Regulation of Feeding Behavior and Hypothalamic Neuropeptide Gene Expression by Melanocortin Analogues

Thesis by
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The Graduate School of Yonsei University

Regulation of Feeding Behavior and Hypothalamic Neuropeptide Gene Expression by Melanocortin Analogues

Directed by Professor Ja-Hyun Baik

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By
Byung-Jin Kim

Brain Korea 21 Project for Medical Sciences

The Graduate School of Yonsei University

A Dissertation for the Degree of Master in Medical Sciences by Byung-Jin Kim has been approved by

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(Supervisory committee, Chairman)

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(Supervisory committee)

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(Supervisory committee)

Brain Korea 21 Project for Medical Sciences

The Graduate School of Yonsei University

December, 2001

감사의 글

항상 지도와 관심으로 보아주시는 백자현 선생님께 먼저 감사를 전합니다. 아울러 논문 심사위원으로 수고해주신 임승길 선생님과 장정원 선생님께도 감사를 전합니다. 듬직한 동료이자 선배였던 수현씨, 이은진 선생님 그리고 조상래 선생님께 감사를 전합니다. 그리고 같은 실험실에 들어와 같이 많은 것을 생각하고 느꼈던 명환, 성재, 용년, 일선, 청섭, 미현, 안연희 선생님께 감사를 전합니다. 아울러 한 식구처럼 대해주신 3층과 5층의 많은 선생님들게 그리고 언제나 많은 얘기를 나누었던 약리학교실의 성지영 선생과 민영규 선생님께도 고마운 마음을 전합니다. 아울러 항상 많은 도움을 주시던 4층의 많은 분들에게 감사를 전합니다.

항상 신나는 격려를 해주던 나우누리 경북대 통신동아리 회원들에게 그리고 지난 16년 동안 한결 같은 친구로 남아준 상욱군과 영돈군, 석창군에게 진심어린 감사를 전합니다. 마지막으로 언제나 염원과 사랑으로 지켜봐 주시는 부모님, 사랑스런 두 여동생 윤경과 윤미 그리고 항상 기도로 돌봐주시는 고모님, 그리고 아프신 몸에도 불구하고 항상 염원해주시는 할머니, 모든 나의 가족들에게 감사를 전하면서 이 논문을 마칩니다.

2001년 12월

김 병 진

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Regulation of Feeding Behavior and Hypothalamic Neuropeptide Gene Expression by Melanocortin Analogues

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Melanocortins are known to be involved in the inhibition of food-intake and energy metabolism. Intracerebroventricular(I.C.V) administration of several different analogues of a-MSH , such as a-MSH, NDP-MSH, a-MSH-ND, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND, which were substituted in the position of His^6 with Gln and Lys, and Cyclic 16k-MSH to C57J/BL6 mice showed that significant inhibition of time course food-intake compared to saline-administerd control. However, truncated form of [Gln^6] a-MSH-ND had no effect on inhibition of food-intake. *In situ*

hybridization and RT-PCR analysis revealed that expression of MCH was significantly decreased by a-MSH, NDP-MSH, a-MSH-ND, [Gln^6] a-MSH, [Lys^6] a-MSH and Cyclic 16k-MSH after 1 and 3hr of administration and expression of AGRP and NPY in hypothalamus was significantly decreased by a-MSH, [Gln^6] a-MSH, [Lys^6] a-MSH and Cyclic 16k-MSH after 3 hr of administration. Administration of NDP-MSH and a-MSH-ND induced a biphasic regulation in expression of AGRP and NPY, showing a decrease after 1hr and an increase after 3hr. Our results suggest that MC3R and MC4R melanocortin receptors mediate hypophagic signaling in association with differential regulation of hypothalamic neuropeptide. Identification of potential target genes in this regulation are currently undertaking to understand the signaling mediated by MC3R and MC4R in the feeding circuitry.

Key Words: Melanocortin receptor, Peptide analogues, Food-intake, Gene Expression, Hypothalamus.

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I. Introduction

Obesity is now a worldwide public health problem owing to increased risk for type II diabetes, hypertention, hyper-lipidaemia, and certain cancers. In recent years, important investigations have been made in identifying the components that regulate body weight including several genes responsible for animal and human obesity. A key element of physiological system that regulates body weight is the hormone, leptin. Leptin is produced by fat tissue and upon

activation of leptin receptor in the brain, a series of neuronal response is required for food intake and energy balance to be affected[1]. Several distinct hypothalamic neuropeptides have emerged as candidate mediators of adiposity signals in CNS. For examples, [2]MCH(melanin-concentrating hormone), [3,4]NPY (neuropeptide Y), [5]AGRP (Agouti-related protein) and [6]galanin are known to stimulate food intake while [7]mahogany, [8]neurotensin, somatostatin, corticotrophin-releasing factor (CRF) and cholecystokinin are known for their anorexigenic effect.

Over recent years, much attention has been drawn to the involvement of the melanocortins in control of feeding behavior. Alpha-melanocyte stimulating hormone (MSH) is peptide hormone with 13 amino acids derived through a series of proteolytic cleavages from the precursor peptide pro-opiomelanocortin (POMC). This peptide mediates its effects through G-protein coupled receptors by stimulating adenylate cyclase. So far, at least five subtypes of melanocortin receptors are identified. Melanocortin 1 receptor (MC1R) is expressed in melanocytes and known to be involved in skin pigmentation and the MC2R is the ACTH receptor and specifically expressed in the adrenal gland.

