvement in the effects of the melanocortins we use AMP-induced hyperlocomotion model in mice. Amphetamine (AMP) may act as psychostimulant, which induces dopamine release from the presynaptic nerve terminals. The further dopamine accumulation in the synaptic cleft leads to enhanced postsynaptic reception. In experimental animals this causes behavioral hyperlocomotion (Segal, 1975). Our data show that in model animals both γ_1-MSH and γ_2-MSH intracisternally reduced the AMP-induced hyperlocomotion in mice. Naturally, we expected that γ_1-MSH will potentate AMP hyperlocomotion, and γ_2-MSH will antagonize AMP effects. Since both peptides attenuated AMP-induced effects, one may suggest that dopaminergic mechanisms are not the unique non-melanocortinergic component for these peptides; however dopamine system may play an essential role for their action.
Still, it is difficult to define precisely whether the MSH peptides are capable to affect the NACC DAergic system directly, or, that is most likely, indirectly. Our further studies were devoted to clarify the mechanisms of MSH peptides in behavioral and neurochemical experiments by use of test-drugs as analogues for different neurotransmitter systems.

6.3 Glutamatergic component

The data obtained in phencyclidine (PCP) model-animals, show that γ-MSH peptides might affect not only DAergic but also activity of the glutamatergic system. PCP is a model-compound used to obtain a 'schizophrenic state' in laboratory animals. This drug is known to induce behavioral hyperactivation by increasing locomotion. PCP is a non-competitive antagonist of glutamate NMDA receptors. Similarly to the data obtained in open field studies, γ-MSH peptides also showed different effects in PCP-model-animals: intracisternal injection of γ_1-MSH potentiated the PCP-hyperlocomotion effects, whereas γ_2-MSH antagonized the PCP effects. Moreover, γ_2-MSH reduced the mentioned γ_1-MSH potentiating effect (γ_1-MSH+PCP). These data show that γ_1-MSH may act as phychoactivating peptide with an ability to enhance the action of glutamate receptor antagonist PCP, whereas γ_2-MSH showed anti-phychotic action. Interestingly, that MC4R antagonist HS014 also was capable to antagonize γ_1-MSH+PCP effects. Similarity in the effects of γ_2-MSH and MC4R antagonist HS014 (antagonism against both γ_1-MSH and γ_1-MSH+PCP) indicates that they may act somehow in similar manner on the glutamatergic processes. Since the influence of PCP on the mesolimbic system *via* DA release in the NACC has been already proved, the modulation of the PCP effects by γ_1-MSH, γ_2-MSH and HS014 allows suggesting their ability to alter this system activity probably *via* glutamate-DA receptor cross-talk mechanisms.
Another evidence suggesting γ-MSHs involvement in the modulation of glutamatergic processes in the brain comes from NMDA-seizure (increased locomotor activity and contra lateral rotations) test. We have shown that intra-VTA pretreatment with γ_2-MSH 5 min prior to intra-VTA administration of NMDA, completely antagonized the NMDA-caused neurotoxic behaviour responses in rats.

6.4 GABAergic component

Behavioral and microdialysis studies indicated that the central effects induced by the melanocortin peptides and their synthetic analogues at least in part can be attributed to the dopaminergic and glutamatergic processes. Further, analgesic studies showed one another neurotransmitterergic system – GABAergic one - crucial for melanocortin actions. MSH peptides (α, γ_1- and γ_2-MSH) were injected intracisternal in mice and examined on pain perception by use of tail flick method. Analgesia study has shown that only γ_2-MSH (at all three doses 0.3, 1 and 3 nmol, i.c.) was capable to cause central analgesic effect. This effect was pronounced and lasted for 90 min. The γ_2-MSH has shown stronger analgesia in comparison even to opiate peptide enkephaline that caused central analgesic effect only for about 5 min (Klusa, 1984). Opiate receptor antagonist naloxone did not affected γ_2-MSH induced analgesia, indicating that opiate receptors are not essential for γ_2-MSH induced analgesia. γ_2-MSH-induced analgesia also was not altered by MC4R antagonist HS014 or by γ_1-MSH peptide, suggesting that melanocortinergic system do not play an important role in analgesic effect of γ_2-MSH. The same was with the dopamine receptor antagonist haloperidol that did not influence the γ_2-MSH analgesia effect.
Surprisingly, only $GABA_A$ receptor ligands affected the γ_2-MSH induced analgesia.

Agonist muscimol potentiated the γ_2-MSH analgesic effect, while antagonist bicuculline completely abolished it. At the same time, γ_2-MSH effect was not influenced by GABA receptor benzodiazepine site ligand diazepam. Intriguingly, γ_1-MSH and also α-MSH significantly reduced analgesic effect caused by diazepam. Thus, γ_2-MSH analgesic effect can be altered only by $GABA_A$ receptor GABA site ligands. The ethanol-induced analgesia was not influenced by α-MSH, however γ_1-MSH reduced and γ_2-MSH increased the ethanol effect. Ethanol is a known modulator of $GABA_A$-receptor (Davies and Alkana, 1998).
Therefore, there is a strict evidence of the diverse interaction shown by MSH peptides on various sites of $GABA_A$ receptor: γ_2-MSH influenced the GABA site ligands muscimol and bicuculline, α-and γ_1-MSH by benzodiazepine site ligand diazepam and both γ-MSHs influenced in opposite manner the ethanol site (Fig.22)

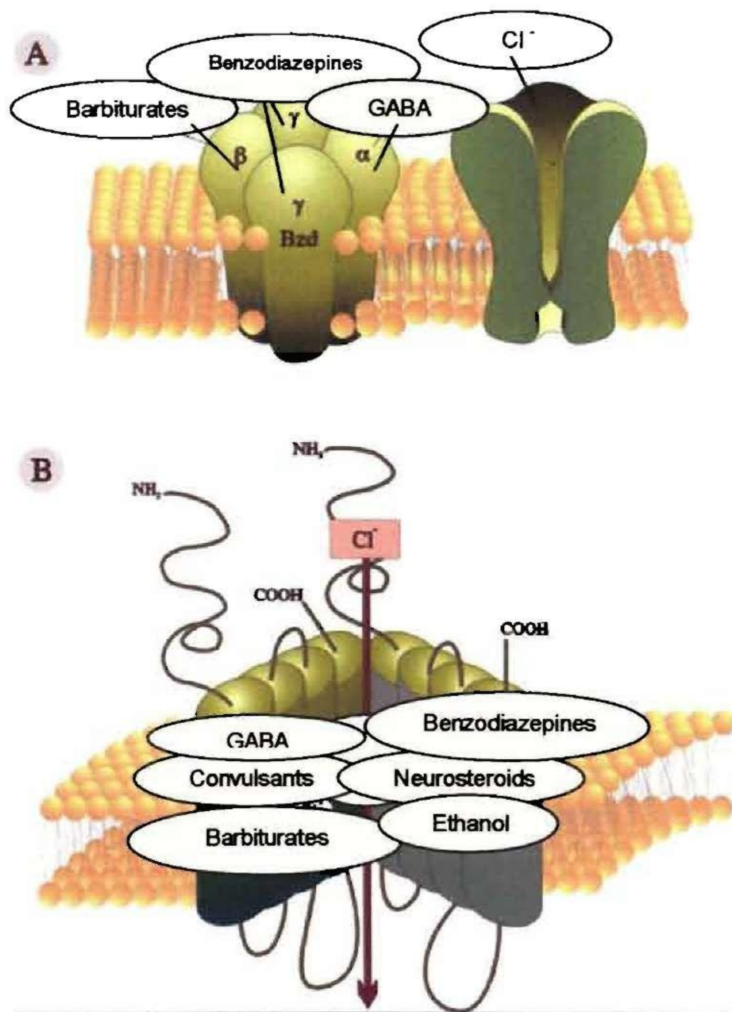

Fig. 22. $GABA_A$-receptor sites: receptor protein organization (A) and cross-section (B).

The short-term hyperalgesia caused by the all three dose of α-MSH must be mention to the central analgesic effects induced by the melanocortins. This effect induced by the intracisternal injection of α-MSH in mice is in line with a previous report where ICV administration of α-MSH caused hyperalgesia in rats (Sandman and Kastin, 1981).

We can conclude that γ_2-MSH induces central analgesia *via* a mechanism that does not involve either melanocortin receptors or opioid receptors. Our data indicate that γ_2-MSH analgesic effect is mediated via direct and/or indirect stimulation of $GABA_A$ receptor GABA site.

The data of present studies indicate that central effects of melanocortin peptides involve not only melanocortinergic (which was tested by other authors as γ-MSH peptides express high affinity to MC3R or MC4R) but also non-melanocortinergic - DAergic, GABAergic and glutamatergic pathways in the central nervous system.

Moreover our studies showed that γ_1-MSH and γ_2-MSH almost in all (the only exception was AMP-induced hyperlocomotion study) demonstrated opposite action: if γ_1-MSH induced pronounced grooming behaviour and increased vertical activity in rats, increased both dopamine and DOPAC levels in the rat NACC, potentiated PCP-induced hyperlocomotion, the γ_2-MSH did not influence grooming behaviour, caused a moderate catalepsy, reduced both dopamine and DOPAC levels in the rat NACC, antagonized PCP-induced hyperlocomotion, abolished NMDA-caused neurotoxic behavior responses in rats, caused a stable analgesic effect mediated *via* $GABA_A$ receptor. The most interesting phenomenon in many studies was a demonstrated γ_2-MSH antagonism towards the effects induced by γ_1-MSH: γ_2-MSH antagonized the γ_1-MSH-induced grooming behavior, abolished the γ_1-MSH-caused increase in dopamine and DOPAC levels and inhibited γ_1-MSH-induced potentiation of the PCP-hyperlocomotion. These behavioral and neurochemical data allow us to suggest that γ_1-MSH acts as psychoactivating whereas γ_2-MSH as antipsychotic peptide.

The functional role of both γ-MSH peptides might be in the maintenance of balanced psychoactivating state regulated either by their possible functional antagonism or their different interaction with melanocortinergic and/or other neurotransmitterergic systems. Hence, present studies have revealed some new aspects of the multifunctional influence of γ-MSH peptides on the various processes of the central nervous system; one may suggest that γ_1-MSH and γ_2-MSH can regulate the mesolimbic dopaminergic system, which is responsible for the development of drug dependence and motivational processes, manifestation of schizophrenic hyperactivation and emotions. Results of our studies may indicate new pharmacotherapeutic strategies in the treatment of drug dependence and other pathological processes associated with a functioning of the reward system.

7. CONCLUSIONS

1. γ_1- and γ_2-MSH injected into the VTA induced an opposite behavioral repertoire in rats: γ_1-MSH caused the increased grooming responses and vertical activity, whereas γ_2-MSH lacked these activities (it caused a moderate catalepsy). Neurochemical data obtained in microdialysis studies also revealed distinct effects, since peptides influenced in different manner the DA and its metabolite content in the rat *nucleus accumbens* after peptide intra-VTA administration: γ_1-MSH increased DA and DOPAC levels whereas γ_2-MSH caused a decrease of both monoamine levels in the rats NACC. These data indicate eventual psychoactivatory role of γ_1-MSH and antipsychotic one of the γ_2-MSH.

2. In both experimental setups in rats (behavioral and microdialysis), γ_2-MSH acted as an antagonist of γ_1-MSH indicating that these peptides may play a role of functional antagonists at the level of the dopaminergic mesolimbic system.

3. Data obtained in the schizophrenia model-mice and NMDA-toxicity tests in rats showed that both γ-MSHs might modulate glutamatergic system. γ_1-MSH potentiated the phencyclidine (PCP)-induced hyperlocomotion, whereas γ_2-MSH significantly reduced the PCP effects (peptides injected intracisternally). In these experiments γ_2-MSH antagonized the γ_1-MSH-induced potentiation of the PCP-hyperlocomotion effects. Intra-VTA injection of γ_2-MSH completely antagonized the NMDA-caused neurotoxic behavioural responses in rats.

4. γ-MSH peptides have a possibility to regulate also GABAergic processes. In analgesia test (tail flick in mice) γ_2-MSH caused a long-lasting central analgesia that was potentiated by muscimol (GABA site agonist of the $GABA_A$ receptor) and completely reduced by bicuculline (GABA site antagonist of the $GABA_A$ receptor) but not altered by other test-drugs like, MC3/MC4 receptor antagonist HS014, opiate receptor antagonist naloxone, γ_1-MSH, and DA receptor antagonist haloperidol. These data indicate GABAergic pathway involved in γ_2-MSH caused central analgesia. Moreover, γ_1-MSH considerably decreased diazepam (benzodiazepine site agonist of the $GABA_A$ receotor) induced analgesia indicating ability of γ_1-MSH to affect benzodiazepine site of $GABA_A$ receptor. Both γ-MSH peptides differentially affected analgesia caused by ethanol, a modulatory site of the $GABA_A$ receptor: γ_1-MSH reduced, whereas γ_2-MSH potentiated the ethanol effect.

5. The data obtained in the present behavioral and neurochemical studies which have shown distinct action of γ_1- and γ_2-MSH, allow to suggest that γ-MSHs play a functional role in regulating/balancing the brain psychoactivating state (mostly the mesolimbic system) by acting not only *via* melanocortin receptors but also by involving non-melanocortinergic mechanisms, at least in part, dopamin-, glutamat- and GABAergic components.

ACKNOLEDGEMENTS

The present study was carried out at the Laboratory of Pharmacology, Latvian Institute of Organic Synthesis (1995-1997); Division of Pharmacology, Department of Pharmaceutical Biosciences, Uppsala Biomedical Center, Sweden (1997-2000), and at the Department of Pharmacology, Faculty of Medicine, University of Latvia (1998-2003).

This thesis is the result of collaboration between several individuals at the different labs of the medical institutions in Latvia and Sweden, to whom I will always be thankful.
However, in particular I want to thank:

Professor Vija Kluša, my supervisor and head of the Department of Pharmacology Faculty of Medicine, University of Latvia, for the constant scientific support and interest throughout these years, for excellent comments concerning the thesis and her great enthusiasm in every day life in general.

I would like to express my sincere gratitude to Professor Jarl Wikberg and Associated Professor Dr. Lena Bergstrom for support in my research providing the necessary things needed for the microdialysis studies during my stay in Uppsala Biomedical Center.

I also thank Associated Professor Ruta Muceniece and Associated Professor Simon Svirskis for sharing their methodological knowledge in neuropharmacology field – from cells to lab animals and creating a pleasant and supportive atmosphere to work.

Many thanks to everybody from the Uppasala Biomedical Center times: Dr. Maija Dambrova, Dr. Peteris Prusis, Ilga Mutule for sharing a lot of laugh. Dr. Jonas Lindblom, Dr. Feliks Mutulis for pleasant collaboration and especially for solving all technical problems occurring with HPCL matters; and from the Department of Pharmacology, Faculty of Medicine, University of Latvia: Juris Rumaks, Raita Brikmane and many others.

Dr. Ingvars and Vita Birznieki, Lena and Andrea, Ieva for their friendship.

My warmest thanks to my husband Martins, my parents and all my relatives for their understanding, support, love and never failing encouragement during this work.

The studies were supported by grants from the Howard Hughes Medical Institute (HHMI 75195-548501; 1995-2000), Swedish Society for Medical Research, Swedish MCR grant 04X-05957, Melacure Therapeutics AB (Uppsala Sweden), a stipend from the Swedish Institute under the *Visby Programme*, the Latvian Council of Science grant for the PhD studies Nr. 586 and Latvian Counsil of Science grant 01.0037.

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Neuropeptides (1998) 32 (6), 573–580
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Evaluation of behavioural effects of neural melanocortin receptor antagonists injected ICV and in VTA in rats

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Summary. The natural melanocortic peptides are known to exert a variety of effects after central administration. Recently, we discovered the first potent and selective substances for the MC4 receptor, i.e. HS964 and HS014. We found HS964 to be an antagonist for the MC1, MC3, MC4 and MC5 receptors in vitro. HS014 is an antagonist for the MC3 and MC4 receptors and a partial antagonist for the MC1 and MC5 receptors. We injected α-MSH and these substances, both intracerebroventricular (ICV) and in the ventral tegmental area (VTA) in rats and scored several behavioural effects. The results show that α-MSH caused intensive grooming which was antagonized by pre-treatment of both HS014 and HS964. The data give further support to the hypothesis that it is the MC4 receptor which mediates grooming in rodents. The grooming effects of α-MSH were more pronounced after intra-VTA administration compared to the ICV administration. Both α-MSH, HS014 and HS964 caused an increase in vertical activity of the rats after intra-VTA administration but not after ICV administration. Horizontal activity was virtually not affected by the administration of the peptides. The data indicate that the neural MC3 and MC4 receptors are not likely to be an important mediators of locomotor activity in rats.

INTRODUCTION

Pro-opiomelanocortin (POMC) is post-translationally cleaved into a variety of peripheral and neuroactive substances including adrenocorticotropin (ACTH) and the α, β-, γ-melanocyte stimulating hormone (MSH). These peptides are commonly termed 'melanocortins'. ACTH stimulates steroidogenesis in the adrenal gland, whereas α-MSH stimulates melanogenesis in melanocytes. Besides these well-known effects, the melanocortins are reported to have a broad array of other effects, e.g. being neurotrophic, induce grooming in rodents, anti-inflammatory, antipyretic, and affecting pain perception, memory, learning, behaviour, blood pressure and events surrounding parturition.[1,2]

Received 8 July 1998
Accepted 9 September 1998

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Five receptors for the melanocortic peptides are cloned.[3-7] The MC1 receptor is expressed in melanocytes[3] and it has a role for pigmentation in several species of vertebrates.[1] The MC2 (or ACTH) receptor is exclusively expressed in the adrenal gland[4,8] and binds ACTH with high affinity but not the MSH peptides.[9] The MC3 receptor is expressed in the brain (predominantly in the VTA, and in few regions of the brain stem), as well as in the periphery where it has been found in the placenta, gut tissues.[5,10,11] The MC4 receptor is predominantly found in the central nervous system, where it is represented in almost every brain region, including the cortex, thalamus, hypothalamus, brain stem and spinal cord.[7,12] The MC4 receptor has recently been knocked out and subsequently related to control of weight homeostasis.[13] The MC5 receptor has widespread peripheral tissue distribution but can also be found in the brain. It is believed that the MC5 receptor plays a role for exocrine gland function.[14]

The natural melanocortic peptides (α-MSH, β-MSH, γ-MSH and ACTH) have a specific affinity profile for each of the MC receptor subtypes although they are not exclusively selective for the different subtypes, with the exception that α-MSH is selective for the MC1 receptor and ACTH is selective for the MC2 receptor.[15-17] The MC2 receptor is distinguishable from the other MC receptors as it does not bind the MSH peptides. The lack of selective compounds have hampered the clarification of the physiological roles especially of the MC3, MC4 and MC5 receptors. SHU9119 was the first potent MC3 and MC4 receptor antagonist.[18] However, SHU9119 is not selective for any of the MC receptors.[18,19] We have recently developed the first selective MC4 receptor substances: HS964 and HS014.[20]

The aim of the present study was to investigate the effects of the new selective MC4 receptor substances HS964 and HS014 on behaviour after both injection into the ventral tegmental area (VTA) and intracerebroventricular (ICV) in rats.

MATERIALS AND METHODS

Chemicals

Chemicals α-MSH were purchased from Neosystem S.A., France. The HS964 and HS014 were synthesized using the solid phase approach applying a Fmoc based Pioneer peptide synthesis system (PerSeptive Biosystems) and purified by HPLC as earlier described.[20] The correct molecular weights of the peptides were confirmed by mass spectrometry. Peptides were dissolved in water and stored frozen in aliquots until used.

Expression of receptor clones

The human MC1[3] and human MC5[7] receptor had earlier been cloned by us into the expression vector pRc/CMV (In Vitrogen). The human MC3 and human MC4 receptor DNAs, cloned into the expression vector pCMV/neo, were gifts from Dr Ira Gantz.[5,6] For receptor expression, COS-1 (CV-1 *Origin*, SV40) cells were grown in Dulbecco's modified Eagle's medium with 10% foetal calf serum. Eighty percent confluent cultures were transfected on 100 mm cell culture dishes with the DNA (approximately 1 μg DNA for every 1 × 10^6 cells) mixed with liposomes in serum free medium. After transfection, the serum-free medium was replaced with growth medium and the cells were cultivated for approximately 48 h. Cells were then scraped off, centrifuged, and used for radioligand binding.

cAMP assay

The transfected cells were harvested and incubated for 30 min at 37°C with 0.05 ml serum free Dulbecco's modified Eagles medium in each tube, containing 0.5 mM IBMX (isobutylmethylxantine) and appropriate concentrations of α-MSH or HS964. After incubation with the indicated drugs, cAMP (adenosine 3′:5′-cyclic monophosphate) was extracted with perchloric acid at a final concentration of 0.4 M. After centrifugation, the protein free supernatants were neutralized with 5 M KOH/1 M Tris (tris-(hydroxymethyl)aminomethane). 0.05 ml of the neutralised cAMP extract or a cAMP standard (dissolved in distilled water) was added to a 96 well microtiter plate. The content of cAMP was then estimated essentially according to Nordstedt & Fredholm,[21] 1990, by adding to each well [^{3}H]cAMP (0.14 pmol, approximately 11,000 cpm, specific activity 54 Ci/mmol, Amersham) and bovine adrenal binding protein and incubating at 4°C for 150 min. Standards containing non-labelled cAMP were also assayed concomitantly with the samples. The incubates were thereafter harvested by filtration on Whatman GF/B filters using a semiautomatic Brandel cell harvester. Each filter was rinsed with 3 ml 50 mM Tris/HCl pH 7.4. The filters were punched out and put into scintillation vials with scintillation fluid and counted. The cAMP assays were performed in duplicate wells and repeated three times.

