Latvijas Universitāte
Medicīnas Fakultāte
Farmakoloģijas docētāju grupa

Melanokortīni – uzvedības un neiroķīmisko procesu regulētāji

Promocijas darbs

Baiba Jansone
(née Opmane)

Rīga
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PROMOCIJAS DARBS

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Promocijas darba vadītāja:

LZA akadēmiķe profesore, Dr. habil. med. Vija Kluša
Latvijas Universitātes
Medicīnas fakultātes
Farmakoloģijas docētāju grupas vadītāja

Habilitācijas un promocijas padomes priekšsēdētāja:

Profesore, Dr. habil. med. Renāte Ligere

Neatkarīgais starptautiskais recenzents:

Profesors, Dr. habil. med. *Alexander Zharkovsky*
Tartu Universitātes
Farmakoloģijas katedras vadītājs

Oficiālie recenzenti:

LZA akadēmiķis, Dr. chem. Ivars Kalviņš
Latvijas Organiskās sintēzes institūta direktors

Profesors, Dr. habil. med. Nikolajs Sjakste
Latvijas Universitātes
Medicīnas fakultātes
Medicīnas bioķīmijas docētāju grupas vadītājs

Dr. pharm. Maija Dambrova
Latvijas Organiskās sintēzes institūta
Farmaceitiskās farmakoloģijas laboratorijas vadītāja

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visiem, kas sekmēja tā realizēšanos

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ANOTĀCIJA

Par būtisku melanokortīnu (α-MSH, β-MSH, γ-MSHs, ACTH) izpētes sākumu var uzskatīt piecu melanokortīnu receptoru subtipu (MC1R-MC5R) atklāšanu, klonēšanu un ekspresijas vietu atrašanu (1992.-1993.). Joprojām visneskaidrākā ir MC3R un γ-MSH peptīdu funkcionālās lomas nozīme centrālajā nervu sistēmā. Taču intriģējošs ir fakts, ka γ-MSH ar augstu afinitāti saistās ar MC3R, un savukārt, MC3R ir plaši pārstāvēts mezolimbiskās dopamīnerģiskās sistēmas struktūrās - *ventral tegmental area* (VTA) un *nucleus accumbens* (NACC), kuras ir atalgojuma sistēmas sastāvdaļas. Dopamīna A10 šūnas, kas veido VTA struktūru, saņem gan inhibējošo GABAerģisko, gan aktivējošo glutamāterģisko interneironu projekcijas.

Mūsu zinātniskais darbs ir veltīts γ-MSH peptīdu (γ_1- un γ_2-MSH) neirofarmakoloģiskajām aktivitātēm, lai vismaz daļēji noskaidrotu šo peptīdu funkcionālo lomu smadzeņu procesu regulācijā. Darbā izmantoti uzvedības un neiroķīmijas testi, un modeļi laboratorijas dzīvniekiem, kā arī dažādu receptoru agonisti un antagonisti.

Uzvedības testos žurkām, intra-VTA ievadot γ_1- un γ_2-MSH, tika novērotas atšķirīgas, pat pretējas uzvedības reakcijas. γ_1-MSH (līdzīgi agrāk izpētītajam α-MSH) izraisīja pastiprinātu *grooming* uzvedību un vertikālo lokomociju, kas liecina par dopamīnerģiskās sistēmas hiperaktivāciju, kamēr γ_2-MSH izraisīja psihodepresijas stāvokli (katalepsiju), kas norāda uz antipsihotisku aktivitāti dopamīnerģiskās sistēmas līmenī. Vēl jo vairāk, γ_2-MSH spēja antagonizēt γ_1-MSH izraisītās uzvedības reakcijas. Tālākie neiroķīmiskie pētījumi (izmantojot smadzeņu mikrodialīzes metodi) parādīja, ka gan α-MSH, gan γ_1-MSH paaugstināja dopamīna un DOPAC koncentrāciju NACC struktūrā, turpretī γ_2-MSH samazināja abu monoamīnu koncentrāciju. Līdzīgi, kā to novēroja uzvedības testos, arī šajā gadījumā γ_2-MSH antagonizēja γ_1-MSH efektus. Šie dati liecina, ka abi γ-MSH peptīdi spēj būtiski modulēt dopamīnerģisko aktivitāti mezolimbiskajā sistēmā, un šīs ietekmes ir ne vien atšķirīgas, bet gan pilnīgi pretējas. Tas norāda uz šo peptīdu funkcionālo lomu, piedaloties smadzeņu savstarpēji regulējošo/normalizējošo dopamīnerģisko procesu balansēšanā, kurā γ_1-MSH darbojas kā psihoaktivējošs, bet γ_2-MSH kā antipsihotisks (antišizofrēnisks?) faktors.

Abu γ-MSH peptīdu (ievadot intracisternāli) pretēja darbība atklājās arī glutamāterģiskās sistēmas līmenī, izmantojot fencikliđīna (PCP) hiperlokomocijas (šizofrenijas) modeli pelēm. Šajā testā γ_1-MSH pastiprināja PCP lokomotoro aktivitāti, turpretim γ_2-MSH to samazināja. Bez tam γ_2-MSH antagonizēja γ_1-MSH potencējošo efektu attiecībā uz PCP darbību. γ_2-MSH pilnīgi antagonizēja inta-VTA ievadītā glutamāta receptora liganda NMDA neirotoksiskos efektus žurkām.

Savukārt analgēzijas pētījumi atklāja, ka γ_2-MSH (bet ne γ_1-MSH) izraisa stabilu un ilgstošu (90 min) neopiātu analgēziju, kura realizējas, ietverot $GABA_A$ receptora mediētos procesus. Tā, $GABA_A$ receptora GABA saita ligands – agonists muscimols potencēja γ_2-MSH analgēziju, bet šā saita antagonists bikukulīns antagonizēja γ_2-MSH analgēziju. Savukārt γ_1-MSH darbojās kā $GABA_A$ receptora benzodiazepīna saita liganda - diazepama darbības antagonizētājs. Abi peptīdi atšķirīgi ietekmē arī $GABA_A$ modulatora etanola efektus: γ_2-MSH potencēja, bet γ_1-MSH antagonizēja etanola analgēziju.

Tādējādi šajā zinātniskajā darbā pirmo reizi parādīta melanokortīnu daudzveidīgā ietekme uz centrālās nervu sistēmas procesiem, kuros iesaistīti ne tikai melanokortīnerģiskie mehānismi, bet arī dopamīn-, glutamāt- un GABAerģisko procesu modulācija. Vissvarīgākais pēc mūsu domām ir atklātais γ_1-MSH un γ_2-MSH pretējas darbības (pat savstarpējā darbības antagonisma) fenomens, ko var uzskatīt par būtisku argumentu mūsu hipotēzei par abu γ-MSH peptīdu funkcionālo lomu regulēt psihoaktivācijas motivācijas un sāpju percepcijas līmeni, kam var būt liela nozīme turpmākā melanokortīnu vai to receptoru ligandu izmantošanā jaunām farmakoterapētiskām stratēģijām psihopatoloģiju koriģēšanā.

PUBLIKĀCIJU SARAKSTS

Šis promocijas darbs ir veidots kā publikāciju kopa un apraksta sekojošas publikācijas (skat. zemāk). Tekstā publikācijas apzīmētas ar attiecīgiem romāņu numerācijas cipariem (I-VII). Promocijas darbam papildus pievienoti arī nepublicēti dati.

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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

SAĪSINĀJUMI

α-MSH	alfa melanocītu stimulējošais hormons
β-MSH	beta melanocītu stimulējošais hormons
γ_1-MSH	gamma$_1$ melanocītu stimulējošais hormons
γ_2-MSH	gamma$_2$ melanocītu stimulējošais hormons
γ_3-MSH	gamma$_3$ melanocītu stimulējošais hormons
AGRP	*agouti* radniecīgais peptīds
AKTH	adrenokortikotropais hormons
ASIP	*agouti* signāl proteīns
AMP	L-amfetamīns
cAMF	cikliskais adenozīnmonofosfāts
CNS	centrālā nervu sistēma
CSF	cerebrospinālais šķīdums
DA	dopamīns
DNS	dezoksiribonukleīnskābe
DOPAC	3,4-dihidroksifeniletiķskābe
EDTA	etilēndiamīna tetraacetiletiķskābe
Fiziol. šķ.	fizioloģiskais šķīdums
GABA	gamma-aminosviestskābe
$GABA_A$	gamma-aminosviestskābes $_A$ tipa receptors
HPLC	augsta spiediena šķidruma hromatogrāfija (*high performance liquid chromatography)*
hGH	cilvēka augšanas hormons
IUPAC-IUB	*International Union of Pure and Applied Chemistry-International Union of Biochemistry and Molecular Biology*
i.c.	intracisternāli
i.p.	intraperitonāli
ICV	intracerebroventrikulāri
IL	interleikīns
IP_3	inozitola 1'4'5'-trifosfāts
K_i	disociācijas konstance
LPH	lipotropīns
LPS	lipopolisaharīds
MC1R	melanokortīnu receptora 1. subtips
MC2R	melanokortīnu receptora 2. subtips
MC3R	melanokortīnu receptora 3. subtips
MC4R	melanokortīnu receptora 4. subtips
MC5R	melanokortīnu receptora 5. subtips
MSH	melanocītus stimulējošais hormons
NACC	*nucleus accumbens*
NF-kB	nukleārais faktors-kappa B
NMDA	N-metil-D-aspargīnskābe
PC	prohormona konvertāze
NO	slāpekļa oksīds
PCP	fenciklidīns
PKA	proteīna kināze A
POMK	proopiomelanokortīns
TNF-α	tumora nekrozes faktors-α
VTA	*ventral tegmental area*

1. IEVADS

Kopš neiropeptīdu ēras sākuma pagājušā gadsimta 70. gados, informācija par peptīdu veidošanos, lokalizāciju, to receptoriem un funkcionālo lomu ir pieaugusi lavīneidīgi. Tā melanokortīni, par kuru nozīmi smadzenēs bija visai maza izpratne, kopš 90. gadu sākuma ir kļuvuši par vienu no visvairāk pētītiem peptīdiem. Pirms 10 gadiem (1992.-1993.g) izdarīto atklājumu var uzskatīt par revolūciju neirozinātnēs, kas parādīja piecu melanokortīna receptoru subtipu (MC1R-MC5R) eksistenci dažādās šūnās. Līdz ar to intensīvāk tika pētītas melanokortīnu "ģimenei" piederošo peptīdu (AKTH, α-, β- γ_1- un γ_2-MSH) farmakoloģiskās īpašības un to līdzdalība dažādās organisma norisēs, kas līdz šim likās pilnīgi nesaistītas ar šiem peptīdiem.

Līdz šim vislielākā uzmanība pievērsta melanokortīnu receptoru 1. un 4. subtipam (MC1R un MC4R, attiecīgi), ar kuriem augstāku saistīšanās spēju uzrāda filoģenētiski vecākais melanokortīnu peptīds - α-MSH, kurš izraisa vardes ādas melanocītu dispersiju un līdz ar to pigmentācijas maiņas. Taču pēdējo 5-7 gadu pētījumi parāda, ka MC1R un α-MSH var piedalīties arī cilvēka melanomas tumoroģenēzē un pretiekaisuma procesu regulācijā. MC4R iesaistās apetītes regulācijā, tādējādi intensīvi tiek pētīts šis receptoru subtips tādu patoloģiju kā anoreksija un aptaukošanās kontekstā. Iegūtie dati liecina, ka MC4R agonisti spēj samazināt apetīti, bet antagonisti stimulē apetīti, izraisa barības uzņemšanas un svara pieaugumu.

Vismazāk ir pētīti γ-melanokortīni, kuri ar augstu afinitāti saistās ar melanokortīnu receptoru 3. subtipu (MC3R). Intriģējoši, ka šis subtips ekspresējas dažādos smadzeņu kodolos, īpaši plaši - smadzeņu dopamīnerģiskajā mezolimbiskājā sistēmā. Zināms, ka dopamīnerģiskā mezolimbiskā sistēma ir iesaistīta atalgojuma un emocionālo procesu regulācijā, tātad vielu atkarības, šizofrēnijas un citu psihoaktivācijas stāvokļu disbalansā. Divas nozīmīgas šīs sistēmas struktūras ir *ventral tegmental area* (VTA) un *nucleus accumbens* (NACC). Dopamīna A10 šūnas, kas veido VTA struktūru, saņem gan inhibējošo (GABAerģisko), gan aktivējošo (glutamāt- un holīnerģisko) interneironu projekcijas, kas modulē dopamīna izdalīšanos NACC. Dopamīna hiperprodukcija VTA šizofrēnijas pacientiem, kā arī personām, kas cieš no vielu atkarības problēmas, var izraisīt psihozes, paranoidālas mānijas un tamlīdzīgas izpausmes.

Latvijā γ-MSH peptīdu pētījumiem uzmanība tika pievērsta kopš 1995. gada profesores Vijas Klušas vadībā. Pētījumi tika uzsākti Latvijas Organiskās sintēzes institūta Farmakoloģijas laboratorijā un turpinās Latvijas Universitātes Farmakoloģijas katedrā. Neapšaubāmi stimulējoša nozīme šajās aktivitātēs ir bijusi sadarbībai ar Upsalas Universitātes zinātnieku grupu profesora Jarla Vikberga vadībā un *Howard Hughes Medical Institute* (ASV) granta būtiskam finansiālam nodrošinājumam (1995.-2000.).

Šis darbs ietver pētījumu rezultātus, kas iegūti, izstrādājot LU bakalaura, maģistra grāda un doktorantūras darbu iepriekš minētajā laboratorijā un katedrā. Darbs veltīts γ-MSH peptīdu centrālo farmakoloģisko efektu izpētei, ietverot peptīdu izraisītās uzvedības reakcijas un neiroķīmiskos mehānismus. Pirmo reizi parādīta šo peptīdu spēja izraisīt savdabīgu laboratorijas dzīvnieku uzvedības repertuāru, kurā iesaistīti ne tikai melanokortīnerģiskie mehānismi, bet arī dopamīn-, glutamāt- un GABAerģiskie procesi. Visinteresantākais pēc mūsu domām ir atklātais γ_1-MSH un γ_2-MSH pretējas darbības (pat savstarpējā darbības antagonisma) fenomens, lai gan abu γ-MSH peptīdu struktūras lielā mērā ir līdzīgas. Daudzos pētījumos atklātā γ_1-MSH un γ_2-MSH pretēju efektu izraisošā iedarbība norāda uz abu peptīdu iespējamo funkcionālo lomu smadzeņu procesu regulācijā, lai nodrošinātu psihoaktivācijas, sāpju percepcijas (un, iespējams, arī motivācijas) procesu homeostāzi.

Iegūtie dati ne tikai paplašina priekšstatus par melanokortīniem un par to endogēno lomu, bet var arī rosināt dizainēt un sintezēt jaunas antipsihotiskas, analgētiskas un motivāciju regulējošas vielas-prototipus, bāzētas uz pilnīgi jauniem mehānismiem, kas atklāti šajos γ-MSH peptīdu farmakoloģiskajos pētījumos.

2. LITERATŪRAS APSKATS

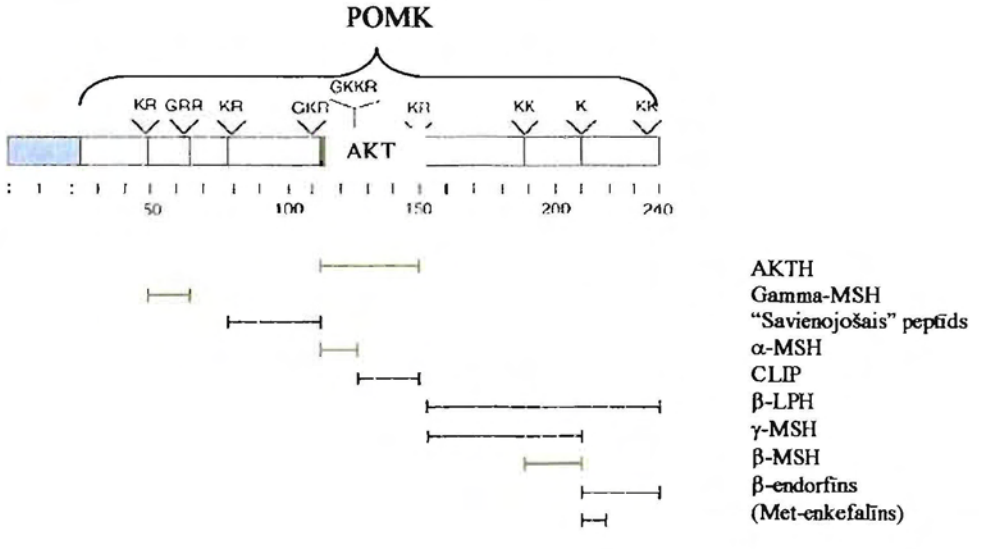
2.1. Melanokortīnu peptīdi

2.1.1. Melanokortīnu atklāšanas vēsture un nomenklatūra

Saskaņā ar IUPAC-IUB konvenciju, hormonu, kas melanocītos stimulē melanīna veidošanos, dēvē par melanocītstimulējošo hormonu jeb saīsināti - MSH. Melanocītstimulējošā hormona triviālais nosaukums ir melanotropīns. Terminu "opiomelanokortīni" lieto attiecībā uz jebkuru peptīdu, kura prekursors ir proopiomelanokortīns (POMK), bet par melanokortīniem dēvē vienīgi AKTH un MSH izcelsmes peptīdus [α-MSH, β-MSH, γ_1-MSH, γ_2-MSH un γ_3-MSH] (Eberle, 1988). 1912. gadā parādījās pirmais zinātniskais raksts par melanokortīnu bioloģisko aktivitāti, parādot hipofīzes ekstrakta ietekmi vardes ādas pigmentācijas procesos (Fuchs, 1912). α-MSH ir viens no pirmajiem peptīdu hormoniem, kas tika atklāts, un to identificēja, izolējot melanofora stimulantus (Lerner and Lee, 1955). Izrādījās, ka α-MSH iesaistās melanocītu dispersijas procesā un ir atbildīgs par ādas tumšošanos. 1960.-to gadu literatūrā atrodami daudzi pētījumi par α-MSH un AKTH funkcionālo lomu. Salīdzinot ar samērā labi identificētiem α-MSH darbības mehānismiem, vēl joprojām maz ir zināms par γ-MSH peptīdu funkcionālo lomu.

2.1.2. Pro-opiomelanokortīns un melanokortīnu veidošanās

Melanokortīni (AKTH, α-MSH, β-MSH un γ-MSH peptīdi), kā arī vairāki citi bioloģiski aktīvi fragmenti, ieskaitot β-endorfīnu un lipotropīnus, organismā tiek enzimātiski sašķelti no kopīga 240 aminoskābju atlikumu gara prekursora - proteīna proopiomelanokortīna (POMK) (Uhler and Herbert, 1983) (1. zīm.). POMK aminoskābju sekvences ir identiskas hipofīzē, citos smadzeņu un perifērajos audos (Smith and Funder, 1988).

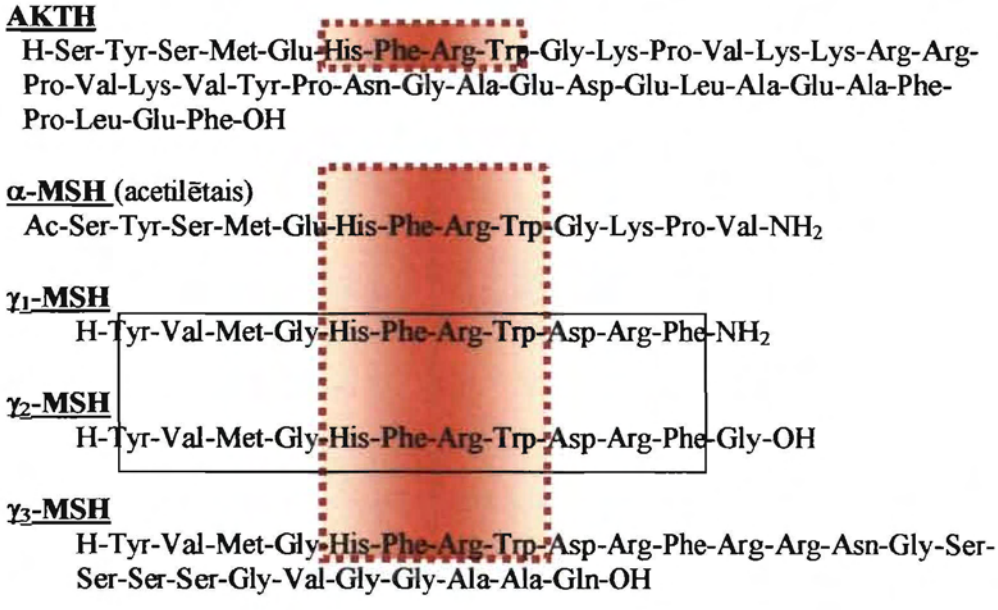


1. zīm. Shematiski attēlota peptīdu veidošanās POMK biodegradācijas rezultātā.

POMK bioprocesings notiek ar prohormona konvertāžu PC1 un PC2 palīdzību. Prohormona konvertāze PC1 ģenerē AKTH, bet PC2, nošķeļot no AKTH pirmās 13 aminoskābes, veido α-MSH peptīdu. PC konvertāzes ir šķīstošas un galvenokārt lokalizētas neironu liela blīvuma serdes vezīkulās un endokrīnajās šūnās. Visbiežāk enzimātiskā šķelšana notiek vietās, kur divas polāras aminoskābes veido peptīda saiti: Arg-Arg (R-R), Lys-Lys (K-K), Arg-Lys (R-K), Lys-Arg (K-R). α-MSH ir atklāts arī hipotalāmā, aknās, nierēs, placentā, aizkuņģa dziedzerī, ādā, zarnās un imunokompetentajās šūnās. Savukārt γ-MSH peptīda klātbūtne noteikta atsevišķos CNS apvidos, zarnu neironos un virsnieru medulārajā daļā (Eberle, 1988). α (α-MSH) norāda uz tridekapeptīda izcelšanos no adrenokortikotropīna (AKTH), β (β-MSH) norāda uz oktapeptīda izcelšanos no lipotropīna (LPH). Centrālajā nervu sistēmā POMK-imunoreaktīvie neironi veido divas nozīmīgas neirālas sistēmas: 1) no hipotalāma mugurējās daļas šūnu ķermeņiem, un 2) smadzeņu stumbra šūnu ķermeņiem, kuru neironi projicējas dažādās CNS daļās, ieskaitot *telencephalon, diencephalon, mesencephalon*, smadzeņu stumbru un muguras smadzenes (Low et al., 1994). Galvenais melanokortīnu avots ir hipofīzes dziedzeris. Tipiskās šūnas, kas ekspresē POMK, ir hipofīzes vidējās daļas melanotropās un priekšējās daļas kortikotropās šūnas. α-MSH un β-lipotropīns ir hipofīzes vidējās daivas galvenie hormoni. Jauns atklājums tika veikts 2003. gadā, kad dzeloņzivs un dzeloņrajas POMC gēnos tika noteikta jauna - delta-MSH peptīda esamība (Dores et al., 2003).

2.1.3. Melanokortīnu primārā struktūra

Melanokortīnu "ģimenes" peptīdi atšķiras viens no otra ar aminoskābju sekvenču saturu un ķēdes garumu (2. zīm.). Taču tie visi satur farmakoforo vienību jeb kopējo tetrapeptīdu His^6-Phe^7-Arg^8-Trp^9.



2. zīm. Melanokortīnu peptīdu primārā struktūra. Brūnā krāsā iekrāsots MSH peptīdu kopējais fragments jeb farmakoforā vienība, γ_1-MSH un γ_2-MSH molekulas homoloģiskā struktūra attēlota rāmī.

α-MSH aminoskābju sekvence ir identiska pirmajām 13 aminoskābēm AKTH ķēdē, tomēr α-MSH N-terminālē ir acetil-grupa un C-terminālē amīda grupa. α-MSH veido 3 endogēnas formas: dezacetilēto-, monoacetilēto- un diacetilēto. Dezacetilētais α-MSH var veidoties par α-MSH jeb acetilēto formu (Verburg-van Kemenade et al., 1987). α-MSH galvenokārt izdalās hipofīzes melanotropās šūnās, savukārt hipotalāmā - α-MSH dezacetilētā forma. γ-MSH peptīdu struktūru var uzskatīt par gandrīz homogēnu, jo tikai viena papildus aminoskābe (glicīns, Gly) γ_2-MSH molekulas C terminālē atšķir šo peptīdu no γ_1-MSH.

2.1.4. Melanokortīnu struktūras īpatnības dažādās sugās

Filoģenētiski α-MSH ir sena molekula, kas būtībā palikusi neizmainīta mugurkaulnieku evolūcijas vēlīnajā laikā. α-MSH aminoskābju secība ir ievērojami līdzīga dažādām sugām. Septiņās zīdītāju sugās (cūkas, vērša, aitas, zirga, pērtiķa, kamieļa un žurkas) izdalītā α-MSH struktūra ir identiska. Putnu (pīles un tītara) α-MSH struktūra ir ievērojami līdzīga zīdītāju α-MSH.
Dažādām sugām γ-MSH peptīdu struktūras noteiktas pēc atbilstošās DNS sekvences (Eberle, 1988).

2.1.5. Melanokortīnu sastopamība CNS un perifērajos audos

α-MSH peptīda imunoreaktivitāte atklāta trīs CNS rajonos: hipotalāma *nucleus arcuatus*, hipotalāma dorsolaterālajā rajonā un *nucleus tractus solitarius* (Eberle, 1988). No šiem smadzeņu rajoniem izejošās šķiedras projicējas visās smadzenēs, ieskaitot hipotalāmu, talāmu, vidussmadzenes, *amygdala*, smadzenītes, muguras smadzenes, hipokampu un lielās smadzeņu puslodes. α-MSH ir difūzi lokalizēts arī perifērijā (1. tabula). γ-MSH imunoreaktivitāte noteikta POMK ekspresējošos neironos - *nucleus arcuatus* un *nucleus cornu commissuralis* (Bloom, 1980), kā arī citos CNS rajonos. Perifērijā γ-MSH atrasts virsnieru dziedzerī, sirdī, nierēs un citur. α-MSH un γ-MSH klātbūtne nervos, kas inervē smadzeņu stumbra baroreceptoru rajonus, norāda uz iespējamo MSH peptīdu ietekmi uz kardiovaskulāro sistēmu (Palkovits, 1987).

1. tabula. Melanokortīnu peptīdu ekspresija hipofīzē un perifērajos audos

PEPTĪDI	**AUDI**
	Hipofīze
α-MSH, γ-MSH	*Pars intermedia*
AKTH	*Pars anterior*
	Perifērie audi
AKTH	Virsnieru dziedzerī, kuņģī, resnajā un tievajā zarnā, aknās, plaušās, olnīcās, liesā
α-MSH	Virsnieru dziedzerī, divpadsmitpirkstu zarnā, sirdī, tievajās zarnās, aknās, plaušās, olnīcās, placentā, liesā, skeleta muskuļos, ādā, muguras smadzenēs, sēkliniekos un vairogdziedzerī
γ-MSH	Virsnieru dziedzera garozā, sirdī, nierēs, olnīcās, kuņģī un asinsvados

Daudzveidīgie stresa apstākļi var izraisīt 2-8 reizes lielu α-MSH un γ-MSH koncentrācijas paaugstināšanos žurku asins plazmā. α-MSH koncentrācija plazmā ir līdzīga γ_3-MSH līmenim, no rīta tā ir zemāka, bet vakarā nedaudz augstāka. α-MSH bazālā rīta koncentrācija asins plazmā svārstās no 10-50pM, vidēji tā ir 28pM.

Žurku asins paraugos α-MSH sadalīšanās ātrums ir vienāds gan endogēniem, gan sintētiskiem peptīdiem. MSH peptīdu pussabrukšanas laiks ir atkarīgs no temperatūras svārstībām: 37°C inkubētiem paraugiem tas ir 39 min, bet uz ledus inkubētiem paraugiem 54 min (Wilson et al., 1982). Sevišķi nenoturīga ir α-MSH Phe^7-Arg^8 saite, kas tiek uzskatīta par primāro šķelšanos vietu. Pārraujot Phe^7-Arg^8 saiti, eksopeptidāzes sadala fragmentus brīvās aminoskābēs.

2.2. Melanokortīnu receptori

2.2.1. Melanokortīnu receptoru atklāšana

Jaunu izpratni par melanokortīnu funkcionālo lomu ienesa pagājušā gadsimta beigās veiktie atklājumi, kad tika atklāti un klonēti pieci melanokortīnu receptoru subtipi (MC1-5R). 1992. gadā divas zinātnieku grupas (prof. *J. Wikberg*, Uppsalas Universitāte, Zviedrijā, un prof. *R. Cone*, Oregonas Universitāte, ASV) neatkarīgi viena no otras, izmantojot melanomu šūnu līniju cDNS, klonēja gēnu, kas kodē ar G proteīnu saistīto MSH receptoru jeb MC1R (Chhajlani and Wikberg, 1992; Mountjoy et al., 1992). Tajā pašā gadā tika klonēts arī strukturāli līdzīgs receptors, ko nodēvēja par MC2R (Montjoy et al., 1992). Pielietojot molekulārās klonēšanas metodes, 1993. gadā tika identificēti trīs garākie melanokortīnu receptoru subtipi - MC3R, MC4R un MC5R (Chhajlani et al., 1993; Gantz et al., 1993).