MC5R is expressed in many peripheral tissues and suggested to regulate hair lipid production and thermal regulation. [9,10] MC3R is expressed in specific brain regions, ventromedial hypothalamus (VMH) and arcuate nucleus(ARC) whereas the MC4R is expressed more widely across the brain and spinal cord. It has been reported that activation of MC4R by a-MSH increases energy expenditure and decreases food intake, genetic disruption of MC4R also caused obesity in mice[11,12]. Therefore, MC4R receptor-selective agonists have been considered as potential candidates for the treatment of abnormal eating behaviors including obesity and anorexia[13,14]. It has been reported recently that genetic disruption of this MC3R in mice caused increase of fat mass and decrease of lean body mass without outstanding increase of food intake and showed distinct feature of obesity as compared to that of MC4R knock-out mice. These reports suggested differential physiological functions of MC3R in obesity with MC4R [15,16] and that melanocortin 3 and 4 receptor play a important mediator of feeding and energy expenditure in hypothalamus.

[17]Previously, we analyzed several a-MSH analogues, such as a-MSH, NDP-MSH, a-MSH-ND, [Gln6] a-MSH-ND, [Lys6] a-MSH-

ND, which were substituted in the position of His^6 with Gln and Lys, and Cyclic 16k-MSH upon stimulation of MC3R and MC4R using a CRE-mediated reporter gene transcription activity assay. In our CRE-mediated gene transcription activity assay, a-MSH -ND was the most efficient a-MSH analogue on MC4R whereas NDP-MSH was the most efficient for MC3R. Changing the His^6 residue of a-MSH -ND to Gln, Lys markedly decreased the CRE-mediated luciferase activity for MC3R as compared to MC4R. In the present study, we have analyzed the effect of these analogues on feeding behavior *in vivo* by [18,19,20] intracerebroventricular(ICV) administration in mice and [21]we also assessed regulation of expression of several neuropeptides involved in the regulation of food-intake, by *in situ* hybridization and RT-PCR analysis.

II. Materials and Methods

1. Animals preparation, intracerebroventricular administrations and food-intake measurements

Male C57/BL6 mice weighing 20-30g (6-8 weeks old) were maintained in individual metabolic cages with free access for feed and water under 12hr light cycle and 12 hr dark cycle(lights on at 0800 hr) with controlled temperature (21℃-23℃). Before ICV administration, mice were fasted for 24hr. Administration was proceeded with Hamilton syringe (26 gauge needle) and performed essentially as described[21]. Peptides(Table.1) was prepared with concentration 3 nmole in 5㎖ volume for 1 administration with same volume of saline control. Administration was performed at the beginning of the light phase and measurement was performed at the end of this period. Food and water intake was measured at 1, 2, 4, 6, 8, 10, 12 and 24hr after each administration.

2. Brain sample preparation and *in situ* hybridization

Animal sacrifice and brain section

Animals were sacrificed 1hr or 3hr after final administration. Brains were immediately excised after sacrifice and stored at –70℃.

Prepared Brains were mounted in frozen stage and serially sectioned of 10㎜ slices with a microtome.

Probe synthesis

Antisense NPY was prepared by linearizing the plasmid pBLNPY-1 with Fsp I, which contains 511 bp of the rat NPY gene(provided by Dr.Steven L.Sabol). Antisense MCH was prepared by linearizing the plasmid pGEM4-MCH with Xba I, which contains 700 bp of the rat MCH gene(provided by Dr.Rebecca M. Demo). Antisense galanin was prepared by linearizing the plasmid pBluescript-galanin with Hind III, which contains 700 bp of the rat galanin gene (provided by Dr.Tom Teal). Antisense AGRP is prepared by linearizing the plasmid pBluescript-AGRP with EcoR I , which contains 300 bp of mouse AGRP gene. [^{35}S] cRNA probes were prepared by transcribing 1㎎ of each linearized DNA with T3 polymerase(NPY), T7 polymerase(galanin and AGRP) and SP6 polymerase(MCH) for 1hr 30 min at 37℃ in a reaction containing [^{35}S]CTP(Amersharm Pharmacia) using an in vitro transcription kit(Promega).

Hybridization

Before hybridization, all sections were fixed with acetone and 37% formaldehyde solution at 4℃. After fixation, sections were acetylated with 0.1M triethanolamine (pH8.0) solution for 5 min and then treated in 50% formamide-1 x SSC solution at 60℃ for 10 min. Hybridization was performed for 24hr at 52℃ with ^{35}S-labeled probes(2.5 x 10^7 cpm/ml with yeast tRNA(50㎎/㎖) in 50% formamide, 0.3M NaCl, 1 x Denhardt's solution, 5 mM EDTA, 1mM sodium phosphate buffer, 10% dextran sulfate, 10mM DTT and 20mM Tris). Sections were then washed with 50% formamide-1 x SSC solution twice at 55℃ for 1 hr. After rinsing with 2 x SSC twice, sections were treated with RNase A(20㎎/ml) and RNase T(1 unit/ml) for 30 min at 37℃. Then sections were treated with 50% formamide-2 x SSC solution twice at 55℃ for each 1 hr. Sections were desalted in a series of washes with 2 x SSC for 15 min at room temperature, 0.1 x SSC for 15 min at 50℃ and 0.1 x SSC for 30 min at room temperature. Dehydration of sections were performed with ascending cold ethanol (30%, 70% and 100%) and sections were dried at room temperature for 40 min.

Section emulsion and quantification

Dried sections were dipped in emulsion solution (Kodak NTB2). Slides were exposed at 4℃ for 1-7 weeks, developed in Kodak GBX developer and counterstained with 0.01% toluidine blue. Quantification of auto-radiogram on X-ray film (Kodak AR) were measured by using TINA 2.0 program and slides were quantified by using the MCID program.

3. RT-PCR and southern Blot Analysis

Total RNA was prepared from isolated hypothalamus of mice brain using LiCl RNA extraction buffer. First strand cDNAs were generated from total RNA using reverse-transcription with random primer by denaturating at 90℃ for 4 min, annealing at room temperature for 10 min and extending at 42℃ for 50 min. Primers for PCR amplification and internal forward primers for southern blot analysis were generated from cDNA sequences in gene bank(NCBI) or related references (Table.2). PCR amplification for each genes was performed with cycle 94℃ 30sec, 55℃ 30 sec and 72℃ 1 min for 35 cycles. Agarose gels with these PCR products were transferred to nylon transfer membrane (hybond-N+,

Amersharm) and membranes were hybridized by using forward primers which were labeled with γ^{32}P-ATP(NEN). Co-amplification of the GAPDH gene was performed to normalize the expression.