Animals

Male Wistar rats were bred at the Breeding House of the Joint Stock Company GRINDEX, Riga, Latvia, and used at weights 300–350 g. The animals were housed in groups of five in a light-dark cycle of 12 h (lights off 19.00–7.00).

Surgical procedures

Cannulas, made from stainless steel needles (15 mm long, 0.56 mm OD), were implanted stereotaxically under Nembutal (CEVA, Sanofi, France) anaesthesia (60 mg/kg i.p.) into the left VTA or left lateral ventricle (ICV) at the following co-ordinates: −5.2 caudal to bregma, 0.8 mm lateral from midline, and −8.0 ventral from dura mater or −1.5, 1.0, and −3.6, respectively (Paxinos and Watson, 1982). Cannulas were kept in place with dental cement (SPOFA Dental) and covered with duracryl (SPOFA Dental). To prevent the clogging of the cannulas, a bent stylet of stainless steel was inserted into each of them, and removed only when the injection took place. After the surgery, the rats were individually housed, given food and water ad libitum and allowed to recover for the following 7 days in a recovery room.

Injections

Drugs were injected into the VTA by means of a 75RN Hamilton Digital Syringe and a very fine polyethylene tube (Clay Adams, PE-10) attached to a 30-gauge needle

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which was inserted into the implanted cannula. On the day of experiment an MSH-peptide aliquot was thawed and dissolved in saline to provide 0.3 and 3 nmol/rat for injections into the VTA or ICV. The total volume injected for each substance was 1 μl and the speed of injection was 0.25 μl min^{-1}. For the combined treatments (HS964/HS014/saline + α-MSH), HS964/HS014/saline was administered 15 min prior to the α-MSH injection.

Behavioural analysis

On day 7 after surgery, the rats were transported from the recovering room to an observation room for 1-day of handling and habituating. Each rat was placed into a Plexiglas observation cage (60 × 40 × 15) and left for at least 1 h to diminish stress reactions to the novel environment. The rat was then placed onto an injection platform for 5 min before drug administration. Immediately after the injection, the rats were placed into observation cage. Observation started on the 5th min after the onset of the injection and continued for 1 h for time-response studies by using a Psion Workabout microcomputer (Noldus, the Netherlands). Grooming activity was expressed in seconds as total duration of the separate grooming reactions (face washing, body licking, scratching, ano-genital grooming, head and wet-dog shakes) for each observation period (0–15 min, 0–30 min, 0–45 min and 0–60 min). Following non-grooming, behavioural events were registered as rearing or vertical activity (VA) and horizontal activity (HA). VA and HA were scored as incidences during all observation periods. In the case of combined drug treatments, the injection schedule was: HS014/saline was injected 15 min prior to α-MSH for both intra-VTA or ICV administration, and the behaviour was observed during 1 h after the last injection. However, with respect to HS964+α-MSH was observed only after intra-VTA administration and during the first 15 min period. The observation sessions were performed between 10.00 and 14.00 Each experimental group consisted of at least six rats.

Statistics

Statistical analysis was done using independent samples t-test or one-way ANOVA with Newman-Keuls test as a post-hoc. Data are expressed as the mean ± S.E.M.

Animal ethics

Experimental procedures were carried out in accordance with guidelines of the European Community, local laws and policies and were approved by Ethics Committee of Animal Experimentation at the Latvian Research Council.

RESULTS

We have earlier characterized how HS014 influences cAMP levels in cells expressing the MC1, MC3, MC4 and MC5 receptors.[20] However, a possible cAMP response after stimulation by HS964 had not been tested. We expressed the DNAs for the above mentioned receptors and measured the cAMP accumulation after addition of α-MSH and HS964. As can be seen in Figure 1, α-MSH stimulated accumulation of cAMP in all the cell types. COS-1 cells which had not been transfected by any of the MC receptors did not respond to α-MSH (data not shown). HS964, in concentrations up to 1 μM, did not affect the cAMP levels of any of the MC receptor expressing cells. Instead, 0.1 μM HS964 was found to completely block the cAMP increase induced by α-MSH for all the four MC receptors.

We then used both these MC4 selective antagonist (HS964 and HS014) for behavioural studies in rats. We injected both of the substances either intra-VTA or ICV in rats and scored grooming behaviour, horizontal and vertical (or rearing) locomotor activities. α-MSH was injected alone as well as with prior treatment with saline, HS964 or HS014. The complete results are shown in Table 1 and Table 2.

The results on grooming behaviour are shown in Figure 2. The results show that α-MSH induced considerable and significant (vs saline control) grooming activities already after 15 min following both intra-VTA and ICV injections, which were sustained for 60 min in a slightly gradually increasing manner. The intensity of the grooming (duration and number of incidences) was more pronounced after intra-VTA administration that after ICV administration. HS964 and HS014 also caused some increase in duration of grooming activity after both intra-VTA and ICV administration. When HS014 was administrated prior to the administration of α-MSH it blocked grooming behaviour, both after ICV and intra-VTA administration. As can be read out of Figure 2, this blocking effect is most pronounced after the intra-VTA administration. HS964 did also block α-MSH induced grooming after intra-VTA administration at 15 min which was the only time period scored for this peptide in this study.

α-MSH caused an approximately two-fold increase (vs saline control) in vertical activity at the 30, 45 and 60 min observation periods after intra-VTA injection, but not after ICV administration. HS014 also caused an increase in vertical activity after intra-VTA administration but not after ICV administration. This increase in vertical activity caused by HS014 was comparable to that of α-MSH. The same pattern was observed for HS964 as for HS014 and α-MSH even though the vertical activity after intra-VTA administration of this peptide was not pronounced.

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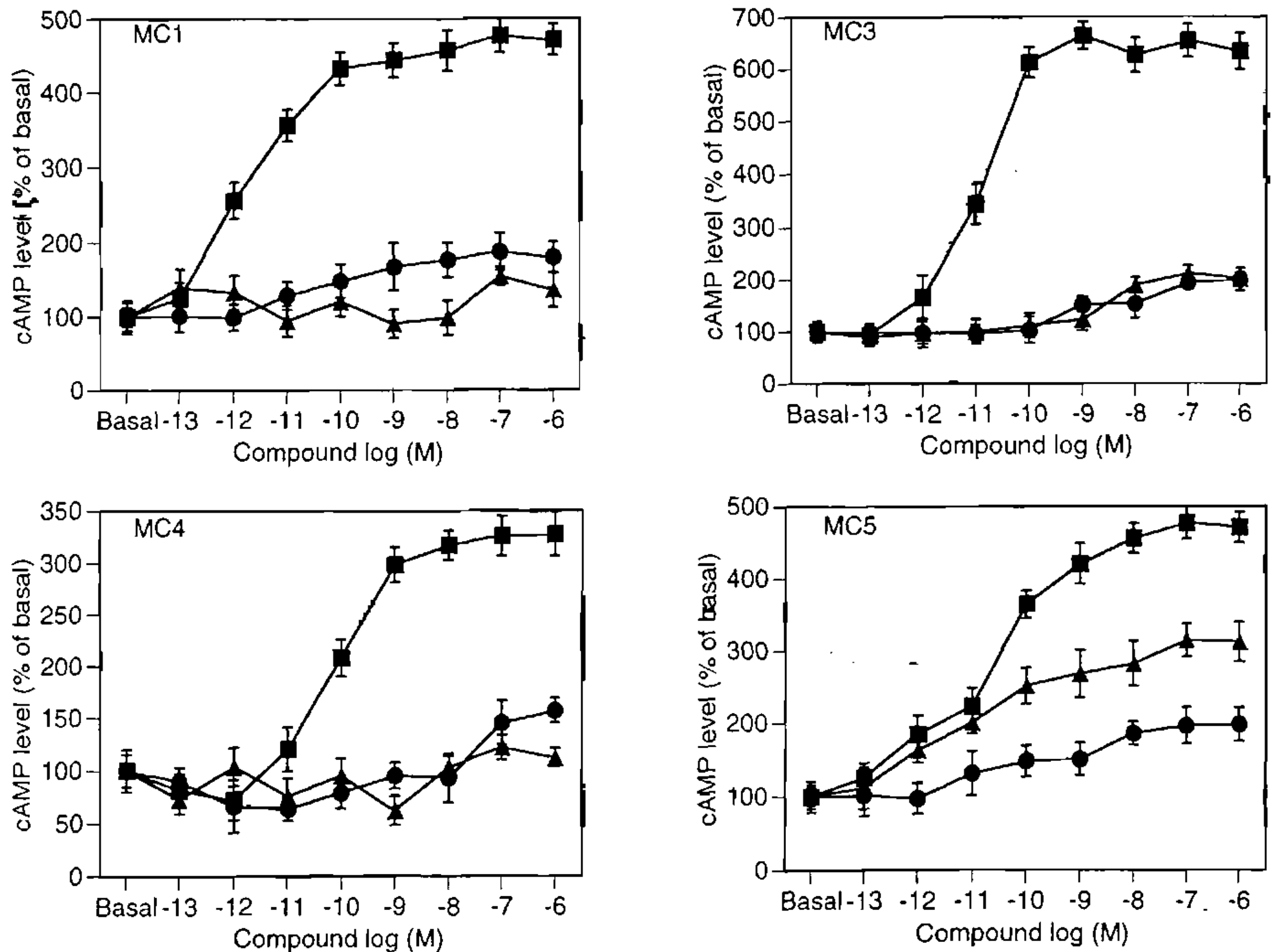

Fig. 1 Generation of cAMP in response to α-MSH (■), HS964 (●) or α-MSH + 1 μM HS964 (▲) for the MC1, MC3, MC4 and MC5 receptors in transfected COS-1 cells. Each point represents the average±SEM (n=6).

No significant alterations in horizontal activity was observed when α-MSH was administrated in either of the routes used. Horizontal activity was not influenced by HS014 after intra-VTA application, however, this activity was slightly (approximately 30–40%) increased during the 15 and 30 min observation periods after ICV injection. Locomotor activities after intra-VTA and ICV injections of HS964 did not differ from the activity of the control rats, with the exception that a delayed (at the 45 and 60 min observation periods) more than two-fold increase in horizontal activity was observed at both administration routes.

DISCUSSION

The MSH peptides were among the first peptide hormones that were discovered.[1] There is a broad array of physiological effects related to them which underlying mechanisms are not well understood. After the cloning of the genes for the MC receptor subtypes and specific binding characterization of each of the subtypes in vitro, it became clear that none of the natural MSH peptides are selective for the newly discovered MC3, MC4 or MC5 receptors. It has therefore not been possible to delineate each of the different physiological effects presumed to be exerted by these receptors by using the natural MSH peptides. Recent discovery of non-selective antagonists for the MC3 and MC4 receptors[18] and the first selective antagonist for the MC4 receptors[20] (HS964 and HS014) have opened new possibilities to explore the underlying mechanism behind the effects of the MSH peptides. HS964, and its more potent and more selective analogue HS014, would therefore be interesting tools to probe the specific effects of the MC receptors in the central nervous system. HS014 has also recently been shown to be highly effective in inducing food intake in free feeding rats, an effect related to the MC4 receptor.[22-23] HS014 has earlier been shown to be potent antagonist for the neural MC3

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Table 1 intra-VTA injections in rats

A) Total grooming, duration, sec

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	56.2 ± 8.4	57.3 ± 7.0	69.2 ± 23.0	77.9 ± 27.9
α-MSH	230.2 ± 37.0*	424.6 ± 108.4*	564.2 ± 167.6*	655.5 ± 173.6*
HS964	118.8 ± 22.4*	123.7 ± 20.6*	169.3 ± 9.6*	210.5 ± 20.7*
HS014	97.3 ± 14.7*	134.3 ± 25.3*	141.1 ± 21.2*	186.2 ± 38.7*
saline+α-MSH	236.0 ± 42.0*	468.4 ± 123.8*	665.0 ± 189.7*	780.7 ± 205.2*
HS964+α-MSH	17.7 ± 10.1+			
HS014+α-MSH	66.7 ± 44.0+	103.6 ± 65.0+	118.7 ± 61.3+	135.0 ± 54.9+

B) Vertical activity (or rearing), incidences

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	16.2 ± 2.1	12.8 ± 3.0	13.0 ± 2.7	15.5 ± 4.5
α-MSH	20.5 ± 4.9	28.2 ± 5.9*	29.2 ± 5.8*	30.9 ± 5.3*
HS964	19.3 ± 2.7	23.5 ± 6.3*	30.5 ± 8.0	33.8 ± 9.8
HS014	30.3 ± 6.7	36.3 ± 9.1*	37.8 ± 8.3*	41.0 ± 6.8*
saline+α-MSH	30.3 ± 5.5*	44.0 ± 8.3*	48.4 ± 10.4	52.1 ± 12.5*
HS964+α-MSH	6.0 ± 2.1+			
HS014+α-MSH	15.3 ± 5.8	23.5 ± 10.5	33.8 ± 16.8	41.5 ± 22.9

C) Horizontal activity, incidences

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	18.6 ± 2.3	19.5 ± 6.2	19.5 ± 6.2	21.0 ± 6.3
α-MSH	22.5 ± 4.0	30.8 ± 6.2	31.6 ± 6.1	32.8 ± 5.8
HS964	16.0 ± 2.0	22.3 ± 6.0	42.6 ± 12.5	51.3 ± 15.4
HS014	28.4 ± 4.5	33.3 ± 8.0	34.8 ± 8.6	38.5 ± 8.5
saline+α-MSH	27.7 ± 4.7	37.3 ± 6.1	39.7 ± 6.6	41.4 ± 7.3
HS964+α-MSH	9.5 ± 4.3+			
HS014+α-MSH	17.0 ± 5.5	21.0 ± 8.0	25.3 ± 9.3	32.0 ± 14.0

*p<0.05 vs saline one-way ANOVA and post-hoc Bonferroni's multiple comparison test, nonparametric Mann-Whitney test
+ p<0.05 vs saline+αMSH

and MC4 receptors and our present data show that also HS964 is a potent antagonist for these receptors in vitro. However, as HS014 were found to be a partial agonist for the MC1 and MC5 receptors,[20] HS964 proved in the present study to be a antagonist for also these two MC receptors.

Grooming is a pattern of behavioural condition which has been described as 'scratching', 'preening', 'rubbing against objects and dust, sand, mud and sun bathing'. This behaviour may seem to be directed to outer body surface but may be more related to the nature of the situation than to the condition of the skin.[24] Grooming is known to be influenced by several central stimulants such as opioids and it has been known for long time that grooming behaviour is induced by central administration of α-MSH.[25] Our present data confirm the grooming effect of α-MSH. The selective MC4 receptor antagonists HS964 and HS014 seem to be potent inhibitors of the α-MSH induced grooming which may indicate that it is indeed the MC4 receptor which mediates the α-MSH induced grooming. This falls in line with earlier data which indicate that the low affinity analogue of ACTH(4–10), stated to be selective for the MC4 receptor, also inhibited α-MSH induced grooming.[26] We have shown earlier that γ-MSH has very low affinity for the MC4 receptor[16] and previous observations indicating that γ-MSH is not capable of causing grooming activity when injected ICV[27] are thus also in line with our present observations. A recent study, using the non-selective analogue SHU9119 and some low affinity ACTH(4–10)

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Table 2 ICV injections in rats

A) Total grooming, duration, sec

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	17.8 ± 3.6	36.1 ± 6.9	40.0 ± 9.3	41.5 ± 10.6
α-MSH	188.5 ± 34.4*	229.3 ± 29.1*	229.7 ± 29.2*	280.9 ± 42.1*
saline+α-MSH	194.3 ± 64.7*	244.1 ± 62.5*	255.6 ± 57.9*	292.2 ± 46.7*
HS964	93.8 ± 8.5*	116.8 ± 8.9*	148.3 ± 33.9*	193.5 ± 71.3*
HS014	114.8 ± 29.2*	224.5 ± 78.7*	237.7 ± 85.0*	283.5 ± 76.3*
HS014+α-MSH	41.7 ± 23.2+	58.4 ± 23.7+	78.9 ± 26.8+	100.3 ± 23.7+

B) Vertical activity (or rearing), incidences

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	18.3 ± 2.4	33.0 ± 5.3	38.3 ± 6.6	41.3 ± 8.7
α-MSH	15.3 ± 3.2	19.4 ± 2.5	21.3 ± 7.3	25.4 ± 4.9
saline+α-MSH	22.8 ± 6.3	27.3 ± 7.7	27.3 ± 7.7	29.4 ± 8.7
HS964	24.3 ± 11.5	37.8 ± 13.1	45.3 ± 14.9	55.9 ± 17.7
HS014	29.5 ± 6.6	33.7 ± 7.3	34.8 ± 7.8	36.0 ± 8.2
HS014+α-MSH	15.5 ± 5.7	29.0 ± 12.9	39.5 ± 19.0	40.5 ± 20.0

C) Horizontal activity, incidences

	Observation periods			
	0–15′	0–30′	0–45′	0–60′
saline	15.8 ± 2.9	25.0 ± 2.7	30.3 ± 3.8	35.9 ± 3.1
α-MSH	14.1 ± 1.8	18.2 ± 2.3	20.1 ± 2.1	26.6 ± 2.5
saline+α-MSH	19.8 ± 4.7	28.3 ± 5.5	28.9 ± 5.1	32.9 ± 6.7
HS964	32.3 ± 14.9	52.4 ± 14.9	63.6 ± 10.4*	72.4 ± 11.5*
HS014	30.8 ± 3.5*	37.3 ± 3.5*	39.5 ± 4.5	46.8 ± 5.3
HS014+α-MSH	19.3 ± 8.1	30.0 ± 8.1	36.3 ± 16.4	38.3 ± 18.3

*p<0.05 vs saline one-way ANOVA and post-hoc Bonferroni's multiple comparison test, nonparametric Mann-Whitney test
+ p<0.05 vs saline+aMSH

analogues as well as analogues of γ-MSH may also support that it might by the MC4 receptor which mediates grooming.[28]

We have not been able to find any earlier reports relating any melanocortic peptides to vertical or horizontal activity. Our data show that there is some increase in vertical activity after intra-VTA administration of α-MSH. However, both HS014 and HS964 show also some increase in vertical activity after administration in this area indicating that there is not any specific activity of α-MSH on the MC3 or MC4 receptors of which these peptides should act as antagonist. It is likely therefore that this effect is non-specific and not mediated through the MC receptor, or alternatively it is mediated through the MC1 or MC5 receptors. There are only two reports about expression of the MC1 receptor in the CNS[29,30] whose data suggest that the expression of the MC1 receptor is very limited and seemingly related to only the periaqual gray area. The MC5 receptor has been found to be present in the brain by several research groups, but is seems that the expression of this receptor is also comparatively limited.[31–35] Our present data can therefore not exclude that the effect of α-MSH on locomotor activity was mediated through any of these receptors.

It is intriguing that our data show that grooming behaviour is increased to a much greater degree when α-MSH is injected intra-VTA in the midbrain than after a ICV administration. The VTA area shows an abundant expression of the MC3 receptor[5,10,11] but expression of the MC4 receptor is also present in this area.[12] Moreover, it seems that the effects of these MSH peptides on locomotor activity seem to be more pronounced after the intra-VTA administration than after the ICV administration. It cannot be concluded that the effects after intra-VTA

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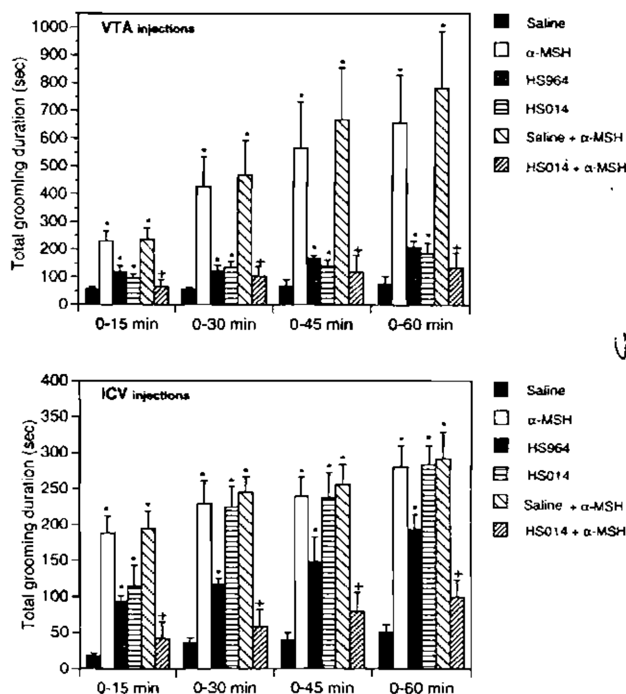

Fig. 2 Effect of the administration of different MSH peptides into A) the left VTA or B) ICV on grooming behaviour of male rats during different observation times.

administration are exclusively due to interactions with MC receptors in this particular area, but it is likely that the activity of MSH peptide administration is, in general, more effective in this area or in adjacent areas in the mid-brain.