2.2.2. Melanokortīnu receptora 1. subtips (MC1R)

MC1R tika atklāts pigmentšūnu ļaundabīgā audzēja jeb melanomas šūnās (Mountjoy et al., 1992; Chhajlani and Wikberg, 1992), kā arī melanocītos un citos audos. Turklāt MC1R ekspresijas līmenis vidēji pieaug 10 līdz 20 reizes situācijā, kad normāli funkcionējošie melanocīti pārveidojas par ļaundabīgās melanomas šūnām (Loir et al., 1999). Vesela pieauguša cilvēka ādā ar imunoreaktivitātes metodi anti-MC1R antivielas tika konstatētas mata folikula epitēlijā, sebocītos un sviedru dziedzera kanāla epitēlijā (Bohm et al., 1999). Šim receptoram ir nozīme ādas pigmentācijas un dzīvnieku apmatojuma krāsas veidošanās procesos. Šobrīd ir klonēts daudzu zīdītāju (piemēram, grauzēju, govs) un nezīdītāju (putnu) sugu MC1R (Wikberg, 1999). MC1R ekspresija tika noteikta arī Leidiga šūnās sēklinieko s, *corpus luteum* luteina šūnās un placentas trofoblastiskās šūnās (Wikberg et al., 2000). MC1R lokalizācija makrofāgos un monocītos (Hartmeyer et al., 1997), neitrofīlos (Catania et al., 1996), endotēlija šūnās, fibroblastos (Boston and Cone, 1996), keratinocītos (Luger et al., 1997) un microglijas šūnu tipos (Wong et al., 1997) pēdējā laikā ir pievērsusi īpašu uzmanību saistībā ar šī receptora iespējamo funkcionālo lomu iekaisuma procesos un MSH peptīdu pretiekaisuma darbību. Izmantojot peļu makrofāgu līnijas, kuras ekspresē MC1R, tika pierādīta α-MSH peptīda un tā fragmentu pretiekaisuma darbība (Mandrika et al., 2001). Izmantojot *in situ* hibridizācijas metodi, MC1R ekspresija smadzenēs tika atklāta tikai žurku un cilvēku smadzeņu garozas pelēkās vielas neironos (Xia et al., 1995) un hipofīzē (Chhajlani, 1996), taču netika konstatēta citos smadzeņu rajonos. Tādējādi tiek pieņemts, ka MC1R nav nozīmīgas ietekmes uz centrālās nervu sistēmas funkcijām. MC1R kodējošais reģions satur būtisku vienkāršu nukleotīdu polimorfismu (SNP's), kā rezultātā veidojas fenotipiskas matu un ādas pigmentācijas variācijas. Atsevišķas MC1R mutācijas ir saistītas ar paaugstinātu melanomas rašanās risku (Wikberg et al, 2000). MC1R saistās ar α-, β- un γ-MSH peptīdiem, taču MC1R un α-MSH mijiedarbība tiek uzskatīta par noteicošo ādas pigmentācijas un melanomas veidošanās procesos.

2.2.3. Melanokortīnu receptora 2. subtips (MC2R)

MC2R tiek saukts arī par AKTH receptoru, jo selektīvi saistās tieši ar AKTH, bet ne ar MSH peptīdiem (Schioth et al., 1996). Vislielākā MC2R koncentrācija ir virsnieru garozā, lielākoties *zona glomerulosa* un *zona fasciculata*, kā arī dažās izkaisītās šūnās virsnieru *medulārajā daļā*, taču MC2R neatrod hipofīzē un hipotalamā (Xia and Wikberg, 1996). Saistoties ar MC2R subtipu, AKTH regulē steroidoģenēzi virsnierēs (Hruby et al., 1995). Dabiska MC2R gēna mutācija varētu būt saistīta ar glikokortikoīdu nepietiekamību (Weber et al., 1993). Peļu, bet ne cilvēku, taukaudos atrasta MC2R ekspresija (Chhajlani, 1996; Boston, 1999), kur šis receptora subtips varētu būt iesaistīts stresa izraisītā lipolīzē, ko mediē no hipofīzes izdalījies AKTH (Chhajlani, 1996; Boston, 1999). Cāļiem atrasta MC2R ekspresija gan liesā, gan virsnieru dziedzerī, kas norāda uz receptora papildus funkcijām putniem salīdzinājumā ar zīdītājiem (Takcuchi et al., 1998). MC2R mRNS noteikta arī cilvēka ādā, tādējādi norādot uz šī receptora subtipa un kortikotropīna iespējamu jaunu lomu ādas fizioloģijā (Slominski et al, 1996).

2.2.4. Melanokortīnu receptora 3. subtips (MC3R)

Sākotnēji MC3R tika uzskatīts par 'orfāna' G-proteīna receptoru un tika raksturots kā nezināms receptors, kurš var tikt izmantots kā polimorfisks marķieris cilvēka hromosomai q20 saistībā ar ne-insulīna-atkarīgo *diabetes mellitus* (Bell et al., 1991; Yamada et al., 1991). 1993. gadā MC3R tika identificēts, klonēts un lokalizēts smadzeņu, placentas un zarnu audos ar RT-PCR metodi (Gantz et al., 1993). MC3R ekspresija tika noteikta arī zīdītāju sirdī, cāļu virsnieru dziedzerī, placentā, zarnās, peļu tīmusā un peritoneālos makrofāgos (Chhajlani, 1996; Takeuchi and Takahashi, 1999; Getting et al., 1999). Šis receptora subtips dominējoši ekspresējas CNS struktūrās: *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). Relatīvi augsts MC3R blīvums ir NACC un VTA (Lindblom et al., 1998). Smadzeņu struktūras VTA un NACC pieder mezolimbiskai dopamīnerģiskai sistēmai, kura savukārt piedalās atalgojuma procesu regulācijā, tādējādi MC3R dominante varētu būt interesanta saistībā ar melanokortīnu sistēmas lomu psihiskās slimībās un atkarības veidošanās procesos. Vairākos pētījumos ir atrasts, ka MC3R proteīns sintezējās lielākos daudzumos, nekā tas varētu veidoties, ņemot vērā MC3R mRNS daudzumu, kā arī MC3R ekspresiju *arcuate nucleus* POMK neironos. Tas liek domāt, ka MC3R varētu būt lokalizēts uz projicējošo neironu nervu galiem, tātad – presinaptiski un funkcionēt kā autoreceptors, regulējot MSH peptīdu izdalīšanos no POMK neironiem (Lindblom et al., 1998). Pētījumos atklāts, ka MC3R ekspresijas blīvums embrija attīstības laikā un nobriedušā organismā ir atšķirīgs. MC3R ekspresija uzreiz pēc dzimšanas ir zema, bet tai ir tendence laika gaitā pakāpeniski pieaugt (Xia and Wikberg, 1997). Pēdējie pētījumi parāda, ka anaboliskie androgenie steroīdi izraisa hipotalāmā spēcīgu MC3R blīvuma pieaugumu (*upregulation*). MSH peptīdi var arī stimulēt dzimumuzvedību (Wikberg et al., 2000). Tomēr joprojām MC3R fizioloģiskās funkcijas ir samērā neskaidri definētas. Radioliganda saistīšanās pētījumos noteikts, ka pie MC3R ar visaugstāko afinitāti saistās tieši γ_1- un γ_2-MSH peptīdi.

2.2.5. Melanokortīnu receptora 4. subtips (MC4R)

Nesenie pētījumi ir uzrādījuši MC4R augsto ekspresiju zīdītāju embrijā visā ontoģenēzes attīstības laikā (Kistler-Heer et al., 1998). MC4R ir plaši izplatīts centrālajā nervu sistēmā un pārstāvēts gandrīz katrā smadzeņu rajonā: galvas smadzeņu garozā, talamā, hipotalamā, hipokampā, smadzeņu stumbrā, muguras smadzenēs, *septum lateralis*, *tuberculum olfactorium* un VTA (Mountjoy et al., 1994). Izpētot cilvēka 20 orgānus, MC4R ekspresija perifērajos audos netika konstatēta (Chhajlani, 1996).

Turpretim daudzviet cāļa perifēros audos tika atklāta MC4R ekspresija (Takeuchi and Takahashi, 1998). Tomēr ir pētījumi, kas parāda MC4R iespējamo ekspresiju cilvēka adipocītos (Chagnon et al., 1997). Šis atklājums ir veicinājis plašu pētījumu veikšanu saistībā ar MC4R un aptaukošanos problēmas risināšanu (Wikberg et al., 2000). MC4R gēna kodējošā reģiona mutācijas ir saistītas ar dominantām autosomāli iedzimtām smagām aptaukošanās formām. Hipotalāma un smadzeņu stumbra MC4R ir nozīmīga loma barības uzņemšanas uzvedības kontrolē. Tieša α-MSH vai AKTH-(1-24) ievadīšana hipotalāmā vai intracerebroventrikulāri (ICV) izraisa ievērojamu barības uzņemšanas inhibīciju (Poggioli et al., 1986). α-MSH uzrāda augstāku afinitāti attiecībā pret MC4R. Saistību starp barības uzņemšanu un melanokortīnu receptoriem atklāja 1994.gadā, kad endogēnam *agouti* peptīdam atklāja antagonistisku iedarbību uz MC4R (Lu et al., 1994). Vēlāk atrada, ka MC4R trūkums pelēm izraisa tukluma sindromu, kas līdzīgs *agouti* tukluma sindromam (Huszar et al., 1997). Arī MC4R antagonists HS014 izraisa apetītes palielināšanos žurkām un smagu aptaukošanos (Kask et al., 1998), tādējādi norādot uz MC4R svarīgo lomu barības uzņemšanas un ķermeņa masas kontrolē. Daudzie pētījumi norāda uz MC4R agonistu iespējamo pielietojumu aptaukošanās ārstēšanā, bet MC4R antagonisti varētu būt izmantojami anoreksijas gadījumos. Hroniska morfīna vai kokaīna ievadīšana izraisa MC4R ekspresijas inhibīciju smadzeņu rajonos, kas saistīti ar opiātu atkarību, norādot uz MC4R varbūtējo lomu atkarības procesos (Alvaro et al., 1996). Daži pētījumi norāda uz MC4R nozīmi kardiovaskulārās sistēmas regulācijā, jo α-MSH un AKTH izraisīto arteriālā spiediena paaugstinājumu iespējams noņemt, intracerebroventrikulāri ievadot endogēno MC4R antagonistu - *agouti* signālpeptīdu (Dunbar and Lu, 1999). Iespējams, ka caur MC4R tiek mediēts arī α-MSH neirotrofais efekts: bojāta perifēra nerva saaugšana. (Van der Kraan et al., 1999). Centrālai MC4R aktivēšanai ir būtiska loma luteinizējošā hormona un prolaktīna sekrēcijas regulēšanā (Wikberg et al., 2000). Galvenā īpatnība, kas atšķir MC4R no pārējiem melanokortīnu receptoriem, ir tā zemā saistīšanās spēja ar γ-MSH peptīdiem, bet nedaudz augstāka affinitāte pret β-MSH, salīdzinot ar α-MSH un AKTH (4.tabula.) (Schioth, 2000).

2.2.6. Melanokortīnu receptora 5. subtips (MC5R)

MC5R ir plaši izplatīts perifērajos audos: virsnieru dziedzerī, taukaudos, aknās, nierēs, plaušās, limfmezglos, kaula smadzenēs, tīmusā, krūšu dziedzeros, sēkliniekos, olnīcās, dzemdē, kuņģī, ādā, liesā, zarnās, skeleta muskuļos, kā arī galvas un muguras smadzenēs (Chhajlani et al., 1993; Labbe et al., 1994). Atšķirībā no pārējiem melanokortīnu receptoriem tikai MC5R subtips tika atklāts skeleta muskuļos (Griffon et al., 1994). MC5R bagātīgi ekspresējas eksokrīno dziedzeru (asaru, prostatas, aizkuņģa dziedzera, Harderiana) audu sekretorajā epitēlijā. Atrasts, ka šis receptora subtips stimulē grauzēju tauku dziedzeru funkcijas (Wikberg et al., 2000). Pelēm MC5R iesaistās tauku dziedzera sekrēta veidošanās un izdalīšanās procesos no tauku dziedzera, kā rezultātā kažoks neizmirkst un notiek termoregulācija (Wikberg, 2001). Līdzīgi MC1R arī MC5R saistās ar α-MSH, AKTH, β-MSH un γ-MSH, un šī saistīšanās ir ar daudz zemāku afinitāti nekā šo peptīdu saistīšanās spēja ar MC1R. α-MSH peptīdam, aktivējot MC5R, var tikt stimulēta lipolīze adipocītos. Eksistē sugu atšķirības lipolīzes stimulācijā adipocītos, piemēram, α-MSH ir daudz jūtīgāka ietekme uz trušiem un jūras cūciņām nekā pelēm, žurkām un primātiem (Boston, 1999). α-MSH peptīds stimulē aldosterona sekrēciju virsnieru dziedzerī, un, iespējams, ka šis efekts tiek mediēts caur MC5R. Interesants ir fakts, ka α-MSH stimulē inozitol-trifosfātu un proteīnkināzes C aktivitāti glomerulozajās šūnās, tādējādi norādot, ka virsnierēs MC5R darbojas caur inozitol-trifosfāta signāla transdukcijas ceļu (Kapas et al., 1996). Jāatzīst, ka dažādos organisma audos MC5R funkcijas joprojām paliek neskaidras.

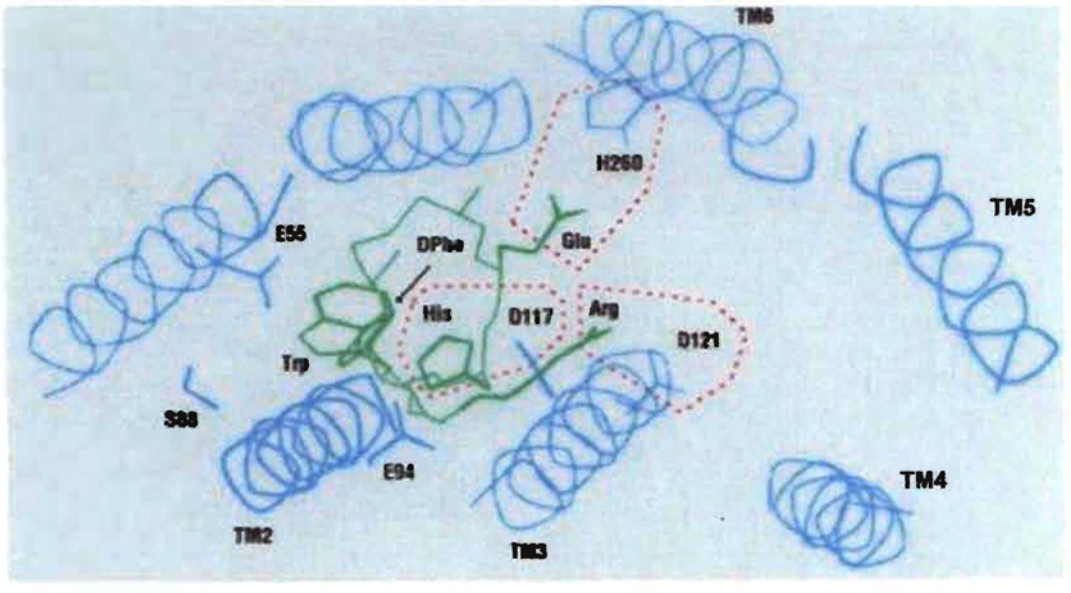
Melanokortīnu receptoru plašā izplatība dažādos audos un atšķirīgā afinitāte attiecībā uz melanokortīnu peptīdiem liecina par MCR daudzveidīgo funkcionālo nozīmi organismā, kuras izzināšanai un noskaidrošanai joprojām nepieciešams veikt vēl daudz pētījumus. Piecu melanokortīnu receptoru subtipu ekspresija dažādos organisma audos attēlota 2. tabulā.

2. tabula. Melanokortīnu receptoru subtipu izplatība audos (Wikberg, 2001)

Receptora subtips	Audi, šūnas
MC1R	Melanocīti, melanomas šūnas, makrofāgi, smadzenes, taukaudi, sēklinieki
MC2R	Virsnieru dziedzeris, taukaudi
MC3R	Smadzenes, placenta, divpadsmitpirkstu zarna, aizkuņģa dziedzeris, kuņģis, sirds
MC4R	Dažādi smadzeņu rajoni
MC5R	Smadzenes, virsnieru dziedzeris, āda, liesa, tīmuss, sēklinieki, olnīcas, muskuļi, plaušas, taukaudi, aknas, dzemde, kuņģis, kaula smadzenes, leikocīti, limfmezgli, piena dziedzeri, vairogdziedzeris

2.2.7. Melanokortīnu receptoru strukturālā uzbūve un signāltransdukcijas ceļi

Melanokortīnu receptori pieder ar G proteīnu saistīto metabotropo receptoru grupai, kam raksturīgi septiņi transmembrānas (7-TM) domēni jeb heptaheliksa struktūra. Ar G proteīnu saistīto metabotropo receptoru grupa ir lielākā 'receptoru ģimene'. Zīdītājos atrasti aptuveni 1000 šīs grupas receptoru tipi. MCR raksturojas ar salīdzinoši īsu aminoskābju sekvenci salīdzinājumā ar pārējiem receptoriem, kas pieder pie G proteīnu saistīto receptoru ģimenes. Cilvēka MC1R, MC2R, MC3R, MC4R un MC5R ir, attiecīgi, 317, 297, 361, 333 un 325 aminoskābju atlikumu sekvenču garumā. MCR ir raksturīgi īsi N-termināles (25-39 aminoskābes) un C-termināles 17-21 aminoskābes rajoni, kā arī ļoti īsa otrā ekstracelulārā cilpa (9 aminoskābes). Visiem MCR subtipiem N termināles domēnos ir vairākas potenciālās N-glikozilēšanās vietas.
MCR C-termināles cisteīnu molekulas nodrošina taukskābju acilēšanos, piesaistot C-termināli pie plazmas membrānas (Tatro, 1996). Tikai pagājušā gadsimta 90. gadu vidū sākās pētījumi par MSH peptīdu piesaistīšanos MCR. Konstatēja, ka α-MSH piesaistei pie MC1R ir būtiskas divas aminoskābes: trešā TM segmenta Asp^{117} (D117) un sestā TM segmenta His^{260} (H260) (Frändberg et al., 1994). Bez tam šīm aminoskābēm ir nozīme kopējās MC1R struktūras uzturēšanā (Schioth et al. 1997a). Lineārie MSH peptīdi ir par kustīgiem, lai panāktu precīzu liganda saistīšanas. Tādēļ tika sintezēta mazu, nekustīgu ciklisku MSH 'serdes' peptīdu sērija, piemēram, [Cys^4, Cys^{10}]α-MSH(4-10).



3. zīm. Kompjūtera modelēšanas trīsdimensiju modelī attēlota MC1R subtipa un cikliskā [Cys^4, Cys^{10}]α-MSH(4-10) mijiedarbība (Prusis et. al., 1997).

3. zīmējumā attēlotie peptīda-receptora saistīšanās modelēšanas dati rāda, ka peptīda saistīšanās notiek 'receptora kabatā', iesaistot gandrīz visus TM domēnus, izņemot TM4 un TM5. Taču neapšaubāmi visi receptora domēni ir būtiski, lai radītu optimālu proteīna konformāciju un nodrošinātu augstu saistīšanās aktivitāti.

Dažādie melanokortīnu receptoru subtipi uzrāda aminoskābju sekvences homoloģiju 40% - 60% robežās (3. tabula). Salīdzinot gēnu homoloģiju atklāja, ka viszemākā homoloģija (38%) ir MC1R un MC2R, turpretī visaugstāko homoloģijas pakāpi (60%) uzrāda MC4R un MC5R. MC2R gēna homoloģijas pakāpe ir samērā līdzīga ar MC3R, MC4R un MC5R (Mountjoy et al., 1994). Pieciem melanokortīnu receptoriem viszemākā aminoskābju sekvences homoloģijas pakāpe atrasta intra-, ekstracelulārajās cilpās un TM4, TM5 domēnos, bet augstāka aminoskābju sekvenču homoloģija noteikta TM1, TM3 un TM7 domēnos.

3. tabula Aminoskābju sekvenču homoloģija (%) pieciem klonētajiem cilvēka melanokortīnu receptoriem (Schiöth et al., 1995; Schiöth et al., 1997)

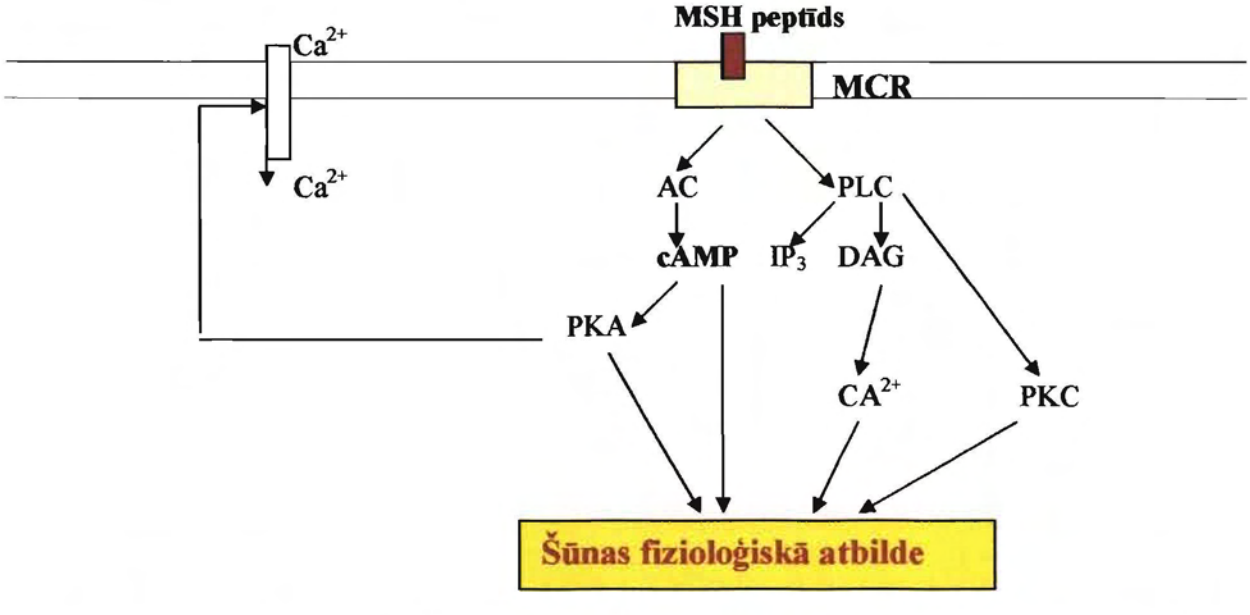
	MC1R	MC2R	MC3R	MC4R	MC5R
MC1R	100	38	45	47	44
MC2R		100	42	46	44
MC3R			100	42	57
MC4R				100	60
MC5R					100

MSH peptīdiem un AKTH piemīt atšķirīga saistīšanās afinitāte pie konkrētā MCR subtipa (4. tabula). Vienīgi AKTH un tā fragmenti (ne citi melanokortīni) saistās ar MC2R. Taču AKTH un tā lielākos fragmentus saista arī pārējie melanokortīnu receptoru subtipi, savukārt AKTH var tikt sašķelts īsākos MSH peptīdos, kuri uzrāda augstāku saistīšanās aktivitāti pie šiem MCR (Schioth et al, 1995).

4. tabula. Ligandu saistīšanās aktivitātes ar melanokortīnu receptoriem (Schiöth et al., 1995; Schiöth et al., 1996; Schiöth et al., 1997)

Melanokortīnu receptoru subtipi	MSH peptīdu saistīšanās tendence ar MCR subtipiem
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

α-MSH diacetilēšanās samazina peptīda afinitāti pret visiem MCR subtipiem, turpretim abas dezacetilētās AKTH un α-MSH formas labāk saistās ar MC3R. α-MSH, salīdzinot ar γ_1-MSH, uzrāda 30 reizes un 2 reizes augstāku saistīšanos ar MC1R un MC5R, attiecīgi. Turpretim γ_1-MSH saistīšanās afinitāte ar MC3R ir 2-3 reizes augstākā nekā α-MSH peptīdam. Ar MC3R vislabāk saistās γ-MSH peptīdi. γ_2-MSH uzrāda līdzīgu vai nedaudz vājāku nekā γ_1-MSH saistīšanās tendenci ar MCR. Atšķirībā no citiem melanokortīnu receptoru subtipiem, MC4R ir zemākā afinitāte attiecībā uz γ-MSH peptīdiem. Tādejādi, α-MSH dominējoši saistās ar MC1R, γ-MSH peptīdi ar MC3R un β-MSH ar MC4R (Schiöth et al., 1995; Schiöth et al., 1996; Schiöth et al., 1997). Visi pieci melanokortīnu receptori stimulē adenilātciklāzi un regulē cAMP veidošanos. Shematiski tas attēlots 4. zīmējumā.



4. zīm. Melanokortīna receptora signāla transdukcijas ceļš, ko ierosina melanokortīnu saistīšanās pie MCR. (Gantz, 1993)
AC, adenilātciklāze; cAMF, cikliskais adenozīnmonofosfāts; DAG, diacilglicerols; IP_3, inozitola 1,4,5-trifosfāts; MCR, melanokortīna receptors; PKA, proteīnkināze A; PKC, proteīnkināze C; PLC, fosfolipāze C.

cAMF veidošanās notiek ātri, sasniedzot maksimumu 15 min laikā. Taču ir arī dati, kuros postulēts MC3R fosfoinozitola signāla transdukcijas ceļš, kura rezultātā veidojas IP_3 (Konda et al., 1994). MC5R varētu būt saistīts arī ar Jak/STAT signāla transdukcijas ceļu (Buggy, 1998). Injicējot cAMF antagonistu pirms α-MSH un AKTH lielās koncentrācijās, iespējams novērst IP_3 samazināšanos un intracelulārā Ca^{2+} līmeņa pieaugumu. Tādejādi, MC3R var būt saistīts ar divām sekundāro 'mesendžeru' sistēmām: PKA/cAMP un IP_3/Ca^{2+} (4.zīm.). IP_3/Ca^{2+} sistēmu kontrolē PKA, iesaistot to fosforilācijas regulēšanā (Wikberg et al., 2000).

2.3. MSH peptīdu bioloģiskie efekti

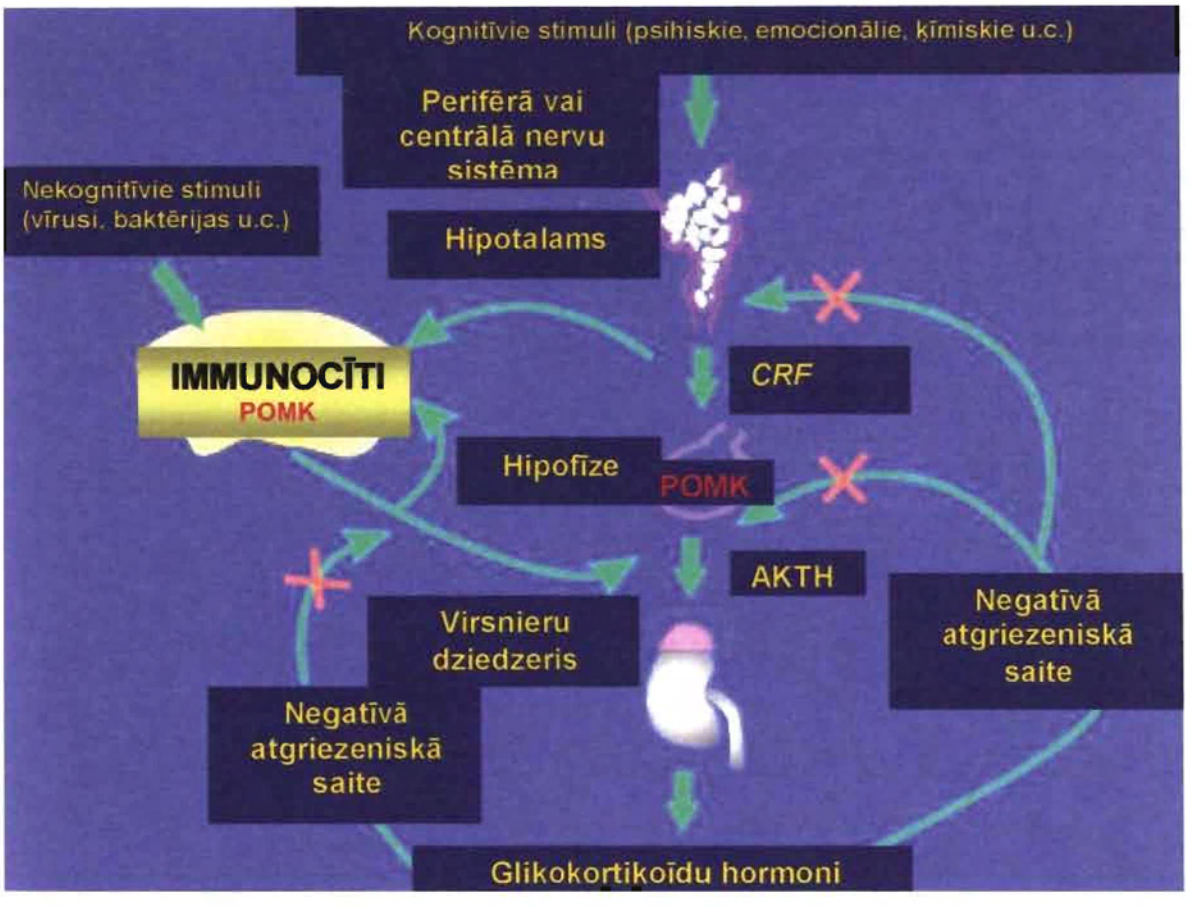
2.3.1. Pigmentācija

Pagājušā gadsimta sešdesmito gadu pētījumi parādīja, ka α-MSH peptīds spēlē būtisku lomu ādas pigmentācijas intensitātes palielināšanā cilvēkiem (Eberle, 1988). α-MSH un AKTH sintezējas cilvēka epidermā kā atbildes reakcija ultravioletās radiācijas ietekmei. Neilgi pēc MC1R subtipa klonēšanas parādījās publikācijas, kurās parādīts, ka MC1R gēns kontrolē zīdītāju apmatojuma krāsu. Populāciju pētījumi atklājuši vairāk nekā 20 alēļu MC1R gēna variantus. MC1R aktivācija stimulē cAMF veidošanos, tālāk notiek pigmentācijas enzīmu (ieskaitot tirozināzi) iesaistīšanās un melanīna veidošanās. MC1R ir arī liela nozīme eumelanīna (melns/brūns) un faeomelanīna (sarkans/dzeltens) pigmentācijas veidošanās regulācijā zīdītāju melanocītos (Schiöth et al., 1999). α-MSH citu sinerģētisku mitogenu faktoru klātienē darbojas kā normālu melanocītu augšanas stimulātors. Tādejādi MSH peptīdiem darbojoties kopā ar citiem faktoriem, ir būtiska nozīme melanocītu šūnu proliferācijas procesos *in vivo* (Halaban, 2000). Melanocītu ļaundabīgā pārveidošanās par melanomu izraisa augšanas autonomiju, kā arī ir saistīta ar neskaitāmu augšanas faktoru un receptoru veidošanos audzēja šūnās. Melanomas audzēja šūnās konstatēts augsts α-MSH imunoreaktivitātes līmenis, un melanoma šūnu līnijas ekspresē 20 reizes augstāku MC1R mRNS, salīdzinot ar normālo melanocītu aktivitāti (Loir et al., 1997). Uz šo brīdi vislabāk ir izpētīta MC1R saistība ar α-MSH, kā rezultātā var rasties ādas pigmentācijas izmaiņas un melanomas tumoroģenēze.