Talble.1 Amino acid sequence of melanocortin and analogues

Peptides	Amino acids												
	1	2	3	4	5	6	7	8	9	10	11	12	13
[Nle^4]α-MSH	Ser	Tyr	Ser	Nle	Glu	His	Phe	Arg	Trp	Gly	Lys	Pro	Val
NDP-MSH	Ser	Tyr	Ser	Nle	Glu	His	D-Phe	Arg	Trp	Gly	Lys	Pro	Val
α-MSH-ND				Nle	Asp	His	D-Phe	Arg	Trp	Lys			
[Gln^6] α-MSH-ND				Nle	Asp	**Gln**	D-Phe	Arg	Trp	Lys			
[Lys^6] α-MSH-ND				Nle	Asp	**Lys**	D-Phe	Arg	Trp	Lys			
[Gln^6] α-MSH-ND (6-10)						Gln	D-Phe	Arg	Trp	Lys			
Cyclic 16k-MSH					Cys	His	D-Phe	Arg	Trp	Cys	Lys		

Talble 2. Primers for PCR amplification and internal primers for southern blot analysis.

		Primer Sequences	Length	References
NPY	Forward	5' CTA GGT AAC AAG CGA ATG GGG 3' (21 mer) : 4～24	286 bp	AF 273768
	Reverse	5' ACA TGG AAG GGT CTT CAA GCC 3' (21 mer) : 269～289		
	Internal	5' ACT ACA TCA ATC TCA TCA 3' : 161～178 (18 mer)		
MCH	Forward	5' GCA AAG ATG ACT CTC TCT TCC 3' (21 mer) : 4～24	492 bp	Molecular & Cellular neuroscience Vol 4 : 271-284 (1993)[23]
	Reverse	5' GAC TTG CCA ACA TGG TCG GTA 3' (21 mer) : 475～495		
	Internal	5' TCC GTA GCC TTC CCA GCT 3' (18 mer) : 331～348		
AGRP	Forward	5' TGA CTG CAA TGT TGC TGA GTT GTG 3' (24 mer) : 5～28	391 bp	NM_007427
	Reverse	5' TAG GTG CGA CTA CAG AGG TTC GTG 3' (24 mer) : 372～395		
	Internal	5' AGA AGA AGT TCT GCT GCA 3' (18 mer) : 174～191		

III. Results

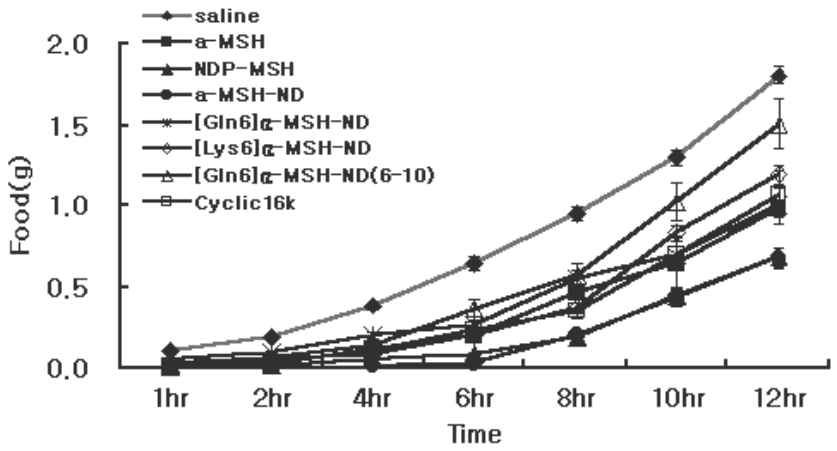
1. Inhibition of feeding by ICV administration of the melanocortin analogues and body weight change.

Mice were induced to feed by food deprivation for 24 hr before intracerebroventricular (I.C.V) administration of a-MSH analogues. Different melanocortin analougues [NDP-MSH, a-MSH-ND, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND, cyclic16k-MSH and [Gln^6] a-MSH-ND (6-10) : Table 1] were administered and their effect on food intake inhibition were analyzed *in vivo*. Food-intake was measured at 1, 2, 4, 6, 8, 10, 12 and 24 hr after administration of peptide. Fig 1 showed the time-course food intake over 12hr(A) and 24hr(B) period and accumulative food intake over 2hr (C) and 10hr (D). NDP-MSH and a-MSH-ND significantly inhibited food intake by 65-70% as compared to saline administered control over 10 hr period (P<0.01). Other peptides, such as a-MSH, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic16k-MSH also significantly inhibited food intake by 36-50% over 10hr period (* P<0.05, n=10) after administration. However, truncated form of [Gln^6] a-MSH-ND showed no significant effect on food intake inhibition (Fig 1). Therefore, NDP-MSH and a-**