ACKNOWLEDGEMENTS

This study was supported by grants from the Swedish MRC (04X-05957), the Swedish CFN, the Groschinsky, Bergwall, and Wiberg foundations, the Royal Swedish Academy of Science, the Howard Hughes Medical Institute (HHMI 75195–548501) and the Swedish Society for Medical Research.

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II

Acta Physiol Scand 1999, 167, 99–104

Behavioural responses of γ-MSH peptides administered into the rat ventral tegmental area

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ABSTRACT

The behavioural effects induced by α-, γ1- and γ2-MSH peptides (0.3 and 3 nmole per rat) injected into the left ventral tegmental area (VTA) of rats were compared. α- and γ1-MSH caused grooming of comparable magnitude, and also additional vertical activity (rearing). By contrast γ2-MSH caused a moderate but stable catalepsy, and practically no grooming. Moreover, intra-VTA pre-treatment with γ2-MSH, 15 min prior to intra-VTA γ1-MSH, markedly attenuated both the γ1-induced grooming and vertical activities. The differences in the behavioural response of the MSH peptides indicate that they act differentially on MC receptors in the VTA.

Keywords behavioural effect, MSH, ventral tegmental area.

Received 29 October 1998, accepted 24 June 1999

Despite many years of investigations, the mechanisms underlying the central effects of melanocortins (i.e. ACTH and α-, β- and γ-MSH peptides) are still poorly understood. However, substantial progress was made with the cloning of five different types of melanocortin receptors, termed MC1, MC2, MC3, MC4 and MC5 (Chhajlani & Wikberg 1992, Mountjoy *et al.* 1992, Chhajlani *et al.* 1993, Gantz *et al.* 1993a, b). Using *in situ* hybridization the distributions of mRNAs for the different MC receptors have been mapped. Thus, MC1 receptor mRNA was detected only in some discrete neurones of the periaqueductal grey (PAG) area (Xia *et al.* 1995). MC2 receptor mRNA seems not to be expressed in the central nervous system, although it is abundantly expressed in cells of the adrenal cortex (Xia & Wikberg 1996). The MC3 receptors are distinctly expressed in several regions of the hypothalamus, thalamus and mesencephalon. In particular neurones of the ventral tegmental area (VTA) of the midbrain show abundant expression of MC3 receptor mRNA (Low *et al.* 1994, Xia & Wikberg 1997). The MC4 receptor is widely expressed in many regions of the CNS although at varying levels (Low *et al.* 1994). The localization of the MC5 receptor in the central nervous system has hitherto not been mapped with histochemical techniques, although Northern blot and RT-PCR analysis indicate that it is present in the brain (Chhajlani *et al.* 1993, Gantz *et al.* 1994, Fathi *et al.* 1995).

ACTH and the α-, β- and γ-MSH peptides are formed from the POMC (pro-opio melanocortin) precursor, and POMC-immunoreactive neurones from two neural systems of the brain, of which one originates in cell bodies localized in the posterior hypothalamus, and the other in cell bodies of the brain stem. These neurones project to distinct regions of the central nervous system including the telencephalon, diencephalon, mesencephalon, brainstem and spinal cord (see Low *et al.* 1994). The functions of the γ-MSH peptide family are not well understood. Evidence exists that the acetylated melanocortin (α-MSH) and the des-acetylated melanocortins (γ-MSH and ACTH) are bioprocessed in different ways in different POMC neurones (Mezey *et al.* 1985). Moreover, in contrast to the acetylated-MSH produced in the pituitary, the hypothalamic α-MSH is not acetylated (Eberle 1988). The pharmacological properties of α- and γ-MSH differ. Using radioligand binding Schiöth *et al.* (1996) found α-MSH to show about 40-fold higher affinity for the human MC4 receptor affinity than γ1-MSH, while γ1-MSH showed about 4-fold higher affinity for the MC3 receptor than α-MSH. In this study the γ2-MSH was found to show a similar affinity profile as γ1-MSH

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although it showed all over slightly (about 3-fold) lower affinities than γ1-MSH for the different MC receptor subtypes.

In the present study we have focused on the behavioural effects induced by α-, γ1- and γ2-MSH peptides injected into the VTA of rats, which is an area where the mRNA for both MC3 and MC4 receptors have been reported (Gantz *et al.* 1993a, Mountjoy *et al.* 1994, Xia & Wikberg 1997). Our present data indicate that the pharmacology of the α-, γ1- and γ2-MSH peptides differs significantly. In the case of γ1- and γ2-MSH these differences are intriguing as γ2-MSH differs only by the extra C-terminal Gly residue compared with γ1-MSH. This is the first time such pharmacological differences of γ1- and γ2-MSH were reported upon their injection into the CNS.

MATERIALS AND METHODS

Animals

Male Wistar rats were bred at the Breeding House of the State Pharmaceutical Company GRINDEX, Riga, Latvia, and used at weights 300–350 g. The animals were housed in groups of five in a light–dark cycle of 12 h (lights off 19.00–7.00 hours).

Surgical procedures

Cannulas made from stainless steel needles (15-mm long, 0.56-mm OD) were implanted stereotaxically under Nembutal (CEVA, Sanofi, France) anaesthesia (60 mg kg^{-1} i.p.) into the left VTA at the following co-ordinates: –5.2 caudal to bregma, 0.8 mm lateral from midline, and –8.0 ventral from dura mater (Paxinos & Watson 1982). Cannulas were kept in place with dental cement (SPOFA Dental) covered with duracryl (SPOFA Dental). To prevent clogging of the cannulas a bent stylet of stainless steel was inserted into them, and removed only when the injection took place. After surgery rats were individually housed and given food and water *ad libitum* and allowed to recover for the following 7 days in a recovery room.

Peptides

α-MSH (N-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), γ1-MSH (H_2N-Tyr-Val-Met-Gly His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2), and γ2-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly OH) peptides were from BACHEM. The peptides were stored frozen in aliquots until used.

Injections

Drugs were injected into the VTA by means of a 75RN Hamilton Digital Syringe and a very fine polyethylene tube (Clay Adams, PE-10) attached to a 30-gauge needle which was inserted into the implanted cannula. On the day of the experiment an MSH-peptide aliquot was thawed and dissolved in saline to provide 0.3 or 3 nmole per rat. The total volume injected was for each substance 1 μL at a speed of 0.25 μL min^{-1}. For the combined treatments (i.e. γ2-MSH + γ1-MSH), the γ2-MSH was administered 15 min prior to the γ1-MSH injection. In the control groups γ2-MSH was substituted with saline (i.e. saline + γ1-MSH).

Behavioural analysis

On day 7 after surgery the rats were transported from the recovering room to an observation room for 1 day of handling and habituating. Each rat was placed into a Plexiglas observation cage (60 × 40 × 15 cm^3) and left for 30 min to diminish stress reactions to the novel environment. The rat was then placed onto an injection platform for 5 min before drug administration. Immediately after the injection the rat was placed in an observation cage. Observation started at the fifth minute after the onset of the injection and continued for up to 1 h using a Psion Workabout microcomputer (Noldus, the Netherlands). Grooming activity (i.e. total grooming) was expressed in seconds as the sum of the durations of the separate grooming reactions (face washing, body licking, scratching, ano-genital grooming, head and wet-dog shakes) recorded during a 15-min period. The following non-grooming behavioural events were registered: vertical activity (i.e. rearing) and catalepsy. Vertical activity was scored as incidences during the total of each observation period. The tests were performed either for one 15-min period, or for four 15-min periods for a total of 60 min. At the start and at the end of, respectively, 15 and 60 min the rats were tested individually for catalepsy in three consecutive tests for maximum 10 s each by: (1) placing the rat with its forepaws on a 7-cm high bar, (2) placing the rat with the hind legs on the same bar, and by (3) placing the rat across two 7-cm high bars distanced at 15 cm. Each of the catalepsy tests 1–3 were given a weighting coefficient, respectively, 0.2, 0.3 and 0.5, and the total catalepsy was scored as total time the animal spent in the separate tests multiplied by its corresponding coefficient. Thus the maximal score for a rat is $10 \times 0.2 + 10 \times 0.3 + 10 \times 0.5 = 10$ (see Sanberg *et al.* 1988). The observation sessions were performed between 10.00 and 14.00 hours. Each experimental group consisted of at least six rats.

Statistics

Statistical analysis was done using independent samples *t*-test or one-way ANOVA and Bonferroni's multiple

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comparison test as a post hoc. Results are expressed as the mean ± SEM.

Animal ethics

Experimental procedures were carried out in accordance with guidelines of the European Community, local laws and policies and were approved by the Ethics Committee of Animal Experimentation at the Latvian Research Council.

RESULTS

Before the onset of the experiments the rats were handled and habituated to the experimental environment in order to minimize the novelty factors as much as possible. We first studied the effect of intra-VTA administration of α-, γ1- and γ2-MSH at the doses 0.3 and 3 nmole during the first 15-min observation period (Fig. 1a). As can be seen 3 nmoles of both α-MSH and γ1-MSH caused significant grooming. However, at the dose 0.3 nmole the effect was significant only for α-MSH. By contrast, neither 0.3 nor 3 nmoles of γ2-MSH affected the total grooming compared with the saline control.

In Fig. 1(b) is shown the vertical activity recorded in the experiment shown in Fig. 1(a). As can be seen 0.3 and 3 nmoles of γ1-MSH significantly increased the vertical activity. Also α-MSH increased vertical activity, although this was significant only at the 3 nmole dose. By contrast, none of the doses of γ2-MSH increased vertical activity. In Fig. 1c is shown the catalepsy scores recorded at the end of the experiment. As can be seen both 0.3 and 3 nmoles of γ2-MSH caused catalepsy (4–5 scores from the maximum 10), whereas α- and γ1-MSH did not.

Time–effect relations were then assessed by administering 3 nmole of each of the peptides and observing motor activities at four consecutive 15-min observation intervals. The data collected for total grooming are represented in Fig. 2a. As can be seen from the figure the intra-VTA injection of either α-MSH or γ1-MSH caused a marked and significant increase in total grooming throughout the 1-h observation period. By contrast the intra-VTA administration of γ2-MSH did not cause any significant increase in the grooming during the first two, or during the last observation period. However, during the third (30–45 min) period γ2-MSH did seem to cause a small, but significant increase of grooming compared with the saline control ($P < 0.05$). With respect to vertical activity α- and γ1-MSH induced a marked increase, but only during the first 15 min (Fig. 2b). γ2-MSH did not cause vertical activity at any of the time periods studied (Fig. 2b).

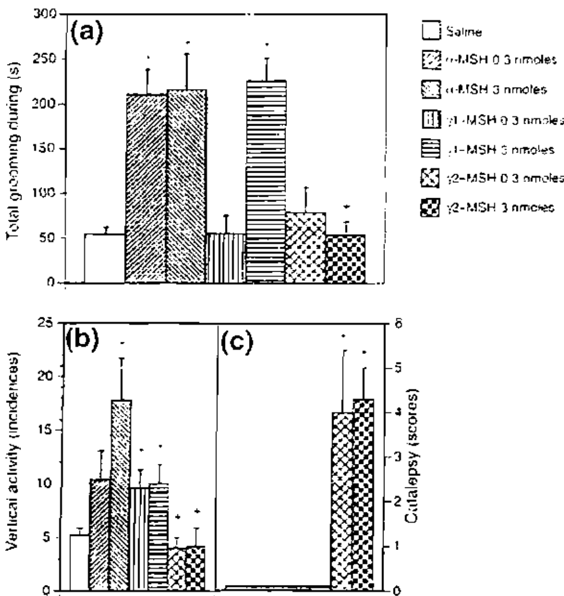

Figure 1 Effect of the administration of 0.3 and 3 nmole of α-, γ1- and γ2-MSH into the left VTA of male rats during 0–15 min after the injections on (a) total grooming, (b) vertical activity and (c) cataleptic activity recorded at the end of the experiment. *Indicates $P < 0.05$ vs. saline control; + indicates $P < 0.05$ vs. α-MSH (see Methods for details of statistical analysis). $n = 8$).

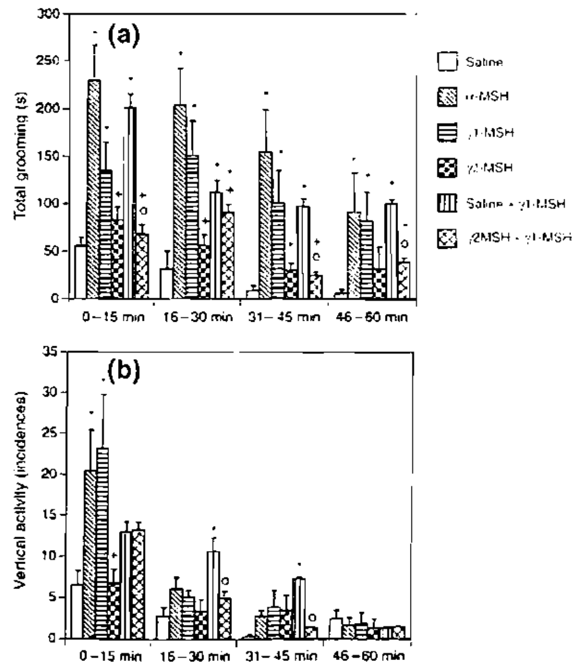

Figure 2 Effect of the administration 3 nmole of α-, γ1- and γ2-MSH, as well as combinations of γ1- and γ2-MSH into the left VTA of male rats during 0–60 min after the injections on (a) total grooming and (b) vertical activity. *Indicates $P < 0.05$ vs. saline control; + indicates $P < 0.05$ vs. α-MSH. O indicates $P < 0.05$ vs. saline + γ1-MSH. ($n = 8$). (For details see text).

In order to assess the effect of combined treatment of γ1- and γ2-MSH the rats were pre-treated with either 3 nmoles of intra-VTA γ2-MSH or saline (serving as control) 15 min prior to intra-VTA administration of 3 nmole γ1-MSH. The results are shown in Fig. 2. As can be seen in Fig. 2a the γ1-MSH given to the saline-treated rats caused a significant increase in total grooming at all time periods studied. For the rats pre-treated with γ2-MSH significant attenuations of the γ1-MSH grooming response were seen for the first and the two last time periods studied. The vertical activity was also increased in the saline pre-treated animals receiving γ1-MSH, and this increase was attenuated by the γ2-MSH pre-treatment, the effects being significant at the second and third 15-min period (Fig. 2B). In order to further assess the inhibitory effect of γ2-MSH on the γ1-MSH, the grooming scores of the experiment shown in Fig. 2 were summed for the whole 60-min period. For the saline + γ1-MSH and γ2-MSH + γ1-MSH-treated groups these sums of scores were 501 ± 96 and 205 ± 45, respectively, a difference being statistically significant ($P < 0.05$). The corresponding numbers for vertical activity were 36.4 ± 4.5 and 19.9 ± 6.6 ($P < 0.05$).

At the end of the experiment shown in Fig. 2 the animals were tested for catalepsy. In these tests it was revealed that only the γ2-MSH treatments induced catalepsy. Thus for the group receiving γ2-MSH alone the catalepsy score was 4.9 ± 0.1, while for the γ2-MSH pre-treated group (i.e. the group that was also given γ1-MSH) the score was 3.1 ± 1.0. (In the former case the time point for recording catalepsy was 65 min after the injection of γ2-MSH, whereas in the latter case the recording was 80 min after the injection of γ2-MSH, and thus 65 min after the injection of γ1-MSH).

DISCUSSION

We have here shown that left intra-VTA administration of α- and γ1-MSH induces marked grooming activity of a similar maximal magnitude, although the γ1-MSH appears to be slightly less potent than α-MSH. The observed grooming activity is typical for the α-MSH induced behavioural repertoire earlier reported (see Gispen *et al.* 1975). In addition both α- and γ1-MSH increased vertical activity.

The α-MSH and γ1-MSH show considerable sequence differences although they share a common core (His-Phe-Arg-Trp), a core which is found in all natural melanocortic peptides. Binding studies using recombinant human MC receptors indicate that γ1-MSH is 3-fold more potent than α-MSH on an MC3-receptor, whereas on an MC4-receptor α-MSH shows 45-fold higher potency than γ1-MSH (Schiöth *et al.* 1995, 1996). In the present study we found that α-MSH was more potent than γ1-MSH. Thus, our present results would speak in favour of the notion that the grooming effect by MSH-peptides is mediated via the MC4 receptor. We have earlier also shown that the intra-VTA administrations of our MC4-receptor blocking compound HS014 lead to attenuation of the grooming responses induced by intra-VTA administration of α-MSH (Klusa *et al.* 1998), an observation which also supports the role of the MC4 receptor in grooming. Support for this contention also comes from observations by Adan *et al.* (1997) demonstrating that MC4-receptor antagonistic ACTH(4–10) analogues are capable of blocking the grooming response of i.c.v. α-MSH. However, functional results from the *in vivo* administration of a peptide might not entirely reflect its potencies at the receptors as factors such as differential susceptibility to breakdown by peptidase, diffusion or other process leading to elimination from the site of injection may affect its concentration. Moreover, the receptor selectivities of presently used peptides are quite low. In this context we would also like to mention that we recently found receptor autoradiographic evidence that there are MSH-binding sites in the VTA which are distinct from the MC3 and MC4 receptors (Lindblom *et al.* 1998). Further studies are warranted before one definitely ascribes a role for the MC4 receptor in the mediation of the grooming responses induced by MSH-peptides.

The results obtained with γ2-MSH were strikingly different from those obtained with α- and γ1-MSH. Thus, the γ2-MSH peptide was practically ineffective in inducing grooming and vertical activity; instead it induced a long-lasting cataleptic state. Our observations are somewhat in line with a previous observation indicating that γ2-MSH is not capable of causing grooming when injected intra-cerebroventricularly (Van Ree *et al.* 1981). However, besides the capability of γ2-MSH to induce a long-lasting cataleptic state, we have in the present study in addition shown that the peptide is capable of attenuating the grooming responses and the vertical activity induced by γ1-MSH.

Our earlier binding studies suggest that γ2-MSH shows an affinity profile for the human MC receptors which is similar to that of γ1-MSH, although the binding affinities of γ2-MSH for human MC receptors are about 2- to 3-fold lower than they are for γ1-MSH (Schiöth *et al.* 1996). It has earlier been hypothesized that γ2-MSH can act as an endogenous opioid antagonist (Van Ree *et al.* 1981), and opiate-induced catalepsy is reported to be comparable with those induced by neuroleptics (Costall & Naylor 1973). Moreover, Oki *et al.* (1980) reported that γ1, γ2 and γ3-MSH, but not α-MSH, prevented the binding [^{3}H]-naloxone to opiate receptors of the rat brain. However, quite high

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concentrations were needed (in the μM range) which corresponds to an about 1000-fold lower affinity when compared with the affinities for e.g. an MC3-receptor. The γ2-MSH shows also lower affinity for the [^{3}H]-naloxone sites than does γ1-MSH (Oki *et al.* 1980). In view of the low affinities of γ2-MSH for the opiate receptors it seems unlikely that these receptors are involved in the γ2-MSH effects of the present study. Perhaps the γ2-MSH is exerting its effect via another pathway that is also distinct from the melancortin receptors.

The only difference between γ1- and γ2-MSH is an extra C-terminal Gly in the γ2-MSH. It is possible that this additional residue, which is expected to be flexible as a 'moving tail', could render the peptide conformation quite different and change its pharmacological properties. For example the γ2-MSH could become antagonistic, or it might even be capable of rapidly bringing an MC receptor into a desensitized state. However, further studies will be required to clarify these issues. In these studies it might be of value to use specific antagonists of the different MC receptors, something which is presently available only for the MC4-receptor (see Schiöth *et al.* 1998).

Lastly, it should also be mentioned that we were recently able to delineate between MC3 and MC4 receptors in the rat brain by using an autoradiographic approach (Lindblom *et al.* 1998). Our studies seem to indicate that the MC3 receptor protein dominates in many areas of the CNS such as the nucleus accumbens, medial pre-optic area and ventromedial nucleus of the hypothalamus, whereas in many other areas there are both MC3 and MC4 receptors present. However, interestingly in our study we found peculiar binding properties of MSH-peptides in the VTA seemingly indicating the presence of yet another MC receptor in this area. These data seem to add to the complexity in the interpretation of the present data and should prompt further investigations to clarify the functional roles of MC receptors in the VTA.

In summary we have here shown that distinct differences exist in the behavioural effects caused by intra-VTA injections of α, γ1- and γ2-MSH. Not only do these peptides cause different spectra of behavioural effects, but we have also found γ2-MSH to be capable of attenuating the grooming responses caused by γ1-MSH.

Supported by Howard Hughes Medical Institute (HHMI 75195-548501) and Swedish MRC (04X-05975).

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III

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Rīga 1999

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MELANOCORTINS AND THEIR RECEPTORS: BEHAVIOUR AND NEUROCHEMISTRY

Introduction

During bioprocessing of the proopiomelanocortin (POMC) molecule several melanocortin peptides (ACTH, α-, β-, and γ-MSH) have been formed [5]. A functional role is well established for ACTH (stimulation of steroidogenesis in the adrenals) and α-MSH (melanogenesis in the melanocytes), whereas β- and γ-MSH still remain as mystery. In the 90th an intense identification and cloning of the melanocortin receptors (MCR) have been started. At present five MCR subtypes are described: MC1R, MC2R, MC3R, MC4R and MC5R [2,3,8,9,20,25]. The MC1R mRNA expression is limited in melanocytes and melanoma tumors [3], and MC1R is considered as crucial for α-MSH-mediated effects (pigmentation). More strongly limited (only in the adrenal tissues) is MC2R mRNA expression; this subtype mediates the ACTH corticotropic effects [20,23,28]. Wide expression of the MC3R mRNA is detected in the brain and peripheral tissues (e.g. placenta, pancreas) [5,9,22]; this receptor subtype binds γ_1- and γ_2-MSH peptides, however a functional role for both peptides and their receptors is still unclear.