2.3.2. Iekaisuma procesi

Melanokortīni var veidoties no POMK ne tikai hipofīzē, bet arī imūnajās šūnās un līdz ar to var piedalīties centrālās nervu sistēmas, imūnās un endokrīnās sistēmas regulācijā. Imunokompetentās šūnas producē no POMK veidojošos peptīdus kā atbildes reakciju uz nekognitīvu (bakteriālu, virālu) stimulu. Izdalījušies peptīdi tālāk ierosina glikokortikoīdu sintēzi, ietverot imuno-adrenālo asi, tādējādi izraisot imunosupresiju un pretiekaisuma iedarbību (5. zīm.). Pagājušā gadsimta astoņdesmito gadu sākumā atklāja, ka α-MSH un AKTH(1-24) ievadīšana var izraisīt hipotermiju un samazināt leikocītu pirogēna izraisīto drudzi (Glyn and Lipton, 1981). Iespējams, ka α-MSH iedarbība ir tieši uz iekaisuma šūnām perifērijā, kā arī iedarbojoties caur centrāliem mehānismiem. Daudzi iekaisuma procesi, piemēram, neirodeģeneratīvas slimības, smadzeņu insults, iekšējās smadzeņu traumas, encefalīts un baktēriju endotoksīnu izraisītais iekaisums smadzenēs tiek raksturoti ar lokālu citokīnu koncentrācijas, īpaši TNF-α, palielināšanos. α-MSH inhibē imunoregulatoro un pro-iekaisuma citokīnu (piemēram, interleikīnu IL-1α, IL-1β, IL2, interferonu-γ un iekaisuma citokīna tumora nekrozes faktora TNF-α) produkciju un aktivitāti (Luger et al., 1997, 1998; Lipton and Catania, 1998).

Makrocīti, monocīti un tuklās šūnas arī producē α-MSH. Paaugstināts plazmas α-MSH līmenis atrasts arī AIDS un sepses pacientiem (Airaghi et al., 1999; Catania et al., 2000). Melanokortīnu peptīdi būtiski inhibē NO un TNF- α izdalīšanos no mikroglijas šūnām.



5. zīm. No POMK veidojošo peptīdu dalība informācijas pārnesē starp CNS un imūno sistēmu (Blalock et al., 1985).

Melanocīti, keratinocīti, citas ādas šūnas un iekaisuma šūnas ekspresē MC1R. MC1R atrasts kā būtisks receptors, kurš mediē α-MSH pretiekaisuma darbību. Nesen atrasts, ka ne tikai α-MSH molekula, bet arī tās īsāki fragmenti (α-MSH(1-10) un α-MSH(11-13)) var būtiski nomākt NF-kB producēšanos MC1R ekspresējošās makrofāgu šūnu līnijās, kuras apstrādātas ar iekaisuma reaģentiem, piemēram, ar LPS un interferonu-γ (Mandrika et al., 2001). γ-MSH imunoreaktivitāte atklāta arī cilvēka neitrofīlajos granulocītos (Johansson et al., 1991).

2.3.3. Uzvedības reakcijas

Melanokortīnu peptīdi spēj izraisīt plaša spektra uzvedības reakcijas, piemēram, uzmanības, atmiņas un mācīšanās procesu stimulāciju, motoros efektus, seksuālās uzvedības un barības uzņemšanas modifikācijas (De Wied and Jolles, 1982). Pētījumu rezultāti liecina, ka α-MSH un γ-MSH peptīdi var uzrādīt pat diametrāli pretēju centrālu iedarbību. Salīdzinot gan kopējo publikāciju skaitu, gan uzvedības pētījumu publikāciju skaitu par α-MSH un γ-MSH peptīdiem, jākonstatē, ka daudz mazāk ir pētīta γ-MSH peptīdu loma CNS un perifērijā. γ-MSH peptīdu izraisīto uzvedības efektu pētīšana ir viens no šīs disertācijas uzdevumiem.

2.3.3.1. *Grooming* uzvedība

Grooming uzvedība zīdītājiem (piemēram, žurkām, trušiem, kaķiem) izpaužas kā raksturīgs kustību repertuārs ar viegli definējamiem komponentiem, kuri vērsti uz dzīvnieka ķermeņa virsmas motorām aktivitātēm, piemēram, sejas apmazgāšanu, ķermeņa tīrīšanu, ķermeņa aplaizīšanu, kasīšanos un ģenetāliju apmazgāšanu. Jauna, nezināma vide ierosina spēcīgu *grooming* uzvedību mazajiem zīdītājiem un putniem. MSH, AKTH un to fragmenti ievadīšana žurkām izraisa intensīvu *grooming* reakciju. Šī reakcija ir īpaši spēcīga pēc α-MSH intracerebroventrikulāras ievadīšanas. Līdzīgi darbojas arī sintētiskie MCR agonisti NDP-MSH, Melanoton-II (Adan et al., 1999) un γ_1-MSH peptīds (skat. rezultātus). α-MSH un AKTH izraisīto *grooming* uzvedību bloķē ar MCR antagonistiem HS014 un HSU9119 (Schioth, 2000). Ir pētījumi, kas liek domāt, ka *grooming* efekts var tikt mediēts, aktivējot MC4R subtipu (Vergoni et al., 1998). α-MSH vai AKTH injekcija tieši smadzeņu kodolos, piemēram, VTA, hipotalama paraventrikulārajā kodolā, hipotalama dorsomediālajā kodolā un hipotalama priekšējā rajonā arī ierosina *grooming* uzvedības izpausmes žurkām (Argiolas et al., 2000). MSH peptīdu efekts tiek bloķēts, ja smadzeņu kodolos injicē MCR antagonistus.

2.3.3.2. Dzimumuzvedība

Pētījumos ar abu dzimumu žurkām atrasts, ka AKTH un MSH peptīdi spēj ietekmēt dzimumuzvedību. Intracerebroventrikulāra AKTH(1-24) un α-MSH ievadīšana žurku, kaķu, suņu un trušu tēviņiem veicina dzimumtieksmes uzvedības izpausmes pat bez pretējā dzimuma klātbūtnes, piemēram, erekciju, ejakulāciju, ģenetāliju apkopšanu (De Wied and Jolles, 1982). Tikpat nozīmīga ietekme uz dzimumuzvedību (erekcijas biežumu) izpaužas pēc AKTH un MSH peptīdu tiešas injekcijas periventrikulārajā hipotalama rajonā (Argiolas et al., 2000). Ir noteikti trīs galvenie veidi erekcijas neirālai autonomai kontrolei: stimulējošais adrenerģiskais, inhibējošie holīnerģiskais un ar NO mediētais. Turpretim, perifēra α-MSH un AKTH injekcija neizraisa erekciju. Dubultaklā pētījumā cilvēkiem ar erektīvo disfunkciju tika izmantots sintētiskais MCR agonists Melanotan-II, kura pielietošana ievērojami palielināja erekcijas biežumu (Wessells et al., 1998). Taču MCR antagonists HS014 ar mazāku efektivitāti nekā *grooming* pētījumos, tikai daļēji antagonizē α-MSH izraisīto erekciju žurkām (Vergoni et al., 1998), tādējādi norādot uz MSH peptīdu un AKTH iespējami atšķirīgajiem molekulārajiem mehānismiem *grooming* uzvedības un dzimumuzvedības regulācijā. Šobrīd vēl nav skaidrs caur kuru receptoru/iem melanokortīni mediē erekcijas efektu, un arī kāda ir šī efekta saistība ar citiem centrāliem regulātoriem (piemēram, dopamīnu, serotonīnu, acetilholīnu, NO, oksitocīnu un opioīdu peptīdiem).

2.3.3.3. Ar barības uzņemšanu saistītā uzvedība

Pētījumi rāda, ka ar barības uzņemšanu saistīto uzvedību regulē kompleksi mehānismi, kur iesaistās dažādi peptīdi un monoamīni no dažādiem CNS rajoniem. Pazīstamākie regulatorie faktori smadzenēs ir neiropeptīds Y, kortikoliberīns, galanīns, oreksīns, melanīnkoncentrējošais hormons, monoamīni, perifērijā - leptīns un insulīns. Adipocītos veidojošais hormons leptīns veido sasaisti starp perifēriju un centrālo barošanās sistēmu. Pagājušā gadsimta astoņdesmitajos gados tika atklāta melanokortīnu loma apetītes centrālajā regulācijā (Vergoni et al., 1986). Pēdējo gadu pētījumi norādījuši uz centrālās melanokortīnu sistēmas efektiem barošanās uzvedības kontrolē, galvenokārt MC4R subtipa lielo nozīmi apetītes regulācijā. Ir zināms, ka α-MSH un *agouti* peptīds (AGRP) saistās ar MC4R hipotalamā, smadzeņu stumbrā un citos CNS rajonos. Pat četras nedēļas pēc α-MSH ievadīšanas novēroja barības uzņemšanas samazināšanos (Lim et al., 2000).

Toties pēc MC4R *knock-out* pelēm novēro ievērojamu barības patēriņa pieaugumu, ķermeņa masas un izmēru palielināšanos (Huszar et al., 1997). Centrāla α-MSH, AKTH(1-24) un Melanotan-II ievadīšana var izraisīt anoreksīvu, turpretim neselektīvs MC4R antagonists SHU9119 un selektīvs MC4R antagonists HS014 – ievērojamu oreksigēnu efektu (Kask et al., 1998). Selektīvais MC4R antagonists HS014 uzrāda bloķējošu iedarbību attiecībā pret α-MSH radīto anoreksīvo efektu, savukārt SHU9119 antagonizē Melanotan-II efektu (Vergoni et al., 1998). Relatīvi selektīvais MC4R agonists β-MSH inhibē badošanās izraisīto barības uzņemšanu, turpretim relatīvi selektīvais MC3R agonists γ_1-MSH neuzrādīja inhibējošu ietekmi uz barības uzņemšanu (Kask et al., 2000). Tādējādi, vairāku pētījumu dati liecina par MC4R nozīmīgo lomu barības uzņemšanas regulācijā.

2.3.4. Kardiovaskulārie efekti

Melanokortīni izraisa dažādus kardiovaskulāros efektus. α-MSH un AKTH preventē nāves iestāšanos anestezētiem dzīvniekiem tilpuma-kontrolētā hemorāģiskā šoka modelī un ilgstošas elpošanas funkciju apturēšanas apstākļos (Ludbrook and Ventura, 1995). Pretšoka efekta mehānisma pētījumi liecina, ka melanokortīni inhibē TNF-α un NO pārprodukciju šoka apstākļos (Guarini et al., 1997). Taču MC4R antagonists HS014 spēj antagonizēt AKTH(1-24) izraisītos efektus šoka stāvoklī žurkām (Guarini et al., 1999). Šajā modelī γ-MSH peptīdi, kuriem ir augstāka saistīšanās pie MC3R, neizraisīja efektu. α-MSH mikroinjekcija žurku *medulla oblongata dorsal-vagal* kompleksā izraisa hipotenzijas efektu un bradikardijas rašanos, ko bloķē ar MCR antagonistu SHU9119 (Li et al., 1996). Turpretim, α-MSH un AKTH centrāla ievadīšana palielina vidējo arteriālo spiedienu un lumbārā simpātiskā nerva aktivitāti. Šo α-MSH un AKTH efektu antagonizē endogēnais MC4R antagonists *agouti* signālproteīns jeb ASIP (Dunbar and Lu, 1999). Intracerebroventrikulāri ievadīts, γ-MSH izraisa ilgstošu pressoru, šo efektu nespēj antagonizēt SHU9119. Intravenoza γ_1-MSH un γ_2-MSH injekcija normotenzīvām žurkām izraisa no devas atkarīgu īslaicīgu asinsspiediena, sirds ritma un pulsa amplitūdas paaugstināšanos. Peptīdi, kuriem C-terminālē nav Arg-Phe sekvences, piemēram, AKTH(1-24), α-MSH un γ_3-MSH, ir vai nu mazāk aktīvi vai neaktīvi (Van Bergen et al., 1995, 1997). Jādomā, ka melanokortīnu peptīdi iesaistās kardiovaskulārās sistēmas regulācijā gan caur melanokortīnu receptoriem, gan ne-melanokortīnu receptoru mehānismiem.

2.3.5. Citas melanokortīnu funkcijas

Melanokortīnu peptīdiem atklāta arī antagonizējoša iedarbība attiecībā uz opiātu izraisīto atkarību, toleranci un spēja inducēt opiātu abstinences efektus (Szekely et al., 1979; Contreras et al., 1984). Uzskata, ka melanokortīnu izraisītais antagonistiskais efekts attiecībā uz opiātu pašievadīšanu ('self-administration'), analgētisko toleranci un fizisko atkarību var mediēties caur MC4R (Alvaro et al., 1997). Melanokortīni (α-MSH) var uzlabot aksonālo reģenerāciju pēc perifērā nerva bojājuma, un pētījumos *in vitro* un *in vivo* stimulēt neirītu atjaunošanos. Ir arī apstiprinājies, ka α-MSH var pastiprināt sensori-motoro funkciju pēc sēdes nerva bojājuma (Van der Zee et al., 1991). Analizējot MC3R, MC4R un MC5R ekspresiju žurkas muguras smadzenēs, dorsālās smadzeņu saknītēs, sēžas nervā un muskulī *m.soleus*, salīdzinājumā ar pārējiem subtipiem, MC4R tika atrasts tikai muguras smadzeņu lumbālajā nodalījumā (Van der Kraan et al., 1999). Centrāla un perifēra α-MSH ievadīšana samazina endotoksīnu (lipopolisaharīdu), IL-1, IL-6 vai TNF-α izraisīto temperatūras paaugstināšanos (Lipton and Catania, 1998). α-MSH samazina arī išēmijas/reperfūzijas šūnas bojājumus pēc išēmijas un inhibē neitrofīlu uzkrāšanos un NO produkciju. α-MSH var mazināt nieru bojājumus, ko saista ar peptīda spēju inhibēt no neitrofīliem neatkarīgu signāltransdukcijas ceļu nieru bojājumu gadījumā (Chiao et al., 1998). Žurku mātītēm, bet ne tēviņiem, α-MSH palielina luteinizējošā hormona (LH) izdalīšanos (Limone, 1997). Galvenie melanokortīnu (α-, β- un γ-MSH) efekti apkopoti 5. tabulā.

5. tabula. Melanokortīnu izraisītie fizioloģiskie efekti (Eberle, 1988)

PEPTĪDI	MĒRĶA ORGĀNS	EFEKTI
α-MSH	Āda	Stimulē melanoģenēzi melanocītos
α-MSH	Adenohipofīze	Inhibē prolaktīna izdalīšanos Modulē LH izdalīšanos Stimulē hGH izdalīšanos
α-MSH, β-LPH	Tauku dziedzeri	Stimulē ādas tauku sekrēciju
α-MSH, β-MSH	Adipocīti (trušiem)	Stimulē lipolīzi
α-MSH, β-MSH γ_3-MSH	Virsnieru garoza	Stimulē aldosterona sekrēciju Modulē AKTH efektus
α-MSH	Imūnā sistēma	Inhibē IL-1 ierosinātās imūmreakcijas (pretiekaisuma īpašības)
α-MSH, γ-MSH	Sirds asinsvadi	Pastiprina sirds kontrakcijas Paaugstina asinsspiedienu
α-MSH, γ_2-MSH	Nieres	Palielina natriurēzi
α-MSH	Redzes orgāns	Stimulē caurlaidības izmaiņas

2.4. Sintētiskie MCR agonisti un antagonisti

Būtiska nozīme melanokortīnu funkcionālās lomas CNS un perifērijā noskaidrošanai ir melanokortīnu receptoru selektīvu agonistu un antagonistu radīšanai, kā arī šo savienojumu farmakoloģiskai, farmakokinētiskai un toksikoloģiskai izpētei. Jau samērā sen tika dizainēts un pārbaudīts liels skaits lineāru un arī ciklisku MSH peptīdu analogu. Paši pirmie jaunu ligandu meklējumi pamatojās uz to spēju izraisīt ādas tumšināšanās efektu amfībijām un ķirzakām. Šobrīd lielākai daļai no šiem peptīdiem ir vēsturiska nozīme, un tikai dažus no tiem joprojām izmanto melanokortīnu receptoru pētīšanai. Intensīvi tiek meklēti jauni selektīvi MCR ligandi.

2.4.1. Agonisti

Viens no pirmajiem un joprojām visvairāk eksperimentos izmantotiem lineāriem sintētiskiem peptīdiem ir NDP-MSH ([Nle^4-D-Phe^7]α-MSH jeb melanotan-I) (6. tabula). NDP-MSH uzrāda augstu saistīšanās afinitāti pret visiem MCR (7. tabula). NDP-MSH plaši izmanto, radiojodētas formas veidā, radioligandu saistīšanās pētījumos (Schiöth et al., 1995; Schiöth et al., 1996). Pagājušā gadsimta deviņdesmitajos gados tika dizainēti 23 cikliski laktāma peptīdi, kuru molekulās būtisko četru aminoskābju farmakofora (His-Phe-Arg-Trp) L-Phe tika aizvietots ar D-Phe. Viens no šiem peptīdiem, kas tiek plaši pielietots melanokortīnu fizioloģisko efektu izpētē ir metaboliski stabilais MCR agonists melanotan-II jeb MTII (Hadley et al., 1989). Arī melanotan-II neselektīvi saistās pie visiem MCR (Wikberg et al., 2000). Eksperimentāli melanotan-II tika izmantots dzimumtieksmes stimulēšanai un melanīna sintēzes izpētei zīdītājiem. Melanotan-II uzrāda augstu afinitāti attiecībā pret smadzeņu MC4R un var izraisīt apetītes samazināšanos žurkām (Fan et al., 1997). Klīnikā melanotan-II pielieto psihogēnas izcelsmes erektīlas disfunkcijas ārstēšanā (Wikberg et al, 2000).

6. tabula. Nozīmīgāko melanokortīnu receptoru ligandu struktūras (Schioth, 2001; Wikberg, 2001)

Peptīdi	Ligandu struktūras
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	Buturil-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^{5-10} trifluoroacetāts
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

Piezīme. Amino skābju atlikumi, kas cikliskajos savienojumos veido gredzenus tiek pasvītroti ar līniju. Nle, norleicīns; D-Nal, β-(-2-naftil)-D-alanīns; dCl-D-Phe, 3,4-dihloro-D-fenilalanīns; D-Phe, D-fenilalanīns; L-Phe, L- fenilalanīns.

Izmantojot MSH peptīdu saistīšanās metodi, nesen tika radīts superselektīvs MC1R agonists MS05 (6. tabula). Tam samērā līdzīgs analogs ir MS09, kas uzrāda izteiktāku agonista iedarbības spektru, bet ir mazāk selektīvs nekā MS05. Gan MS05, gan MS09 spēj inhibēt iekaisuma mediatora NO produkciju makrofāgu šūnās (Szardenings et al., 2000). Šos savienojumus varētu padziļinātāki pētīt kā potenciālās pretiekaisuma vielas. Pēdējā laikā sintezēts daudzsološs MC4R agonists RO27-3325 (Benoit et al., 2000), taču šis savienojums uzrāda salīdzinoši zemu afinitāte pret cilvēka MC4R (Schiöth et al., 1996). Radioligandu saistīšanās pētījumos RO27-3325 uzrādīja tikai 40% no tās cAMF stimulācijas, ko izraisa α-MSH (Szardenings et al., 2000).

7. tabula. Sintezēto peptīdu saistīšanās afinitāte (K_i) ar cilvēka MC1R, MC3R, MC4R un MC5R (Wikberg et al., 2000)

	MC1R	MC3R	MC4R	MC5R
Agonisti				
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
Antagonisti				
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. Antagonisti

Pēdējā laikā ir dizainēti un sintezēti dažādi melanokortīnu antagonisti. Viens no pirmajiem sintezētajiem MCR antagonistiem bija cikliskais MSH peptīdu analogs SHU9119, kurš darbojas gan kā neselektīvais MC3R/MC4R antagonists, gan daļējs agonists attiecībā pret MC1R un MC5R (Hruby et al., 1995). Tā kā SHU9119 neuzrāda selektivitāti ne pret vienu MCR, tā pielietošana eksperimentālos pētījumos MCR klasifikācijai ir ierobežota. Tālākā ciklisko peptīdu dizainēšana atklāja pirmo selektīvo MC4R antagonistu HS014 (Schiöth et al., 1998b). Taču tas līdzīgi SHU9119 darbojas arī kā MC1R un MC5R agonists un uzrāda antagonistiskas īpašības pret MC3R (Schiöth et al., 1999). Žurkām intracisternāla SHU9119 un HS014 ievadīšana izraisa apetītes un ķermeņa masas palielināšanos (Kask et al., 1999). MC4R antagonists HS014 bloķē leptīna izraisīto ķermeņa svara samazināšanās efektu eksperimentālajiem dzīvniekiem. Cits ciklisks melanokortīnu peptīdu analogs ir HS024, kas atšķirībā no HS014 uzrāda apmēram 10 reizes lielāku saistīšanos ar MC4R. Taču, diemžēl, HS024 darbojas kā antagonists pret visiem MCR (7. tabula) Intracerebroventrikulāri ievadot HS024, tas izraisa no devas atkarīgu barības patēriņa palielināšanos žurkām (Kask et al., 1998). Interesanti ir dati par 1,4-dihidropiridīnu savienojumu cerebrokrastu, kas uzrādījis augstu afinitāti pret MC4R (Jansone et al., in press) norādot, ka cerebrokrasta struktūra varētu optimāli atbilst MC4R saistīšanās vietām.
Nesen sintezētie MCR ligandi lielākoties tiek izmantoti eksperimentālos pētījumos kā MSH peptīdu modeļanalogi. 2002. un 2003. gados tika dizainēti un sintezēti vairāki selektīvi MC3R un MC4R agonisti un antagonisti (Grieco et al., 2002; Kavarana et al., 2002; Balse-Srinivasan et al., 2003). Diemžēl, uz šo brīdi selektīvie MCR agonisti/antagonisti nav vēl komerciāli pieejami. Tas lielā mērā apgrūtina pētījumus.

3. PROMOCIJAS DARBA PĒTĪJUMU MĒRĶIS UN UZDEVUMI

Noteikt melanokortīnu γ_1-MSH un γ_2-MSH izraisīto uzvedības repertuāru un tā neiroķīmisko pamatojumu, un, vismaz daļēji, noskaidrot γ-MSH peptīdu funkcionālo lomu centrālajā nervu sistēmā.

UZDEVUMI

1. Veikt uzvedības un mikrodialīzes eksperimentus laboratorijas dzīvniekiem, salīdzinot γ-MSH peptīdu darbību ar standarta peptīda α-MSH efektiem (peptīdi ievadīti intra-*ventral tegmental area*, intra-cerebroventrikulāri un intracisternāli).

2. Izpētīt melanokortīnu receptora 4. subtipa (MC4R) antagonistu HS014, HS964 un HS131 ietekmi uz MSH peptīdu izraisītajiem centrālajiem efektiem.

3. Izpētīt α-MSH, γ_1-MSH un γ_2-MSH izraisīto centrālo efektu mehānismus, izmantojot dažādu neirotransmiteru receptoru (dopamīna, GABA, glutamāte, opiāta) agonistus un antagonistus kā vielas-analizātorus.

4. MATERIĀLI UN METODIKA

Pētījumos izmantotās metodes sīkāk aprakstītas sekojošās publikācijās (I-VII).

4.1. Dzīvnieki

Eksperimentos izmantoja Wistar līnijas (GRINDEX Eksperimentālo dzīvnieku audzētava, Rīga, Latvija) un Sprague-Dawley līnijas (Beco, Zviedrija) žurku tēviņus svarā 270-350g (attiecīgi, I, II un IV, VII publ.). Analgēzijas pētījumos izmantotos BALB/c peļu tēviņus svarā 20±2 ieguva no Mikrobioloģijas un Virusoloģijas institūta, Rīga, Latvija (V publ.). Fenciklidīna (PCP) un L-amfetamīna (AMP) hiperlokomocijas testos izmantoja BALB/c peļu tēviņus (GRINDEX Eksperimentālo dzīvnieku audzētava, Rīga, Latvija) svarā 19±1g (III publ.). Dzīvnieki tika turēti grupās pa 4 (žurkas) un 8 (peles), konstantā temperatūrā (21±1°C), relatīvais gaisa mitrums 55±10%, mākslīgais gaismas-tumsas diennakts cikls 12:12 stundas (gaisma no 07.00 līdz 19.00). Dzīvniekiem bija pieejama standartizēta sausā barība un ūdens *ad libitum*. Eksperimenti tika veikti diennakts cikla gaismas fāzē starp plkst. 09.00-14.00. Dzīvnieku skaits katrā grupā bija žurkām 4-10 un pelēm 7-9. Visi pētījumi, kuros izmantoja laboratorijas dzīvniekus tika veikti ar Latvijas un/vai Zviedrijas Ētikas komitejas akceptu.

4.2. Eksperimentos izmantotie reaģenti

γ_2-MSH tika pirkts no SIGMA CHEMICALS BACHEM, Šveice. Savienojumi HS964, HS131, HS014 (sintētisko peptīdu struktūras skat. nodaļā 2.10.), α-MSH un γ_1-MSH tika sintezēti Uppsalas Universitātes, Farmaceitiskās farmakoloģijas departamentā, Uppsalā, Zviedrijā. Peptīdus sintezēja, izmantojot 'Fmoc-based Pioneer' peptīdu sintēzes sistēmu (PerSeptive Biosystems) ar cietās fāzes pieeju un attīrīja ar augsta spiediena šķidruma hromatogrāfijas (HPLC) metodi. Precīzu peptīdu molekulmasu apstiprināja ar masas spektometrijas metodi. Peptīdus izšķīdināja sterilā ūdenī un uzglabāja sasaldētu šķīdumu veidā līdz eksperimenta sākumam. Pirms eksperimenta peptīdu šķīdumi tika atlaidināti un atšķaidīti mākslīgajā cerebrospinālajā šķīdumā (CSF) vai fizioloģiskajā šķīdumā līdz vajadzīgajai koncentrācijai. α-MSH izmantoja kā standarta peptīdu.

Dopamīnu (DA) un 3,4-dihidroksifeniletiķskābi (DOPAC) ieguva no SIGMA CHEMICALS (St Louise, ASV). DA un DOPAC pamata atšķaidījumus veica sterilā ūdenī un uzglabāja sasaldētus līdz lietošanas brīdim. Pirms eksperimenta DA un DOPAC pamata atšķaidījumus atlaidināja un veidoja vajadzīgo koncentrāciju atšķaidījumus CSF. CSF sastāvs bija sekojošs: 8.65g/l NaCl; 201.31mg/l KCl; 176.42mg/l $CaCl_2$; 172.81mg/l $MgCl_2$, sterils ūdens (Apoteket, Produktion&Laboratorium, Umeå, Zviedrija). Fenciklidīnu (PCP), L-amfetamīnu (AMP), naloksona hidrohlorīdu, bikukulīnu iegādājās no SIGMA CHEMICALS (St Louise, ASV). Haloperidolu (0,5% šķīdumu) un diazepamu (5% šķīdumu) nopirka no Gedeon Richter, Ungārija, bet muscimolu no Fluka AG, Šveice.

4.3. Uzvedības reakciju pētījumi (I, II, III publ.)

4.3.1. Žurkas

4.3.1.1. Intra-*ventral tegmental area* (VTA) vai intracerebroventrikulāra (ICV) kanulēšana un vielu ievadīšana (I, II publ.)