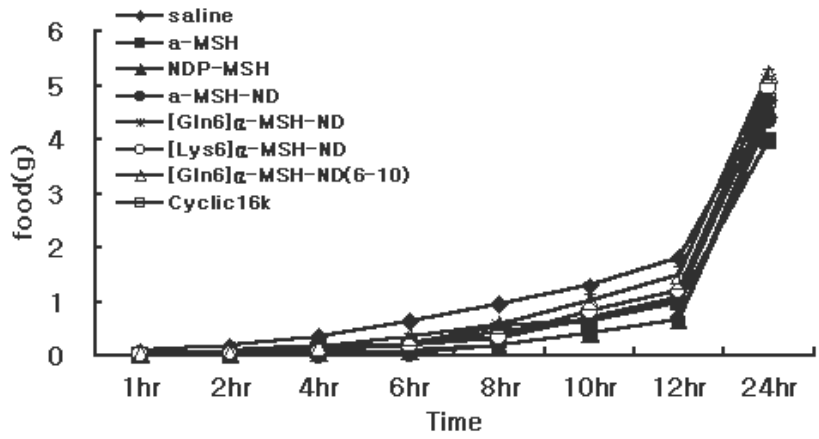
MSH-ND were the most efficient peptides for inhibition of food intake and [Gln6] a-MSH-ND, [Lys6]□a-MSH-ND and cyclic16k-MSH also showed significant inhibition on food intake whereas truncated form of [Gln6] a-MSH-ND did not show significant effect on food intake inhibition. These data are consistent with our previous results with CRE-luciferase activity assay *in vitro*, where the activity of these analogues were accessed on the basis of G protein coupling efficiency. These results also suggested the structural importance of the core-sequence of melanocortin analogues(Asp-His-D-Phe-Arg) for binding and activation of MC3R and MC4R .

Body weight of each peptide administrated mice were measured at every 24hr after administration(Fig 2). Slight but not statically significant decrease of body weight(7-8%) were showed by administration of a-MSH and NDP-MSH in 5th days after administration and other peptides, a-MSH-ND, [Gln6] a-MSH-ND, [Lys6] a-MSH-ND and cyclic 16k-MSH showed no significant changes in body weight over 5 days administration period. (Fig.2)

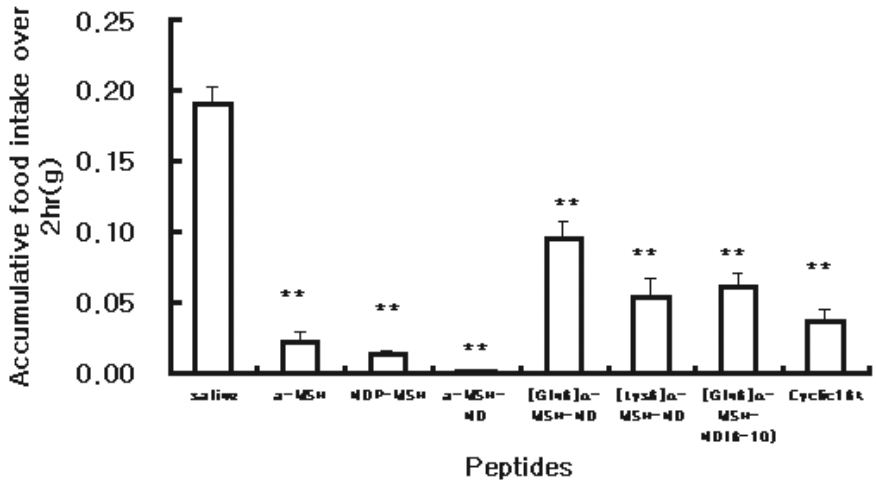
(A) Food intake over 12hr period



(B) Food intake over 24hr period



(C) Accumulative Food intake over 2hr



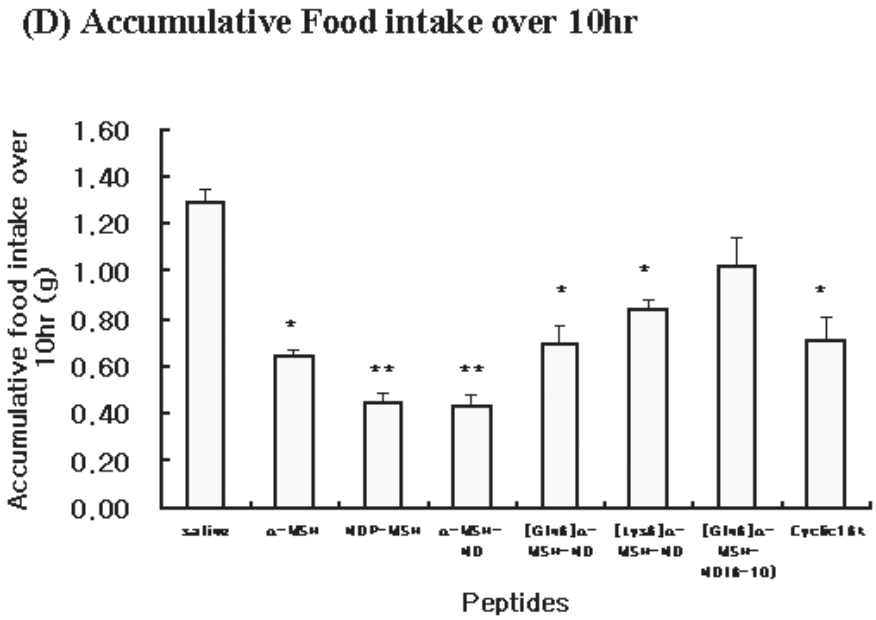


Fig 1. Inhibition of feeding by ICV administration of several melanocortin analogues. α-MSH, NDP-MSH, α-MSH-ND, [Gln^6] α-MSH-ND, [Lys^6] α-MSH-ND and cyclic16k-MSH(3nmole) produced the significant food-intake inhibition effect whereas truncated form of [Gln^6] α-MSH-ND(3nmole) had no significant effect on food intake inhibition. (A), time course food intake over 24hr period, (B) time course food-intake over 12hr period. Accumulative food intake over 2hr (C) and 10 hr (D) from start of administration. All value are mean±SEM, n=10 per each group. Data were analyzed by mean of ANOVA followed by Dunnett test for individual comparisons.(*P<0.05, **P<0.01 vs. saline)