MC4R mRNA expression is found in brain tissues and this receptor subtype is capable to bind β-MSH (but not γ-MSH) [2,9,19,24]. Expression of the MC5R mRNA is distributed in many central and peripheral tissues and its overlapping with MC3R, MC4R and MC5R mRNA expressions is detected [7,10].

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In a framework of collaboration between the Uppsala University and the Latvian Institute of Organic synthesis, the studies were focused on γ_1-MSH and γ_2-MSH to clarify their pharmacological and neurochemical effects, possible functional role of these peptides and their receptors. Since MC3R mRNA expression is detected to a greater extent in the ventral tegmental area (VTA) [22,29], the peptides were microinjected in this brain structure. The first data were striking: although the chemical structures of both γ-MSHs are very similar (γ_1-MSH: H_2N–YVMGHFRWDRF–OH; γ_2-MSH: H_2N–YVMGHFRWDRFG–OH) and they differ by only one extra amino acid (Gly) residue at the C-terminal of γ_2-MSH, these peptides showed different behaviour spectrum. [16]. If γ_1-MSH injected intra-VTA in rats induced excessive grooming and intensification of vertical activity, γ_2-MSH in opposite, caused hypoactivation, even moderate catalepsy. Moreover, γ_2-MSH acted as antagonist by reducing the γ_1-MSH-induced grooming responses. Therefore the question can be arisen whether these opposite effects of γ_1-MSH and γ_2-MSH are mediated via the same specific MC3R and its different activation, or other receptor subtype – MC4R– which expression is recently detected in the VTA, is also involved in peptide actions. Besides, one should be taken into account that the VTA belongs to one of the major dopaminergic pathways – the mesolimbic system – which transfers signals from the VTA to the nucleus accumbens (NAAC). This system is considered as "reward system" and intense dopamine release in the NACC is taken as crucial neurochemical basis which explain motivation of chronic opiate, alcohol use and development of the drug dependence [17]. Dopamine over-release in the NACC and behavioural hyperactivation/hyperlocomotion is found in schizophrenic patients during psychotic states [6].

The present study analyses the influence of γ_1-MSH and γ_2-MSH on "psychotic states" in schizophrenic model animals. Psychoactivation was induced by phencyclidine and amphetamine, which may intensify dopaminergic system and cause an increase in locomotor activity.

Methods

Animals

The experiments were performed in BALB/c male mice (19–21g) obtained from the Breeding Facility of the Joint Stock Company GRINDEX (Riga, Latvia). Mice were kept at +21 t, conditioned air, humidity 60 ± 10%, light cycle from 6.00 a.m to 6.00 p.m., standard diet (Altromin Standard Diets1320), water ad libitum.

Drugs and injection procedure

γ_1-MSH and γ_2-MSH (BACHEM), MC4R antagonist HS014 from the University of Uppsala [26], were injected intracisternally (ic) in mice at a dose of 0.3 nmole/mouse (in a volume 10 µl).

Phencyclidine (PCP) synthesized at the Latvian Institute of Organic Synthesis; L–amphetamine (AMP) purchased from SIGMA; both drugs were administered peripherally 5 min prior to peptide injections: PCP 5 mg/kg intraperitoneally, AMP 5 mg/kg subcutaneously. Peptides were dissolved in saline. Mice of the control groups received saline ic in the volume of 10 ml/mouse.

Assessment of the locomotor activity

The mice were placed in the Activity Cage (Ugo Basile, Cat.7400) and locomotor activity was registered from the 30th to 60th min after PCP or AMP administration.

Statistics

The results are calculated as mean values ± SEM and significance was evaluated at $p<0.05$ (Student's t test). Inter-group statistics was evaluated by ANOVA followed by Student's t test).

Ethics

The experimental procedures were carried out in accordance to the EU recommendations and accepted by the Ethics Committee on laboratory animal use at the Latvian Science Council.

Results and discussion

In model animals which have received AMP as psychoactivation-mimicking drug, both γ-MSH reduced the AMP-induced hyperlocomotion. As it is well-known, AMP acts as dopamine releaser from the presynaptic nerve terminals resulting in dopamine accumulation in the synaptic cleft, which in turn leads to enhanced postsynaptic reception. This manifests as behavioural hyperactivation and increased locomotion [27].

In case of PCP-model the peptides acted differentially: γ_1-MSH potentiated the PCP-effects, whereas γ_2-MSH antagonized the PCP-effects. Moreover, γ_2-MSH reduced the mentioned γ_1-MSH potentiating effect (γ_1-MSH+PCP).

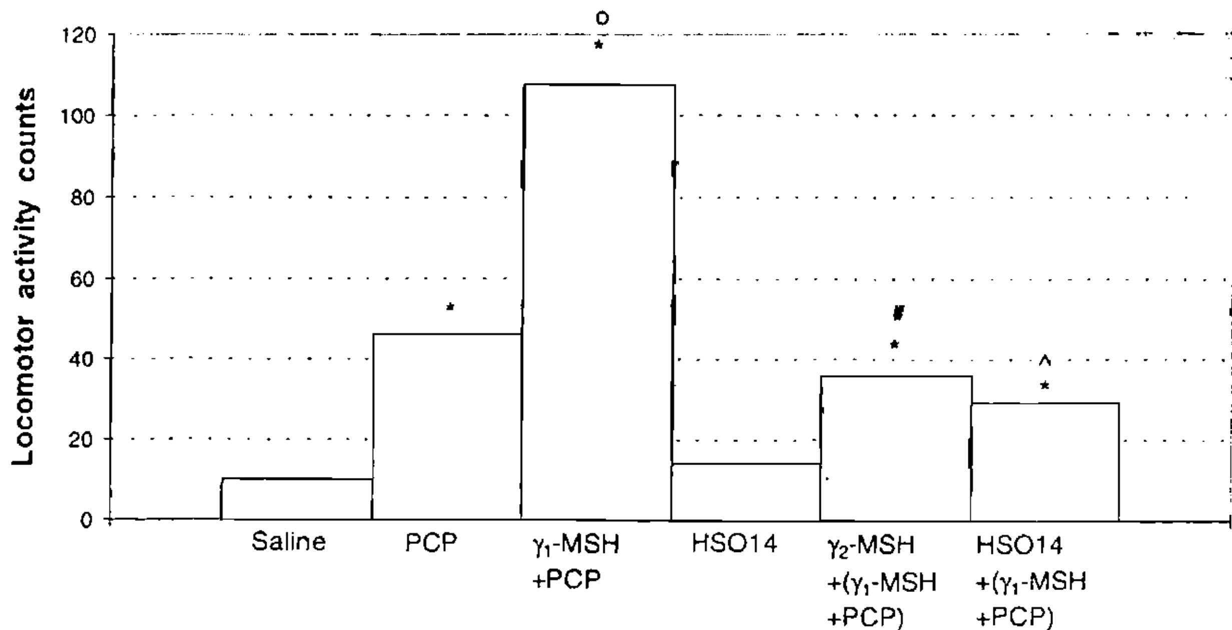
These data indicate that γ_1-MSH may act as psychoactivating, whereas γ_2-MSH as anti-psychotic peptide. However, there is no clear understanding of their mechanisms of action, since PCP is a representative of the opiate sigma receptor ligand [18], either a non-competitive NMDA receptor antagonist [1]. The question of the participation of melanocortin receptors in γ-MSH-induced influence on PCP effects, could had be solved by using selective MCR antagonists. Unfortunately, there is a problem to design selective MC3R antagonist, however HS014 was found [26] as effective MC4R antagonist [13. 14]. It seems that detection of MC4R mRNA in the VTA has a great importance, although MC4R has lower affinity to γ-MSHs in comparison to that of MC3R. Recently we have shown that MC4R antagonist HS014 acted as strong antagonist towards the a-MSH-induced grooming [14]. In light of these results there is surprisingly novel data from the present experiments: similarly to γ_2-MSH, HS014 was capable also to antagonise γ_1-MSH+PCP effects (Fig.). One may suggest whether MCRs can mutually interact (cross-talk?) or γ-MSHs and HS014 may affect mesolimbic dopaminergic processes. Our previous neurochemical data [15] are in line of that suggestion, as γ_1-MSH and γ_2-MSH injected intra-VTA in rats considerably influenced dopaminergic processes in the mesolimbic system (NACC and tuberculum olfactorium), particularly by intensifying dopamine metabolism (elevation of DOPAC concentration) in 15 min after administration. Despite short half-life of the pep-

tides, their neurochemical effects were long-lasting that manifested as a propagation (in 1h after injection) of the alterations also in other adjacent structures (nigrostriatal) and other neurotransmitter contents (e. g. sharp reduction of serotonin in the striatum).

Thus, the data obtained show that γ-MSHs are capable to interfere with various neurotransmitter systems. Obviously that may explain a variety of different central effects caused by γ-MSH peptides, e.g. influence on blood pressure, pain perception, memory [15, 21]. So, in our experiments γ_1-MSH enhanced the activity of glutamate receptor antagonist PCP, on one hand, and antagonised effects of the dopamine releaser AMP, on the other hand. Besides one can be taken into consideration that γ_1-MSH has high affinity to MC3R. In turn, similarity in the effects of γ_2-MSH and MC4R antagonist HS014 (antagonism against both γ_1-MSH and γ_1-MSH+PCP) indicates that they may act in similar manner on the glutamatergic processes. It remains as mystery how one C-terminal residue which makes γ_2-MSH structure longer than that of γ_1-MSH may cause so great difference in their behavioural activities and probably in modifying the melanocortinergic and glutamatergic systems. Since PCP influence on the mesolimbic system (stimulation of DA release in the NACC) can be taken as proved [14], the modulating influence of the PCP effects by γ_1-, γ_2-MSH and HS014 may indicate their ability to alter effectively this reward system and appropriate processes. The effectiveness of HS014 in the stimulation of feeding behaviour [12] allows to suggest food intake as reward process. One may expect that γ_2-MSH will be capable to express similar to HS014 activity but opposite (anorexic) from γ_1-MSH. The elucidation of the roles of melanocortins and their receptors are under intense studying.

Acknowledgements

The studies are supported by the Howard Hughes Medical Institute, grant HHMI 75195–548501. Authors are grateful to Professor G. Duburs (Latvian Institute of Organic Synthesis) for kind supply of PCP.



Influence of γ_2-MSH and HSO14 on the γ_1-MSH+PCP effect.

* $P < 0.05$ vs saline

o $P < 0.05$ γ_1-MSH+PCP vs PCP

# $P < 0.05$ γ_2-MSH+(γ_1-MSH+PCP) vs γ_1-MSH+PCP

^ $P < 0.05$ HSO14+(γ_1-MSH+PCP) vs γ_1-MSH+PCP

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IV

The MC_4 receptor mediates α-MSH induced release of nucleus accumbens dopamine

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Received 28 March 2001; accepted 2 May 2001

Strong evidence suggests a functional link between the melanocortin and dopamine systems. α-Melanocyte stimulating hormone (α-MSH) induced grooming behaviour, which can be blocked by dopamine receptor antagonists, is associated with increased dopaminergic transmission in the striatal regions. Whether this effect is mediated specifically by melanocortin (MC) receptors has not previously been established. Using *in vivo* microdialysis on anesthesized rats we have shown that α-MSH administered into the ventral tegmental area induced a significant increase in dopamine and DOPAC levels in the nucleus accumbens. This increase was completely blocked by pre-treatment with the MC_4 receptor selective antagonist HS131, indicating that the effects of α-MSH on dopamine transmission may be mediated by the MC_4 receptor. *NeuroReport* 12:2155–2158 © 2001 Lippincott Williams & Wilkins.

Key words: Dopamine; HS131; MC_4-receptor; Microdialysis; α-MSH

INTRODUCTION

Melanocortin peptides, such as α-melanocyte stimulating hormone (α-MSH) and adrenocorticotropic hormone (ACTH), are derived from the pro-hormone pro-opiomelanocortin (POMC). Central POMC containing neurons project from areas in the hypothalamus and brain stem to many areas of the central nervous system. The melanocortin peptides are known to act on five subtypes of the melanocortin receptors. Two of them, the MC_3 and MC_4 receptors, are claimed to be dominant in the CNS [1]. The behavioural effects of the melanocortin peptides are well documented, and include avoidance, feeding, stretching-yawning and grooming [2]. In some recent studies we used the MC_4 receptor selective antagonist HS014 and showed that it could block the grooming behaviour elicited by α-MSH, indicating that melanocortin induced grooming may be mediated by the MC_4 receptor [3,4].

There are several reports suggesting the existence of a functional link between the melanocortin and dopamine systems [1]. The two systems are anatomically overlapping, and some dopamine synthesizing regions, such as the VTA [5], also express melanocortin receptors [6]. Intracerebroventricular administration of melanocortin receptor agonists not only induce excessive grooming, but also causes elevated concentrations of caudate nucleus dopamine levels [7]. Elevations in DOPAC/DA ratios in the caudate putamen and nucleus accumbens have also been observed after administration of α-MSH into the ventral tegmental area (VTA) [8]. Grooming may also be induced by administration of dopamine D_1 agonists [9]. Moreover, the dopamine D_1-receptor selective antagonist SCH 23390 inhibits the grooming behaviour induced by ACTH, suggesting that melanocortin induced grooming, and perhaps other behaviours, are at least partially mediated by central dopamine [9,10].

Whether the effects of melanocortin peptides on striatal dopamine levels is specifically mediated by melanocortin receptors has not been established. The aim of this study was to test the hypothesis that MC_4-receptors in the VTA causes increased release of dopamine in the nucleus accumbens.

MATERIALS AND METHODS

α-MSH (Ac-Ser-Tyr-Ser-Met-Glu-His-L-Phe-Arg-L-Trp-Gly-Lys-Pro-Val-NH_2) and HS131 (cyclo (S-S)-Ac-L-Cys^5, Gly^6,D-Nal^7,L-Cys-$NH_2{}^{10}$)α-MSH^{5-10} trifluoroacetate [11]) were synthesized using the solid phase approach applying a Fmoc-based Pioneer peptide synthesis system (PerSeptive Biosystems) and purified by HPLC. The correct molecular weights of the peptides were confirmed by mass spectrometry. Dopamine and DOPAC (3,4-dihydroxyphenylacetic acid) were obtained from Sigma-Aldrich, Tyresö, Sweden.

The rat MC_3 and MC_4 receptor clones were generously provided by Dr RD Cone, Vollum Institute, USA and Dr R Duman, Yale University, USA, respectively. The receptor clones were transiently expressed in COS cells [12] and competition curves were made for HS131 with a constant concentration of [^{125}I][Nle^4, D-Phe^7]α-MSH as described previously [13]. The radioligand binding assays were performed in duplicate and repeated 3–4 times.

In order to establish the antagonistic/agonistic properties of HS131 on the rat MC_4-receptor, a cAMP assay was performed by applying eight different concentrations of α-MSH alone or in the presence of 30 nM HS131 to COS cells transiently expressing the rat MC_4 receptor. Incubations with ligands were performed for 20 min before quenching the cells with perchloric acid/Tris–KOH and assaying cAMP using a protein binding assay essentially as described [14].

Male Sprague–Dawley rats (Beco, Sweden; $n = 21$, 270–340 g) were housed in groups of four at a temperature of 20–22°C and a relative humidity of 55% under an artificial light:dark cycle (lights on 07.00–19.00 h). The animals had unlimited acess to food (R36 food pellets, Labfor, Lactimin, Vadstena, Sweden) and water. The study was approved by the local ethical committee.

The animals were divided into four groups; saline ($n = 6$), α-MSH ($n = 6$), HS131 ($n = 5$) and α-MSH + HS131 ($n = 4$). They were anaesthetized with inactin (80 mg/kg, i.p.) and positioned in a Kopf stereotaxic frame. Body temperature was kept at 37°C using a HB 101/2 temperature control unit (Letica Scientific Instruments, Barcelona, Spain). The skull was exposed and two holes were drilled for the placement of a MAB microdialysis probe (cut-off 20 kDa PES; AgnTho's AB, Lidingö, Sweden) in the left nucleus accumbens (coordinates from bregma: B +2.2, L −1.5, V −7.1) and for a guide cannulae (made from stainless steel syringes, length 15 mm, o.d. 0.56 mm) into the left VTA (B −5.0, L −0.9, V −7.2). The guide cannula was implanted 2 mm over the VTA for later insertion of a microinjection needle and kept in place with dental cement (De Trey, Sevriton, Germany).

The microdialysis probe was perfused with artificial cerebrospinal fluid (CSF; Apoteket Produktion and Laboratorier, Umeå, Sweden) and inserted slowly into the nucleus accumbens in order to minimize tissue damage caused by penetration of the probe. The implanted probe was used to deliver extracellular dopamine and DOPAC. A constant flow of 2 µl/min was maintained with a microdialysis pump (Univentor 684 Syringe pump, Bulebel Industrial Estate, Malta). Two hours after implantation of the probe, 20 min dialysate samples were collected from the outlet line in the Microsampler (Univentor 810 Microsampler, Bulebel Industrial Estate, Malta) into polyethylene microcentrifuge tubes. Three basal samples were collected with < 15% variation in DA and DOPAC levels before drug administration.

α-MSH and HS131 were respectively dissolved in sterile artificial CSF. CSF (0.5 µl), α-MSH (10 nmol/0.5 µl) and HS131 (1 nmol/0.5 µl), respectively, were injected manually into the VTA via the guide cannula using a Hamilton Mirolitre syringe (Hamilton-Bonadaz AG, Switzerland) and a fine polyethylene tube (Clay Adams, PE-10) attached to a 30-gauge microinjection needle that extended 1.2 mm deeper than the guide cannula, i.e. 0.8 mm over the VTA. For the combined treatments HS 131 was administered 40 min prior to the injection of α-MSH. The microinjection needle was left in place after injection to limit drug efflux up the cannula shaft. Sampling was continued for 4 h.

The concentrations of DA and DOPAC were determined using HPLC with electrochemical detection. A reversed-phase column (ReproSil-Pur C-18-AQ, 150 ×3 mm, particle size 5 µm) was used to separate the biogenic amines, and a coulometric-electrochemical detection system (ESA Inc, Chelmsford, MA, USA) utilizing two electrodes was used to oxidize the amines. Preinjection part guard electrode voltage was +0.4 V (ESA, guard cell Model 5020) and working electrode voltage was +0.34 V (ESA, analytical cell Model 5011). The mobile phase consisted of 2 g/l $CH_3COONa.H_2O$, 38.75 mg/l 1-octanesulfonic acid, 3.7 mg/l EDTA and 100 ml/l methanol at pH 4. The HPLC pump (LKB 2150 HPLC pump, Bromma, Sweden) was 0.6 ml/min. Chromatograms were recorded using a MEGA series integrator (Carlo ERBA, Strumentazione, USA). The limit of detection was 0.4 nM for both dopamine and DOPAC.

After the experiment, the animals were killed by decapitation. The brains were removed, frozen in cold (between −20 and −30°C) 2-methylbutane, mounted on a cryostat microtome and sectioned (35 µm). The sections were collected on gelatine coated slides and stained with Mayer hematoxylin (Histolab Products AB, Sweden). The stained sections were digitized in a video camera (CCD-72, Dage-MTI, Michigan City, IN), and the positioning of the microdialysis probe and the guide cannula was confirmed using NIH-Image software (NIH Image 1.54, NIMH, Bethesda, MD) and a brain atlas as a reference [15]. Only animals with correctly implanted probe and cannula were included in the statistics.

For statistical analysis, cAMP data were tested with a repeated measurements ANOVA, followed by Fisher's protected least significant difference (PLSD) test where appropriate. Changes in DA and DOPAC levels were represented as a mean of the three post-injectional samplings relative to the three pre-injection samplings. Intergroup comparisons were made with a factorial ANOVA test followed by Fisher's PLSD test where appropriate. All statistics were performed using the StatView 4.51 software for Macintosh. $p < 0.05$ was used as the criterion of statistical significance.

RESULTS

The results from the radioligand binding experiment are shown in Table 1. HS131 displayed a 150-fold selectivity for the recombinant rat MC_4 receptor over the recombinant rat MC_3 receptor. Measurements of cAMP indicated that 30 nM HS131 blocked the increase of intracellular cAMP levels induced by α-MSH in cells expressing the recombinant rat MC_4 receptor (Fig. 1). Repeated measurements ANOVA analysis indicated that HS131 significantly ($F(1,6) = 14.1$, $p < 0.001$) blocked the α-MSH effect. *Post-hoc* analysis (Fischer's PLSD) showed that the effect was

Table 1. Binding constants (pK_i, mean ± s.e.m., and K_i values) of HS131 for the rat MC_3 and MC_4 receptors determined by radioligand binding on receptors expressed in COS cells and using [^{125}I]-NPD-MSH as radioligand

HS131	rMC_3	rMC_4
pK_i	6.5 ± 0.1	8.7 ± 0.3
K_i (nM)	331	2.12
n	3	4

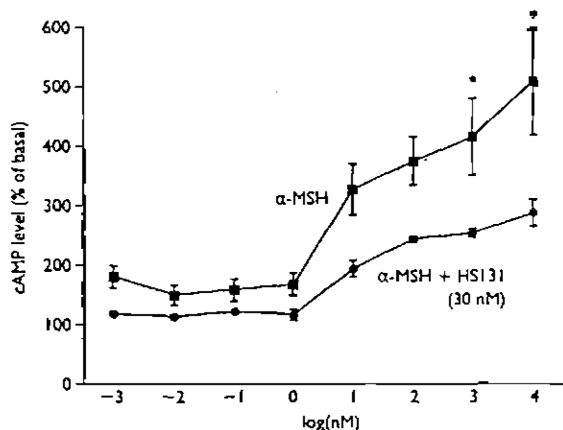

Fig. 1. Generation of cAMP in COS-1 cells expressing the rat MC_4 receptor in response to α-MSH ($n=5$) and α-MSH+HS131 ($n=3$). Each point represents mean ± s.e.m. * $p<0.05$.