Intracerebrālās kanulas (garums 15mm, ārējais ∅ 0,56mm) tika izgatavotas no nerūsējoša tērauda šļircu adatām un lēnām implantētas ar stereotakses aparāta palīdzību žurku kreisajā VTA: koordinātes no bregmas: kaudāli -5,2mm, laterāli 0,8mm un ventrāli -8,0mm vai kreisajā laterālajā ventrikulī: koordinātes no bregmas: kaudāli -1,5mm, laterāli 1,0mm un ventrāli -3,6mm (Paxinos and Watson, 1982). Anestēzijā izmantots nembutāls (60 mg/kg, i.p.; CEVA, Sanofi, Francija). Intracerebrālās kanulas implantēja 2mm virs VTA struktūras, ar nolūku, lai mikroinjekcijas adata tiktu ievadīta tieši VTA. Kanulas pie eksperimentālo dzīvnieku galvaskausa fiksēja ar dentālo zobu cementu (De Trey, Sevriton, Vācija), ko pārklāja ar durakrilu (SPOFA Dental, Čehijas Republika). Lai novērstu kanulu aizsērēšanu ar neiroglijas ieaugumiem, tajās tika ievietota ieliekta, smalka nerūsējoša tērauda mandrēna, kuru izņēma pirms vielu ievadīšanas caur kanulu.
Pēc kanulēšanas operācijas katrs dzīvnieks tika turēts atsevišķā būrī ar brīvu pieeju sausajai barībai un ūdenim. Pēcoperācijas atkopšanās periods ilga 7 dienas. Eksperimentos izmantotās α-MSH, HS964, HS014, γ_1-MSH vai γ_2-MSH peptīdu devas bija 0,3 un 3 nmoli/0,5µl/žurkai. Kontroles dzīvnieku grupai tika izmantots fizioloģiskais šķīdums 0,5µl/žurkai. Kombinētās vielu ievadīšanas gadījumā vielas HS964, HS014, γ_2-MSH vai fizioloģiskais šķīdums (kontroles grupai) tika injicētas 15 minūtes pirms α-MSH vai γ_1-MSH. Peptīdus ievadīja manuāli caur kanulu VTA vai ICV, izmantojot 30-kalibra mikroinjekcijas adatu, savienotu ar tievu politelēna caurulīti (PE-10, Clay Adams, Zviedrija), kura bija savienota ar 75 RN Hamiltona digitālo mikrošļirci (Hamilton-Bonadaz AG, Šveice). Kopējais injicējamais tilpums katrai vielai bija 0,5 vai 1µl, ar ievadīšanas ātrumu smadzeņu struktūrā 0,25 µl/min. Pēc injekcijas veikšanas mikroinjekcijas adata tika atstāta kanulā apmēram 5min, lai novērstu vielu noplūdi augšup pa kanulu.

4.3.1.2. Uzvedības reakciju testi (I, II publ.)

Vienu dienu pirms uzvedību testu veikšanas Wistar (300-350g) un Sprague-Dawley līnijas (270-340g) žurku tēviņus pārveda no pēcoperācijas telpas uz uzvedības testu veikšanas telpu, lai dzīvnieki adaptētos jaunai videi, ka arī veica *handling* jeb dzīvnieku pieradināšanu pie eksperimentātora. Eksperimenta veikšanas dienā katru žurku ievietoja uz 30min adaptācijai organiskā stikla būrī (60cm x 40cm x 15cm), kurā pēc pētāmo vielu ievadīšanas novēros žurku uzvedību. Dzīvnieku uzvedības testu uzsāka 5min pēc MSH peptīdu injekcijas VTA vai ICV. Uzvedības reakcijas reģistrēja ar mikroskaitļotāju Psion Workabout (Noldus, Nīderlande) vienas stundas garumā. *Grooming* aktivitāte tika izteikta sekundēs un sastāvēja no atsevišķu *grooming* reakciju (sejas mazgāšanas, ķermeņa apkopšanas, kasīšanās u.c.) summārā ilguma. *Grooming* aktivitātes rezultāti attēloti zīmējumos kā četri reģistrācijas periodi: 0-15min, 16-30min, 31-45min un 46-60min periodi (II publ.) vai summēti kā 0-15min, 0-30min, 0-45min un 0-60min periodi (I publ.). Bez *grooming* uzvedības tika reģistrēta arī žurku vertikālā (VA), horizontālā (HA) aktivitāte un katalepsija. VA un HA tika attēlota kā kopējais lokomociju gadījumu skaits uzvedības testa veikšanas laikā. Katalepsijas testā žurkas novērtēja pirms vielu ievadīšanas un uzvedības testa beigās. Katalepsijas stiprums tika vērtēts trīs secīgos testos, nosakot, cik ilgi dzīvnieks saglabāja nekustīgu ķermeņa pozu ar 10s maksimuma laiku katram testam: (1) dzīvnieku novietoja ar priekškājām uz 7cm augstas barjeras; (2) ar pakaļkājām uz tās pašas barjeras; (3) žurka tika novietoja uz 7cm augstām barjerām starp kurām bija 15cm distance (Kobayashi et al., 1997).

Katram katalepsijas testam tika pieņemts nozīmīguma koeficients 0,2, 0,3 un 0,5, attiecīgi. Kopējā katalepsija tika izteikta kā summārais laiks (s), ko dzīvnieks pavadīja visos 3 testos kopā, pareizināts ar attiecīgo koeficientu (Sanberg et al., 1988).

4.3.1.3. NMDA-toksicitātes tests (nepublicēti dati)

Wistar līnijas žurku tēviņiem (270-340g) injicēja γ_2-MSH (0,3 nmol/0,5µl; intra-VTA) 5min. pirms NMDA (10µg/0,5µl; intra-VTA). Kontroles grupas žurkām intra-VTA ievadīja fizioloģisko šķīdumu vai NMDA. Reģistrēja šādas uzvedības reakcijas: horizontālo aktivitāti, vertikālo aktivitāti, ipsilaterālas un kontralaterālas rotācijas.

4.3.2. Peles

4.3.2.1. Lokomotorās aktivitātes tests (III publ.)

BALB/c peļu tēviņiem (19-21g) intracisternāli (i.c.) ievadīja γ_1-MSH, γ_2-MSH vai MC4R antagonistu HS014 (katrs devā 0,3 nmoli/10µl/pelei). Peptīdi tika izšķīdināti fizioloģiskajā šķīdumā. Fenciklidīnu (jeb PCP; 5 mg/kg; i.p.) vai L-amfetamīnu (jeb AMP; 5 mg/kg; subkutāni) ievadīja 5 minūtes pirms peptīda injekcijas. Kontroles grupas pelēm ievadīja i.p. fizioloģisko šķīdumu 10µl/pelei. Eksperimentālos dzīvniekus ievietoja aparātā (Active Cage, Ugo Basile, Cat. 7400), kur lokomotorā aktivitāte tika reģistrēta no 30. līdz 60. minūtei pēc PCP vai AMP ievadīšanas.

4.3.2.2. Analgēzijas *tail-flick latency* tests (V publ.)

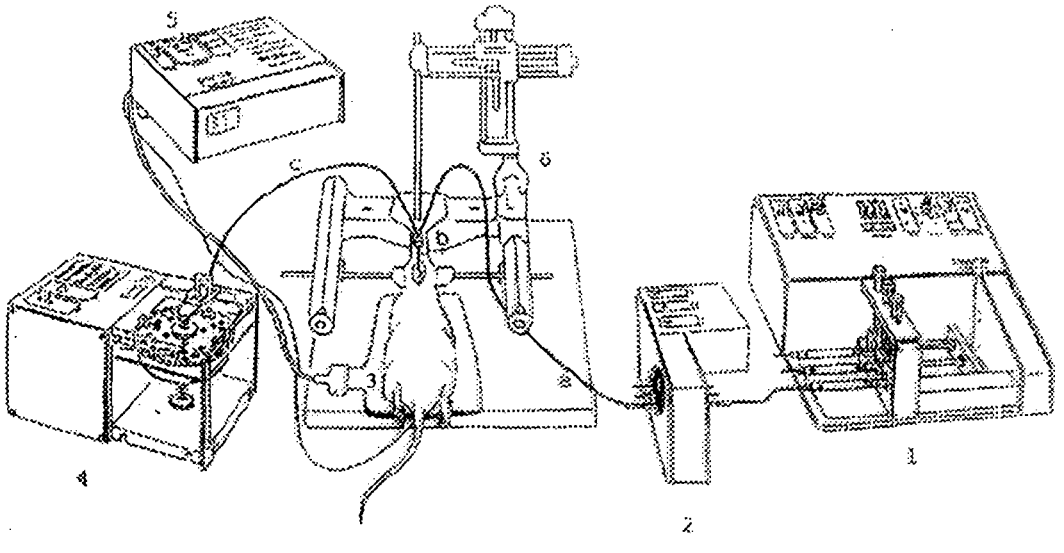
α-MSH, γ_1-MSH, γ_2-MSH un HS014 (katrs devā 0,3, 1 un 3 nmoli/10µl) tika izšķīdināti fizioloģiskajā šķīdumā un ievadīti (i.c. *cisterna magna*) BALB/c peļu tēviņiem caur J-veida adatu, kas savienota ar Hamiltona šļirci (metode aprakstīta Takagi et al., 1979). Fizioloģiskajā šķīdumā izšķīdinātos naloksona hidrohlorīdu (2 mg/kg), haloperidolu (0,5 un 1 mg/kg; 0,5% šķīdumā), bikukulīnu (0,5 mg/kg), diazepāmu (10 mg/kg; 5% šķīdumā), muscimolu (1 mg/kg) un etanolu (10% etanola šķīdums) izmantoja kā vielas-analizātorus un injicēja i.p. pelēm pirms melanokortīnu ievadīšanas. Analgētisko efektu novērtēja ar *tail flick* testu (Dewey, 1981). Peles aste tika novietota uz aparāta (MODEL DS20 SOCREL, Ugo Basile, Itālija) fotoelementa lodziņa. Infrasarkano staru fokusēja uz iezīmētu punktu dzīvnieka astē, orientējoši 2cm no astes pamata. *Tail flick* aparāts fiksēja laika intervālu (s), kurā pele reaģēja uz radīto sāpju stimulu, paceļot asti no infrasarkano staru iedarbības zonas. Lai neradītu audu bojājumus, maksimālais sāpju stimula ilgums nepārsniedza 15 sekundes. Peles *tail flick* testā tika pārbaudītas 30min, 60min, 90min, 2h, 3h un 24h pēc melanokortīnu vai vielu-analizātoru injekcijas.

4.4. Neiroķīmiskie eksperimenti - mikrodialīzes pētījumi anestezētām žurkām (IV, VI, VII publ.)

4.4.1. Ķirurģiskās procedūras

Smadzeņu mikrodialīze ir biogēno paraugu savākšanas metode *in vivo*, kad eksperimentālajiem dzīvniekiem noteiktā smadzeņu struktūrā ievieto mikrodialīzes zondi ar puscaurlaidīgu membrānu zondes galā. Vispārējai anestēzijai žurkām tika izmantots Inaktīns^R (80 mg/kg, i.p.).

Žurkas tika fiksētas stereotakses aparātā (David Kopf Instruments, CA, ASV) un tām nodrošināja konstantu (37C°) ķermeņa temperatūra ar speciālu ķermeņa temperatūru kontrolējošu sistēmu (Temperature Control Unit HB 101/2, Lsi LETICA, Barselona, Spānija). Mikrodialīzes zonde (MAB, Agn Tho's AB, Lidingo, Zviedrija) tika lēnām ievadīta žurkas kreisajā NACC; koordinātes no bregmas: kaudāli +2,2 mm, laterāli -1,5 mm un ventrāli -7,1 mm (Paxinos and Watson, 1982). No NACC savāktajos mikrodialīzes paraugos izanalizēja ektracelulāro DA un tā metabolīta DOPAC koncentrācijas. Mikrodialīzes zondes ārējais ∅ bija 0,6mm, mikrodialīzes membrānas garums 2mm, un tai bija 15 000 Daltons's cutoff PES (Polyethen Sulphone) membrāna. Mikrodialīzes zonde tika perfuzēta ar mākslīgo cerebrospinālo šķīdumu (Apoteket, Produktion & Laboratorium, Umeå, Zviedrija), izmantojot mikrodialīzes sūkni (Univentor 684 Syringe pump, Buledel Industrial Estate, Malta) ar plūsmas ātrumu 2 µl/min. Divas stundas pēc mikrodialīzes zondes implantēšanas mikrodialīzes paraugi tika savākti ar mikrosūkni (Univentor 810 Microsampler, Bulebel Industrial Estate, Malta) polietilēna mikrocentrifūgas mēģenēs ik pa 20 minūšu intervāliem 1 stundas garumā. Savāktie paraugi tika nekavējoties analizēti augsta spiediena šķidruma hromatogrāfā (skat. zemāk). Peptīdu ievadīšanu caur implantētu kanulu VTA uzsāka tad, ja trīs *pamata* mikrodialīzes paraugu DA un DOPAC koncentrācijas svārstības bija <15%. 6. zīmējums attēlo smadzeņu mikrodialīzes eksperimenta veikšanai nepieciešamo aparatūru.



6. zīm. Shematiski attēlota smadzeņu mikrodialīzes aparatūra.
(1) mikrodialīzes sūknis; (2) injekcijas šļirču pārslēdzējs; (3) ķermeņa apsildāmais paliktnis; (4) mikrodialīzes paraugu savācējs; (5) ķermeņa temperatūru kontrolējošais aparāts un (6) stereotakses aparāts ar fiksētu mikrodialīzes zondes sistēmu: (a) mikrodialīzes zondē ieejošā caurulīte; (b) mikrodialīzes zondes fiksētājs un (c) no mikrodialīzes zondes izejošā caurulīte.

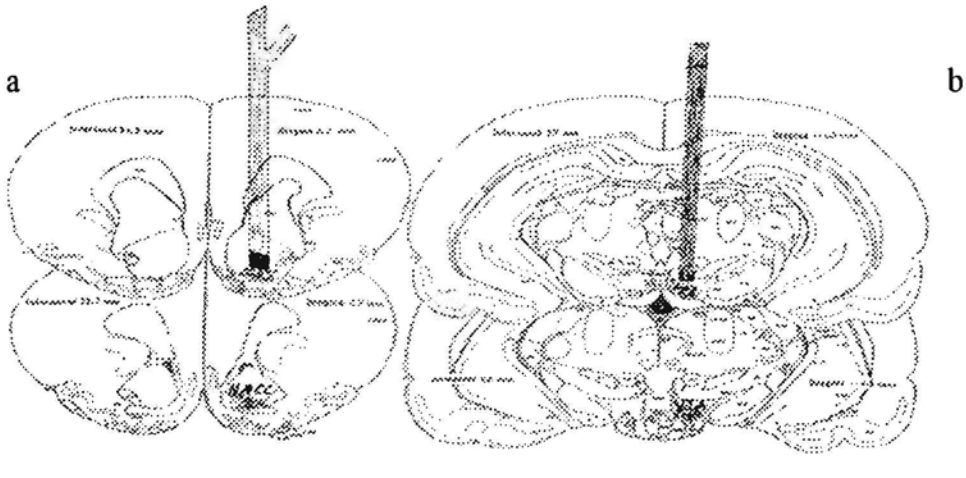
Intracerebrālā kanula (garums 15 mm, ārējais ∅ 0,56 mm) tika implantēta ar stereotakses aparāta palīdzību žurku kreisajā VTA; koordinātes no bregmas: kaudāli -5,0mm, laterāli -0,9mm un ventrāli -7,2mm (Paxinos and Watson, 1982). Detalizētāka VTA kanulēšana aprakstīta 4.3.1.1. α-MSH (10 nmoli/0,5µl/žurkai), γ_1-MSH (3 nmoli/0,5µl/žurkai), γ_2-MSH (3 nmoli/0,5µl/žurkai) un HS131 (1 nmols/0,5µl/žurkai) tika izšķīdināti mākslīgajā cerebrospinālajā šķīdumā un ievadīti žurku VTA. Kombinētās ievadīšanas gadījumā γ_2-MSH vai HS131 tika injicēti 40min pirms γ_1-MSH vai α-MSH, attiecīgi. Kontroles dzīvnieku grupai VTA tika injicēts mākslīgais cerebrospinālais.

4.4.2. Ekstracelulārās dopamīna un DOPAC koncentrācijas noteikšana ar HPLC (augsta spiediena šķidruma hromatogrāfijas) metodi

HPLC metodi ar elektroķīmisko detektēšanu izmantoja, lai noteiktu DA un tā metabolīta DOPAC ekstracelulāro koncentrāciju smadzeņu NACC mikrodialīzes paraugos (metode aprakstīta Gamache et al., 1993). Biogēno monoamīnu atdalīšanai, 40µl mikrodialīzes paraugus injicēja apgrieztās fāzes kolonas (ReproSil-Pur C18-AQ, 150x3mm, daļiņu izmērs 5µm) *Rheodyne* injekcijas ventilī savienotā ar 100µl garu cilpu. Monamīnu oksidēšanai izmantoja kulonometriskās elektroķīmiskās sistēmas divus elektrodus. Elektroķīmiskā detektora (ESA, Inc, Chelmsford, MA, ASV) pirms-injekcijas daļas aizsargšūnas spriegums bija +0,4V (ESA, Guard Cell Modelis 5020), bet analītiskā elektroda spriegums bija +0,34V (ESA, Analytical cell Model 5011). HPLC sistēmas recirkulējošā mobilā fāze sastāvēja no 900ml ūdenī (filtrēta caur Milipora filtru) izšķīdinātiem 2g $CH_3COONa \bullet H_2O$, 38,75mg 1-oktānsulfonskābes, 3,7mg EDTA un 100ml metanola (pH 4). Pēc pagatavošanas mobilā fāze tika filtrēta caur Milipora stikla šķiedras filtru. HPLC sūkņa (LKB 2150 HPLC sūknis, Bromma, Zviedrija) plūsmas ātrums bija 0,6ml/min. Hromatogrammu pierakstīšanai izmantoja Mega sērijas integratoru (Carlo ERBA, Strumentazione, ASV). DA un DOPAC detektēšanas robeža bija 0,4nM.

4.4.3. Histoloģiskā pārbaude

Mikrodialīzes eksperimenta beigās veica histoloģisko pārbaudi, lai pārliecinātos par mikrodialīzes zondes pareizu atrašanās vietu NACC, kā arī par kanulas atrašanās vietu nedaudz virs VTA struktūras. Žurkas tika dekapitētas, smadzeņu audi sasaldēti aukstā (starp -20C° un -30C°) 2-metilbutāna šķīdumā. Sasaldētās žurku smadzenes tika sagrieztas kriostata mikrotoma aparātā (MICROM HM 500 OMV, Laborgerate GmbH, Walldorf, Vācija), plānos (35µm) frontālos griezumos. Smadzeņu audu griezumus piestiprināja uz iepriekš ar želatīnu apstrādātiem priekšmetstikliņiem un iekrāsoja ar Mayer hematoksilīnu (Histolab Products AB, Zviedrija). Iekrāsoto smadzeņu griezumu palielinājumus apskatīja mikroskopā, kas savienots ar video kameru (CCD-72, Dage-MTI, Mičigāna, IN, ASV). Mikrodialīze zondes un kanulas atrašanās vietas pārbaudīja, izmantojot *NIH-Image software* programmu (NIH Image 1,54, NIMH, Bethesda, MD) un žurku smadzeņu atlasu (Paxinos and Watson, 1982) (7. zīm.).



7. zīm. Žurkas smadzeņu frontālie griezumi (pēc Paxinos and Watson, 1982), kuros redzamas mikrodialīzes zondes NACC (a) un kanulas VTA (b) atrašanās vietas attiecīgajās struktūrās.

Statistikas aprēķinos tika izmantoti tikai to eksperimentālo dzīvnieku mikrodialīzes pētījuma rezultāti, kuru mikrodialīzes zonde un intracerebrālā kanula bija precīzi ievadītas atbilstošajā smadzeņu struktūrā.

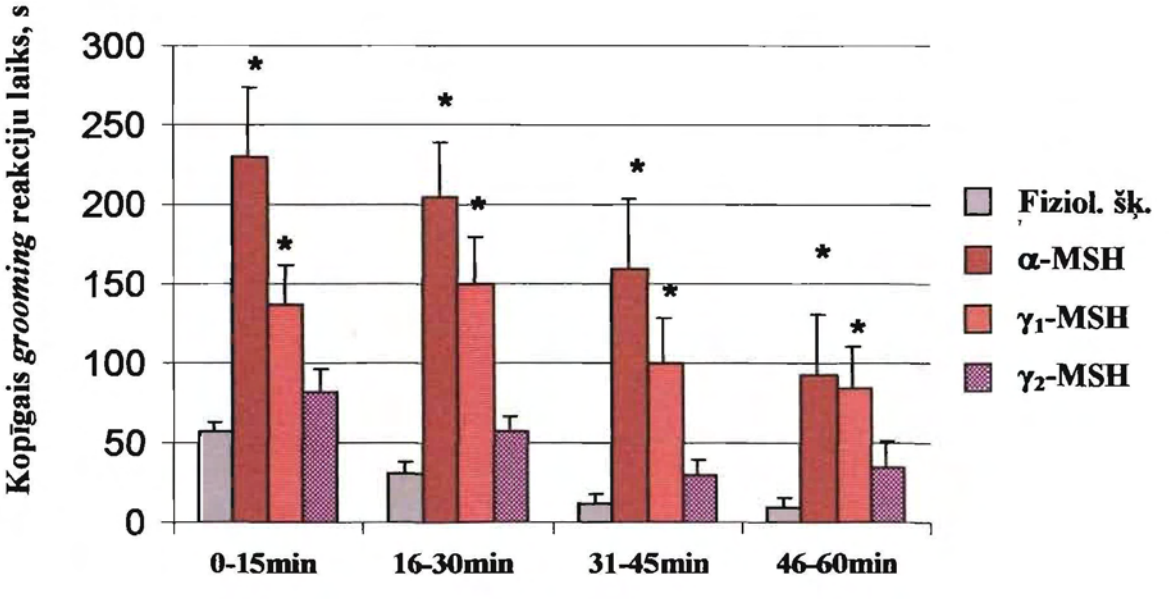
4.5. Statistiskie aprēķini

Uzvedības un analgēzijas pētījumu (I, II, V publ.) datu analizē pielietoja vienfaktora ANOVA testu, un statistisko ticamību starp grupām noteica ar *Newman-Keuls* vai *Bonferoni* testiem. Eksperimentālie dati tika izteikti kā vidējie aritmētiskie lielumi ± standartkļūda (SEM).
Lokomotorās aktivitātes testa rezultātus izteica kā vidējos aritmētiskos lielumus ± standartkļūda (SEM), un ticamības pakāpi izvērtēja ar ANOVA, un ar sekojošu *Student's* testu. Mikrodialīzes pētījumu (IV, VII publ.) datu trīs bazālo paraugu vidējā koncentrācija tika pieņemta kā kontroles vērtība un pielīdzināta 100%. Eksperimentālie dati tika izteikti kā vidējie aritmētiskie lielumi ± standartkļūda (SEM). Mikrodialīzes datu (VII publ.) statistiskai aprēķinu veikšanai izmantoja vienfaktora ANOVA testu. Ticamību starp grupām noteica ar *Newman-Keuls* un *paired t* testiem. Datu analīzei pielietoja statistisko programmu *Prism 3.0* (graph Pad). Starp-grupu statistiskam salīdzinājumam (IV publ.) pielietoja ANOVA testu un *Fisher's* PLSD testu. Statistiskos datu analīzei izmantoja *Macintosh* programmu *StatView 4.51 software*. Statistiski nozīmīgas p-vērtības bija vienādas un zemākas par 0,05.

5.1. Centrāli ievadīto melanokortīnu un to analogu izraisītās uzvedības reakciju pētījumi žurkām (I, II publ.)

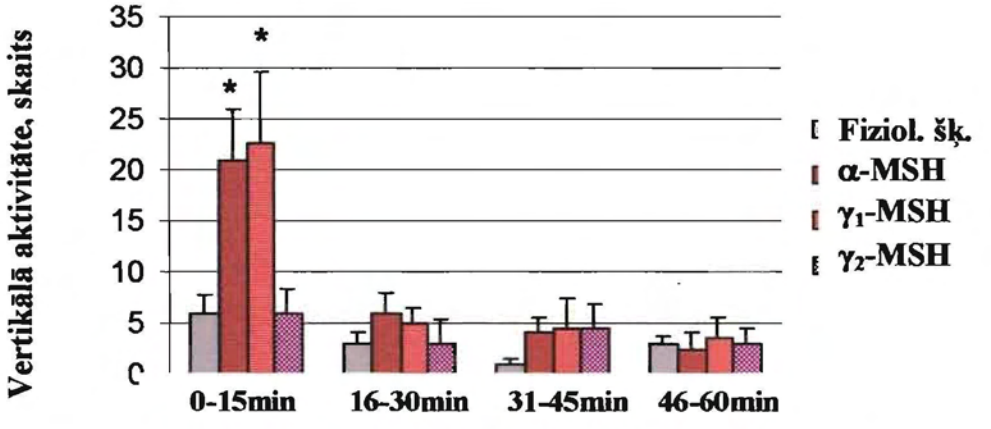
5.1.1. α-MSH, γ_1-MSH un γ_2-MSH efekti (II publ.)

Intra-VTA vai ICV ievadītie melanokortīnu peptīdi (α-, γ_1- un γ_2-MSH) tika pētīti *open field* testā žurkām. Vispirms tika atrasta uzvedības pētījumiem optimālā peptīdu deva. α-, γ_1- un γ_2-MSH ievadīja intra-VTA devās 0,3 un 3nmoli/žurkai. *Grooming* uzvedības reakcijas un vertikālo aktivitāti žurkām reģistrēja 15 minūtes. Tikai α-MSH, bet ne γ-MSH peptīdu mazākā deva izraisīja būtisku *grooming* efekta palielinājumu žurkām (dati nav attēloti zīmējumā). Tā kā izteiktāku efektu uzrādīja peptīdu lielākā deva - 3nmoli, tad šo devu izvēlējāmies turpmākajiem eksperimentiem, lai noteiktu efekta ilguma dinamiku, reģistrējot uzvedības reakcijas (*grooming* un vertikālo aktivitāti) 1 stundas laikā ik pa 15 minūšu intervāliem: 0-15min, 16-30min, 31-45min un 46-60min (8. un 9. zīm.). Gan α-MSH, gan arī γ_1-MSH devā 3nmoli uzrādīja nozīmīgu *grooming* pastiprināšanās efektu pirmajās 15min, un efekts saglabājās līdz uzvedības reakciju reģistrēšanas beigām 1 stundas garumā (8. zīm.). Atšķirīgi darbojās γ_2-MSH (0,3 un 3nmoli), kura efekts neatšķīrās no kontroles dzīvnieku grupas (kurai tika injicēts fizioloģiskais šķīdums) *grooming* uzvedības (8. zīm).



8. zīm. Melanokortīnu α-MSH, γ_1-MSH un γ_2-MSH (visi peptīdi devā 3 nmoli/0,5µl/ žurkai) ietekme uz *grooming* uzvedības reakcijām žurkām vienas stundas laikā pēc peptīdu intra-VTA ievadīšanas. n=8.
* $p < 0,05$ vs kontrole (fiziol. šķ.).

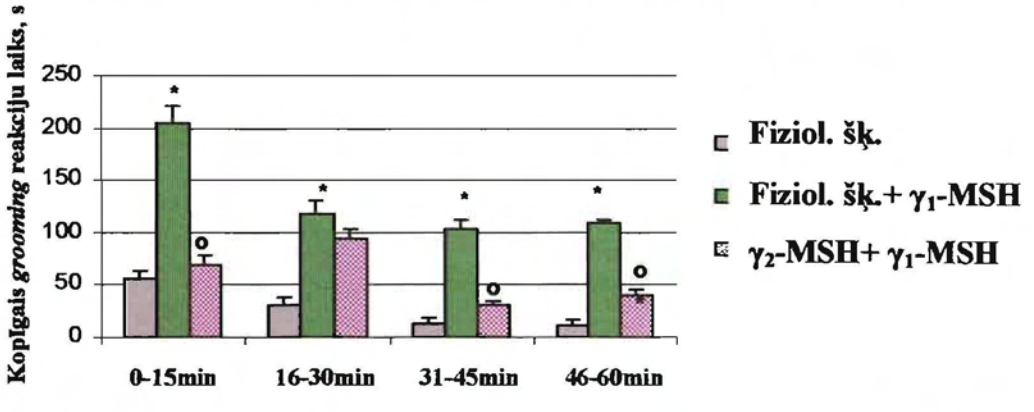
Arī attiecībā pret vertikālo aktivitāti melanokortīnu peptīdi eksperimentālajiem dzīvniekiem uzrādīja dažādus efektus: γ_1-MSH (0,3 un 3nmoli) un α-MSH (3nmoli) intra-VTA būtiski palielināja vertikālo aktivitāti tikai pirmajās 15 minūtēs pēc ievadīšanas, turpretim neviena no γ_2-MSH pārbaudītajām devām neietekmēja vertikālo aktivitāti nevienā no stundas garumā mērītajiem četriem laika periodiem (9. zīm.).



9. zīm. Melanokortīnu α-MSH, γ_1-MSH un γ_2-MSH (visi peptīdi devā 3 nmoli/ 0,5µl/ žurkai) ietekme uz vertikālo aktivitāti (pacelšanās uz pakaļkājām) vienas stundas laikā pēc peptīdu intra-VTA ievadīšanas. n=8.
* $p< 0,05$ vs kontrole (fiziol. šķ.).