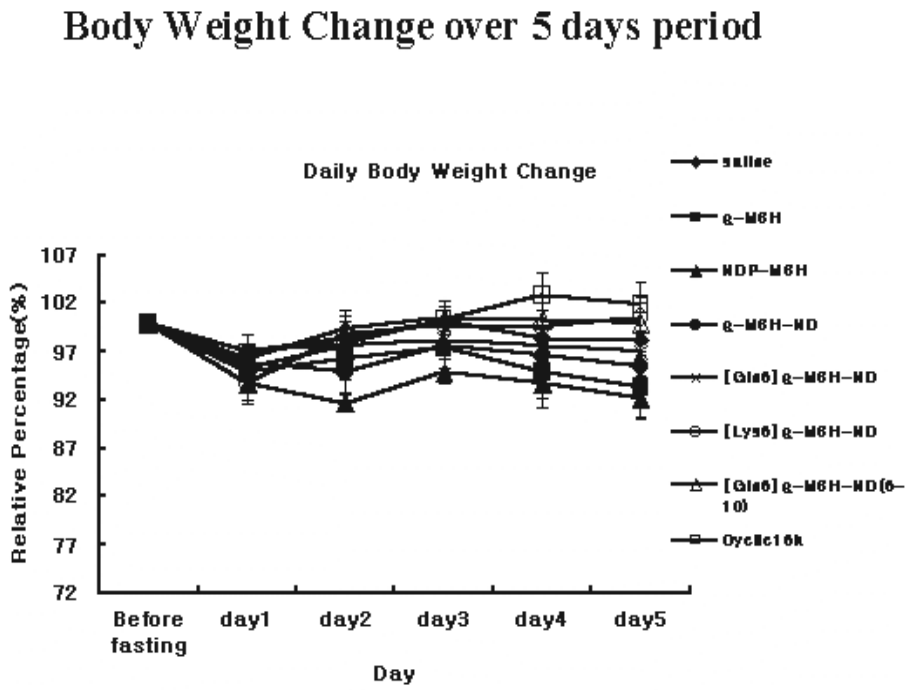


Fig 2. Daily body weight change by ICV administration of melanocortin analogues. All values are mean±SEM n=10 per each group. Data were analyzed by mean of ANOVA followed by Dunnett test for individual comparisons.

2. Effect of melanocortin analogues on the expression of hypothalamic neuropeptides.

Expression of several hypothalamic neuropeptides, such as MCH, AGRP and NPY, after 3hr of administration was analyzed by in *situ* hybridization and RT-PCR and expression of these genes after 1hr of administration was analyzed by RT-PCR to investigate the regulation of these genes by melanocortin analogues. Administration of a-MSH induced a decrease of expression of MCH by 10% (* P<0.05) and 12-17% of MCH expression were significantly decreased by NDP-MSH, a-MSH-ND [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic16k-MSH administration (P<0.01). Expression of MCH was significantly decreased in ventromedial hypothalamus (VMH) and lateral hypothalamus (LH) region showing a close association with the food intake inhibition. However, MCH expression was not affected by administration of truncated form of [Gln^6] a-MSH-ND (Fig 3. (A) and (B)).**

Expression of AGRP was decreased significantly by 13-15% in brain arcuate nucleus (ARC) by administration of [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic 16k-MSH (* P<0.05). However, administration of NDP-MSH induced a significant increase of AGRP expression by 18% (P<0.01) and similar effect was**

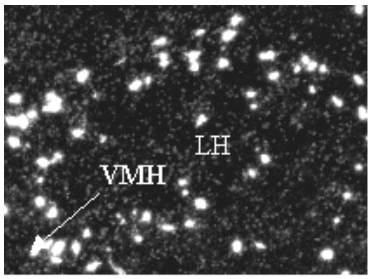
observed in a-MSH-ND administered group. Expression of NPY in ARC was significantly decreased by 21% by administration of a-MSH , [Gln^6] a-MSH-ND and cyclic16k-MSH but also by the truncated form of [Gln^6] a-MSH-ND administration(P<0.01). Whereas, administration of NDP-MSH and a-MSH-ND did not induce significant change in NPY expression (Fig 5 (A) and (B)).**

In parallel, expression of hypothalamic mRNA corresponding to neuropeptide, MCH, AGRP and NPY, after 3hr of administration was analyzed by RT-PCR (Fig 6). Expression of MCH was decreased by a-MSH, NDP-MSH, a-MSH-ND [Gln^6] a-MSH-ND, cyclic16k-MSH, and [Gln^6] a-MSH-ND(6-10). AGRP expression was decreased in a-MSH, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic16k-MSH-administered group and increased in NDP-MSH and a-MSH-ND-administered group. NPY expression was decreased by a-MSH, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic16k-MSH administration and increased by NDP-MSH and a-MSH-ND administration. Therefore, RT-PCR analysis supported our observations made by *in situ* hybridization .

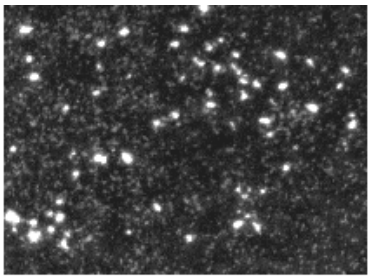
Expression of MCH, AGRP and NPY after 1hr of NDP-MSH and a-MSH-ND administration was analyzed by RT-PCR.

Expression of MCH was decreased by 10-20% by NDP-MSH and a-MSH-ND (Fig 7. A) and expression of AGRP and NPY was decreased by 40-70% after 1hr of administration of NDP-MSH and a-MSH-ND (Fig 7. B, C).