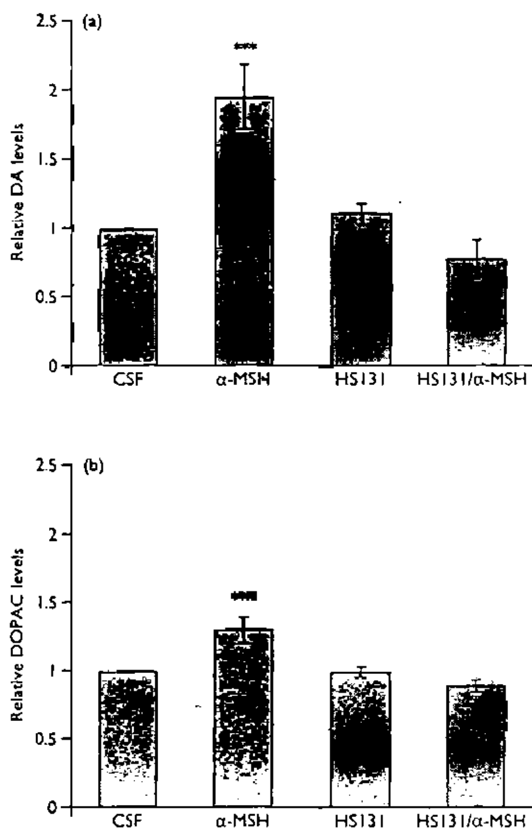

Fig. 2. Average levels of DA (a) and DOPAC (b) in the nucleus accumbens measured by microdialysis after administration of artificial CSF, 10 nmol α-MSH, 1 nmol HS131 or 10 nmol α-MSH after pretreatment with 1 nmol HS131 into the VTA. The results are presented as mean ± s.e.m. of DA and DOPAC 60 min after administration relative to baseline levels. *** $p<0.005$ vs CSF, HS131 and HS131/α-MSH.

significant at the two highest concentrations of α-MSH tested, i.e. 1 and 10 μM ($F(1,6)=9.413$, $p<0.05$ and $F(1,6)=7.184$, $p<0.05$).

Baseline dialysate levels of DA and DOPAC were 0.67 ± 0.05 and 369 ± 29 nM, respectively. The results from the measurements of DA ($F(3,17)=12.63$, $p<0.0001$) and DOPAC ($F(3,17)=9.40$, $p<0.001$) by microdialysis are shown in Fig. 2. *Post hoc* analysis (Fischer's PLSD) indicated that α-MSH injected into the VTA caused a significant increase in the levels of DA ($p<0.001$) and DOPAC ($p<0.005$) in the nucleus accumbens during the 60 minutes following the injection. Pre-treatment with HS131 into the VTA completely abolished the effect of α-MSH on both DA and DOPAC. HS131 administered alone into the VTA did not induce any effect on DA or DOPAC levels.

DISCUSSION

In this study we investigated the involvement of melanocortin receptors in the VTA for the melanocortin induced release of DA in the nucleus accumbens. We used *in vivo* microdialysis to measure the levels of extracellular DA and DOPAC in the nucleus accumbens following the intra-VTA injections of the MCR agonist α-MSH and the MC_4-selective antagonist HS131. In agreement with previous findings [7,8], α-MSH caused an increase in the levels of DA and DOPAC in the nucleus accumbens. The effect of HS131 was similar to that of CSF alone, but pre-treatment with HS131 completely abolished the effect of α-MSH. This is in support of the hypothesis that α-MSH mediates its effect on nucleus accumbens dopamine by activating melanocortin receptors.

α-MSH is a non-selective agonist for both the rat MC_3 and MC_4 receptors, showing a K_i of about 10 nM for each of these receptors in binding assays [16]. Our present results, on the other hand, show that the MCR antagonist HS131 has a 150-fold binding preference for the MC_4 receptor compared to the rat MC_3 receptor. Moreover, our present results show that HS131 is a blocker to the agonistic activity of α-MSH on the rat MC_4 receptor. It seems thus likely that the quenching effect of HS131 on the α-MSH induced release of dopamine is due to blockade of MC_4 receptors, but due to the fact that the concentration of HS131 at the site of action in the VTA is unknown, it cannot be rigorously excluded that some other subtype of MC receptor is blocked.

Previous evidence indicate that stimulation of central MC_4 receptors may induce grooming behaviour [3,4]. Moreover, earlier studies show that ACTH-induced grooming may be blocked by dopamine receptor antagonists [10]. Therefore, it is reasonable to suggest that MC_4 receptor mediated release of dopamine might be a causative event in the induction of grooming behaviour by the melanocortins.

The mesolimbic dopamine system originates in the ventral tegmental area (VTA) and project axons to the limbic system, including the nucleus accumbens. The role of the mesolimbic dopamine system is supposedly that of a

mediator of rewards; hence it is often referred to as the reward system. The reward system is believed to be involved not only in the hedonic impact of natural stimuli (such as food), but also in the development of drug addiction [17]. In a recent study, the availability of dopamine D_2 receptors were shown to be reduced in the striatum of obese patients, suggesting a role of the central dopamine system in the pathology of obesity [18]. Dopamine is also essential for the hyperphagia in leptin-deficient mice [19]. The melanocortin system is strongly implicated in the regulation of energy homeostasis, and the mechanism of action of melanocortin peptides on food intake involves the complex signaling of several hypothalamic nuclei [20]. However, the observed action of melanocortins on the mesolimbic dopamine system suggests that some of the effect of melanocortin peptides on food intake may be attributed to alterations in the subjective value of food (for a discussion on possible involvements of dopamine in feeding effects of the melanocortins see [21]). Moreover, the melanocortin system appears to have a role as a functional antagonist to opiate action [22]. Although this may seem somewhat paradoxical, due to the fact that opiates, in a similar fashion as the melanocortins, stimulate the release of nucleus accumbens dopamine [23], it may nevertheless provide yet a link between the melanocortic and reward systems. Interestingly, several of the other behaviours elicited by melanocortins, e.g. passive avoidance [24] and stretching-yawning [25] may also involve the central dopamine system. It is thus possible that these melanocortin induced actions are also, at least partly, mediated by dopamine release.

CONCLUSION

We have here provided evidence that melanocortin receptors, with the MC_4 receptor being a likely candidate, may mediate the elevated release of dopamine seen in the nucleus accumbens after administration of α-MSH into the VTA. It is likely that MC receptor-mediated dopamine transmission is responsible for melanocortin-induced grooming behaviour, and possibly also in other melanocortin effects, such as feeding behaviour, passive avoidance and stretching-yawning.

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Acknowledgements: This research was supported by grants from the Swedish MCR (04X-05957), the Wiberg fund and Melacure Therapeutics. Baiba Opmane was supported by a stipend from the Swedish Institute under the Visby Programme.

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Neuropeptides (2001) 35 (1), 50–57
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doi: 10.1054/npep.2000.0843, available online at http://www.idealibrary.com on

The γ_2-MSH peptide mediates a central analgesic effect via a GABA-ergic mechanism that is independent from activation of melanocortin receptors

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SUMMARY Using the latency for tail-flick after thermal stimulation we have assessed the effects of α-, γ_1- and γ_2-MSH on nociceptive threshold in the mice. Intracisternal injections of γ_2-MSH induced a distinct analgesia, while γ_1-MSH in the same doses gave only a minor analgesia. Intracisternal α-MSH instead gave a short-term hyperalgesia. The effect of γ_2-MSH was not blocked by any of the MC_4/MC_3 receptor antagonist HS014, naloxone or by the prior intracisternal administrations of γ_1-MSH. However, the γ_2-MSH analgesic response was completely attenuated by treating animals with the $GABA_A$ antagonist bicuculline. The γ_2-MSH analgesic effect was moreover additive to the analgesia afforded by muscimol and ethanol, but not to that afforded by diazepam. In addition both γ_1- and γ_2-MSH induced moderate catalepsy, but could at the same time attenuate haloperidol induced catalepsia. We conclude that γ_2-MSH mediates a central analgesic effect via GABA-receptor dependent pathway that is distinct from melanocortic- and opioid-receptors. Moreover, the mechanism for γ_2-MSH's analgesic effect appears to be distinct from that causing moderate catalepsia by γ-MSH's. © 2001 Harcourt Publishers Ltd

INTRODUCTION

The melanocortic peptides (ACTH and the α-, β- and γ-MSH peptides) are derived from the POMC (pro-opio melanocortin) precursor and have a wide-spread distribution in the body. In the central nervous system POMC-immunoreactive neurones form essentially two neural systems: one originates in cell bodies localised in the posterior hypothalamus, and the other in cell bodies of the brain stem. These neurones project to distinct regions of the central nervous system including the telencephalon, diencephalon, mesencephalon, brainstem and spinal cord (Eberle, 1988).

Received 23 August 2000
Accepted 20 September 2000

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Over the last few years the mode of central actions of the melanocortins have started to become increasingly understood. Five different types of melanocortin receptors, MC_{1-5}, that are responsive to the melanocortic peptides have been cloned (Wikberg, 1999). Using in situ hybridization the distribution of mRNAs for the different MC receptors were mapped. The MC_1 receptor mRNA was detected only in some discrete neurones of the periaqueductal gray (PAG) area (Xia et al., 1995). MC_2 receptor mRNA seems not to be expressed in the central nervous system, although it is abundantly expressed in cells of the adrenal cortex (Xia and Wikberg, 1996). The MC_3 receptors are distinctly expressed in several regions of the hypothalamus, thalamus and mesencephalon. In particular neurones of the ventral tegmental area (VTA) of the midbrain show abundant expression of MC_3 receptor mRNA (Low et al., 1994; Xia and Wikberg, 1997). The MC_4 receptor is widely expressed in many regions of the CNS

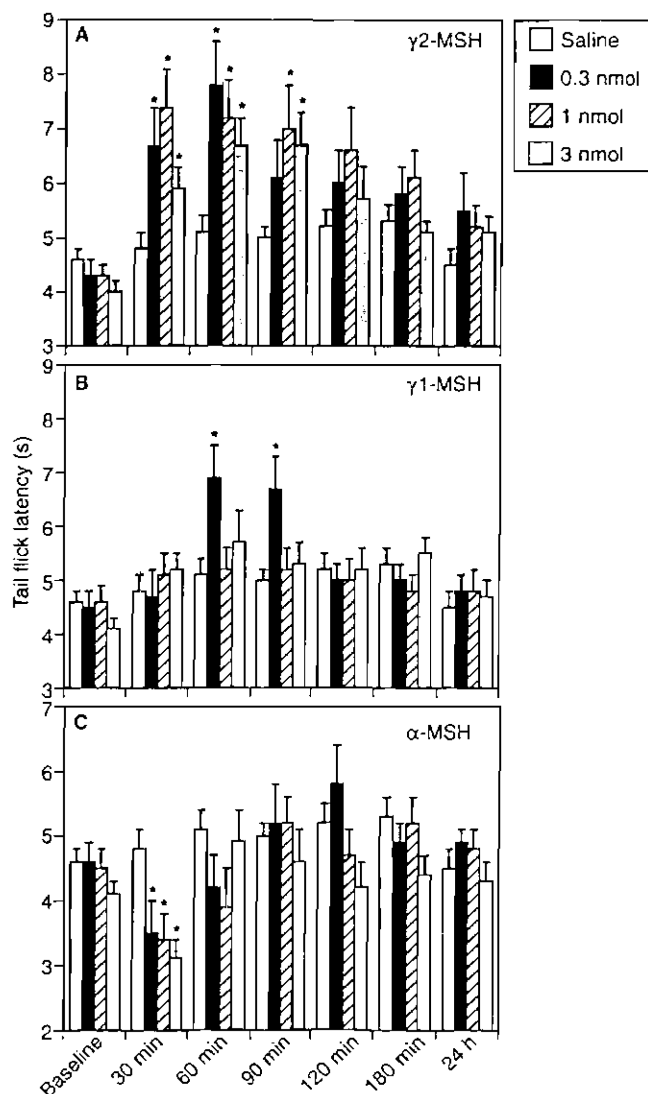

Fig. 1 A–C Effects of γ_2-, γ_1- and α-MSH peptides on tail-flick latencies in BALB/c mice. Peptides were administered intracisternally at doses 0.3, 1 or 3 nmol and the tail-flick latencies were recorded for up to 24 h. Saline indicates controls injected with the same volume (10 μl) of solvent used for peptides (saline). Baseline represent tail flick responses recorded 30 min prior to administration of saline or peptides. Each bar represents the mean ± S.E.M from measurements of 7–9 different animals. * indicates $P < 0.05$ vs saline.

of Animal Experimentation at the Latvian Research Council.

RESULTS

Comparison of the effects of γ_2-MSHs, γ_1-MSH and α-MSH on tail-flick latencies

Results for the effect of intracisternal injections of 0.3, 1 and 3 nmol of, respectively, γ_2-MSH, γ_1-MSH and α-MSH on tail-flick latencies are shown in Fig. 1 All three doses of γ_2-MSH significantly increased the tail-flick latencies during the 30–90 min following the injections; the peak response being seen at 60 min and maximally amounting to an approximately 70% increase compared to the baseline level (Fig. 1A). By contrast, only the 0.3 nmol dose of γ_1-MSH gave a significant increase of the tail flick latencies at 60–90 min following its intracisternal injection, while at other time points, or at the 1 or 3 nmol doses no significant effects were seen (Fig. 1B). α-MSH gave a completely different picture as it caused hyperalgesia. Thus, 30 min after the injection of α-MSH all three tested doses reduced significantly the tail flick latencies, the maximal decrease being about 35% from the baseline level (Fig. 1C).

Effect of HS014 on tail-flick latencies

The possible effect of the MC_4/MC_3 blocker HS014 on tail-flick latencies was assessed after intracisternal injections of 0.3, 1 or 3 nmol of the compound (Fig. 2A). As seen the HS014 induced no or only very minor effects. Only at 60 min and 24 h after its injection minor significant effects were seen at 1 and 3 nmol, respectively. These minor effects may presumably be regarded as spurious responses. Thus, HS014 appears to be essentially devoid of effects on tail-flick latency.

Effects of HS014 and γ_1-MSH pre-treatments on the effect of γ2-MSH on tail-flick responses

Animals were pre-treated by intracisternal administration of either 1 nmol of HS014 or 1 nmol γ_1-MSH 30 min prior to the administration of 1 nmol of γ2-MSH, and the tail-flick latencies were assessed (Fig. 2B). As can be seen from the figure the HS014 per-se did not affect tail-flick latencies, while γ1-MSH caused a minor significant increase 30 min after its injection. The γ_2-MSH, on the other hand, caused the expected increase in tail-flick latencies; its effect being significant 30–90 min following its injection. However, neither the HS014 nor the γ_1-MSH pre-treatment affected the response induced by γ2-MSH (Fig. 2B).

Effect of haloperidol and γ2-MSH on tail-flick responses

Animals were pre-treated by i.p. administration of 1 mg/kg mg of haloperidol 30 min prior to the intracisternal administration of 1 nmol of γ2-MSH, and the effects on tail-flick latencies were assessed (Fig. 3A). Controls were injected with saline via the appropriate route. The haloperidol pre-treatment, per-se, induced a clear and significant increase in the tail-flick latency already at the start of the assessment period (i.e. 30 min after haloperidol administration, corresponding to the zero time point recorded just before the saline/γ_2-MSH injection) (Fig. 3A). The tail-flick latencies then remained significantly increased up until 90 min,

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although at varying levels (Low et al., 1994). The localisation of the MC_5 receptor in the central nervous system has hitherto not been mapped with histochemical techniques, although Northern blot and RT-PCR analysis indicate its presence in the brain (Chhajlani et al., 1993; Gantz et al., 1994; Fathi et al., 1995).

The melanocortic peptides induce a variety of central effects, which include alterations in motor and sexual behaviour, analgesia, improvement of memory, antipyretic effects, and regulation of feeding behaviour (Wikberg, 1999). Some of these effects have been possible to tie to distinct sub-types of the MC-receptors. Thus, the MC_4 receptor appears to have a prominent role in the control of feeding homeostasis (Wikberg, 1999). The excessive grooming behaviour induced upon central administration by α-MSH is also mediated by MC-receptors as it is blocked by MC-receptor selective antagonists (Klusa et al., 1998), but the exact receptor(s) involved is still not entirely clear. In a recent study we found that γ-MSH's showed quite distinct differences on motor behaviours induced upon its injection into the ventral tegmental area of the rat, when compared with α-MSH (Klusa et al., 1999). Thus, γ_2-MSH produced slight catalepsy and hypoactivation while α-MSH (and γ_1-MSH) induced excessive grooming. Moreover, γ_2-MSH was capable to antagonise the γ_1-MSH induction of grooming. These, results prompted us to investigate further the behavioural pharmacology of γ-MSH peptides. In the course of these studies we found that the γ_2-MSH peptide is capable of inducing a powerful analgesic effect on its central administration to the mice. In the present study we set forth to characterise these effects. The most prominent result emerging from our study is that, while the analgesic effect of γ_2-MSH remains completely untouched by administration of the MC-receptor blocker HS014 or the opioid-receptor blocker naloxone, it is completely attenuated by the $GABA_A$-receptor antagonist bicuculline, as well as augmented by the $GABA_A$ agonist muscimol.

MATERIALS AND METHODS

Animals

Male BALB/c mice were bred at the Breeding House of the A. Kirhenstein Institute of Microbiology and Virusology, University of Latvia, Riga, Latvia, and used at weights 20 $\pm$ 2 g. The animals were housed in groups of five, using a light–dark cycle of 12 h (lights off 19.00–7.00). At the time of experiments the animals were arranged in groups consisting of 6–10 mice.

Drugs

α-MSH (N-Acetyl-Ser-Tyr-Ser-Met-Glu-His-Phe-Arg-Trp-Gly-Lys-Pro-Val-NH_2), γ_1-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-NH_2), and γ_2-MSH (H_2N-Tyr-Val-Met-Gly-His-Phe-Arg-Trp-Asp-Arg-Phe-Gly-OH) peptides were from BACHEM. The HS014 peptide was synthesised as described (Schiöth et al., 1998). Peptides were stored frozen in aliquots until used. Naloxone hydrochloride (substance) was from Sigma Chemicals Co, USA; haloperidol (0.5% solution) from Gedeon Richter, Hungary; (+)bicuculline (substance) from Sigma Chemicals Co, USA; muscimol (substance) from Fluka AG, Switzerland; diazepam (5% solution) from Gedeon Richter, Hungary.

Drug administrations

Peptides were dissolved in saline and injected intracisternally (ic) into the cisterna magna in conscious mice via a J-shape needle connected to a Hamilton syringe, as described (Takagi et al., 1979). Intracisternal injection volumes never exceeded 10 µl. Drugs were dissolved in saline and ethanol in water (10% ethanol), and injected intraperitoneally.

Tail flick test

Tail flick tests were carried out in accordance to the method described elsewhere (Dewey, 1981), with minor modifications. In brief, the tail of the mouse was placed on the photoelement window of a MODEL DS20 SOCREL tail flick apparatus (Ugo Basile, Italy) and an infrared beam was focused on the tail area, 2 cm from its basis. The latency for the mouse to react to the pain stimuli was recorded. To avoid tissue damages, maximal exposure to the pain stimuli was restricted to 15 s.

Catalepsy test

Test of catalepsy was carried out essentially as described (Kobayashi et al., 1997). In brief, catalepsy was evaluated by placing both forepaws of the mouse over a horizontal bar (diameter 0.2 cm), elevated 15 cm from floor, and the time (in s) during which the animal maintained this position was recorded.

Statistics

Statistical analysis was done using independent samples *t*-test or one-way ANOVA and Bonferroni's multiple comparison test as a post-hoc. Results are expressed as the mean $\pm$ S.E.M.