5.1.2. Kombinētās ievadīšanas efekti (γ_2-MSH+γ_1-MSH)

Lai noteiktu γ_1-MSH un γ_2-MSH kombinētās ievadīšanas efektus, γ_2-MSH (3 nmoli) vai fizioloģiskais šķīdums tika ievadīts žurkām intra-VTA piecpadsmit minūtes pirms γ_1-MSH (3 nmoli) peptīda. Kontroles dzīvnieku grupa (fiziol. šķ.+γ_1-MSH) uzrādīja nozīmīgu *grooming* uzvedības intensitātes un vertikālās aktivitātes palielināšanos uzvedības testā 1 stundas laika periodā. Toties γ_2-MSH iepriekšēja ievadīšana pirms γ_1-MSH (γ_2-MSH+γ_1-MSH) nozīmīgi antagonizēja gan γ_1-MSH izraisīto *grooming* uzvedību 0-15min, 31-45 un 46-60min laika periodos vienas stundas garumā (10. zīm.), gan vertikālo aktivitāti (dati nav attēloti).

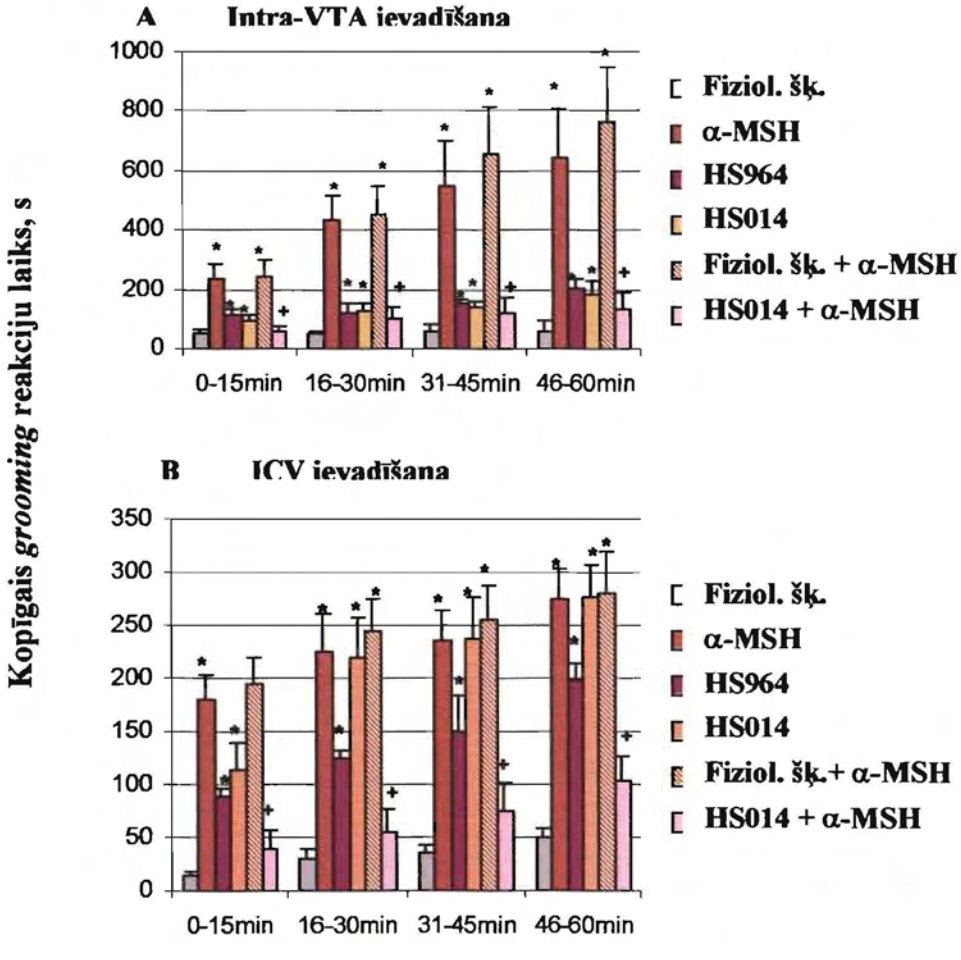


10. zīm. Kombinētā ievadīšana: γ_2-MSH vai fizioloģiskā šķīduma ietekme uz γ_1-MSH izraisīto *grooming* uzvedību žurkām (abi peptīdi devā 3 nmoli/ 0,5µl/ žurkai) vienas stundas laikā pēc intra-VTA ievadīšanas. n=8. * $p< 0,05$ vs kontrole (fiziol. šķ.), $^0p<0,05$ vs fiziol. šķ.+ γ_1-MSH.

Žurkas tika pārbaudītas katalepsijas testā 15min un 1 stundu pēc intra-VTA injekciju veikšanas. Vienīgi γ_2-MSH abās devās (0,3 un 3nmoli) izraisīja katalepsiju. Intra-VTA ievadītā γ_2-MSH izraisītā katalepsijas intensitāte gan pēc 15min, gan pēc vienas stundas atbilda vidēji no četrām līdz piecām ballēm (no teorētiski iespējamajām desmit ballēm). Kombinētās ievadīšanas gadījumā (γ_2-MSH+γ_1-MSH) žurkām konstatēja 3,1 balles intensīvu katalepsiju.

5.1.3. MC4R antagonistu HS964 un HS014 efekti un to ietekme uz α-MSH izraisītiem uzvedības efektiem (I publ.)

Pētījuma mērķis bija noteikt MC4R antagonistu HS964 un HS014 uzvedības reakcijas (*grooming* uzvedību, horizontālo un vertikālo lokomotoro aktivitāti) žurkām, tos ievadot ICV vai intra-VTA. α-MSH tika izmantots kā standartviela. Iegūtie dati liecina, ka gan ICV, gan intra-VTA α-MSH ievadīšana izraisīja nozīmīgu *grooming* uzvedības aktivitātes (vs fiziol.šķ. kontrole) pastiprināšanos jau testa pirmo 15 minūšu laikā (11. zīm.).



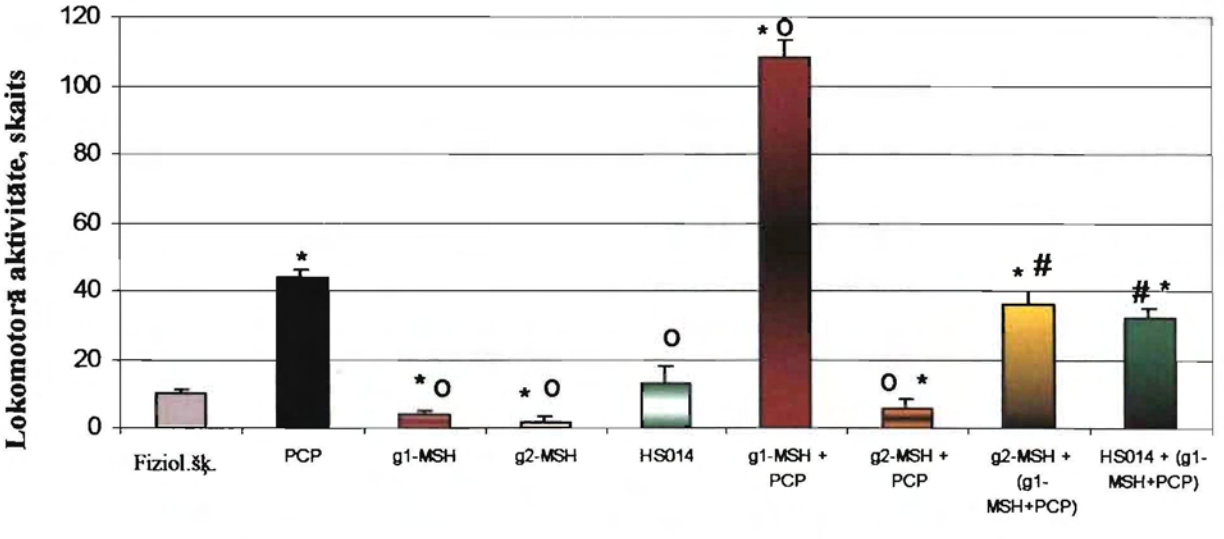
11. zīm. Intra-VTA (A) vai ICV (B) ievadītu α-MSH, HS964, HS014 un kombinēti ievadītu HS014 vai fiziol. šķ. + α-MSH (visi peptīdi devā 3 nmol/0,5µl) izraisītie *grooming* uzvedības efekti žurkām. n=6-10.
*$p< 0.05$ vs fiziol. šķ. kontrole, $^{+}p< 0.05$ vs fiziol. šķ.+ α-MSH.

α-MSH izraisītā *grooming* aktivitāte žurkām pakāpeniski pieauga 1h laika periodā, un šis pieaugums bija izteiktāks pēc peptīda intra-VTA ievadīšanas. Pēc melanokortīnu receptoru antagonistu HS964 un HS014 ievadīšanas žurku VTA un ICV konstatēja salīdzinoši nelielu, vienmērīgu *grooming* aktivitātes pieaugumu. HS014 (salīdzinot ar HS964) ievadīšana ICV izraisīja izteiktāku *grooming* aktivitātes kāpumu nekā peptīda intra-VTA injekcija. Ievadot HS014 piecpadsmit minūtes pirms α-MSH, tika panākta α-MSH izraisītā *grooming* uzvedības efekta samazināšanās gan pēc ICV, gan intra-VTA peptīdu ievadīšanas (11. zīm.). HS964 antagonizēja α-MSH izraisītā *grooming* uzvedības efektu tikai pirmajā 15 minūšu periodā pēc peptīdu ievadīšanas intra-VTA (dati nav attēloti).
Vertikālā aktivitāte žurkām palielinājās pēc α-MSH ievadīšanas intra-VTA, bet ne pēc peptīda ICV ievadīšanas. Vertikālās aktivitātes intensitāte pēc HS014 peptīda ievadīšanas bija līdzīga α-MSH izraisītajam efektam. Ne α-MSH, ne HS964 neietekmēja horizontālo lokomotoro aktivitāti žurkām. Pēc peptīda HS014 ICV ievadīšanas (bet ne intra-VTA) novēroja nelielu horizontālās aktivitātes pieaugumu (lokomotorās aktivitātes dati nav attēloti).

5.2. γ_1-MSH un γ_2-MSH ietekme uz psihoaktivējošo vielu izraisīto hiperlokomociju

5.2.1. Fenciklidīna (PCP) un amfetamīna (AMP) hiperlokomocijas tests pelēm (III publ.)

Intraperitonāla PCP (5mg/kg) injekcija izraisīja hiperlokomociju (horizontālo aktivitāti) pelēm. γ-MSH peptīdi, ievadīti intracisternāli pelēm, uzrādīja atšķirīgu darbību hiperlokomocijas modelī: γ_1-MSH potencēja PCP izraisītos efektus, turpretim γ_2-MSH tos antagonizēja (12. zīm). Kombinētās ievadīšanas gadījumā pelēm gan γ_2-MSH, gan MC4R antagonists HS014 uzrādīja antagonizējošu iedarbību uz γ_1-MSH+PCP izraisītās hiperlokomocijas potencēšanos (12. zīm.).

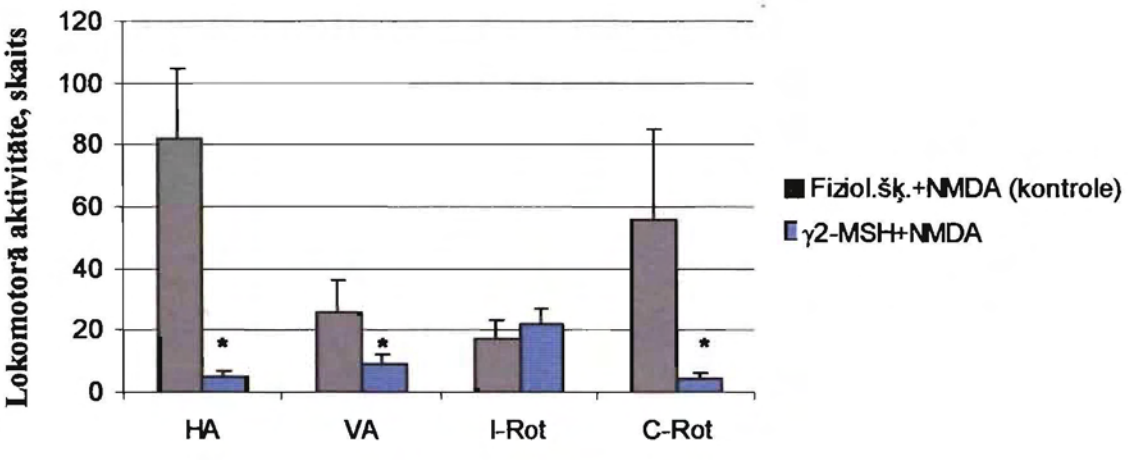


12. zīm. γ_1-MSH, γ_2-MSH un HS014 (peptīdi ievadīti intracisternāli, devā 0,3 nmoli/10μl/pelei) ietekme uz fenciklidīna (PCP) izraisīto hiperlokomociju BALB/c pelēm. PCP (5mg/kg, i.p.) ievadīts 5 min pirms peptīda. Lokomotorā aktivitāte reģistrēta no 30 min līdz 60 min pēc PCP ievadīšanas. n=9.
* $p< 0,05$ vs kontrole (fiziol. šķ.), $^0p< 0,05$ vs PCP, $^{\#}$ $p<0,05$ γ_1-MSH + PCP.

Abu peptīdu - gan γ_1-MSH, gan γ_2-MSH intracisternāla ievadīšana BALB/c pelēm samazināja AMP (5mg/kg, subkutāni, ievadīts 5min pirms peptīda) izraisīto hiperlokomociju (dati nav attēloti).

5.2.2. γ_2-MSH ietekme uz NMDA izraisītām uzvedības izmaiņām žurkām (nepublicēti dati)

Ievadot NMDA (10μg) žurku *ventral tegmental area*, šī aktivējošā aminoskābe izraisīja dramatiskas izmaiņas žurku uzvedībā: palielinājās lokomotorā aktivitāte un parādījās rotācijas. γ_2-MSH intra-VTA ievadīšana 5 minūtes pirms NMDA pilnīgi preventēja NMDA izraisītās neirotoksiskās uzvedības reakcijas (13. zīm.).

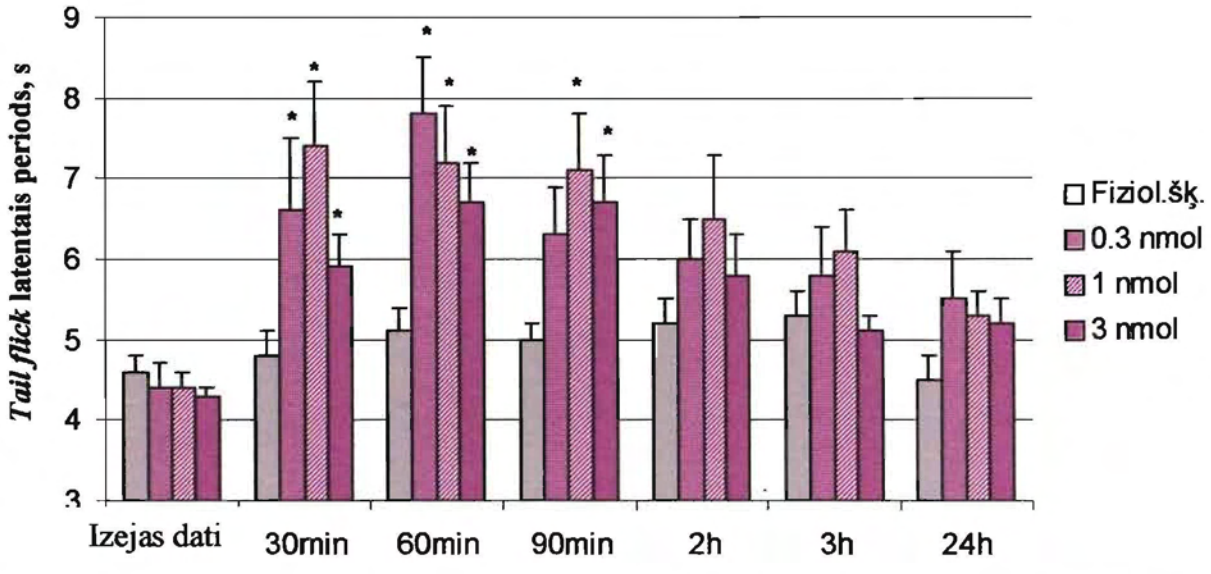


13. zīm. γ_2-MSH (0,3 nmoli/0,5 μl, kreisajā VTA) ietekme uz NMDA (10 μg/0,5 μl/žurkai) izraisīto lokomotoro aktivitāti. NMDA ievadīts 5 min pēc γ_2-MSH injekcijas. n=4.
HA - horizontālā aktivitāte, VA - vertikālā aktivitāte, I-Rot – ipsilaterālas rotācijas, C-Rot – kontralaterālas rotācijas.
* $p < 0,05$ vs fiziol.šķ. + NMDA (kontroles grupa).

5.3. Melanokortīnu analgētisko efektu pētījumi (V publ.)

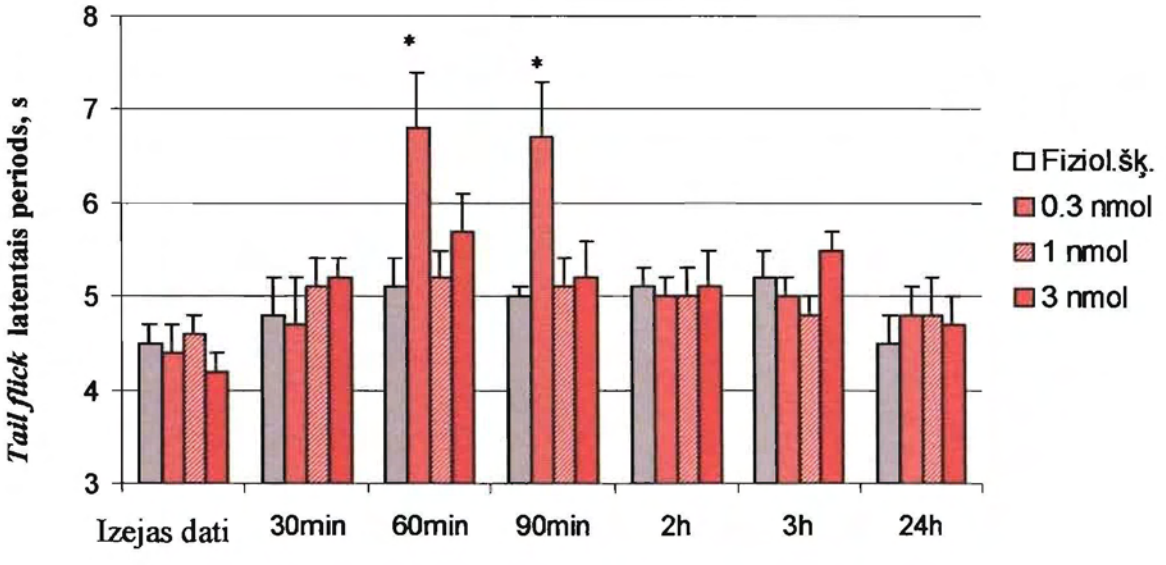
5.3.1. α-, γ_1-, γ_2-MSH un HS014 efekti

Analgēzijas pētījumos α-, γ_1-, γ_2-MSH un MC4R antagonistu HS014 injicēja intracisternāli (i.c.) pelēm. Šajos pētījumos tika izmantotas arī dažādas vielas-analizātori (haloperidols, naloksons, bikukulīns, muscimols, etanols un diazepams), lai noteiktu analgētiskās darbības mehānismus. Analgētisko efektu noteica pelēm *tail flick* testā. Iegūtie rezultāti parāda, ka injicējot pelēm i.c. γ_2-MSH (0,3, 1 un 3 nmoli), statistiski ticami pagarinājās latentais periods no sāpju stimula (infrasarkanais stars) pielikšanas līdz atbildes reakcijai uz to. Šis analgētiskais efekts sasniedza maksimumu pēc 60 minūtēm un bija stabils, ilgstošs līdz 90 minūtēm pēc γ_2-MSH ievadīšanas (14. zīm.).



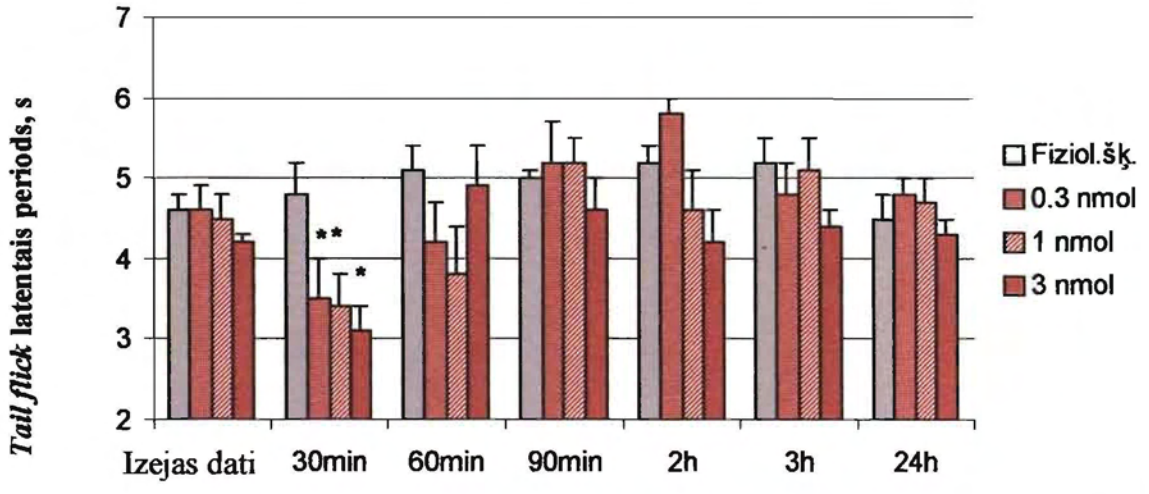
14. zīm. γ_2-MSH (0,3, 1 un 3nmoli, i.c.) izraisītais efekts *tail flick* testā BALB/c pelēm. n=7-9.
* $p < 0,05$ vs fiziol. šķ. (kontroles grupa).

γ_1-MSH analgētisko efektu uzrādīja tikai devā 0,3 nmoli, kad tas izpaudās 60-90min periodā (15. zīm.).



15. zīm. γ_1-MSH (0,3, 1 un 3nmoli, i.c.) izraisītais efekts *tail flick* testā BALB/c pelēm. n=7-9.
* $p < 0,05$ vs fiziol. šķ. (kontroles grupa).

Taču pilnīgi pretēju efektu salīdzinot ar γ_1-MSH un γ_2-MSH, uzrādīja α-MSH. Intracisternāla α-MSH (0,3, 1 un 3 nmoli) injekcija pelēm izraisīja hiperalgēziju 30min mērījumu periodā: latentais periods uzrādīja apmēram par 35% pazeminājumu, salīdzinot ar izejas datiem (16. zīm.).



16. zīm. α-MSH (0,3, 1 un 3nmoli, i.c.) izraisītais analgētiskais efekts *tail flick* testā BALB/c pelēm. n=7-9.
* $p < 0,05$ vs fiziol. šķ. (kontroles grupa).

MC4R antagonista HS014 (0,3, 1 un 3 nmoli) i.c. injekcija pelēm neizraisīja efektu *tail flick* analgēzijas testā (dati nav attēloti).

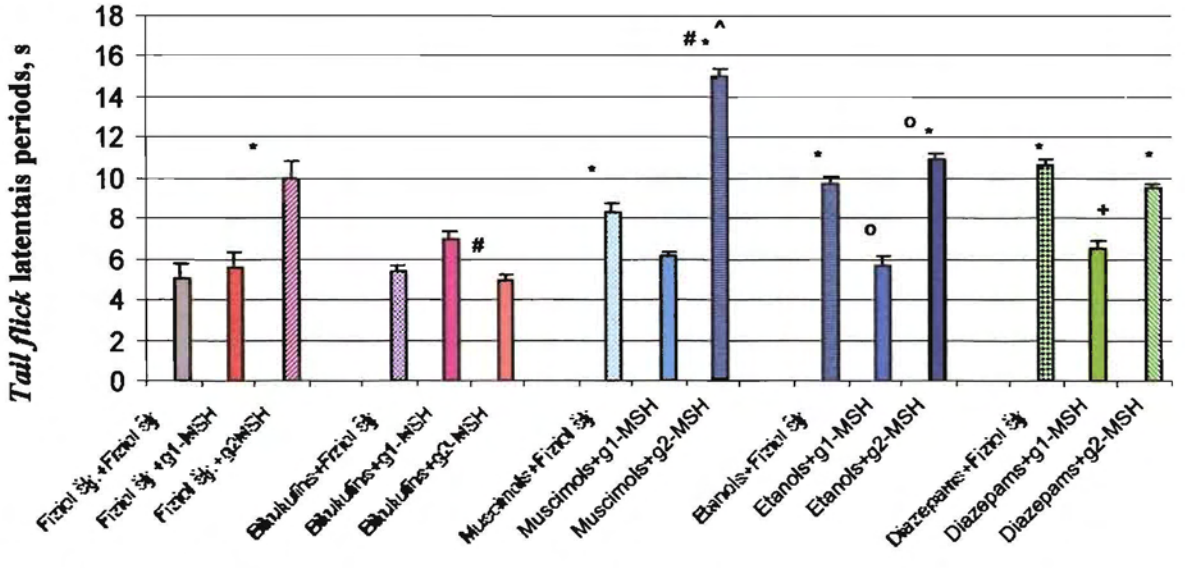
5.3.2. Dažādu vielu-analizātoru ietekme uz γ_2-MSH izraisīto analgētisko efektu

Injicējot pelēm i.c. HS014 (1nmols) vai γ_1-MSH (1nmols) 30 minūtes pirms γ_2-MSH, konstatēja, ka šie peptīdi neietekmēja γ_2-MSH izraisīto analgēzijas efektu *tail flick* testā. Dopamīna receptora antagonists haloperidols (0,5 un 1mg/kg, i.p.) uzrādīja būtisku latentā perioda pagarinājumu *tail flick* testā, taču tas nozīmīgi neizmainīja γ_2-MSH izraisītās latentā perioda izmaiņas.
Opiātu receptora antagonista naloksona (2mg/kg, i.p.) ievadīšana neizraisīja izmaiņas sāpju atbildes latentajā periodā *tail flick* testā, un līdzīgi haloperidolam arī naloksons (naloksons ievadīts 30min pirms γ_2-MSH devā 1nmols ievadīšanas) neietekmēja γ_2-MSH izraisīto analgēziju.
Injicējot pelēm etanolu (4g/kg, i.p.), novēroja izteiktu analgēzijas efektu. Kombinētās ievadīšanas gadījumā etanols (4g/kg, i.p.) tika injicēts 10 min pirms intracisternālas α-MSH, γ_1-MSH vai γ_2-MSH (peptīdi devā 1 nmols) injekcijas. Konstatēja, ka α-MSH būtiski neietekmēja etanola radīto analgēzijas efektu, taču γ_1-MSH nozīmīgi samazināja etanola izraisīto analgēziju. Turpretim γ_2-MSH būtiski palielināja etanola izraisīto analgēzijas efektu (17. zīm.).
$GABA_A$ receptora antagonists bikukulīns (0,5mg/kg, i.p.) *per se* neizraisīja analgēziju pelēm. Kombinētās ievadīšanas gadījumā bikukulīnu ievadīja 5min pirms intracisternālas α-MSH, γ_1-MSH vai γ_2-MSH (peptīdi devā 1nmols) ievadīšanas.

Bikukulīns pilnīgi antagonizēja γ_2-MSH izraisīto analgēzijas efektu. Taču kombinētās ievadīšanas gadījumā bikukulīns neizmainīja ne α-MSH, ne γ_1-MSH efektus *tail flick* testā (17. zīm.).

$GABA_A$ receptora agonists muscimols (1mg/kg, i.p.) pats izraisīja nelielu analgētisko efektu. Kombinētās ievadīšanas gadījumā muscimolu ievadīja 5min pirms intracisternālas α-MSH, γ_1-MSH vai γ_2-MSH (peptīdi devā 1nmols) ievadīšanas. Kombinētās ievadīšanas gadījumā muscimols neizraisīja izmaiņas ne α-MSH, ne γ_1-MSH *tail flick* testa latentajā periodā. Turpretī γ_2-MSH izraisīto analgētisko efektu muscimols nozīmīgi potencēja (17. zīm.). Kā vielu-analizatoru izmantojām arī $GABA_A$ receptora benzodiazepīna saita ligandu diazepamu (10 mg/kg, i.p.). Tas *per se* uzrādīja analgētisko efektu pelēm *tail flick* testā. Kombinētās ievadīšanas gadījumā diazepamu ievadīja 5min pirms intracisternālas α-MSH, γ_1-MSH vai γ_2-MSH (peptīdi devā 1nmols) ievadīšanas un konstatēja, ka α- un γ_1-MSH būtiski samazināja diazepama iedarbību, taču γ_2-MSH neietekmēja diazepama analgētisko efektu.

17. zīmējums attēlo γ_1-MSH, γ_2-MSH analgētisko efektu un to izmaiņas ar $GABA_A$ receptora ligandiem.