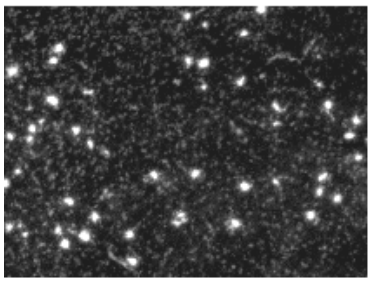
(A) Regional expression of MCH



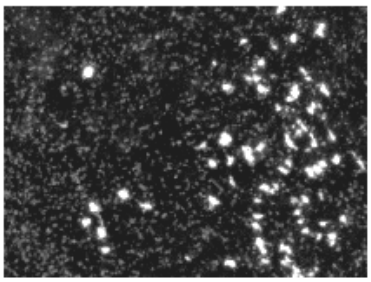
Saline



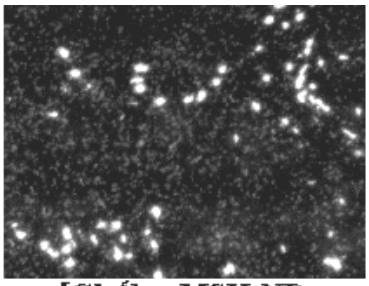
α-MSH



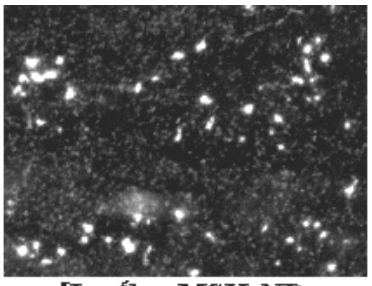
NDP-MSH



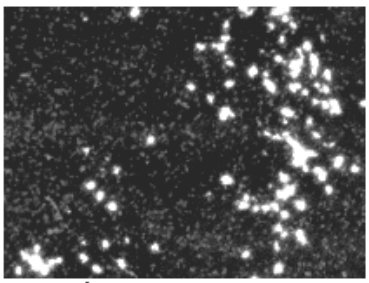
α-MSH-ND



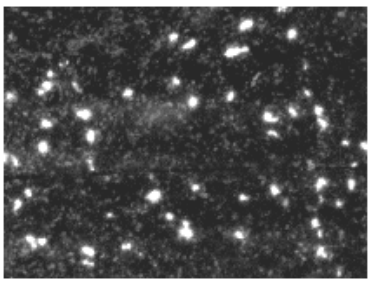
[Gln^6] α-MSH-ND



[Lys^6] α-MSH-ND



[Gln^6] α-MSH-ND (6-10)



Cyclic 16k-MSH

(B)

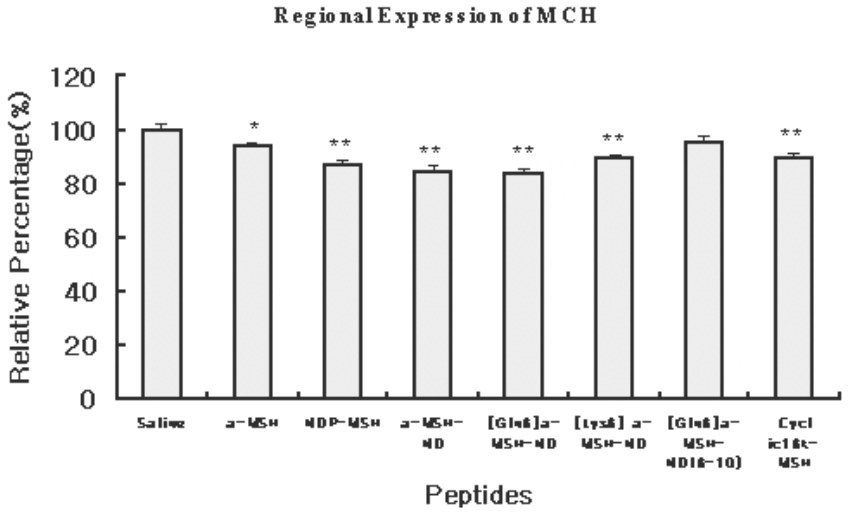
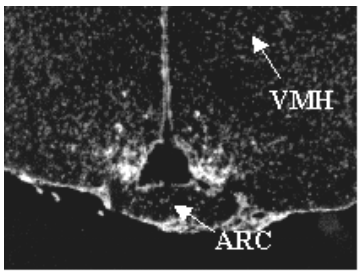
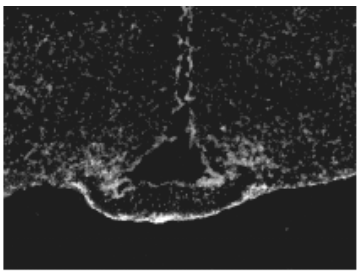


Fig 3. Photogram and graph of in situ hybridization for MCH expression in ventromedial hypothalamus(VMN) and lateral hypothalamus(LH). Expression of MCH was decreased significantly with by administration of melanocortin analogues. All values are mean±SEM. n=3 per individual group. Data were analyzed by mean of ANOVA followed by Dunnett test for individual comparisons.(*P<0.05, **P<0.01 vs. saline)

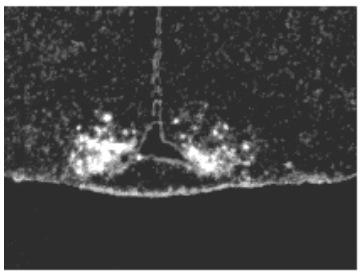
(A) Regional expression of AGRP



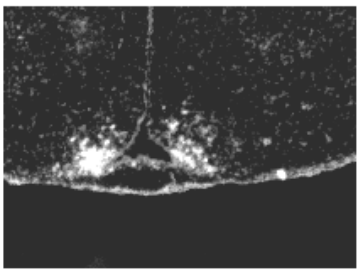
Saline



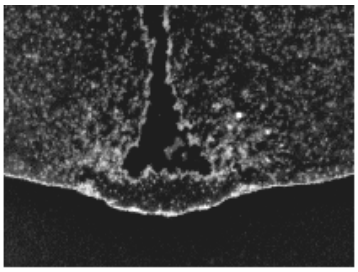
α-MSH



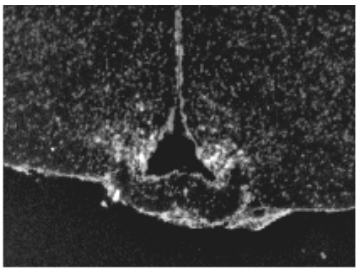
NDP-MSH



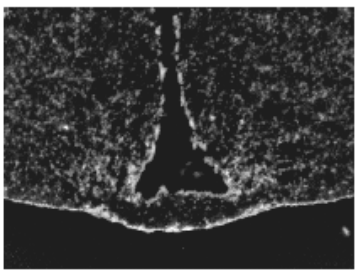
α-MSH-ND



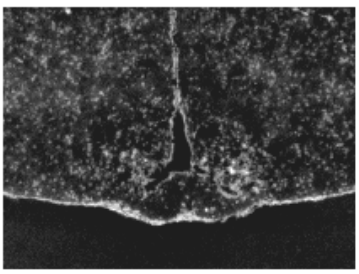
[Gln^6] α-MSH-ND



[Lys^6] α-MSH-ND



[Gln^6] α-MSH-ND (6-10)