Animal ethics

Experimental procedures were carried out in accordance with guidelines of the European Community, local laws and polices and were approved by the Ethics Committee

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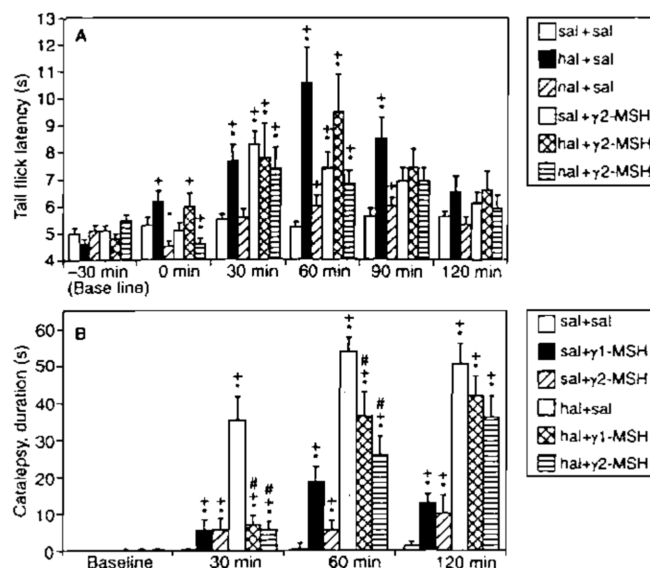

Fig. 3 A. Effect of haloperidol (hal), naloxone (nal) and γ_2-MSH on tail-flick latencies in BALB/c mice. Haloperidol (1 mg/kg) or naloxone (2 mg/kg) were given i.p. at 30 min prior (− 30 min) to the injection of γ_2-MSH (1 nmol). Sal indicates controls injections with the same volume of solvent as used for respective agent. Baseline represents tail-flick responses recorded just immediately prior to the − 30 min time point. $n = 8$. * indicates $P < 0.05$ vs saline. + indicates $P < 0.05$ vs baseline. **B.** Assessment of cataleptic effects of haloperidol (hal), γ_1- and γ_2-MSH in BALB/c mice. Haloperidol (0.5 mg/kg) was given 30 min prior to the intracisternal injection of 0.3 nmol of respective peptide. Sal indicates controls injections with the same volume of solvent as used for respective agent. Baseline represents the catalepsy recorded just immediately prior to administration of haloperidol. $n = 10$. * indicates $P < 0.05$ vs saline. + indicates $P < 0.05$ vs baseline. # indicates $P < 0.05$ vs hal + sal.

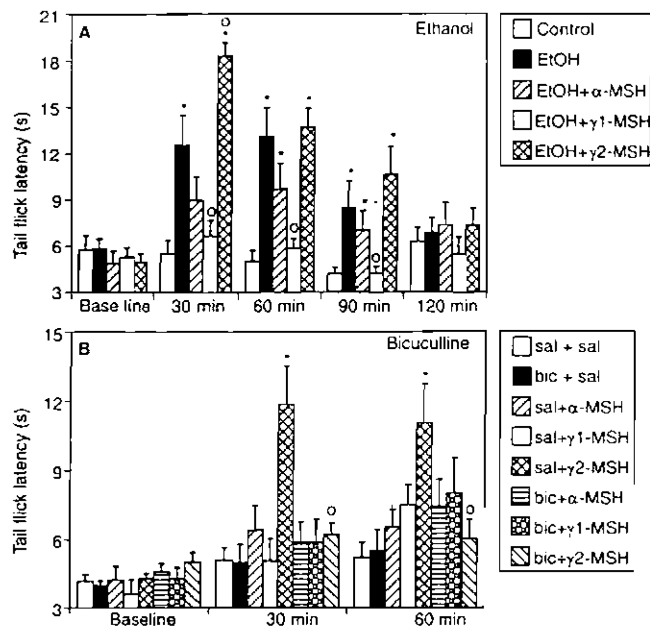

Fig. 4 A. Effect of ethanol (EtOH) and MSH-peptides on tail-flick latencies in BALB/c mice. Ethanol (4.0 g/kg) was given i.p. 10 min prior to intracisternal injections of α-, γ_1 or γ_2-MSH (1 nmol in 10 µl). The Control represents animals injected i.p. with the same volume of saline as used for ethanol. The animals of the plain EtOH group were given intracisternal saline (10 µl). $n = 9$–10. * indicates $P < 0.05$ vs saline. o indicates $P < 0.05$ vs EtOH ctrl. **B.** Effect of bicucculine (bic) and MSH-peptides on tail-flick latencies in BALB/c mice. Bicuculline (0.5 g/kg) was given i.p. 5 min prior to intracisternal injections of α-, γ_1- or γ_2-MSH (each 1 nmol). Sal indicates control injections with the same volume of solvent as used for respective agent. $n = 8$. * indicates $P < 0.05$ vs saline. o indicates $P < 0.05$ vs sal + γ_2-MSH. Baselines represent tail-flick responses recorded just immediately prior to the i.p. injections.

Assessment of cataleptic activities

Fig. 3B shows the effect of i.p. administration of 0.5 mg/kg haloperidol on the cataleptic duration in the mice, as well as the influences of 0.3 nmol of, respectively, γ_1- and γ_2-MSH. Haloperidol was administered 30 min prior to the intracisternal injection of peptides or saline vehicle and the presence of catalepsy was then assessed 30, 60 and 120 min after the intracisternal injections. As seen haloperidol caused a distinct catalepsy throughout the experiment, while both γ_1- and γ_2-MSH caused weak cataleptic activities (Fig. 3B). Interestingly, at the 30–60 min both γ_1- and γ_2-MSH significantly attenuated the catalepsy induced by haloperidol (Fig. 3B). As seen from the Fig. 3B this effect was very marked at the 30 min time point, as the γ_1- or γ_2-MSH peptides attenuated the haloperidol catalepsy down to the same level as that seen when the γ_1- or γ_2-MSH were administered alone.

Effect of combined treatments of ethanol and MSH-peptides on tail-flick responses

Animals were given 4.0 g/kg of ethanol i.p. 10 min prior to intracisternal injections of 1 nmol of, respectively, α-, γ_1 or γ_2-MSH, and the tail-flick latencies were assessed 30–120 min following the intracisternal injections. Results are shown in Fig. 4A. The ethanol pre-treatment per-se caused significant increase in tail-flick latencies during the 30–90 min observation periods. α-MSH did not significantly alter the ethanol response, while γ_1-MSH significantly attenuated the ethanol response at the 30–90 min periods. By contrast, γ_2-MSH significantly potentiated the ethanol-induced increase in tail-flick latencies, at the 30 min time point (Fig. 4A).

Effect of combined treatments of bicuculline and MSH-peptides on tail-flick responses

In the experiment shown in Fig. 4B the effects of the GABA receptor blocker bicuculline were assessed. Animals were given 0.5 mg/kg of bicuculline i.p., 5 min prior to intracisternal injections of 1 nmol of, respectively, α-, γ_1- or γ_2-MSH and the tail-flick latencies were assessed during a 30–60 min period. Bicuculline, per-se, did not induce any significant effects on the tail-flick latencies. γ_2-MSH alone induced a clear and significant increase in the tail-flick latencies at both the 30 and 60 min periods, as expected.

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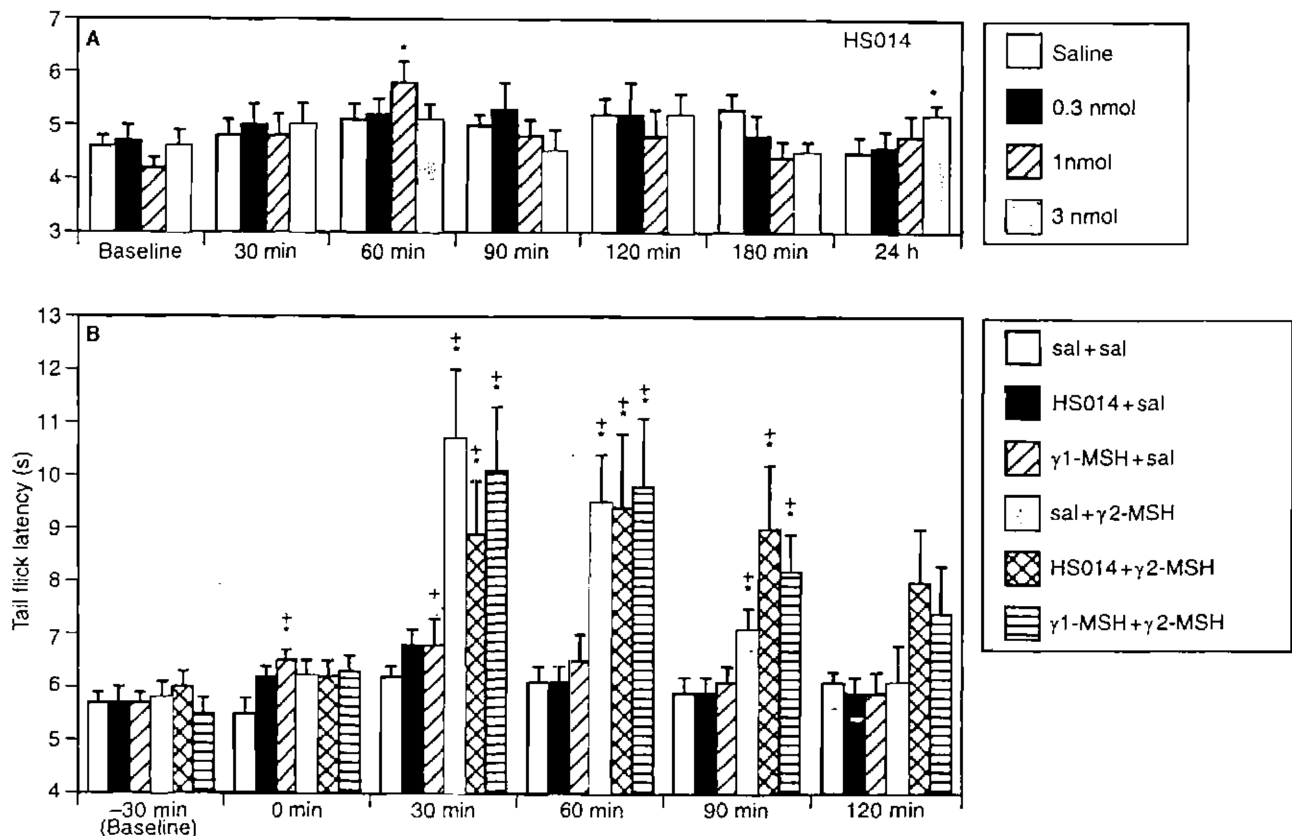

Fig. 2 Effects of HS014 and combinations of HS014 and γ_1- and γ_2-MSH peptides on tail-flick latencies in BALB/c mice. **A.** Effect of HS014 administered intracisternally at doses of 0.3, 1 and 3 nmol on tail-flick responses for up to 24 h after administration. Saline indicates controls injected with the same volume of solvent used for peptides (i.e. 10 μl of saline). Baseline represent tail-flick responses recorded 30 min prior to administration of HS014 or saline. $n = 7$–9 * indicates $P < 0.05$ vs saline. **B.** Effect of pre-treatment with HS014 or γ_2-MSH on the effect of γ_2-MSH on tail-flick latencies. HS014 or γ_1-MSH were given intracisternally, each at a dose of 1 nmol (in 5 μl), 30 min prior (−30 min) to the intracisternal administration of 1 nmol of γ_2-MSH (in 5 μl at '0 min') and the tail-flick latencies then recorded for up to 120 min following the injection of γ_2-MSH. Sal indicates controls injected with the same volume of solvent used for peptides (saline). Baseline and '0 min' represent tail flick responses recorded just immediately prior to the −30 min and zero time points recorded just immediately prior to injection of peptides. $n = 8$. * indicates $P < 0.05$ vs saline. + indicates $P < 0.05$ vs baseline.

with the peak effect being seen at the 60 min and amounting approximate to a doubling over the basal (Fig. 3A). In animals pre-treated with i.p. saline, the intracisternal injection of γ_2-MSH induced the expected increase in tail-flick latencies, with the peak effect being reached at 30–60 min. In the haloperidol pre-treated animals γ_2-MSH elicited essentially the same increase in tail-flick latencies as in the haloperidol controls (i.e. animals given i.p. saline). There were no significant differences between the haloperidol pre-treated animals, γ_2-MSH treated animals and the animals receiving combined treatment with haloperidol and γ_2-MSH (Fig. 3A).

In another series of experiments the animals were instead pre-treated with 0.5 mg/kg of i.p haloperidol and then injected intracisternally with 0.3 nmol of γ_2-MSH, using a similar protocol as for the tests of the previous paragraph. In these tests essentially the same pattern as above was seen. Thus, haloperidol, γ_2-MSH and haloperidol $+\gamma_2$-MSH significantly increased the tail-flick latencies to the same degree during 30–60 min following the intracisternal injections (data not shown).

Effect of naloxone pre-treatment on the effect of γ_2-MSH on tail-flick responses

Animals were pre-treated by i.p. administration of 2 mg/kg of naloxone 30 min prior to the intracisternal administration of 1 nmol of γ_2-MSH, and the effects on tail-flick latencies were assessed (Fig. 3A). (The naloxone tests were done concomitantly with the tests of haloperidol, allowing these two groups to share the same controls). As seen from the figure naloxone per-se caused no, or only marginal, effects on the tail-flick latencies. Moreover, the naloxone pretreatment did not affect the increase in tail-flick latency induced by the intracisternal injection of γ_2-MSH (Fig. 3A).

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whereas γ_2-MSH induced a distinct analgesic effect, γ_1-MSH was almost devoid of effect. It is well known that γ_1-MSH show preference for the MC_3 receptor over the MC_4 receptor in binding tests. (γ_1-MSH binds with high affinity to MC_1 receptors as well). The affinity profile of γ_2-MSH for MC receptors is quite similar to that of γ_1-MSH, although γ_2-MSH shows all over about 3-fold lower affinities for MC receptors compared with γ_1-MSH (Wikberg, 1999). In view of the affinity profiles of γ_1-MSH and γ_2-MSH, the unique ability of γ_2-MSH to induce analgesia is thus not congruent with the idea that the effect is mediated via melanocortic receptors. Moreover, the hypothesis that γ_1-MSH binds to an identical receptor as γ_2-MSH, but is devoid of agonistic effect may be ruled out, as we demonstrated in the present study that γ_1-MSH did not antagonise the γ_2-MSH mediated analgesia. (See also Oosterom et al., 1998).

In our earlier study (Klusa et al., 1999) we observed that the γ_2-MSH induced a cataleptic effect in the rat. It seemed essential to elucidate whether or not γ-MSH peptides were also cataleptic in mice. Thus, in the present study we found that both γ_1- and γ_2-MSH induced a moderate catalepsy. In comparison to the cataleptic activity induced by haloperidol these effects of γ_1- and γ_2-MSH appeared weak. Moreover, the cataleptic activity induced by γ_1-MSH appeared equally strong (or perhaps even more pronounced) compared to that induced by γ_2-MSH. Since γ_1-MSH showed only minor analgesic effects it seems very unlikely that the analgesia induced by γ_2-MSH is directly due to the cataleptogenic effect. It was also an interesting finding that γ_1- and γ_2-MSH were capable of attenuating the catelepsy induced by haloperidol. The mechanism underlying these effects of γ-MSH peptides are presently not well understood.

However, the data of the present study lend strong support to the notion that $GABA_A$-receptors are involved in the γ_2-MSH mediated analgesia. Strongest support for this idea comes from the observation that the $GABA_A$-receptor antagonist bicuculline can completely abolish γ_2-MSH induced analgesia. Moreover, the effect of γ_2-MSH appeared to be additive to the analgesia caused by pretreatment of the $GABA_A$-receptor agonist muscimol. Moreover the γ_2-MSH also appeared additive to the analgesic effect induced by ethanol, the latter which is a known potentiator of GABA activation of $GABA_A$-receptors (Davies and Alkana, 1998). However, the analgesic effect of γ_2-MSH appeared not to be additive to that induced by diazepam, a ligand capable of binding to the $GABA_A$ receptor at a site distinct from that which binds GABA. Another interesting observation was that α- and γ_1-MSH reduced the analgesia induced by diazepam. There are some reports suggesting interactions between benzodiazepines and the α-MSH. Thus, diazepam was reported to significantly decrease α-MSH-induced grooming (Cremer et al., 1995), while another benzodiazepine, clonazepam, was reported to inhibit the release of α-MSH from the neurointermediate lobe in vitro (Tonon et al., 1989). Moreover, chlordiazepoxide was reported to inhibit the basal release of α-MSH from hypothalamic slices obtained from rats (Mabley et al., 1991).

The finding of the present study that α-MSH injected intracisternally induced a short-term hyperalgesia is also notable. The mechanism for this action of α-MSH is presently unknown; perhaps it might be mediated by some of the known subtypes of MC receptors. Anyhow, this observation of the present study is in line with a previous study where hyperalgesia was seen after i.c.v. administration of α-MSH in rats (Sandman and Kastin, 1981). However, it shall also be noted that some contradiction exist as to the effect of α-MSH in rats (Sandman and Kastin, 1981). Contradiction also exist as to the effect of α-MSH on pain perception, since α-MSH was reported to be analgesic in mice, as could be assessed by use of the hot-plate test (Ohkubo et al., 1985).

To summarise, the most pertinent finding of the present study is that the γ_2-MSH induces a central analgesia via a mechanism that does appear neither to involve melanocortin receptors nor opioid receptors. Instead our data indicate that the analgesia is mediated via a mechanism involving the stimulation of $GABA_A$ receptors. However, whether the γ_2-MSH effect is mediated directly on $GABA_A$ receptors or indirectly via stimulation of GABAergic pathways remains to be studied. The present study thus reinforces the complexity for the pharmacology of melanocortic peptides, findings which are in accord with our earlier study (Klusa et al., 1999) showing that distinct differences exist in the behavioural pharmacology of the α-, γ_1- and γ_2-MSH peptides.

ACKNOWLEDGEMENTS

A preliminary account of part of the present studies was presented at the 29th Annual Meeting, Society for Neuroscience, Miami, USA, 1999. Supported by the Howard Hughes Medical Institute (HHMI 75195–548501), the Swedish MRC (04X–05957) and Melacure Therapeutics AB, Uppsala, Sweden.

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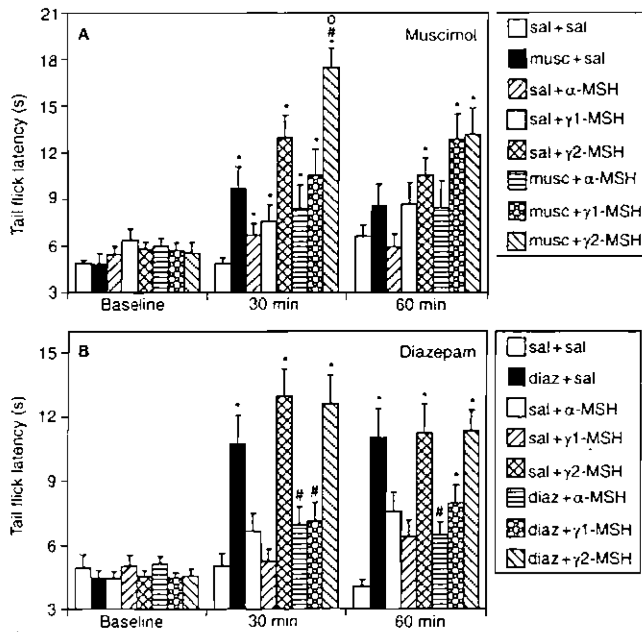

Fig. 5 A. Effect of muscimol (musc) and MSH-peptides on tail-flick latencies in BALB/c mice. Musicmol (1 mg/kg) was given i.p. 5 min prior to the intracisternal injections of α-, γ_1- or γ_2-MSH (1 nmol each). Sal indicates control injections with the same volume of solvent as used for respective agent. $n = 8$. * indicates $P < 0.05$ vs saline. # indicates $P < 0.05$ vs musc + sal. o represents $P < 0.05$ vs sal + γ_2-MSH. **B.** Effect of diazepam (diaz) and MSH-peptides on tail-flick latencies in BALB/c mice. Diazepam (10 mg/kg) was given i.p. 5 min prior to the intracisternal injections of α-, γ_1- or γ_2-MSH (1 nmol each). Sal indicates control injections with the same volume of solvent as used for respective agent. $n = 7$–16. * indicates $P < 0.05$ vs saline. # indicates $P < 0.05$ vs diaz + sal. Baselines represent tail-flick responses recorded just immediately prior to the i.p. injections.

However, in animals pre-treated with bicuculline the increase in tail-flick latency induced by γ_2-MSH was completely attenuated, at both 30 and 60 min. These effects were significant at both the 30 and 60 min time points. By contrast, neither α- nor γ_1-MSH, gave any significant effects on the tail-flick response. Moreover, no effects were seen by α- or γ_1-MSH in animals pre-treated with bicuculline.

Effect of combined treatments of muscimol and MSH-peptides on tail-flick responses

The GABA receptor agonist muscimol was given i.p at a dose of 1 mg/kg, 5 min prior to intracisternal injections of α-, γ_1- or γ_2-MSH (1 nmol each), and the tail-flick latencies were assessed during the following 30–60 min period (Fig. 5A). Muscimol, per-se, induced a significant increase in tail-flick latency at 30 min, but not at 60 min. γ_2-MSH, per-se, also significantly increased tail-flick latency time at both time periods. At 30 min with muscimol the tail-flick latency induced by γ_2-MSH was significant higher than in the absence of muscimol. The levels for tail-flick latencies seen after α- and γ_1-MSH were similar as reported for other experiments above, although at the 30 min time point there appeared to be a minor (probably spurious) increase in tail-flick latency induced by both α- and γ_1-MSH. Neither α- nor γ_1-MSH gave any significant alterations of the response induced by muscimol.