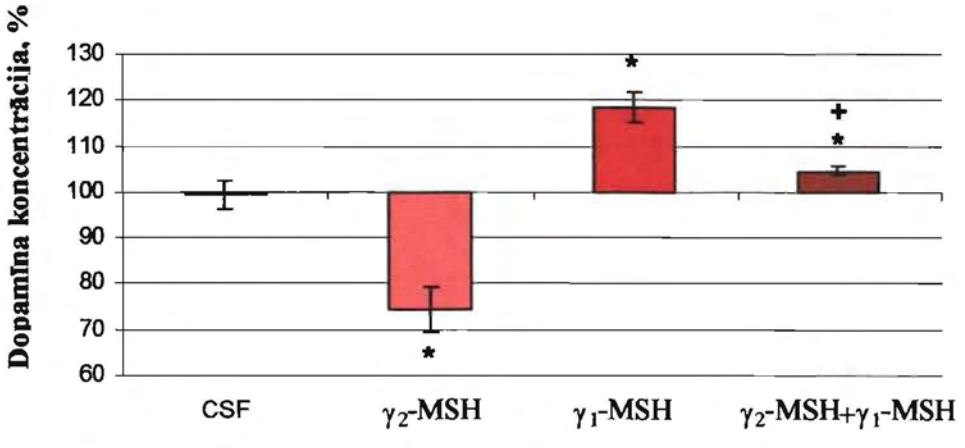
17. zīm. γ_1-MSH un γ_2-MSH (abi peptīdi devā 1nmols, i.c.) analgētiskie efekti un to izmaiņas kombinējot ar bikukulīnu (0,5mg/kg, i.p.), muscimolu (1mg/kg, i.p.), etanolu (4g/kg, i.p.), diazepama (10mg/kg, i.p.) BALB/c pelēm *tail flick* testā. n=7-9

*p< 0,05 vs fiziol.šķ. (kontrole), $^{\#}$ p<0,05 vs γ_2-MSH, ^p<0,05 vs muscimols + fiziol.šķ., ^{0}p<0,05 vs etanols + fiziol.šķ., $^{+}$p<0,05 vs diazepams + fiziol.šķ..

5.4. Intra-VTA ievadīto melanokortīnu un MC4R antagonista HS131 ietekme uz NACC ekstracelulāro dopamīna un DOPAC koncentrācijas izmaiņām anestezētām žurkām mikrodialīzes pētījumos (IV, VII publ.)

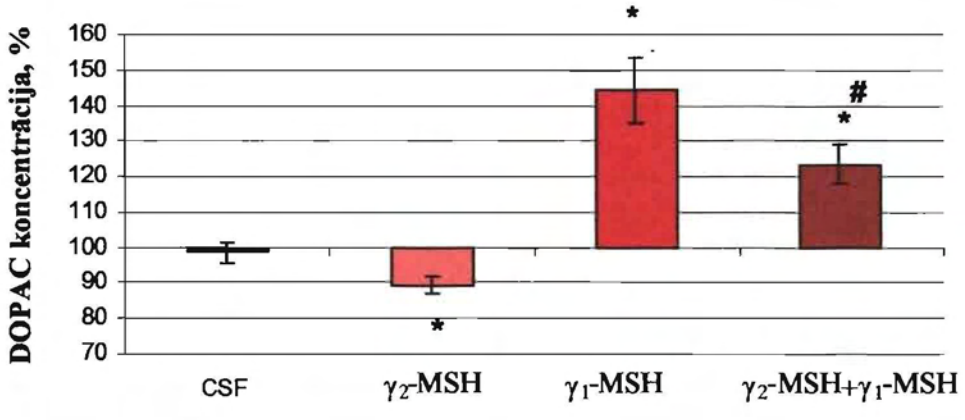
5.4.1. γ_1-MSH un γ_2-MSH efekti (VII publ.)

Tā kā melanokortīnu deva 3nmoli/0,5µl uzrādīja būtiskus efektus uzvedības pētījumos, tā tika izvēlēta arī mikrodialīzes pētījumos. γ_1-MSH un γ_2-MSH (abi devā 3nmoli/0,5µl) tika ievadīti kreisajā VTA anestezētām žurkām, un, izmantojot smadzeņu mikrodialīzes metodi, tika noteiktas ekstracelulārā DA un tā metabolīta DOPAC koncentrācijas līmeņa izmaiņas kreisajā NACC. Mākslīgo cerebrospinālo šķīdumu (CSF) injicēja kreisajā VTA kontroles dzīvnieku grupai. Kombinētās ievadīšanas gadījumā γ_2-MSH ievadīja intra-VTA 40 minūtes pirms γ_1-MSH injekcijas. DA un DOPAC pamata līmenis mikrodialīzes paraugos bija 32,3±0,3fmol/40µl and 24,6±1 nmol/40µl, attiecīgi. CSF injekcija anestezētu žurku kreisajā VTA neradīja izmaiņas NACC ekstracelulārā DA un DOPAC koncentrācijā. Toties γ-MSH peptīdu ievadīšana VTA izraisīja pretējus efektus attiecībā uz ekstracelulārā DA un DOPAC līmeņa izmaiņām NACC. γ_1-MSH izraisīja DA koncentrācijas palielināšanos žurku NACC. Salīdzinājumā ar γ_1-MSH izraisīto monamīna līmeņa palielināšanos, γ_2-MSH ievadīts intra-VTA, pazemināja DA koncentrāciju NACC mikrodialīzes paraugos. Intriģējoši, ka γ_2-MSH iepriekšēja ievadīšana 40min pirms γ_1-MSH (γ_2-MSH + γ_1-MSH), nozīmīgi antagonizēja γ_1-MSH izraisīto DA līmeņa pieaugumu (18. zīm.).



18. zīm. γ_1-MSH, γ_2-MSH un kombinētās ievadīšanas γ_2-MSH + γ_1-MSH (peptīdi 3 nmoli/0,5µl/ žurkai, intra-VTA) ietekme uz ekstracelulārā dopamīna koncentrāciju *nucleus accumbens* anestezētām žurkām. Peptīdu izraisītais efekts ir attēlots procentuāli (±SEM), attiecinot pret dopamīna izejas līmeni (aprēķināts kā vidējais lielums no 3 pamata paraugiem pirms peptīdu intra-VTA ievadīšanas). n=7.
*$p<0,05$ vs CSF (kontroles grupa), $^{+}p< 0,05$ vs γ_1-MSH.

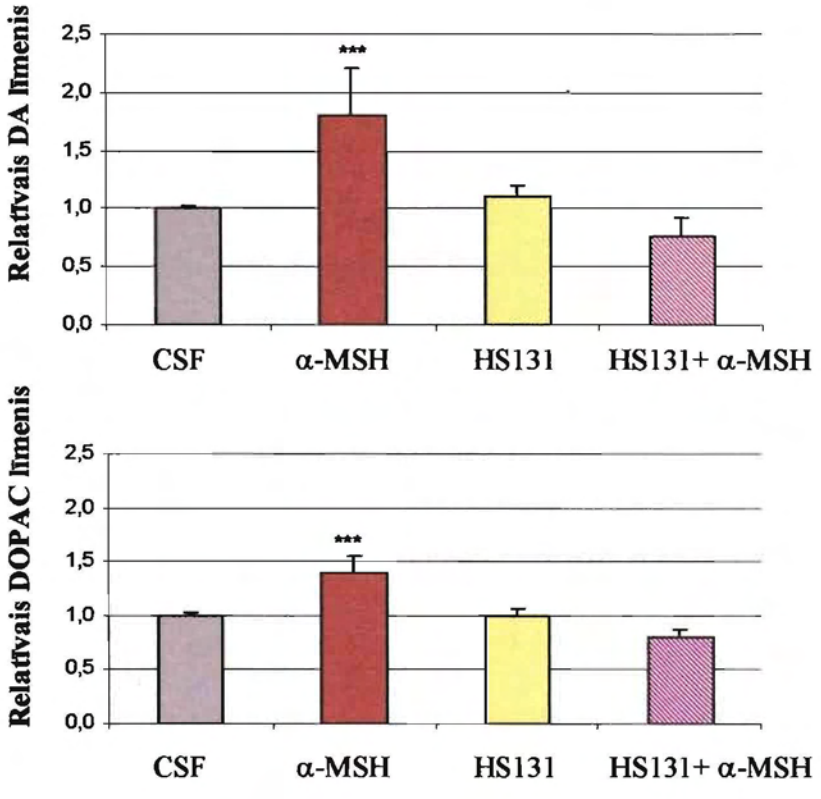
γ_1-MSH ievadīšana kreisajā VTA izraisīja arī ekstracelulārā DOPAC līmeņa pieaugumu NACC, un šī pieauguma intensitāte bija ievērojami lielāka nekā DA līmeņa pieaugums (18. un 19. zīm.). γ_2-MSH intra-VTA ievadīšana, līdzīgi kā DA, pazemināja arī DOPAC koncentrāciju NACC. γ_2-MSH ievadīšana 40min pirms γ_1-MSH (kombinētā ievadīšana γ_2-MSH + γ_1-MSH), nozīmīgi pazemināja γ_1-MSH izraisīto DOPAC pieaugumu (19. zīm).
Mikrodialīzes pētījumu rezultāti, līdzīgi kā uzvedības eksperimentu dati, apstiprina, ka abi γ-melanokortīni uzrāda atšķirīgus, pat pretējus efektus, kā arī var darboties savstarpēji antagonistiski.



19. zīm. γ_1-MSH, γ_2-MSH un kombinētās ievadīšanas γ_2-MSH + γ_1-MSH (visi peptīdi 3 nmoli0,5µl/ žurkai, intra-VTA) ietekme uz ekstracelulārā DOPAC koncentrāciju *nucleus accumbens* anestezētām žurkām. Peptīdu izraisītais efekts ir attēlots procentuāli (±SEM), attiecinot pret dopamīna izejas līmeni (aprēķināts kā vidējais lielums no 3 pamata paraugiem pirms peptīdu intra-VTA ievadīšanas). n=7.
*$p<0,05$ vs CSF (kontroles grupa), #$p<0,05$ vs γ_1-MSH.

5.4.2. MC4R antagonista HS131 efekti mikrodialīzes pētījumos (IV publ.)

MC4R antagonists HS131 (1 nmols/0,5µl) un α-MSH (10 nmoli/0,5µl) tika ievadīti kreisajā VTA anestezētām žurkām un, izmantojot smadzeņu mikrodialīzes metodi, tika noteiktas ekstracelulārā DA un tā metabolīta DOPAC koncentrācijas līmeņa izmaiņas kreisajā NACC. CSF kreisajā VTA tika injicēts kontroles dzīvnieku grupai. HS131 ievadīšana intra-VTA neizmainīja DA un DOPAC līmeni žurku NACC. α-MSH ievadīšana kreisajā VTA izraisīja gan ekstracelulārā DA, gan DOPAC līmeņa palielināšanos. Kombinētās ievadīšanas gadījumā HS131 (ievadīts intra-VTA 40 min pirms α-MSH) pilnīgi antagonizēja α-MSH izraisīto ekstracelulārās DA un DOPAC koncentrācijas paaugstināšanos (20. zīm).



20. zīm. HS131 (1 nmoli/0,5µl/ žurkai, intra-VTA), α-MSH (10 nmoli,0,5µl/ žurkai, intra-VTA) un kombinētās ievadīšanas (HS131 + α-MSH) ietekme uz ekstracelulārā DA un DOPAC koncentrāciju *nucleus accumbens* anestezētām žurkām. Rezultāti izteikti kā DA un DOPAC vidējais lielums (±SEM) 60min pēc peptīdu ievadīšanas attiecinot pret izejas DA un DOPAC datiem. n=4-6.
***p<0,05 vs CSF (kontroles grupa), HS131 un HS131+ α-MSH.

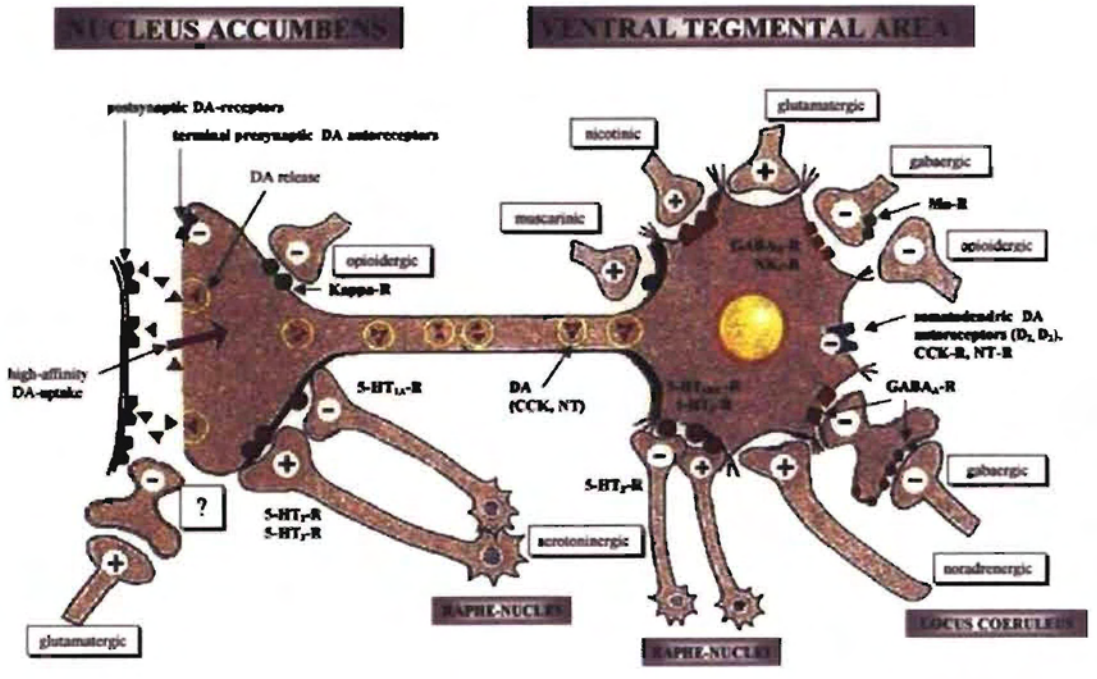
6. DISKUSIJA

Melanokortīnu peptīdi bija vieni no pirmajiem peptīdu hormoniem, kurus atklāja jau pagājušā gadsimta sākumā (Eberle, 1988). Neskatoties uz daudzu gadu pētījumiem, mehānismi, kas regulē melanokortīnu peptīdu izraisītos daudzveidīgos fizioloģiskos efektus gan smadzeņu audos, gan organismā kopumā, vēl joprojām ir samērā maz izprasti. Salīdzinot ar tādiem melanokortīnu peptīdiem kā AKTH un α-MSH, joprojām īpaši maz ir zināms par gamma-melanokortīnu (γ_1-MSH un γ_2-MSH) lomu smadzeņu funkciju regulēšanā.

Pēdējo 5-10 gadu laikā zinātnisko publikāciju skaits, kas veltīts melanokortīnu fizioloģiskās lomas organismā izpētei, ir pieaudzis lavīnveidīgi. Tas noticis, pateicoties pagājušajā gadsimtā (1992.-1993.) izdarītajiem nozīmīgajiem atklājumiem – piecu melanokortīnu receptoru (MCRs) subtipu (MC1R, MC2R, MC3R, MC4R un MC5R) atklāšanai, klonēšanai un raksturošanai. Šie atklājumi tika veikti neatkarīgi divos zinātniskos centros: Upsalas Universitātē (Zviedrijā) prof. J. Vikberga vadībā (Chhajlani and Wikberg, 1992; Chhajlani et al., 1993) un Oregonas Universitātē (ASV) prof. R. Kona vadībā (Mountjoy et al., 1992; Gantz et al., 1993). MCRs ir plaši lokalizēti dažādos audos, taču smadzeņu audos dominē MC3R un MC4R subtipi. Nesenie autoradiogrāfijas pētījumi parādīja, ka MC3R subtips dominē daudzās žurku smadzeņu struktūrās, piemēram, *nucleus accumbens* (NACC), *ventral tegmental area* (VTA), mediālajā preoptiskajā rajonā, ventromediālā hipotalama kodolā (Lindblom et al., 1998). Melanokortīnu receptoru subtipu atrašana smadzeņu audos ļauj daudz mērķtiecīgāk pievērsties melanokortīnu peptīdu izraisīto centrālo efektu pētīšanai. Dažādi melanokortīni uzrāda savu specifisko saistīšanās spēju attiecībā uz noteiktu MCR subtipu smadzenēs. Piemēram, γ_1-MSH ar trīsreiz augstāku efektivitāti nekā α-MSH saistās pie MC3R, bet α-MSH uzrāda 45 reizes augstāku saistīšanos pie MC4R subtipa, salīdzinot ar γ_1-MSH saistīšanās afinitāti (Schiöth et al., 1995, 1996). Peptīda γ_2-MSH saistīšanās pie cilvēka MCR subtipiem ir 2-3 reizes zemāka nekā γ_1-MSH peptīdam (Schiöth et al., 1996). Tomēr neviens no endogēnajiem MSH peptīdiem neuzrāda augstu selektivitāti attiecībā pret MC3R un MC4R subtipiem. Tas, protams, apgrūtina šo receptoru lomas izzināšanu, taču rada nepieciešamību sintezēt selektīvus MCRs antagonistus, lai tos izmantotu MSH peptīdu izraisīto efektu farmakoloģisko mehānismu analīzei. Šobrīd pieejami tikai MCR antagonisti, piemēram HS014, HS964 un HS131, kas ir neselektīvi MC3R/MC4R antagonisti (Schiöth et al., 1998b). 2002-2003. gadā sintezētie augsti selektīvie MC3R un MC4R antagonisti, diemžēl, šobrīd nav komerciāli pieejami (Grieco et al., 2002; Balse-Srinivasan et al., 2003).

Šajā darbā mūsu interese bija fokusēta uz γ_1-MSH un γ_2-MSH funkcionālās lomas izpēti, ņemot vērā šo peptīdu augsto saistīšanās spēju ar MC3R subtipu (Schiöth et al., 1995) un MC3R augsto ekspresijas pakāpi VTA. Kā zināms, VTA sastāv no A10 mezolimbiskajām dopamīnerģiskām šūnām, kuru aksoni projicējas NACC, kas ir galvenā šo neironu terminālā struktūra. A10 dopamīnerģisko šūnu vispārējās aktivitātes pastiprināšanās veicina dopamīna izdalīšanos NACC struktūrā. Arī bioloģiski aktīvas vielas, kas laboratorijas dzīvniekiem izraisa hiperlokomociju un pastiprinātas, stereotipiskas uzvedības izpausmes (piemēram, izpētes reakcijas, *grooming*) ir saistītas ar pastiprinātu dopamīna izdalīšanos. Dopamīna izdalīšanās NACC ir nozīmīgs faktors arī kognitīvo, emociju un atalgojumu procesu regulācijā (Spanagel et al., 1992). Tiek uzskatīts, ka viens no faktoriem šizofrēnijas izcelsmē ir dopamīnerģiskā hiperaktivitāte VTA-NACC signālu vadīšanas ceļā, kas var izraisīt psihozes, paranoidālas mānijas u.c. Citoarhitektoniski VTA struktūra ir ļoti komplicēta, jo tā ietver arī citu sistēmu, piemēram, GABA, glutamāta, opiātu vai holīnerģisko, ietekmi uz VTA dopamīna neironiem (21. zīm.). VTA A10 dopamīnerģiskās šūnas aktivitāti ietekmē neskaitāmi interneironi, kas var modulēt dopamīna izdalīšanos NACC (Spanagel and Zieglgansberger, 1997).

Šī iedarbība var būt gan ar aktivējošo aminoskābi – glutamātu, kas iedarbojoties uz NMDA un/vai ne-NMDA receptoriem, gan ar inhibējošo aminoskābi – GABA, kas saistoties ar $GABA_A$ receptoriem, izraisa inhibējošu ietekmi uz DA šūnu aktivitāti (21. zīm).



21. zīm. Mezolimbiskā dopamīnerģiskā sistēma: VTA DA neirona aktivitāte un DA izdalīšanās NACC tiek modulēta ar aktivējošo un inhibējošo interneironu izdalītajiem neirotransmiteriem (Rommelspacher H, 1997).

Ņemot vērā komplekso VTA-NACC regulējošo sistēmu un augsto MC3R un MC4R ekspresiju šajās struktūrās, kā arī to, ka γ-MSH peptīdi vislabāk saistās ar MC3R salīdzinājumā ar citiem MCR subtipiem, mūsu pētījumiem tika izvēlēti γ_1-MSH un γ_2-MSH peptīdi. Pētījumu aktualitāti pamato arī tas, ka par γ-MSH peptīdu funkcionālo lomu vēl joprojām nav īsta priekšstata.
Šajā darbā par galveno uzdevumu izvēlējāmies pētīt minēto peptīdu uzvedības reakciju spektru un to neiroķīmiskos mehānismus. γ_1-MSH un γ_2-MSH, kā arī MC4R agonists α-MSH un MCR antagonisti tika ievadīti eksperimentālo dzīvnieku smadzenēs, galvenokārt VTA struktūrā. α-MSH tika izmantots kā standarta peptīds.

6.1. MSH peptīdu uzvedības repertuārs

Uzvedības pētījumu rezultāti rāda, ka α-MSH ievadīšana žurku VTA izraisīja pastiprinātu *grooming* uzvedību. *Grooming* uzvedība raksturojas kā laboratorijas dzīvnieka aktivitāte, kas vērsta uz ķermeņa virsmas apkopšanu (sejas mazgāšanu, ķermeņa apkopšanu, kasīšanos u.c.). No agrākām zinātniskām publikācijām redzam, ka α-MSH peptīda injekcija ICV var izraisīt intensīva *grooming* uzvedību grauzējiem (Gispen et al., 1975; Torre and Cellis, 1988). Mūsu rezultāti apstiprināja, ka arī intra-VTA α-MSH ievadīšana izraisa *grooming* uzvedību, pie kam peptīda izraisītais *grooming* efekts ir daudz spēcīgāks nekā pēc peptīda ICV injekcijas. MC4R antagonista HS014 intra-VTA ievadīšana pirms α-MSH bloķē α-MSH izraisīto intensīvo *grooming* uzvedību, kas norāda, ka α-MSH izraisītā *grooming* uzvedība, iespējams var realizēties caur MC4R subtipu.

Citu pētnieku publikācijas rezultāti pamato šo domu, jo cits MC4R antagonistiskās darbības peptīds ACTH(4-10) spēj bloķēt ICV ievadīta α-MSH izraisīto *grooming* uzvedību (Adan et al., 1997). Mūsu pētījumi parādīja, ka arī MC4R antagonists HS014 spēj samazināt ICV ievadīta α-MSH intensīvo *grooming* uzvedību. Pārsteidzoši, bet intra-VTA un ICV ievadīti MC4R antagonisti (HS014 un HS964) paši uzrāda nelielu *grooming* uzvedības pastiprināšanos, palielinātu vertikālo un horizontālo aktivitāti. Izteiktāku *grooming* uzvedības efektu MC4R antagonisti HS014 un HS964 uzrādīja pēc ICV, nevis intra-VTA injekcijas. Šo fenomenu grūti izskaidrot, taču MC4R antagonistu duālistiskā darbība (arī agonisms!) ir nenoliedzams. Tas varētu būt gan kā iedarbības rezultāts MC4R līmenī, gan arī kā cita – tieša vai netieša mijiedarbība ar dažādām nemelanokortīnerģiskajām sistēmām.

Salīdzinot abu γ-MSH peptīdu uzvedības spektru ar α-MSH darbību, esam atraduši, ka γ_1-MSH izraisītais *grooming* pastiprināšanās efekts bija nedaudz vājāks nekā α-MSH izraisītais. Abi peptīdi pastiprināja ne tikai *grooming* uzvedību, bet arī vertikālo aktivitāti. Intriģējoši, ka γ_2-MSH uzrādītie uzvedības efekti žurkām bija diametrāli pretēji tiem, ko izraisa α-MSH un γ_1-MSH: intra-VTA ievadot γ_2-MSH, nenovēroja intensīvu *grooming* uzvedību un vertikālās aktivitātes pieaugumu. Atšķirība izpaudās arī tajā faktā, ka tikai γ_2-MSH, bet ne γ_1-MSH un α-MSH, izraisīja vidēji spēcīgu, ilgstošu katalepsijas stāvokli eksperimentālajiem dzīvniekiem. Agrākās zinātniskās publikācijās atrodam, ka intracerebroventrikulāra γ_2-MSH injekcija neizraisa *grooming* uzvedības izpausmes (Van Ree et al., 1981). Vēl jo vairāk, mūsu pētījumu rezultāti pirmo reizi atklāja, ka γ_2-MSH, kas ievadīts intra-VTA pirms γ_1-MSH peptīda, spēja antagonizēt γ_1-MSH izraisīto *grooming* uzvedību un vertikālo aktivitāti. Dažādie γ_1-MSH un γ_2-MSH uzvedības efekti bija ļoti pārsteidzoši, jo strukturāli abu peptīdu vienīgā atšķirība ir viena papildus glicīna molekula γ_2-MSH peptīda C terminālē. Iespējams, ka tieši šis kustīgais glicīna atlikums γ_2-MSH molekulā var izmainīt peptīda konformāciju un tādējādi arī peptīda farmakoloģiskās īpašības.

Mūsu pētījumos iegūtās melanokortīnu inducētās ietekmes uz lokomociju un stereotipiskām uzvedības izpausmēm ļauj spriest, ka šie peptīdi var ietekmēt mezolimbiskās sistēmas dopamīnerģisko aktivitāti. α-MSH un γ_1-MSH izraisītais *grooming* pastiprināšanās efekts lielā mērā ir līdzīgs dopamīna receptora agonista amfetamīna stereotipiskai uzvedībai, bet γ_2-MSH katalepsija – dopamīna antagonista haloperidola katalepsijai. Tādējādi, γ_1-MSH darbojas kā psihoaktivējoša viela, bet γ_2-MSH uzrāda anti-psihotisku raksturojumu. Fenomens, ka γ_2-MSH darbojas kā γ_1-MSH antagonists, iespējams, atspoguļo šo abu peptīdu funkcionālo lomu smadzeņu funkcionālās aktivitātes regulēšanā: γ-MSH peptīdu pretējā darbība var nodrošināt sabalansētu psihoaktivācijas stāvokli mezolimbiskās sistēmas neironālajos ceļos. Šobrīd gan vēl pāragri spriest, vai MSH peptīdu izraisītie uzvedības efekti ir tiešas mijiedarbības ar zināmajiem MCR rezultāts, vai iesaistīts ir vēl kāds līdz šim nezināms MCR subtips, vai arī melanokortīnu uzvedības efektu izpausmēs ņem dalību citas neirotransmiteru sistēmas. Agrākās publikācijās atrodam datus, ka γ_1-, γ_2- un γ_3-MSH, bet ne α-MSH spēj aizkavēt [^{3}H]-naloksona saistīšanos pie smadzeņu opiātu receptoriem, bez tam γ_2-MSH uzrāda zemāku afinitāti nekā γ_1-MSH (Oki et al., 1980). Tomēr γ-MSH peptīdu afinitāte attiecībā uz opiātu receptoriem ir 1000-kārt zemāka nekā pret MC3R. Tādējādi, ir maz ticams, ka opiātu receptori ir iesaistīti intra-VTA ievadīto γ-MSH peptīdu izraisītajās uzvedības reakcijās. Samērā nesen veikts interesants atklājums izmantojot autorodiogrāfisko metodi Upsalas Biomedicīniskā centrā, kas norādīja uz vēl līdz šim nezināma MCR klātbūtni VTA struktūrā (Lindblom et al., 1998).
Mūsu veiktajos uzvedības pētījumos iegūtie dati norādīja uz tālāku pētījumu nepieciešamību, lai noskaidrotu neiroķīmiskos mehānismus, kas ir MSH peptīdu atšķirīgo uzvedības izpausmju pamatā. Nākošā nodaļa sniedz datus par veiktajiem neiroķīmiskajiem pētījumiem.