Cyclic 16k-MSH

(B)

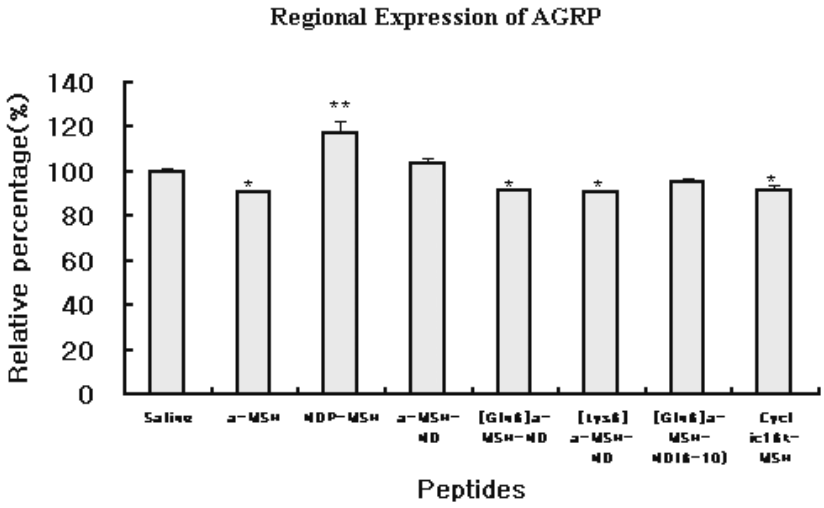
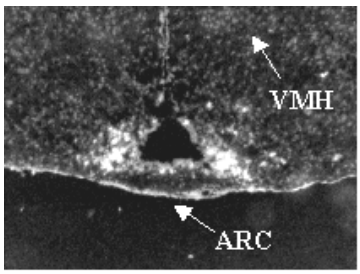
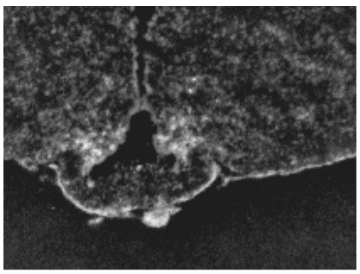


Fig 4. Photogram and graph of in situ hybridization for AGRP expression in arcuate nucleus(ARC). Expression of AGRP was decreased significantly by a-MSH, [Gln6] a-MSH-ND, [Lys6] a-MSH-ND and cyclic16k-MSH whereas expression was increased significantly by NDP-MSH. All values are mean±SEM. n=3 per individual group. Data were analyzed by mean of ANOVA followed by Dunnett test for individual comparisons.(*P<0.05, **P<0.01 vs saline)

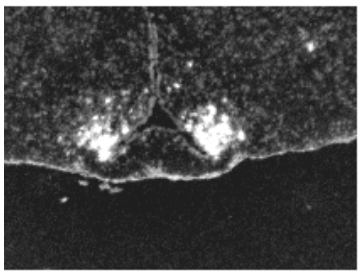
(A) Regional expression of NPY



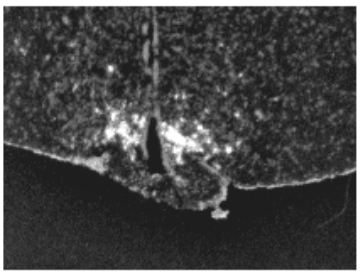
Saline



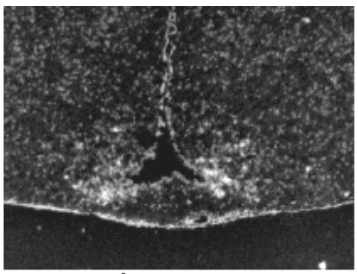
α-MSH



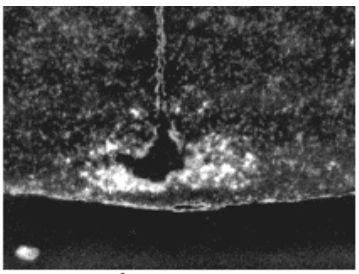
NDP-MSH



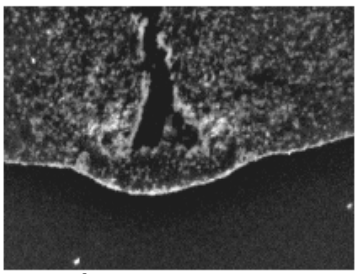
α-MSH-ND



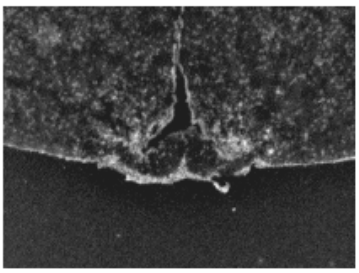
[Gln^6] α-MSH-ND



[Lys^6] α-MSH-ND



[Gln^6] α-MSH-ND (6-10)



Cyclic 16k-MSH

(B)