Effect of combined treatments of diazepam and MSH-peptides on tail-flick responses

Diazepam (10 mg/kg) was given i.p., 5 min prior to intracisternal injections of α-, γ_1- or γ_2-MSH (1 nmol), and the tail-flick latencies were assessed during the following 30–60 min (Fig. 5B). Diazepam, per-se, induced a significant increase in tail-flick latencies at both 30 and 60 min. γ_2-MSH, per-se, also significantly increased the tail-flick latencies, as expected. However, the combined treatment of diazepam and γ_2-MSH did not alter significantly the tail-flick latencies, compared to any of these respective treatments given alone (Fig. 5B). Neither α- nor γ_1-MSH when given alone caused any significant effects on tail-flick latencies. Interestingly, however, both the α- and γ_1-MSH treatments significantly attenuated the increase in tail-flick latencies caused by diazapam. For α-MSH these effects were significant at both the 30 and 60 min time periods, while for γ_1-MSH it was significant only at the 30 min time point.

DISCUSSION

The present study reinforce the complex and multifaceted central nervous system pharmacology of melanocortic peptides. Previous studies have indicated that some of the central effects induced by MSH peptides, and analogues thereof, are clearly linked with activation of melanocortin receptors. These effects include the intense grooming activity induced upon icv administration of α-MSH, which may be blocked by prior administration of the MC_4/MC_3-blocker HS014 (Klusa et al., 1998). Another is the inhibition of ingestive behaviour by MSH peptides, where a large body of accumulating evidence suggest the effect is mediated by activation MC_4 receptors located to hypothalamic areas. For example icv administered α-MSH and β-MSH (but not γ_1-MSH) inhibited spontaneous food intake in food deprived rats (Kask et al., 2000) while HS014 increased food intake (Kask et al., 1998). (See Wikberg, 1999, for a full discussion on the role of MC_4 receptors for control of feeding behaviour).

However, the data of the present study do not lend support to the idea that the analgesic effect of γ_2-MSH is mediated via the activation of melanocortic receptors. One reason being that the analgesic effect of γ_2-MSH was not affected by HS014. HS014 is a blocker of both MC_4 and MC_3 receptors (albeit with a slight selectivity for the MC_4 receptors [Schiöth et al., 1999]). Another reason is that

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VI

THE FUNDAMENTAL ROLE OF MELANOCORTINS IN BRAIN PROCESSES

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Abstract
The discoveries of the latest ten years have shed new light in understanding the roles of melanocortins and their receptors in brain functions and in the development of different pathologies. Since 1992 when genes encoded melanocortin receptor five subtypes were identified, cloned and characterized, the molecular mechanisms underlying different effects such as skin darkening, behaviour, food intake, anti-inflammatory action, analgesia have been clarified. The contribution of melanocortins and their receptors in the physiological control of organism homeostasis has become as the background for the search of agonists and antagonists of separate receptor subtypes, that can be targeted to the melanocortin receptors and used as therapeutic drugs for the treatment of psychoneuroendocrine and immune system diseases.

1. What are Melanocortin Peptides?

1.1 BIOSYNTHESIS AND STRUCTURES

The melanocortins (or melanocyte stimulating hormones, or MSH peptides) are derived from the precursor, a 31-36 kDa glycosylated protein called pro-opio-melanocortin or POMC (Fig.1), during its biodegradation or proteolytic processing [1]. The full amino acid sequence was discovered only at the end of the 1970th by cloning of its cDNA [2]. POMC generates a large array of biologically active peptides, including the adrenocorticotropin (ACTH), MSH peptides and β-endorphin, β-lipotropin, γ-lipoptropin. Proteolytic cleavage occurs mostly at sites where two polar amino acids form a peptide bound, e.g., Arg-Arg, Lys-Lys, Arg-Lys, Lys-Arg. At present, the following peptides are attributed to the melanocortin family: α-MSH, ACTH, β-MSH, γ1-MSH, γ2-MSH, γ3-MSH. These peptides differ from each other by their amino acid sequences, however they share a common pharmacophoric unit (underlined), a tetrapeptide His-Phe-Arg-Trp (Table 1). Interestingly, that α-MSH molecule completely coincides with the first 13 amino acid sequence of the ACTH, however α-MSH has acetyl group in the N-terminus and amide group in the C-terminus.

M.P. Schneider (ed.), Chemical Probes in Biology, 255–267.
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TABLE 1. Primary structures of peptides of melanocortin family

Peptide	Amino acid sequences
ACTH:	H_2N-SYSMEHFRWGKPVGKKRRPVKVYPNGAEDESAEAFPLEF-OH
α-MSH:	N-Acetyl-SYSMEHFRWGKPV-NH_2
β-MSH:	H_2N-AEKKLEGPYRMEHFRWGSPPKD-OH
γ1-MSH:	H_2N-YVMGHFRWDRF-NH_2
γ2-MSH:	H_2N-YVMGHFRWDRFG-OH
γ3-MSH:	H_2N-YVMGHFRWDRFGRRNGSSSSGVGGAAQ-OH

The POMC derived peptides have diffuse distribution in the central nervous system, as well as in peripheral organs [for review see 3]. α-MSH is mostly synthesized in the intermediate lobe, ACTH in the anterior lobe of hypophysis, however POMC is also synthesized and processed in other brain areas, especially in the hypothalamus.

1.2. HISTORY OF THE DISCOVERY OF MELANOCORTINS

In the beginning of the 20th century, melanocortins were originally recognized at the intermediate lobe of hypophysis, and the influence of pituitary extracts on melanocyte dispersion in frog skin and, hence its pigmentation (darkening) was observed [4]. 43 years later, the α-MSH molecule was identified [5] and it was obtained that this is the right peptide which is responsible for frog skin darkening. Only in the 1990th a role of α-MSH in melanoma formation in human beings was detected [6-8]. Therefore phylogenetically, α-MSH is an ancient molecule that has remained essentially unchanged during late vertebrate evolution, and this molecule is remarkably conserved across different species. In the 1960th a lot of studies was devoted to clarification of the functional role α-MSH and ACTH. For instance, the scientists' group from the Netherlands, headed by Professor De Wied have obtained an influence of ACTH, its fragments and analogues on rodents' behavior [9]. They have found that these peptide can induce excessive grooming when administered intracerebroventricularly, and may improve short-term memory. One of the most remarkable discovery made by E.W.Sutherland (Nobel Prize winner, 1971), was that the ACTH-induced steroidogenesis is mediated *via* intracellular mechanisms that involve cAMP, a second messenger molecule formation. However knowledge of melanocortins and their functional role has increased tremendously over the last 10 years when five melanocortin receptor subtypes were identified, cloned and characterized.

2.Melanocortin Receptors (MCRs)

2.1. RECEPTOR SUBTYPES

2.1.1. MC1R

In 1992 the genes encoding G protein coupled MSH receptor, termed MC1R, were cloned independently by two groups (Prof. Jarl Wikberg, Uppsala University, Sweden, and Prof. Rogers Cone, Oregon, USA) [10, 11].The localization of this receptor subtype firstly was found in melanocytes of the skin and in solid melanoma tumor cells, lately in many other cell types including those of different brain areas [12, 13], fibroblasts [14], keratinocytes [15], macrophages and monocytes [16, 17], neutrophiles [18], endothelial and glial cells [19]. Now the mostly clarified function of MC1R is its binding with α-MSH that leads to skin darkening and melanoma tumorogenesis. The new area that is being studied intensively over the last 5 years is anti-inflammatory action where probably MC1R and α-MSH play a crucial role (see below "The Newest Findings...").

2.1.2. MC2R

MC2R which was discovered at the same time as MC1R, is proved to be the ACTH receptor of the adrenals, which controls steroidogenesis processes [20]. This receptor subtype considerably differs from other melanocortin receptor subtypes, since it binds only with ACTH but not with any other melanocortin molecules [21]. Recently, the expression of MC2R was identified in the skin [22] that indicates eventual role of MC2R and ACTH in the physiology of skin.

2.1.3. MC3R

In 1993 the genes encoding MC3R were cloned and their localization was identified in the brain, placenta and gut [23]. Lately its expression in the heart was shown [13]. There are two very important matters which seems to be put forward in studies of MC3R functional role: 1) γ-MSH peptides have high affinity to MC3R, particularly γ1-MSH shows 40-fold selective affinity for rat MC3R vs MC4R [24]; 2) MC3R can be abundantly expressed in the brain structures that belong to the mesolimbic system, i.e., the *ventral tegmental area* [25] and the *nucleus accumbens* [3]. The functional role of MC3R and also of γ-melanocortins is comparatively less studied. However, recent data (see below "The Newest Findings...") show a considerable influence of γ-melanocortins on the functions mediated via the mesolimbic system, particularly those attributed to the psychoactivation states.

2.1.4. MC4R

MC4R was found to be expressed in all brain regions of mammals [26]. The high levels of MC4R is detected in the *nucleus accumbens* [26, 27]. Unlike MC3R, MC4R was not essentially found in the periphery. However, recent findings showed evidence that MC4R may be present in peripheral tissues [28]. The main difference between the MC4R and the other receptors is its particularly low affinity for the γ-MSH peptides, and slightly higher affinity for β-MSH than α-MSH and ACTH [29]. Recently relationship between feeding control and MC4R have been studied (see below "The Newest Findings...").

2.1.5. MC5R

The physiological role of MC5R is still obscure. This subtype is widely expressed in many peripheral tissues, particularly in the exocrine glands (e.g., adrenal glands, prostate, pancreas). There are evidence suggesting that MC5R plays a role in regulation of functions of exocrine glands. For instance, MC5R in mice is involved in production of sebum from sebaceous glands resulted in water repulsion of their furs and thermoregulation [30].

2.2. MCR STRUCTURE AND SIGNAL TRANDUCTION

The MCRs belong to the class of G protein coupled 7-transmembrane (TM) region or heptahelix receptors. All the receptors have several glycosylation sites in their N-terminal domain, and conserved cysteins in the C-terminal part, which may serve as sites for fatty acid acylation anchoring the C-terminus to the plasma membrane. Modelling of the peptide-receptor binding by use of cyclic heptapeptide [Cys^4, Cys^{10}]α-MSH as a model compound, shows that receptor binding site forms a binding pocket by involving most TM domains with exception of TM4 and TM5 [31]. Obviously all domains are necessary to arrange optimal protein conformation to provide high binding activity.

A full lengths of the primary structures of MCR family shows that receptor subtypes share about 40-60% homology of their sequences (Table 2) [32].

TABLE 2. Amino acid identity (in %) between the five cloned human MC receptor subtypes [32]

	MC1R	MC2R	MC3R	MC4R	MC5R
MC1R	100	38	45	47	44
MC2R		100	42	46	44
MC3R			100	42	57
MC4R				100	60
MC5R					100

The signal transduction mechanisms provided *via* MCRs are mostly regarded to stimulatory pathways resulting in second messenger cAMP formation [33], however there is some evidence indicating the phosphoinositol pathway can also be involved in the signaling of the MC3R.

2.3. BINDING AFFINITIES

The evaluation of binding affinities (Table 3) showed that ACTH binds only MC2R, whereas MC3R shows a relative preference for γ-MSH peptides. The other receptor subtypes bind natural MSH peptides with an order of potency which can be seen in Table 4 [34,35].

TABLE 3. Binding affinities for melanocortins, obtained in MCR transfected COS cells [34, 35]

Ligand	MC1	MC3	MC4	MC5
α-MSH	0.12 ± 0.023	20.7 ± 3.7	641 ± 104	8240 ± 1670
β-MSH	1.17 ± 0.27	13.4 ± 6.4	446 ± 96.5	14400 ± 1670
γ1-MSH	2.68 ± 0.35	7.06 ± 2.90	29001 ± 1791	42600 ± 6600
γ2-MSH	11.2 ± 5.4	17.7 ± 1.9	>100000	>100000

TABLE 4. Comparative pharmacology of melanocortin receptors [34, 35]

Receptor (MCRs)	Potency of POMC peptides
Human MC1R	α-MSH>ACTH>β-MSH>>γ-MSH
Human MC2R	ACTH
Human MC3R	γ-MSH= α-MSH>ACTH
Human MC4R	α-MSH >ACTH= β-MSH> γ-MSH
Human MC5R	α-MSH> β-MSH>>γ-MSH

3. The Newest Findings Of The Functional Roles of Melanocortins And Their Receptors

Over the recent 5-10 years informations about functional roles of melanocortins and their receptors have increased considerably, however the newest findings show complexity of melanocortinergic processes and their link to the non-melanocortinergic pathways and molecules.

3.1. MCR1 AND α-MSH

MCR1 was recently demonstrated to have indirect roles for immune responses. α-MSH may exert anti-inflammatory effects by reducing the production of pro-inflammatory cytokines [18], and inflammatory mediator NO in macrophages [15], as well as by suppressing of the expression of leukocyte adhesion molecules in vascular epithelium [36]. That is in good line with previously described findings [37] demonstrated that POMC-derived peptides may be produced in immunocompetent cells when they receive non-cognitive (bacterial, viral) stimuli. The peptides then can be released to initiate glucocorticoid synthesis along the immuno-adrenal axis, hence induce immunosuppression and anti-inflammatory action. Recent data [38] show that not only entire molecule of α-MSH but also its shorter fragments (α-MSH 1-10 and α-MSH 11-13) may considerably suppress NF-κB production in macrophage cell line expressing MCR1, when they are

exposed to inflammatory agents such as LPS and interferon-γ. These and other data give enough evidence to suggest that MCR1 and α-MSH play a crucial role for providing anti-inflammatory effects by involving pathways from the activation of receptor, production of second messengers to the activation of transcription factors and induction of gene expression (Figure 1).

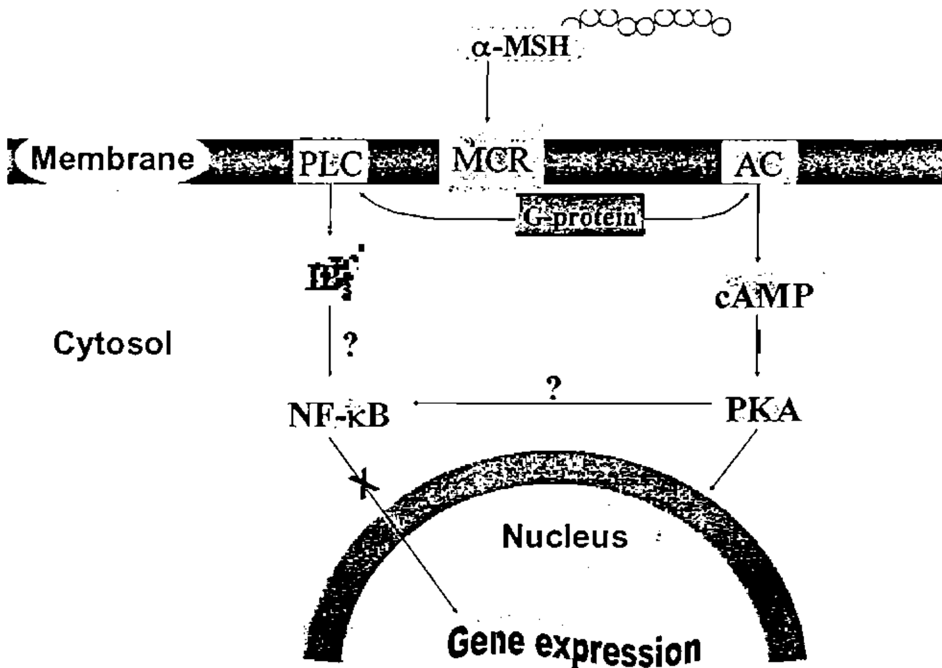

Figure 1. Model for the regulation of α-MSH-induced anti-inflammatory effect. α-MSH binds to MCR and possibly activates both signal transduction pathways (*via* cAMP and IP_3 production) resulted in inhibition of NF-κB translocation into the nucleus, caused by inflammatory cytokines (LSP+INF-γ).

3.2. MCR4 AND α-MSH

MC4R is ubiquitously expressed in the brain and for long time it was considered that this receptor subtype is essentially absent in periphery. However recently MCR4 is found in human adipose tissue, that initiates an interest for the MCR4 as important factor in control of body weight [39]. Now there is huge of data demonstrating that MCR4 plays an important role in feeding behaviour, and a lot of reviews are devoted to this subject [3, 29, 30, 32, 40]. So, already in the 1980th it was shown that α-MSH and ACTH (1-24) injected into the hypothalamus caused a marked inhibition of food intake [41]. In the 1990th intriguing data were added, for instance that MCR4 knockout mice developed morbid obesity [42]. Moreover, several MCR4 mutations in human population were found as essentially associated with obesity [40]. A link of melanocortinergic processes with others

involved in feeding control was confirmed when it was found that agouti peptide acted as MCR4 endogenous antagonist [43]. Afterwards intriguing observations confirmed idea that the selective MC4 antagonists induce overeating and severe obesity. In contrast, agonists of this receptor subtype exerts anorexive effects. That stimulates intensive drug design to obtain selective MCR4 agonists and antagonists. For instance, intracerebroventricular administration of novel highly selective MCR4 antagonists HS014 and HS024 were found to cause 2-4-fold increase in food intake in rats [29, 44]. MC4R is suggested also to be involved in opiate addiction [45]. α-MSH interaction with MC4R probably mediates also regulation of cardiovascular system [46]. α-MSH probably *via* MCR4 can stimulate nerve regeneration after nerve injury [46,47].

3.3. MCR3 AND γ-MSHs

The functional role of both MCR3 and γ-MSHs is less studies and less understandable. An attention can be paid to structures of γ1- and γ2-MSH peptides, which show surprising homology of their amino acid sequences (see Table 1) with exception of extra C-terminal glycine residue in γ2- MSH molecule that differs these peptides from each other. Besides, γ-MSHs have the highest binding activity to MCR3 which is abundantly expressed in the dopaminergic mesolimbic system, that in turn belongs to the reward system involved in drug dependence and motivational processes, manifestation of schizophrenic hyperactivation, and emotions. This system involves two very important brain structures playing an essential role in regulation of dopaminergic pathways: they are the *ventral tegmental area* (VTA) and the *nucleus accumbens* (NACC). The cytoarchitectony of the VTA is very complicated, since dopamine DA cells receive a lot of interneurons both the inhibitory (e.g.GABAergic) and the excitatory (e.g. glutamatergic) that may modulate dopamine (DA) release in the NACC [48]. If DA cells are stimulated the DA release in the NACC is increased and that coincides with hyperlocomotion and stereotypical behavioural responses (e.g. grooming) in laboratory animals. In schizophrenic patiens or drug addicted persons, DA hyperproduction in the VTA may lead to psychoses, paranoidal delutions etc. Logically, the questions can be arisen concerning the role of melanocortins in these processes:

1) are there only melanocortinergic mechanisms involved in γ-MSH effects?
2) may dopaminergic and/or other neurotransmitter systems contribute to γMSH action?
3) what is the neurochemical basis for γ-MSHs effects?
4) what is the endogenous role of these peptides?

We have tried to find answers to these questions for a period since 1995 in collaboration with scientists group headed by Professor Jarl Wikber (Uppsala University) and having five-year financial support from the Howard Hughes Medical Institute (USA). What have we found? First of all, almost in all experiments we have found different behavioural repertoire of both peptides, however their structures are very similar. So, if, γ1-MSH injected into the VTA induced psychoactivation in rats behaviour (excessive grooming, increase in vertical locomotor activity), in contrary, γ2-MSH lacked these activities and instead it caused a moderate catalepsy. Moreover, γ2-MSH completely antagonized the, γ1-MSH-induced behavioural responses (Klusa et al., 1999). These data indicate that γ1-MSH may act as psychoactivatory peptide probably by stimulating effect on DAergic system, whereas γ2-MSH acted in opposite manner – as antipsychotic substance. These suggestions were confirmed by neurochemical data obtained by peptide administrations into the VTA and assessment of DA and its metabolite DOPAC in the NACC [50]: γ1-MSH induced a

considerable increase in DA and DOPAC levels, while γ2-MSH caused a decrease in these levels (Figure 2).

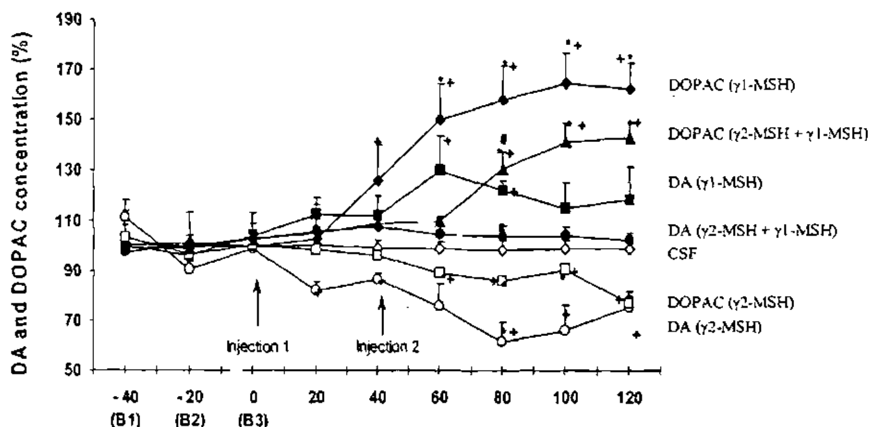

Figure 2. Influence of the intra-VTA administered γ1-MSH, γ2-MSH and γ2-MSH+γ1-MSH on the dopamine (DA) and DOPAC concentrations in the rat nucleus accumbens. Dose of peptides was 3nmol/rat. * $p<0.05$ vs CSF (artificial cerebrospinal fluid), + $p<0.05$ vs control, i.e. basal values (B1+B2+B3)/3, ♦ $p<0.05$ vs γ1-MSH. Injection 1: administration of γ1-MSH or γ2-MSH or saline. Injection 2: regards only to the combined administration γ2-MSH+γ1-MSH (arrow at Injection 2 shows administration of γ1-MSH after pretreatment of γ2-MSH (arrow at Injection 1).