6.2. Dopamīnerģiskā komponenta neiroķīmiskais pamatojums

Lai pierādītu MSH peptīdu (α-MSH, γ_1-MSH un γ_2-MSH) ietekmi uz mezolimbisko DAerģisko sistēmu, izmantojām smadzeņu mikrodialīzes metodi anestezētām žurkām. α-MSH, γ_1-MSH, γ_2-MSH un MC4R antagonists HS131 tika ievadīti anestezētu žurku VTA struktūrā, pēc tam nosakot NACC dopamīna (DA) un tā metabolīta DOPAC ekstracelulāro koncentrāciju mikrodialīzes paraugos. Mikrodialīzes pētījumu rezultāti parādīja, ka intra-VTA α-MSH un γ_1-MSH ievadīšana izraisīja ekstracelulāro DA un DOPAC koncentrācijas paaugstināšanos NACC. α-MSH ievadīšana izraisīja nedaudz lielāku ietekmi uz dopamīna līmeņa izmaiņām nekā DOPAC gadījumā. Turpretim γ_1-MSH (kas līdzīgi kā α-MSH izraisīja izteiktu *grooming* uzvedību žurkām) uzrādīja daudz izteiktāku ietekmi uz dopamīna metabolīta DOPAC izdalīšanās paaugstinājumu NACC mikrodialīzes paraugos, norādot uz γ_1-MSH spēju ierosināt spēcīgus un ilgstošus DA metabolisma procesus. Interesanti, ka neiroķīmiskos pētījumos līdzīgi kā uzvedības pētījumos, γ_2-MSH ievadīts intra-VTA, darbojās atšķirīgi no α-MSH un γ_1-MSH: γ_2-MSH samazināja gan dopamīna, gan DOPAC ekstracelulāro koncentrāciju žurku NACC mikrodialīzes paraugos. γ_2-MSH ietekme uz ekstracelulāro DA koncentrācijas pazemināšanos bija izteiktākā nekā uz DOPAC līmeņa pazemināšanos. Kombinētās ievadīšanas gadījumā γ_2-MSH injicēja pirms γ_1-MSH, un līdzīgi kā uzvedības pētījumos tika novērots antagonizējošs fenomens starp abiem γ-MSH peptīdiem: γ_2-MSH pilnīgi normalizēja γ_1-MSH izraisītās izmaiņas dopamīna un daļēji - DOPAC koncentrācijā. Šie rezultāti liecina, ka α-MSH, γ_1-MSH un γ_2-MSH var tieši vai netieši modulēt DAerģisko sistēmu. Neiroķīmiskos pētījumos iegūtie dati labi papildināja uzvedības pētījumu rezultātus: 1) α-MSH un γ_1-MSH ierosinātā hiperaktivitāte (pastiprināta *grooming* un lokomotorā uzvedība) var tikt saistīta ar mezolimbiskās dopamīnerģiskās sistēmas aktivitātes palielināšanos. Neiroķīmiskie dati apstiprina šo domu, jo α-MSH un γ_1-MSH intra-VTA ievadīšana izraisīja ekstracelulārā DA un DOPAC koncentrācijas palielināšanos; 2) tai pašā laikā, γ_2-MSH inhibējošā iedarbība uz NACC dopamīnerģiskajiem procesiem varētu būt par pamatu šī peptīda izraisītās katalepsijas izpausmes. Vēl jo vairāk, γ_1-MSH un γ_2-MSH pretējā iedarbība uz DAerģiskajiem procesiem, un tas, ka γ_2-MSH darbojas kā spēcīgs antagonists attiecībā uz γ_1-MSH izraisītajiem efektiem, liek domāt, ka šie peptīdi ir nepieciešami, lai darbotos kā endogēnie (iespējams, funkcionālie) antagonisti. Tā kā šis neiroķīmiskais pētījums veikts smadzeņu struktūrā, kas attiecas uz mezolimbisko dopamīnerģisko sistēmu, tad γ_1-MSH un γ_2-MSH antagonistiskās attiecības liecina par abu peptīdu iespējamo lomu šīs sistēmas aktivitātes uzturēšanā sabalansētā līmenī, īpaši modulējot DA metabolisma procesus.
MC4R antagonista HS131 intra-VTA ievadīšana neizraisīja ekstracelulārā DA un DOPAC koncentrācijas izmaiņas NACC mikrodialīzes paraugos, taču, ievadot HS131 pirms α-MSH, novēroja pilnīgu α-MSH efekta bloķēšanu. Tas varētu pamatot hipotēzi, ka α-MSH izraisītais ekstracelulārās DA un DOPAC koncentrācijas pieaugums NACC varētu realizēties caur melanokortīnu receptoru aktivēšanu, galvenokārt, caur MC4R subtipu.

DAerģiskā komponenta pētīšanā izmantojām arī amfetamīna (AMP)- izraisīto hiperlokomocijas modeli pelēm. Amfetamīns darbojas kā psihostimulējoša viela, kas izraisa dopamīna izdalīšanos no presinaptiskajiem nervu galiem. Tālāka dopamīna uzkrāšanās sinaptiskajā spraugā palielina postsinaptisko jutīgumu. Šie procesi eksperimentālos dzīvniekos izsauc pastiprinātu lokomotoro uzvedību (Segal, 1975). Mūsu iegūtie rezultāti modeļdzīvniekos parādīja, ka gan γ_1-MSH, gan γ_2-MSH intracisternāla ievadīšana pelēm samazināja AMP-izraisīto hiperlokomociju. Dabiski, mēs gaidījām, ka γ_1-MSH varētu palielināt AMP-izraisīto hiperlokomociju, bet γ_2-MSH - antagonizēt AMP efektus. Taču tā kā abi peptīdi darbojās šai testā līdzīgi, samazinot AMP-izraisīto hiperlokomociju, tas norāda ne tikai uz dopamīnerģiskā komponenta nozīmīgumu, bet arī uz šo procesu komplekso raksturu, savstarpēji mijiedarbojoties melanokortīn- un dopamīnerģiskajiem procesiem, un, iespējams, vēl citu neirotransmiteru mediētiem procesiem.

Šobrīd ir grūti definēt, vai melanokortīnu ietekme uz NACC mezolimbisko dopamīnerģisko sistēmu ir tieša vai netieša. Šī jautājuma noskaidrošanai mūsu tālākie pētījumi bija vērsti uz MSH peptīdu izraisīto uzvedības un neiroķīmisko efektu mehānismu noteikšanu, izmantojot dažādu neirotransmiteru sistēmu vielas-analizātorus.

6.3. Glutamāterģiskais komponents

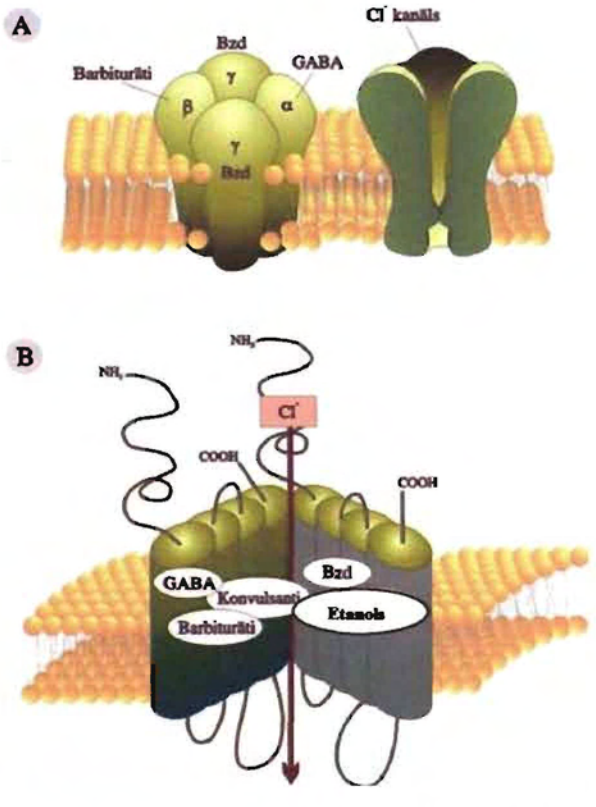
Pētījumi ar modeļdzīvniekiem, kuros izmantojām fenciklidīnu (PCP) – NMDA receptora nekonkurējošo antagonistu, parādīja, ka γ-MSH peptīdi spēj modulēt ne tikai DAerģiskās, bet arī glutamāterģiskās sistēmas aktivitāti. PCP tiek uzskatīts par modeļsavienojumu šizofrēnijas imitācijai grauzējiem. Šī viela izraisa kustību aktivitātes pastiprinājumu, īpaši horizontālās aktivitātes pieaugumu. Uzskata, ka tas saistīts ar PCP spēju izraisīt pastiprinātu DA izdalīšanos NACC struktūrā. Tāpat kā iepriekšējos *open field* testa pētījumos ar žurkām, arī PCP modelī pelēm abi γ-MSH peptīdi (ievadīti intracisternāli) demonstrēja pretēju iedarbību: γ_1-MSH injekcija pastiprināja PCP-izraisīto lokomotoro aktivitāti, turpretim γ_2-MSH preventēja PCP izraisītos efektus. Bez tam, γ_2-MSH spēja antagonizēt minēto γ_1-MSH potencējošo (γ_1-MSH +PCP) efektu. Tādējādi, arī šie rezultāti liecina, ka γ_1-MSH piemīt psihoaktivējoša iedarbība, un tas var palielināt glutamāta receptora antagonista PCP aktivitāti, bet γ_2-MSH uzrādīja anti-psihotisku iedarbību. Interesanti, ka arī MC4R antagonists HS014 spēja antagonizēt γ_1-MSH+PCP izraisīto hiperlokomociju. γ_2-MSH un MC4R antagonista HS014 līdzīgie antagonizējošie efekti attiecībā pret γ_1-MSH un γ_1-MSH +PCP pastiprināto lokomotoro efektu, norāda uz γ_2-MSH un HS014 līdzīgo iedarbību uz glutamāterģiskajiem procesiem organismā. γ_1-MSH, γ_2-MSH un HS014 ietekme uz fenciklidīna efektu modulēšanu liecina, ka šie peptīdi, iespējams, izmaina mezolimbiskās DAerģiskās sistēmas aktivitāti caur glutamāta-DA receptoru *cross-talk* mehānismiem.

γ_2-MSH spēja modulēt glutamāterģisko sistēmu izpaudās arī eksperimentos ar žurkām, kurām kreisajā VTA tika ievadīta neiroaktīvā aminoskābe NMDA. Tā izraisīja pastiprinātu lokomotoro aktivitāti un kontra-laterālas rotācijas. γ_2-MSH, ievadīts intra-VTA 5 minūtes pirms NMDA intra-VTA injekcijas, pilnīgi preventēja NMDA-izraisītās neirotoksiskās uzvedības reakcijas.

6.4. GABAerģiskais komponents

Ja uzvedības un mikrodialīzes pētījumu rezultāti norādīja, ka daļa no centrāliem efektiem, ko ierosina MSH peptīdi un to sintētiskie analogi, ir saistīti ar dopamīn- un glutamāterģiskajiem procesiem, tad analgēzijas pētījumu rezultāti uzskatāmi norādīja uz sarežģīto melanokortīnu multifunkcionālo lomu centrālās nervu sistēmas procesos, un par vismaz vēl vienas neirotransmiteru sistēmas - GABAerģiskās - būtisko lomu melanokortīnu izraisītajos efektos.
α-MSH, γ_1-MSH un γ_2-MSH tika injicēti intracisternāli pelēm un reģistrēta to ietekme uz sāpju percepciju *tail flick* testā. Analgēzijas pētījumi parādīja, ka tikai γ_2-MSH (visās trijās devās 0,3, 1 un 3 nmoli, i.c.) spēja izraisīt izteiktu, stabilu analgēziju, kas ilga līdz 90 min. γ_2-MSH analgētiskais efekts bija daudz spēcīgāks nekā opiātu peptīda enkefalīna izraisītā centrālā analgēzija, kas ilga tikai 5 minūtes (Klusa, 1984).
Interesanti, ka γ_2-MSH analgēzijas efekts nerealizējās caur opiātu receptoriem, jo šo receptoru antagonists naloksons neietekmēja γ_2-MSH izraisīto analgēziju. Šis efekts nerealizējas arī caur melanokortīnu receptoru aktivēšanu, jo ne MC4R antagonists HS014, ne arī γ_1-MSH neuzrādīja ietekmi uz γ_2-MSH izraisīto analgētisko efektu. Arī dopamīna receptora antagonists haloperidols neietekmēja γ_2-MSH uzradīto analgēziju *tail flick* testā pelēm.

Tālākie pētījumi ar vielām-analizātoriem atklāja, ka vienīgi $GABA_A$ receptora ligandi ietekmēja γ_2-MSH analgētisko efektu. $GABA_A$ receptora agonists muscimols potencēja γ_2-MSH analgētisko efektu, turpretī $GABA_A$ receptora antagonists bikukulīns pilnībā preventēja γ_2-MSH analgēziju. Taču γ_2-MSH analgētisko efektu pilnīgi neietekmēja $GABA_A$ receptora benzodiazepīna saita ligands diazepams. Tādējādi, varam secināt, ka γ_2-MSH analgēziju ietekmē vienīgi $GABA_A$ receptora GABA saita ligandi. Interesanti, ka γ_1-MSH un arī α-MSH būtiski samazināja paša diazepama izraisīto analgētisko efektu. Etanola izraisīto analgēzijas efektu neietekmēja intracisternāla α-MSH ievadīšana, turpretim γ_1-MSH to samazināja, bet γ_2-MSH palielināja etanola izraisīto analgēziju pelēm. Kā zināms, etanols darbojas kā $GABA_A$ receptora modulātors, pastiprinot GABA ietekmi uz receptoru (Davies and Alkana, 1998). Analgēzijas pētījuma dati liecina par γ-MSH peptīdu izraisīto efektu dažādību, ietekmējot $GABA_A$ receptora atšķirīgo saitu ligandu efektus. Tas ļauj postulēt, ka γ_2-MSH spēj modulēt analgēzijas efektus, kas mediējas caur $GABA_A$ receptora GABA saitu, α-MSH un γ_1-MSH - benzodiazepīna saitu un abi γ-MSH peptīdi pretēji, ietekmējot etanola saitu. 22. zīmējums attēlo $GABA_A$ receptora saitus: GABA, benzodiazepīna (Bzd), etanola u.c.



22. zīm. $GABA_A$ receptora saiti: receptora proteīna organizācija (A) un tās attēlojums šķērsgriezumā (B).

Interesants ir arī fakts, ka α-MSH (visās trīs devās 0,3, 1 un 3nmoli, i.c.) spēja izraisīt īslaicīgu hiperalgēziju. Šis hiperalgēzijas efekts sasaucas ar agrāk publicētiem datiem, ka α-MSH ievadīšana ICV žurkām izraisa hiperalgēziju (Sandman and Kastin, 1981). Apkopojot mūsu rezultātus par melanokortīnu izraisītajiem analgēzijas efektiem, jāsecina, ka γ_2-MSH centrālā analgēzija neiesaista ne melanokortīnerģisko, ne opiāterģisko, ne DAerģisko sistēmu, bet šis analgēzijas efekts tiek realizēts ar tiešu un/vai netiešu $GABA_A$ receptora GABA saita stimulācijas starpniecību.

Rezumējot visus mūsu pētījumu rezultātus redzam, ka melanokortīnu izraisītajos centrālos efektos (gan uzvedības, gan neiroķīmiskos) iesaistās ne tikai melanokortīnerģiskā, bet arī citas CNS neirotransmiteru sistēmas - DAerģiskā, GABAerģiskā un glutamāterģiskā. Tas nozīmē, ka melanokortīnu funkcionālā nozīme vēl ir tālu līdz tās precīzai identificēšanai, jo to efekti saistīti ne tikai, mijiedarbībojoties ar melanokortīnu receptoru subtipiem, bet arī ar klasisko neirotransmiteru sistēmām. Tas ir īpaši nozīmīgi, ja ņem vērā plašo MCR izplatību smadzenēs, sevišķi mezolimbiskajā sistēmā, kas nodrošina atalgojuma procesu realizāciju, emociju un kognīcijas, motivācijas izpausmes. Vēl jo svarīgāk ir izprast, kas uztur balansētā stāvoklī šo sistēmu. Šajā kontekstā interesanti ir mūsu atrastie fakti par γ_1-MSH un γ_2-MSH atšķirīgo iedarbību uz melanokortīnerģisko un/vai citām neirotransmiteru sistēmām. Mūsu pētījumi ir atklājuši jaunus aspektus γ-MSH peptīdu multifunkcionālai ietekmei uz dažādiem centrālās nervu sistēmas procesiem, kas liecina par γ_1-MSH un γ_2-MSH regulējošo lomu mezolimbiskās dopamīnerģiskās sistēmas līmenī. Iespējams, ka γ-MSH peptīdu funkcionālā loma ir uzturēt balansējošu psihoaktivējošu stāvokli smadzenēs, kuru regulē abu peptīdu funkcionālais antagonisms, kurā γ_1-MSH uzrāda psihoaktivējošu, bet γ_2-MSH pretēju antipsihotisku darbību. Mūsu pētījumu rezultāti var dot impulsu jaunām farmakoterapetiskām stratēģijām, kas regulētu psihopataloģiskus stāvokļus, saistītus ar mezolimbiskās sistēmas disregulāciju (vielu atkarību, šizofrēniju u.c.). Mūsu pētījumu rezultāti norāda arī uz nepieciešamību veikt pētījumus ar augsti selektīviem MCR agonistiem un antagonistiem, kā arī veltīt turpmākos pētījumus mehānismu noskaidrošanai, īpaši atalgojuma sistēmas funkcionēšanas, vielu atkarības procesu regulācijas un melanokortīnerģiskās sistēmas kontekstā.

7. SECINĀJUMI

1. γ_1- un γ_2 -MSH (ievadīti intra-VTA) izraisīja pretējas darbības uzvedības repertuāru žurkām: γ_1-MSH palielināja *grooming* reakcijas un vertikālo aktivitāti, turpretim γ_2-MSH neietekmēja šīs reakcijas, bet izraisīja vidēja stipruma katalepsiju. Mikrodialīzes pētījumu neiroķīmiskie dati arī atklāja atšķirīgus γ-MSH peptīdu efektus uz NACC dopamīnerģiskās mezolimbiskās sistēmas aktivitāti: γ_1-MSH paaugstināja dopamīna un DOPAC līmeni, kamēr γ_2-MSH to pazemināja. Uzvedības un mikrodialīzes pētījumu dati liecina, ka γ_1-MSH darbojas kā psihoaktivējošs, bet γ_2-MSH kā antipsihotisks peptīds.

2. Gan uzvedības, gan mikrodialīzes pētījumos žurkām γ_2-MSH antagonizēja γ_1-MSH-izraisītos efektus, norādot, ka šie peptīdu varētu darboties kā funkcionālie antagonisti dopamīnerģiskās sistēmas līmenī.

3. Šizofrēnijas modeļdzīvniekiem (pelēm) fenciklidīna (PCP) hiperlokomocijas un NMDA-toksicitātes tests parādīja, ka γ-MSH peptīdi (ievadīti intracisternāli) var modulēt smadzeņu glutamāterģisko sistēmu. γ_1-MSH potencēja PCP izraisīto hiperlokomociju, turpretīm γ_2-MSH reducēja PCP efektus. Šajos eksperimentos γ_2-MSH antagonizēja γ_1-MSH izraisīto potencējošo ietekmi uz PCP hiperlokomociju. Intra-VTA γ_2-MSH ievadīšana pilnīgi inhibēja NMDA (ievadīti intra-VTA) izraisītos neirotoksiskos uzvedības efektus žurkām.

4. γ-MSH peptīdi atšķirīgi spēj regulēt arī GABAerģiskos procesus. Analgēzijas testā (*tail flick* pelēm) Tikai γ_2-MSH izraisīja ilgstošu centrālo analgēziju, kuru mediē GABAerģiskie procesi: analgēziju pastiprināja muscimols ($GABA_A$ receptora GABA saita agonists), bet pilnīgi reducēja bikukulīns ($GABA_A$ receptora GABA saita antagonists). Taču γ_2-MSH analgētisko efektu neietekmēja citas vielas-analizātori, piemēram, MC3R/MC4R antagonists HS014, opiātu receptora antagonists naloksons, DA receptora antagonists haloperidols un γ_1-MSH. Toties γ_1-MSH (bet ne γ_2-MSH) nozīmīgi samazināja $GABA_A$ receptora benzodiazepīna saita agonista diazepama izraisīto analgēziju, norādot γ_1-MSH spēju ietekmēt $GABA_A$ receptora benzodiazepīna saita agonista efektus. Abi γ-MSH peptīdi atšķirīgi ietekmēja $GABA_A$ receptora modulējošā saita liganda etanola izraisīto analgēziju: γ_1-MSH to samazināja, bet γ_2-MSH potencēja etanola analgētisko darbību.

5. Uzvedības un neiroķīmijas pētījumos iegūtie dati rāda, ka γ_1-MSH un γ_2-MSH darbojas atšķirīgā veidā. Tas liecina par γ-MSH peptīdu nozīmīgo funkcionālo lomu smadzeņu psihoaktivējošā stāvokļa regulēšanā/balansēšanā, iedarbojoties ne tikai uz melanokortīnu receptoriem, bet iesaistot arī ne-melanokortīnerģiskos mehānismus, vismaz daļēji, dopamīn-, glutamāt- un GABAerģiskos komponentus, kuri spēj būtiski ietekmēt mezolimbisko dopamīnerģisko sistēmu.

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SUMMARY

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.

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Evaluation of behavioural effects of neural melanocortin receptor antagonists injected ICV and in VTA in rats

V. Klusa[1], S. Svirskis[1], B. Opmane[1], R. Muceniece[1,2], A. Skujins[1], F. Mutulis[2], J.E.S. Wikberg[2], H.B. Schiöth[2]

[1]Laboratory of Pharmacology, Latvian Institute of Organic Synthesis, Riga, Latvia
[2]Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden

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]

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Correspondence to: Dr Helgi B. Schiöth, Uppsala University, Department of Pharmaceutical Pharmacology, Biomedical Center, Box 591, 751 24 Uppsala, Sweden. Fax: + 46 18 559718, E-mail: helgis @bmc.uu.se

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

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.

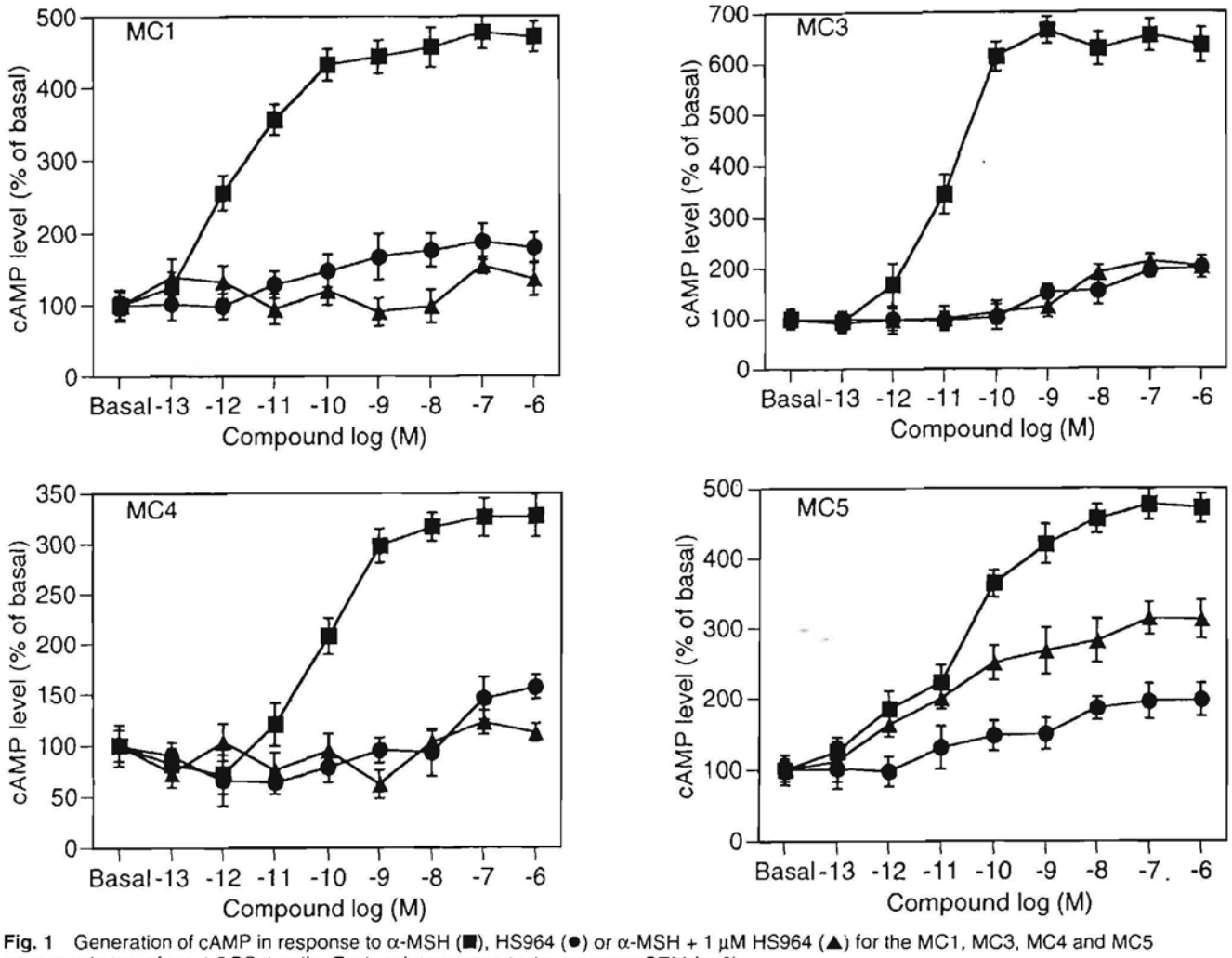

Fig. 1 Generation of cAMP in response to α-MSH (■), HS964 (●) or α-MSH + 1 μM HS964 (▲) for the MC1, MC3, MC4 and MC5 receptors in transfected COS-1 cells. Each point represents the average±SEM (n=6).

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

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)

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

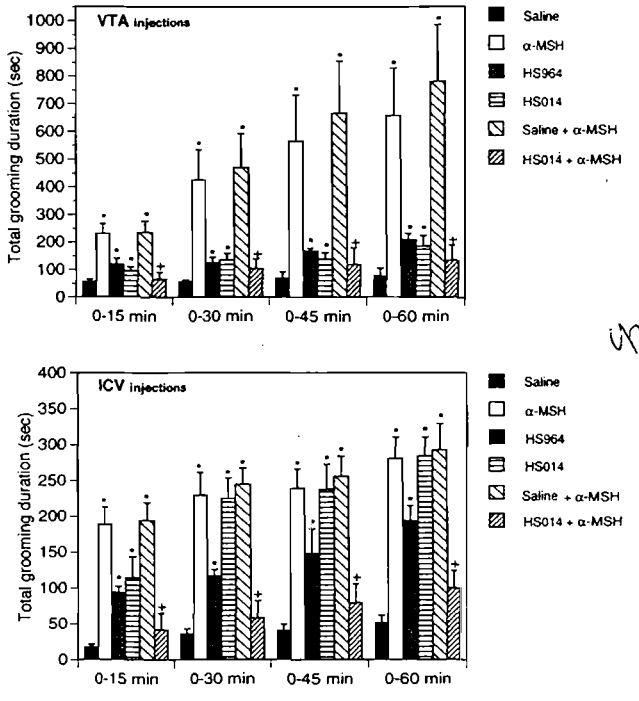

Fig. 2 Effect of the administration of different MSH peptides into A) the left VTA or B) ICV on grooming behaviour of male rats during different observation times.

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.

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II

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

V. KLUSA,[1] S. SVIRSKIS,[1] B. OPMANE,[2] R. MUCENIECE[2] and J.E.S. WIKBERG[2]

1 Laboratory of Pharmacology, Latvian Institute of Organic Synthesis, Riga, Latvia
2 Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden

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.

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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 desacetylated 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

Correspondence: Jarl Wikberg, Pharmaceutical pharmacology, Box 591, BMC, S-751 24 Uppsala, Sweden.

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 × 0.2 + 10 × 0.3 + 10 × 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

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).

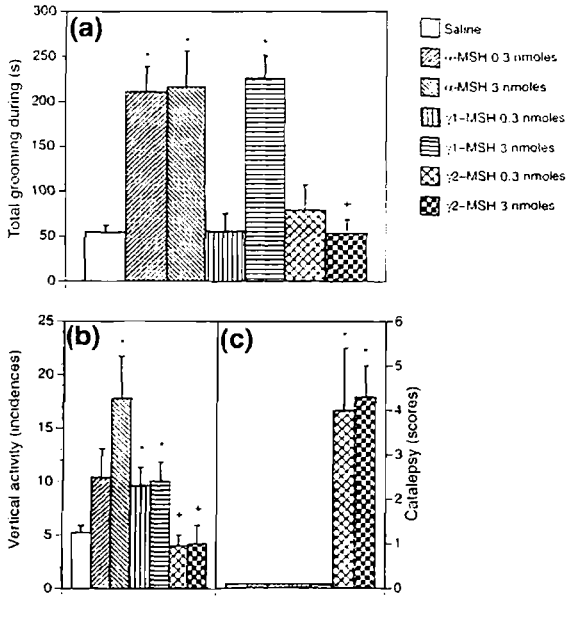

Figure 1 Effect of the administration of 0.3 and 3 nmole of α-, γ1- and γ2-MSH into the left VTA of male rats during 0–15 min after the injections on (a) total grooming, (b) vertical activity and (c) cataleptic activity recorded at the end of the experiment. *Indicates $P < 0.05$ vs. saline control;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

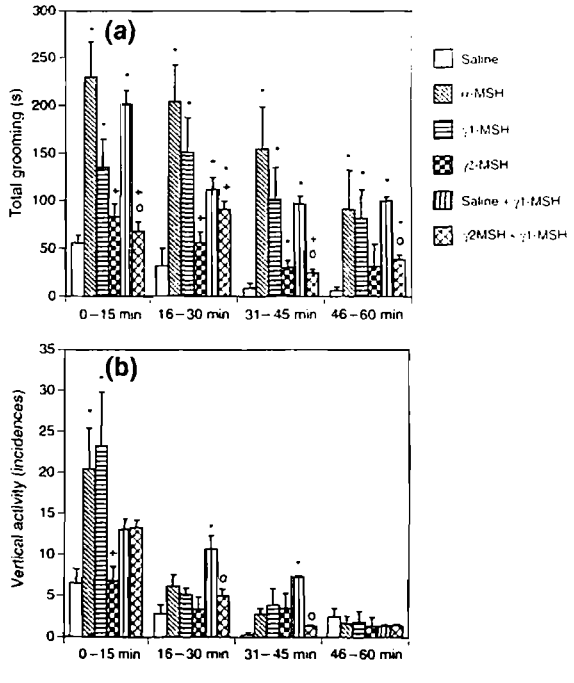

Figure 2 Effect of the administration 3 nmole of α-, γ1- and γ2-MSH, as well as combinations of γ1- and γ2-MSH into the left VTA of male rats during 0–60 min after the injections on (a) total grooming and (b) vertical activity. *Indicates $P < 0.05$ vs. saline control; + indicates $P < 0.05$ vs. α-MSH,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

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.