The same antagonizing phenomenon between both peptides was observed: γ2-MSH abolished the γ1-MSH-induced neurochemical changes in the content of DA and DOPAC (Figure 2). These findings allowed us to suggest that the relationships between both γ-MSH peptides may be considered as functional antagonism (?) based on their opposite influence on the mesolimbic dopaminergic system, particulary on their ability to influence DA metabolism.

It is very important that imbalanced DAergic system may lead to the development of psychiatric disorders. For instance, schizophrenia can be considered as manifestation of hyperactivation of the DAergic system and hypoactivation of the glutamatergic system. Modeling of schizophrenic state in laboratory animals (mice) by use of phencyclidine (PCP), a drug capable to act as non-competetive antagonist of glutamate NMDA receptors, again showed different effects of both γ-MSH peptides injected intracisternally (Figure 3): γ1 MSH potentiated the PCP-hyperlocomotion effects, whereas γ2-MSH reversed up to the control level the locomotor responses increased by PCP [51].

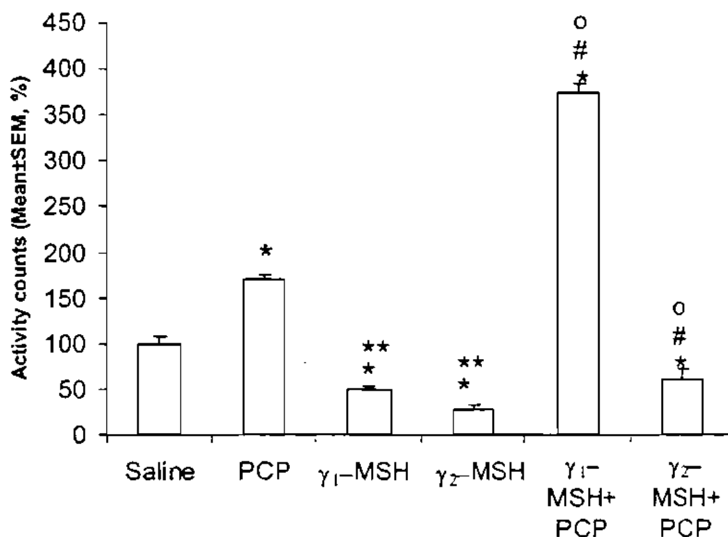

Figure 3. Influence of γ1-MSH and γ2-MSH (0.3nmol/mouse, i.c.) on phencyclidine (PCP)-stimulated spontaneus locomotor activity in BALB/c mice (n=9). PCP, 5 mg/kg, i.p., 5min prior to γ1-MSH or γ2-MSH injection. Activity counts measured in 30-60min period after γ-MSH peptide administrations. * $P<0.05$ vs saline, ** $P<0.05$ γ1-MSH or γ2-MSH vs PCP, # $P<0.05$ γ1-MSH+ PCP or γ2-MSH+PCP vs PCP, ° $P<0.05$ γ1-MSH+ PCP or γ2-MSH+ PCP vs γ1-MSH or γ2-MSH , resp.

These data indicate that both γ-MSH peptides may also modulate glutamatergic system. Moreover, examination of the influence of both peptides on pain perception by use of tail flick method in mice has found that γ2-MSH (but not γ1-MSH) was capable to cause a considerable analgesic effect that is mediated *via* GABAergic mechanisms [52]. γ2-MSH-induced analgesia was not altered by naloxone (opiate receptor antagonist), haloperidol (DA receptor antagonist), HS014 (melanocortin receptor antagonist), neither by γ1-MSH (peptide which antagonized γ2-MSH-induced behavioural and neurochemical responses). At the same time bicuculline (GABA site antagonist of the GABA-A receptor) reduced completely the γ2-MSH-analgesia, and muscimol (GABA site agonist of the GABA-A receptor) augmented this effect. γ2-MSH-analgesia was not influenced by diazepam (benzodiazepine site agonist of the GABA-A receptor), while analgesic effect was increased in ethanol-pretreated rats, indicating that ethanol modulatory site of the GABA-A receptor may be influenced by γ2-MSH. As to γ1-MSH (which does not produce analgesic effect), it exerted antagonizing effects against analgesia caused by diazepam and ethanol [52] Thus these data allow to suggest that γ2-MSH influences GABA site of the GABA-A receptor, whereas γ1-MSH may modulate benzodiazepine site of the GABA-A receptor; both γ-MSHs in opposite manner may modulate ethanol site of the GABA-A receptor.

Summarizing the newest data concerning γ-MSH peptides one may consider that they may be involved in many pleiotropic functions, such as modulation of dopamine-, glutamate- and GABAergic processes, regulation of pain perception and psychoactivation, and probably act as endogenous mutual antagonists (schizophrenic/anti-schizophrenic?). Probably these peptides may play a role also in regulation of the mesolimbic reward system.

4. Drug Design Based On MCR-MSH Binding Data

Knowledge about the melanocortins and their receptors stimulates to modulate melanocortin receptor signaling by a design of new molecules that act as agonists or antagonists at the receptor levels. The newest data indicate that new drug design based on melanocortin and their receptor subtype binding can open new vistas in understanding of both the formation of pathologies where these molecules play an essential role, and the new strategies how to treat diseases. The most promising tendencies are given below (Table 5).

TABLE 5. The new strategies in drug design

MCR subtype	Agonists/antagonists	Usefulness in the treatment of pathology
MCR1	agonists	anti-inflammatory drugs immunoregulators
MCR1	antagonists	melanoma suppressors
MCR3	agonists/antagonists	regulation of drug dependence psychoregulators (antipsychotic drugs?) analgesic drugs blood pressure regulation (pressor activity?)
MCR4	agonists	anorexive drugs (anti-obesity)
	antagonists	orexigenic drugs blood pressure regulation (depressor effect?)
MC5R	agonists/antagonists	regulation of excretory gland functions

The cloning of melanocortin receptors has shed a new light in understanding of the functional role of melanocortins and their receptors. That in turn stimulates rationale new drug design which can be useful in the treatment in different diseases, particularly those attributed to the central nervous system pathologies. This is attractive and rapidly developing field with promising opportunities.

Acknowledgements

Authors thank Professor Jarl Wikberg, Dr.Helgi Schioth, Dr. Lena Bergstrom, Dr.Jonas Lindblom, Dr.Peteris Prusis, Dr.Felikss Mutulis (all of them from the Uppsala University), and Dr. Vijay Chhajlani (Sweden) for fruitful collaboration. These studies were supported by grants from the Howard Hughes Medical Institute (1995-2000), the Latvian Council of Science and the Swedish Society for Medical Research.

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Opposite effects of γ_1- and γ_2-melanocyte stimulating hormone on regulation of the dopaminergic mesolimbic system in rats[*1]

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Available online 9 April 2004.

Abstract

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Author Keywords: Author Keywords: Melanocyte stimulating hormone γ_1; Melanocyte stimulating hormone γ_2; Microdialysis; Ventral tegmental area; Nucleus accumbens; Dopamine; 3,4-Dihydroxyphenylacetic acid

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*1 This article is dedicated to Professor Manfred Zimmermann's 70th birthday with best wishes for his continued vitality.

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Neuroscience Letters xx (2003) 1–4

Neuroscience Letters

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Opposite effects of γ_1- and γ_2-melanocyte stimulating hormone on regulation of the dopaminergic mesolimbic system in rats☆

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Abstract

By use of the brain microdialysis technique we show that administration of γ_1-melanocyte stimulating hormone (γ_1-MSH) into the ventral tegmental area of anaesthetized rats causes an increase in the release of extracellular dopamine and its metabolite 3,4-dihydroxyphenylacetic acid in the nucleus accumbens, while γ_2-MSH causes the opposite effect. Moreover, γ_2-MSH pre-treatment considerably reduced the γ_1-MSH-induced effects. Our findings suggest an opposing action of two γ-MSH-activate pathways on the mesolimbic dopaminergic system, which could be important in the maintenance of a balanced psychoactivation state.

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Pro-opiomelanocortin (POMC) is a precursor protein which is processed into several neuropeptides that include the melanocortin peptides (α-, β-, γ-melanocyte stimulating hormone (MSH) and adrenocorticotropic hormone), as well as lipotropin and endorphin molecules [2]. Since the discovery of five subtypes of melanocortin receptor (MCR_{1-5}) [1,3,9,10,16], tremendous progress has been made to clarify the endogenous roles of melanocortins and their receptors. Among all melanocortins, γ-MSH peptides are less studied and their functional roles are least understood. However, some light has been shed by findings that demonstrate an abundant expression of MCR_3 and MCR_4 in the ventral tegmental area (VTA) and in the nucleus accumbens (NACC) [12,14]. Moreover, a high affinity of MCR_3 for γ-MSH peptide binding [8,12] and comparatively low affinity of MCR_4 for γ-MSH peptide binding [8] has been demonstrated. Furthermore, γ1-MSH shows a 40-fold increase in selective affinity for rat MCR_3 vs. MCR_4 [8]. These data suggest the existence of a functional link between the melanocortin and dopamine systems since these two systems overlap to some extent anatomically: firstly, mesolimbic dopamine (DA) neurons of the VTA project to the NACC and secondly, γ-MSHs display high binding activity for MCR_3.

We have found previously [6] that γ_1- and γ_2-MSH, peptides with strikingly similar structures (γ1-MSH: H_2N-YVMGHFRWDRF-NH_2 and γ2-MSH: H_2N-YVMGHFRWDRFG-OH) induce different, even opposite behavioural responses in rats after their intra-VTA administration: while γ_1-MSH causes pronounced grooming and vertical activity (similarly to α-MSH), while γ_2-MSH lacked these effects; instead it induced moderate catalepsy. Moreover, we showed that γ_2-MSH acted as antagonist of γ_1-MSH [6]. These d: . indicated that the mesolimbic DAergic system might be involved, at least in part, in the mediation of the behavioural effects induced by melanocortic peptides. In earlier studies we have shown that the grooming effect caused by intracerebroventricular, as well as intra-VTA injections of α-MSH, is blocked by an MCR_4 antagonist [5], and that injection of α-MSH into the VTA mediates release of DA in the ipsilateral NACC [7].

The present study was designed to clarify the influence of γ_1- and γ_2-MSHs on the dopaminergic mesolimbic system, and to identify the peculiarities of the behavioural repertoire induced by these peptides. Therefore, we investigated the influence of γ_1- and γ_2-MSH administered intra-VTA on the release of DA and its metabolite DOPAC in the NACC using the microdialysis technique in rats.

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0304-3940/03/$ - see front matter © 2004 Published by Elsevier Ireland Ltd.
doi:10.1016/j.neulet.2003.12.006

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Male Sprague–Dawley rats weighing 270–340 g were housed with free access to food and water at 21 ± 1 °C, lights on 07:00–19:00 h. Rats were anaesthetized with Inactin (80 mg/kg per rat, intraperitoneally) and placed in a Kopf stereotaxic frame. Stereotaxic surgery was performed according to method described elsewhere [7]. Peptides (from Sigma) were injected manually into the VTA at the dose of 3 nmol/0.5 μl cerebrospinal fluid (CSF) via a guide cannula with bregma as reference: B −5.0 mm, L −0.9 mm and V − 7.2 mm [11], by use of a Hamilton Microliter syringe. Animals received CSF injections served as a control group. The microdialysis probe (cut-off 20 kDa PES; AgnTho's AB, Lidingo, Sweden) was placed into the left NACC with bregma as reference: B +2.2 mm, L − 1.5 mm and V −7.1 mm, and perfused with artificial CSF at 2 μl/min. Peptide administration was not started until three samples at 20-min intervals showed less than 15% variation of the DA and DOPAC content. The total time for collection of samples after the injection of a peptide was 2 h. DA and DOPAC were immediately determined by high-performance liquid chromatography using electrochemical detection (ReproSil-Pur C18-AQ; ESA Inc. detector; guard cell electrode voltage +0.4 V; working electrode voltage +0.34 V). The recycled mobile phase used was 2 g/l of sodium acetate monohydrate, 38.75 mg 1-octanesulfonic acid, 3.7 mg EDTA in 900 ml H_2O/100 ml methanol, pH 4, and the flow rate was 0.6 ml/min. Only animals with a histological verification (correctly implanted probe and cannula) were included. The average of three baseline samples was considered as control level and was taken to represent a level of 100%. The data were statistically analysed using one-way analysis of variance followed by the Newman–Keuls Multiple Comparison test and paired t-test.

The dose of 3 nmol (in 0.5 μl)?for γ-MSH peptides was selected because of the pronounced behavioural responses we previously observed it to elicit [6]. Baseline dialysate contents of DA and DOPAC were 32.3 ± 0.3 fmol and 24.6 ± 1 nmol, respectively, in 40-μl samples. Administration of γ_1-MSH into the VTA resulted in a significant increase in the extracellular DA and DOPAC release in the NACC, and the influence on DOPAC level was markedly higher than that on DA level (Fig. 1a,b). By contrast, the intra-VTA injection of γ_2-MSH caused a pronounced decrease in the DA and DOPAC contents in the NACC dialysates, the decrease in the DOPAC levels being more pronounced than the decrease in DA. Intra-VTA pre-treatment with γ_2-MSH (γ_2-MSH injected 40 min prior to γ_1-MSH) significantly attenuated the effect of γ_1-MSH on DA and DOPAC (Fig. 1a,b).

Over the last 5–10 years, the functional roles of melanocortins and their receptors has been considerably clarified; however, the newest findings show complexity of melanocortinergic processes on the one hand, and their link to the non-melanocortinergic pathways and molecules on the other hand. Particularly, interest has been focused on the dopaminergic mesolimbic system, in whose structure the melanocortin receptor subtypes 3 and 4 (MCR_3 and MCR_4) are expressed abundantly (for reviews see [14,15]) and their affinities for γ-MSH peptides binding have been demonstrated. In turn, the dopaminergic mesolimbic system belongs to the reward system which is involved in drug dependence and motivational processes [13], as well as in manifestations of schizophrenic hyperactivation and emotions. This system involves two very important brain structures playing an essential role in regulation of dopaminergic pathways: they are the ventral tegmental area (VTA) and the nucleus accumbens (NACC). The cytoarchitectony of the VTA is very complicated, since A10 DA cells receive numerous interneurones, both inhibitory (e.g. GABAergic) and excitatory (e.g. glutamatergic), that may modulate dopamine (DA) release in the NACC [13]. If DA cells are stimulated, the DA release in the NACC is increased which coincides with hyperlocomotion and stereotypical behavioural responses (e.g. grooming) in laboratory animals. In schizophrenic patients or drug-addicted persons, DA overactivity may lead to psychoses, paranoid delusions, etc. In the present study we have investigated how the dopaminergic system contributes to the γ-MSH-induced behavioural effects. As was shown earlier, intra-VTA administration of γ_1-MSH induced marked grooming and rearing activities in the rat, whereas γ_2-MSH lacked these responses and caused a moderate catalepsy; it acted also as a γ_1-MSH antagonist [6]. Although both the γ_1-and γ_2-MSH peptides studied herein are agonists on the rat melanocortin MC_3 and MC_4 receptors [15], our findings dispute a simple model wherein MC_3 and/or MC_4 receptors become activated only by the MSH peptides upon their intra-VTA administration. Despite the fact that γ_1- and γ_2-MSH structures are almost identical, differing from each other only by an extra C-terminal Gly in the γ_2-MSH molecule, we have demonstrated a striking difference in the effects of γ_1-MSH versus γ_2-MSH. While intra-VTA administration of γ_1-MSH increased the release of DA and DOPAC in the NACC, γ_2-MSH decreased it. Moreover pre-treatment with γ_2-MSH considerably reduced the γ_1-MSH-induced alterations in DA and DOPAC concentrations. These opposite effects on mesolimbic dopamine transmission correlate well with our previous observations on the peptide's behaviour. It is plausible that the γ_1-MSH-induced grooming and rearing hyper-reactivity can be attributed to a stimulating action on the dopaminergic mesolimbic system. The cataleptic state caused by γ_2-MSH, on the other hand, may be attributed to the inhibition of the dopaminergic mesolimbic system. Thus, our findings indicate a distinct behavioural and neurochemical repertoire of both γ-MSH peptides: psychoactivation of the γ_1-MSH and in contrast, antipsychotic action of the γ_2-MSH. A most intriguing phenomenon obtained in the above studies is an antagonistic relationship between γ_1- and γ_2-MSH peptides. The latter findings allowed us to suggest that the relationships between both γ-MSH peptides may be considered, at least in part, as

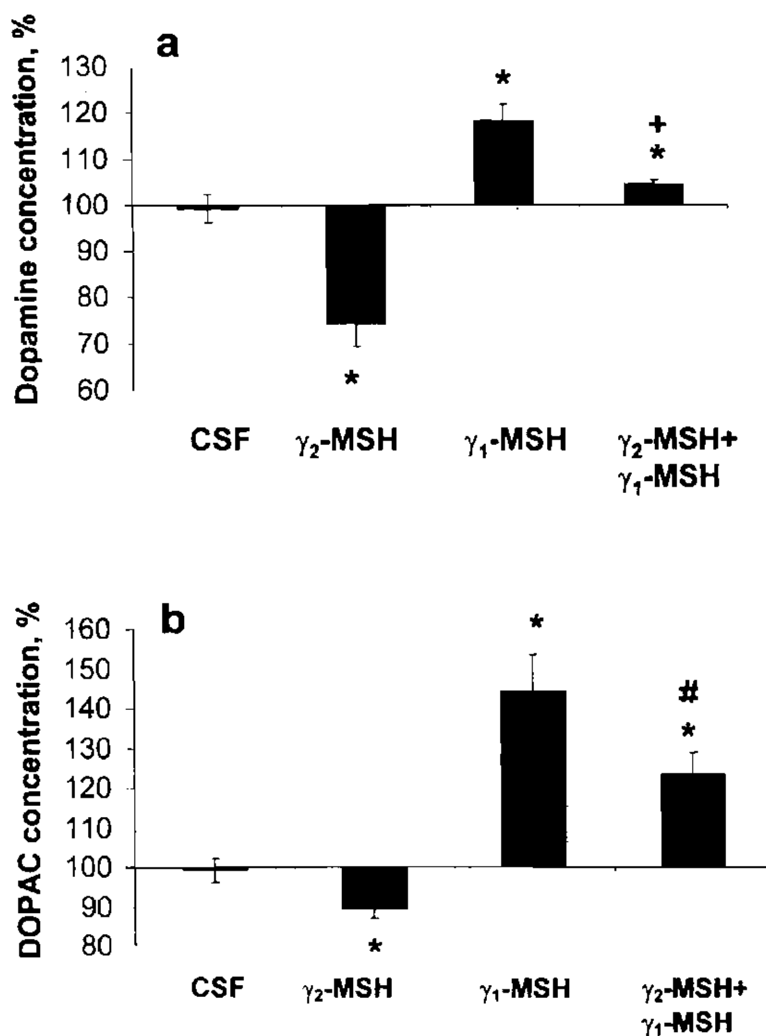

Fig. 1. Average levels of extracellular DA (a) and DOPAC (b) in the anaesthetized rats nucleus accumbens measured by microdialysis following intra-VTA administration of artificial CSF (control), γ_1-MSH and γ_2-MSH (each peptide 3 nmol), and influence of the pre-treatment of γ_2-MSH (3 nmol) on the effect of γ_1-MSH (3 nmol). Changes are expressed as percentages (± S.E.M.) from basal levels of DA and DOPAC (calculated as the mean of the three samples before the treatment of MSH peptides and amounting to for DA 32.3 ± 0.3 fmol/40 μl and for DOPAC 24.6 ± 1 nmol/40 μl). $n = 7$. *$P < 0.05$ vs. CSF. $^+P < 0.05$ vs. γ_1-MSH (a), $^\#P < 0.05$ vs. γ_1-MSH (b).

functional antagonism based on their opposite influence on the mesolimbic dopaminergic system, particularly on their ability to influence DA metabolism in a distinct manner. Differing effects were found also in studies of the capacity of γ_1- and γ_2-MSHs to induce analgesia in mice [4]. Thus, intracisternal injection of γ_2-MSH caused a stable and long-acting central analgesia mediated via γ-aminobutyric $acid_A$ ($GABA_A$) receptor, whereas γ_1-MSHs induced only a negligible effect. The data of the present and our previous studies show that both γ-MSH peptides may be involved in such functions as modulation of dopamine- and GABAergic processes, regulation of pain perception and psychoactivation, probably by acting as endogenous antagonists. We suggest an important functional role of γ-MSH peptides in maintaining a balanced psychoactivation in normal and pathological states mediated by their opposite actions at least at the level of the dopaminergic mesolimbic system. How the interaction between melanocortinergic and non-melanocortinergic systems can be realized remains to be elucidated.

Acknowledgements

Supported by the Latvian Council of Science PhD fellowship grant No. 586, Swedish MRC (04X-05957) and Melacure Therapeutics AB.

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