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III

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622. sējums

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*Vija Kluša, Šimons Svirskis**
*Skaidrīte Ģērmane, Baiba Opmane, Ruta Muceniece***
*Helgi B. Schiöth, Jarl E. S. Wikberg****

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].

* University of Latvia, Faculty of Medicine
** Latvian Institute of Organic Synthesis, Laboratory of Pharmacology
*** University of Uppsala, Department of Pharmaceutical Biosciences

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

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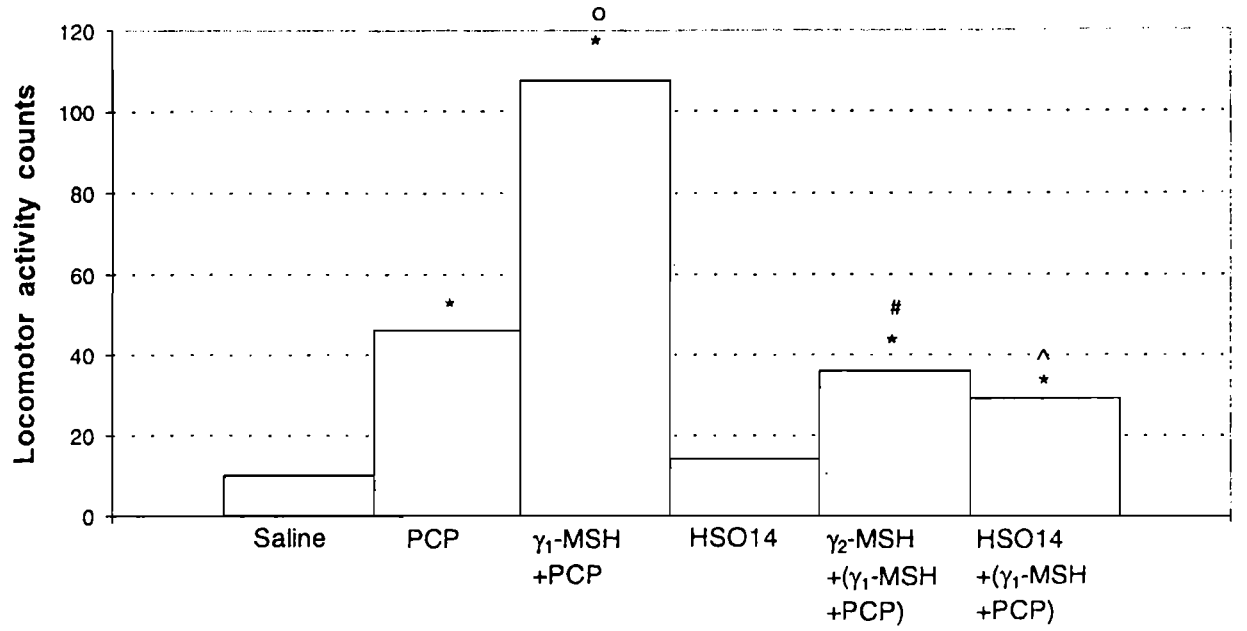

Influence of γ_2-MSH and HSO14 on the γ_1-MSH+PCP effect.

* 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

Jonas Lindblom,[1] Baiba Opmane,[1,2] Felikss Mutulis[1] Ilze Mutule,[1] Ramona Petrovska,[1] Vija Klusa,[2] Lena Bergström[1] and Jarl E. S. Wikberg[1,CA]

[1]Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University, Box 591, BMC, SE-751 24 Uppsala, Sweden; [2]Department of Pharmacology, Faculty of Medicine, University of Latvia, Riga, Latvia

[CA]Corresponding Author

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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

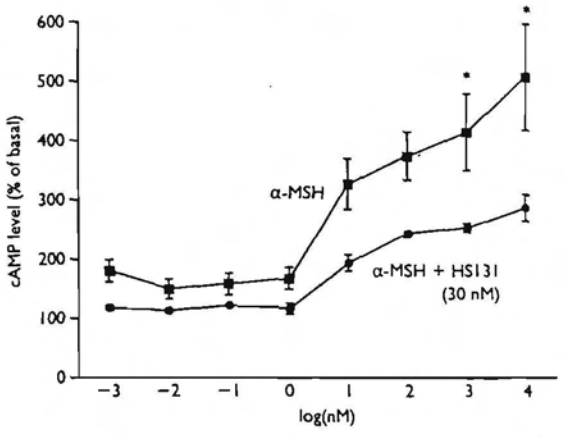

Fig. 1. Generation of cAMP in COS-1 cells expressing the rat MC$_4$ receptor in response to α-MSH ($n = 5$) and α-MSH + HS131 ($n = 3$). Each point represents mean ± s.e.m. * $p < 0.05$.

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.

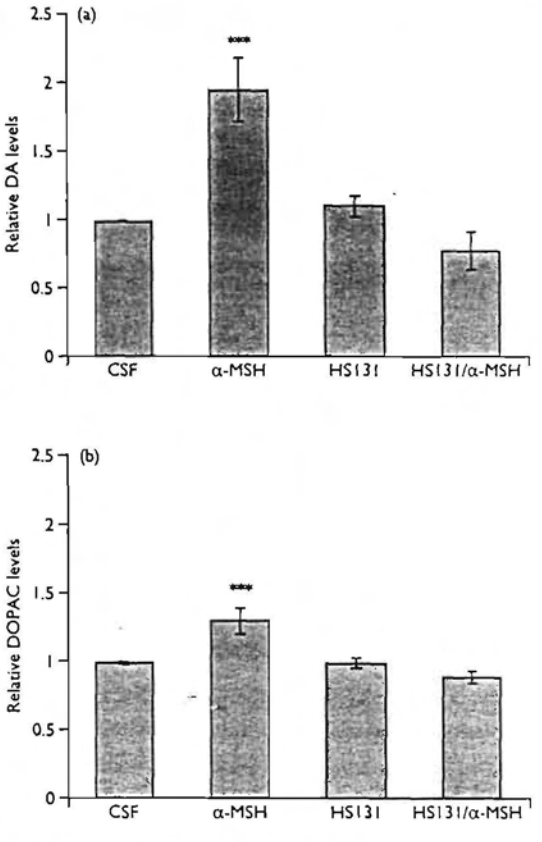

Fig. 2. Average levels of DA (**a**) and DOPAC (**b**) in the nucleus accumbens measured by microdialysis after administration of artificial CSF, 10 nmol α-MSH, 1 nmol HS131 or 10 nmol α-MSH after pretreatment with 1 nmol HS131 into the VTA. The results are presented as mean ± s.e.m. of DA and DOPAC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

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

V. Klusa,[1,2] S. Germane,[2] S. Svirskis,[1,2] B. Opmane,[2]
J. E. S. Wikberg[3]

[1]Department of Pharmacology, Faculty of Medicine, University of Latvia, Riga, Latvia
[2]Laboratory of Pharmacology, Latvian Institute of Organic Synthesis, Riga, Latvia
[3]Department of Pharmaceutical Pharmacology, Uppsala University, Uppsala, Sweden

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).

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Correspondence to: Jarl Wikberg, Pharmaceutical Pharmacology, Box 591, BMC, S-751 24 Uppsala, Sweden; Tel.: +46 18 4714238; Fax: +46 18 559718; E-mail: Jarl.Wikberg@farmbio.uu.se

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

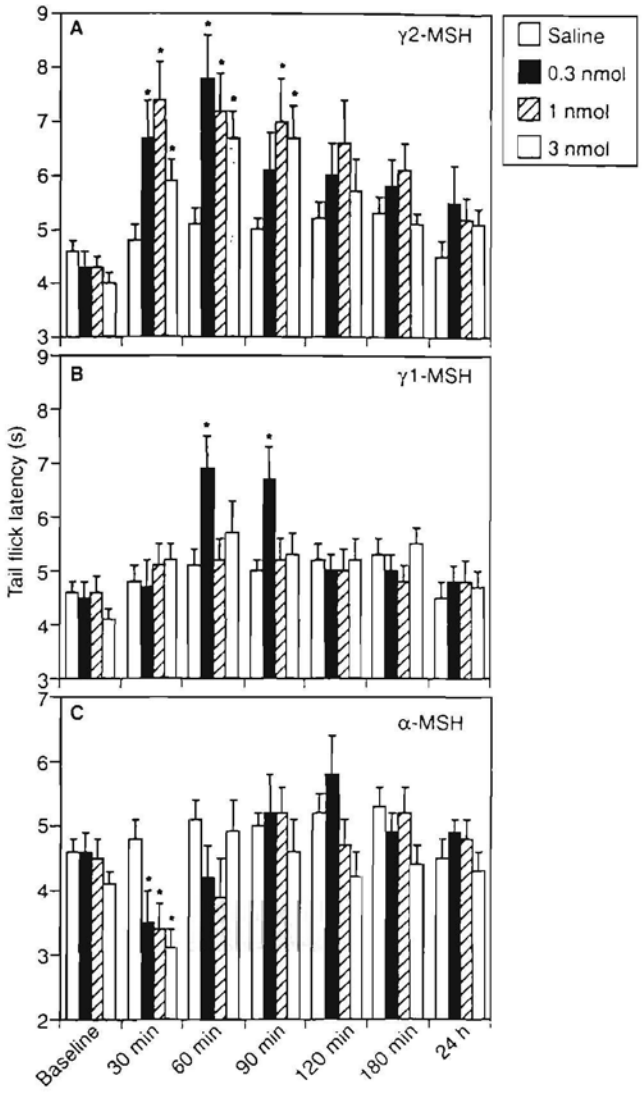

Fig. 1 A–C Effects of γ_2-, γ_1- and α-MSH peptides on tail-flick latencies in BALB/c mice. Peptides were administered intracisternally at doses 0.3, 1 or 3 nmol and the tail-flick latencies were recorded for up to 24 h. Saline indicates controls injected with the same volume (10 µl) of solvent used 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 pretreatment, 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,

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 ± 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 ± 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

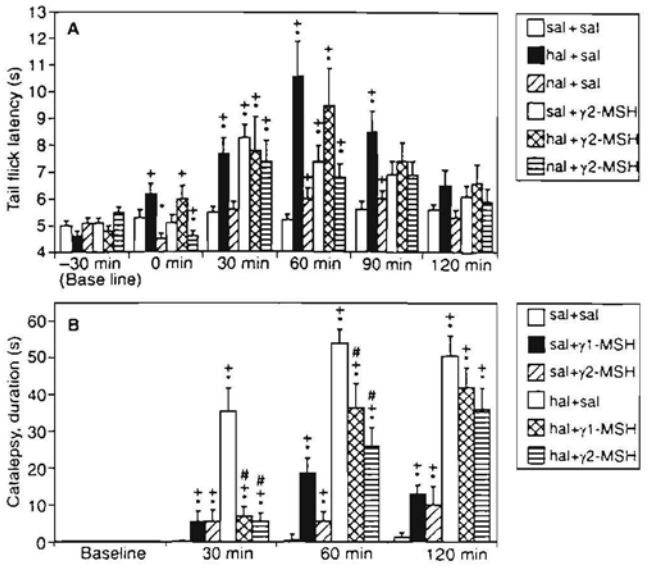

Fig. 3 A. Effect of haloperidol (hal), naloxone (nal) and γ_2-MSH on tail-flick latencies in BALB/c mice. Haloperidol (1 mg/kg) or naloxone (2 mg/kg) were given i.p. at 30 min prior (−30 min) to the injection of γ_2-MSH (1 nmol). Sal indicates controls injections with the same volume of solvent as used 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.

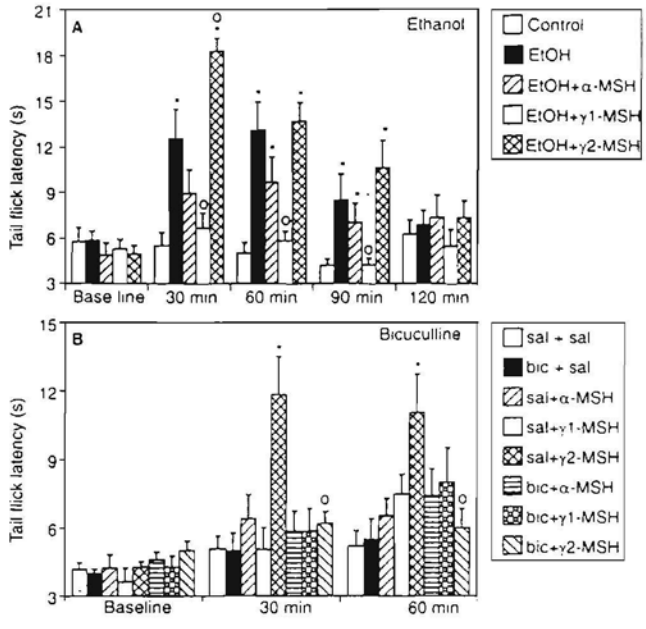

Fig. 4 A. Effect of ethanol (EtOH) and MSH-peptides on tail-flick latencies in BALB/c mice. Ethanol (4.0 g/kg) was given i.p. 10 min prior to intracisternal injections of α-, γ_1 or γ_2-MSH (1 nmol in 10 µl). The Control represents animals injected i.p. with the same volume of saline as used for ethanol. 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.

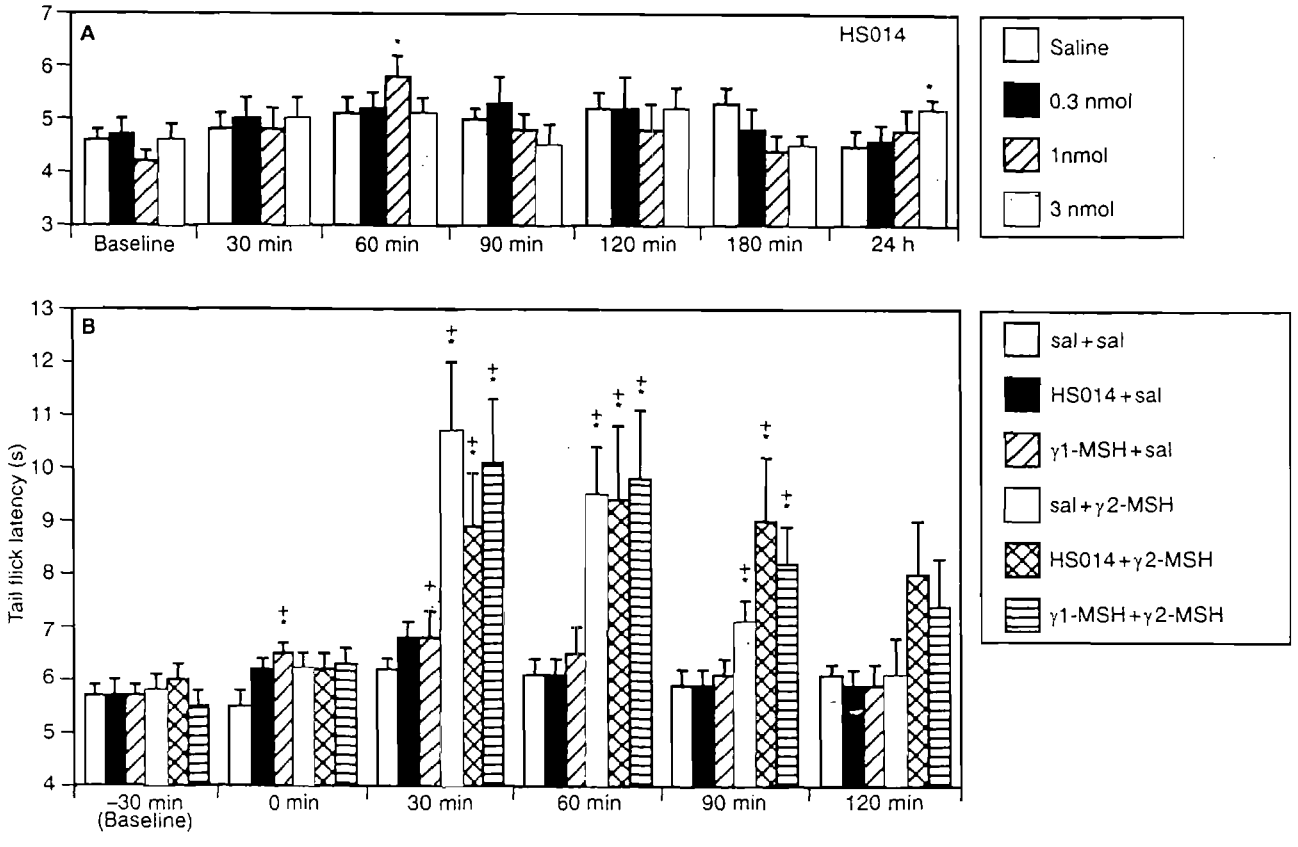

Fig. 2 Effects of HS014 and combinations of HS014 and γ_1- and γ_2-MSH peptides on tail-flick latencies in BALB/c mice. **A.** Effect of HS014 administered intracisternally at doses of 0.3, 1 and 3 nmol on tail-flick responses for up to 24 h after administration. *Saline indicates controls* injected 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 +γ_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 pre-treatment did not affect the increase in tail-flick latency induced by the intracisternal injection of γ_2-MSH (Fig. 3A).

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.

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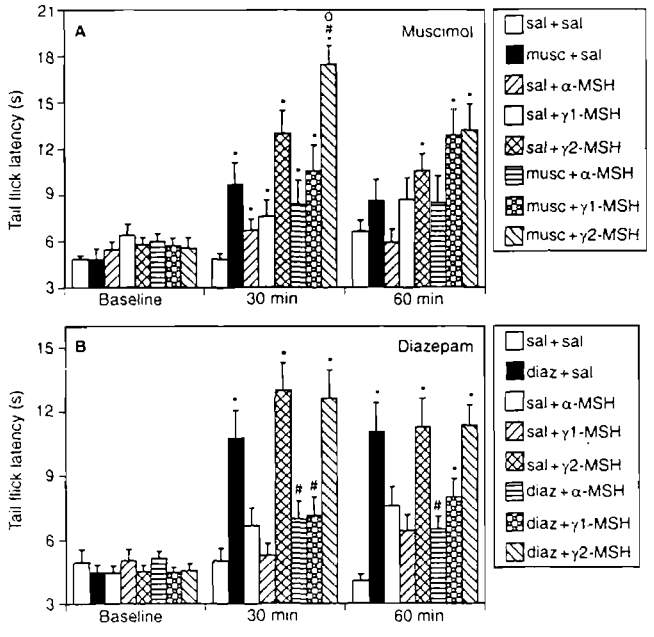

Fig. 5 A. Effect of muscimol (musc) and MSH-peptides on tail-flick latencies in BALB/c mice. Musicmol (1 mg/kg) was given i.p. 5 min prior to the intracisternal injections of α-, γ_1- or γ_2-MSH (1 nmol each). Sal indicates control injections with the same volume of solvent as used for respective agent. $n = 8$. * 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

V. KLUSA, B. JANSONE, S. SVIRSKIS,
J. RUMAKS, R. MUCENIECE*
Department of Pharmacology
*and * Division of Pharmacy*
Faculty of Medicine, University of Latvia
1A, Sarlotes St.
LV-1001 Riga, Latvia
E-mail: Vijaklus@latnet.lv

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.

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).

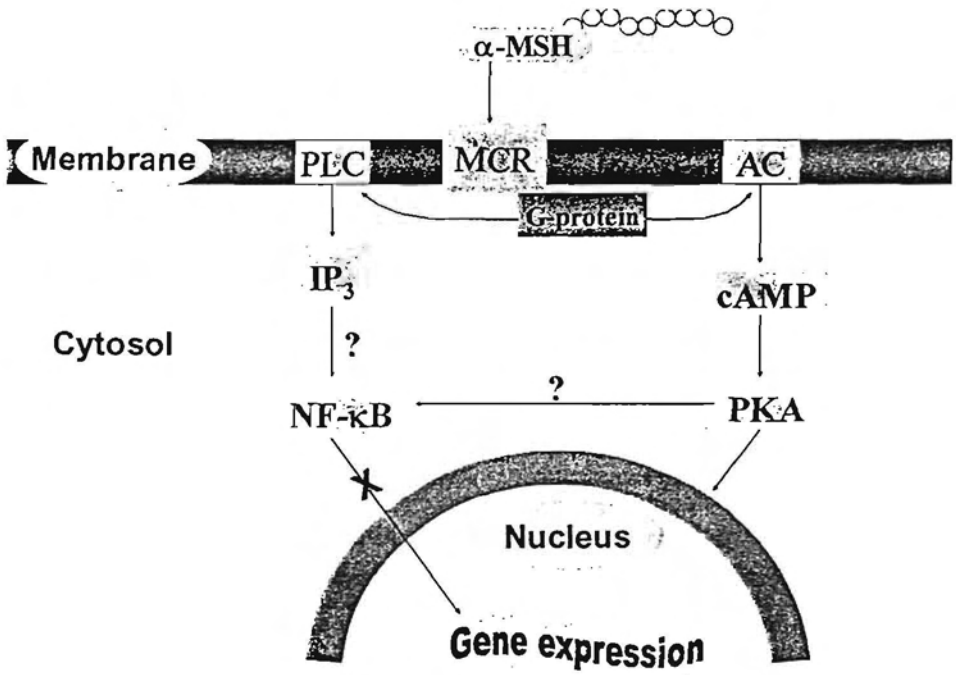

Figure 1. Model for the regulation of α-MSH-induced anti-inflammatory effect. α-MSH binds to MCR and possibly activates both signal transduction pathways (*via* cAMP and IP_3 production) resulted in inhibition of NF-κB translocation into the nucleus, caused by inflammatory cytokines (LSP+INF-γ).

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).

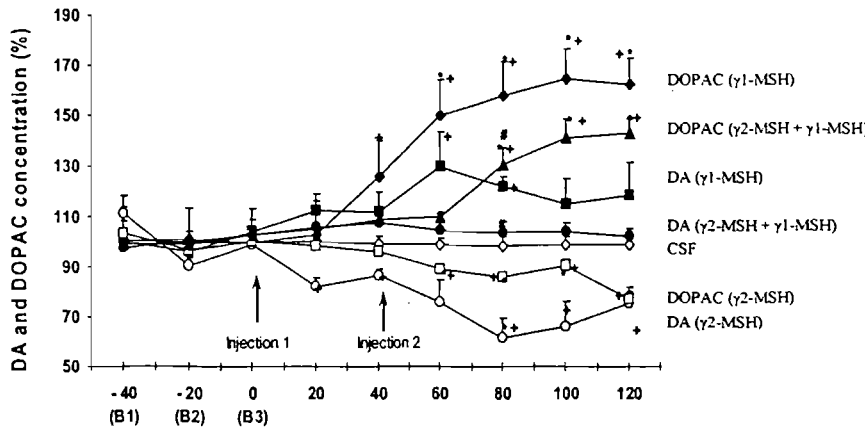

Figure 2. Influence of the intra-VTA administered γ1-MSH, γ2-MSH and γ2-MSH+γ1-MSH on the dopamine (DA) and DOPAC concentrations in the rat nucleus accumbens. Dose of peptides was 3nmol/rat. * $p<0.05$ vs CSF (artificial cerebrospinal fluid), $^{+}$ $p<0.05$ vs control, i.e. basal values (B1+B2+B3)/3, $^{\#}$ $p<0.05$ 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].

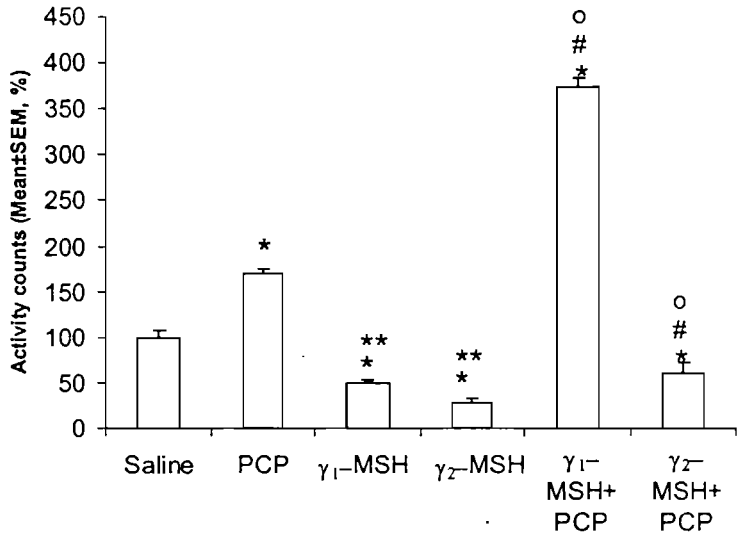

Figure 3. Influence of γ1-MSH and γ2-MSH (0.3nmol/mouse, i.c.) on phencyclidine (PCP)-stimulated spontaneus locomotor activity in BALB/c mice (n=9). PCP, 5 mg/kg, i.p., 5min prior to γ1-MSH or γ2-MSH injection. Activity counts measured in 30-60min period after γ-MSH peptide administrations. * P<0.05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

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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]

Baiba Jansone [a], Lena Bergstrom [b], Simons Svirskis [a], Jonas Lindblom [b], Vija Klusa [a] and Jarl E. S. Wikberg [b]

[a] Department of Pharmacology, Faculty of Medicine, University of Latvia, 1A Šarlotes Street, LV-1001, Riga, Latvia

[b] Department Pharmaceutical Biosciences, Division of Pharmacology, University of Uppsala, POB 591, S-75124, Uppsala, Sweden

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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-activated pathways on the mesolimbic dopaminergic system, which could be important in the maintenance of a balanced psychoactivation state.

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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Corresponding author. Tel.: +371-927-6263; fax: +371-736-6306

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

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

Baiba Jansone[a], Lena Bergstrom[b], Simons Svirskis[a], Jonas Lindblom[b], Vija Klusa[a,*], Jarl E.S. Wikberg[b]

[a]*Department of Pharmacology, Faculty of Medicine, University of Latvia, 1A Šarlotes Street, LV-1001 Riga, Latvia*
[b]*Department Pharmaceutical Biosciences, Division of Pharmacology, University of Uppsala, POB 591, S-75124 Uppsala, Sweden*

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-activated pathways on the mesolimbic dopaminergic system, which could be important in the maintenance of a balanced psychoactivation state.

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

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 data 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.

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

* Corresponding author. Tel.: +371-2-927-6263; fax: +371-2-736-6306.
E-mail address: vijaklus@latnet.lv (V. Klusa).

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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

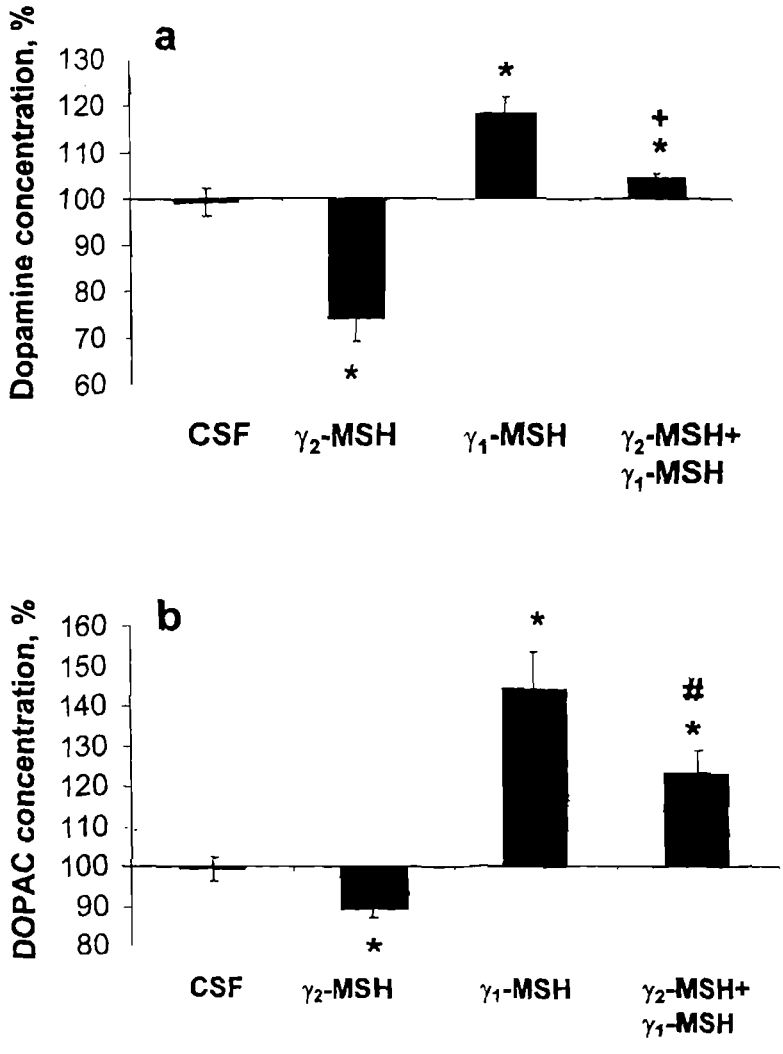

Fig. 1. Average levels of extracellular DA (a) and DOPAC (b) in the anaesthetized rats nucleus accumbens measured by microdialysis following intra-VTA administration of artificial CSF (control), γ_1-MSH and γ_2-MSH (each peptide 3 nmol), and influence of the pre-treatment of γ_2-MSH (3 nmol) on the effect 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.

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