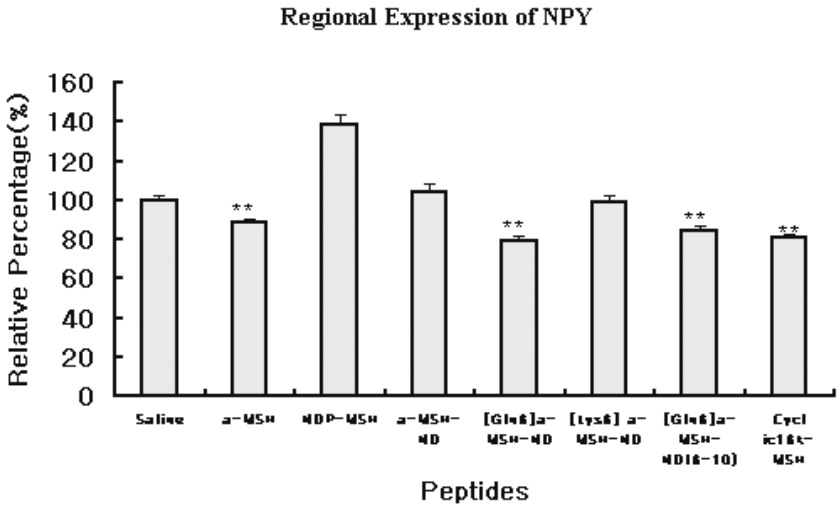
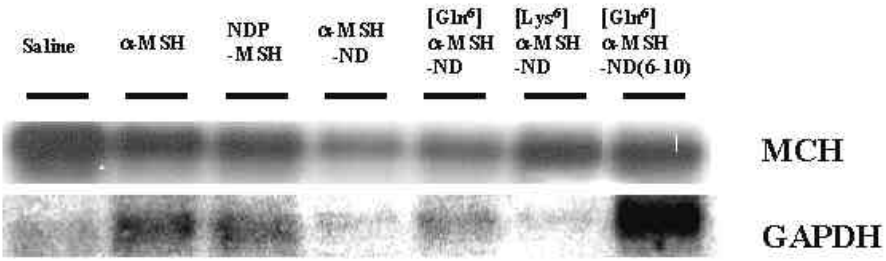
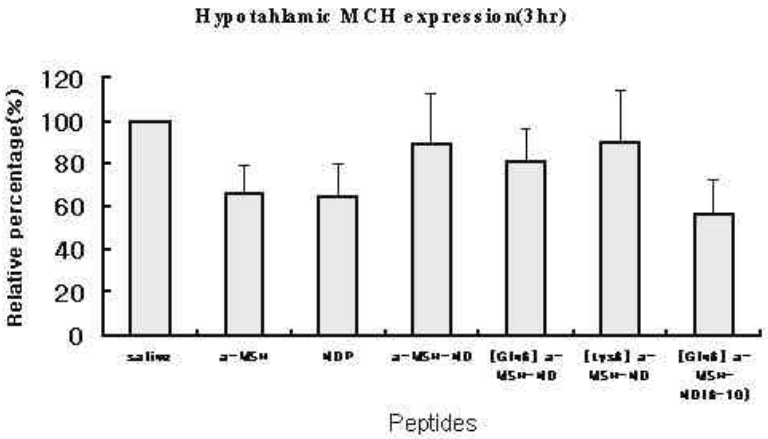


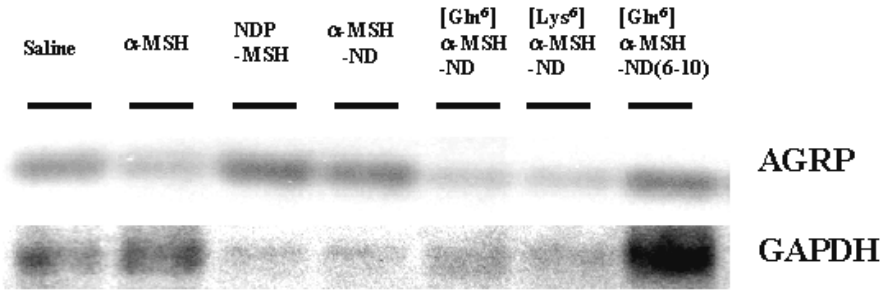
Fig 5. Photogram and graph of in situ hybridization for NPY expression in arcuate nucleus(ARC). Expression of NPY was decreased significantly by a-MSH, [Gln6] a-MSH-ND, truncated form of [Gln6] a-MSH-ND and cyclic16k-MSH whereas expression was increased by NDP-MSH. All values are mean±SEM. n=3 per individual group. Data were analyzed by mean of ANOVA followed by Dunnett test for individual comparisons.(*P<0.05, **P<0.01 vs. saline)

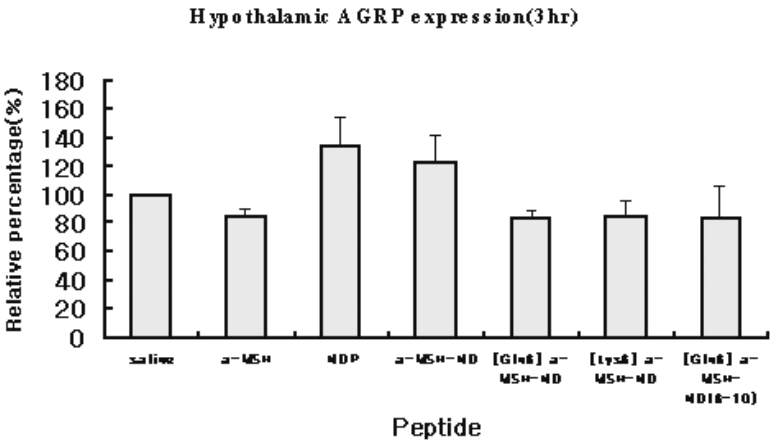
(A)


Saline
α-MSH
NDP -MSH
α-MSH -ND
[Gln6] α-MSH -ND
[Lys6] α-MSH -ND
[Gln6] α-MSH -ND(6-10)
MCH
GAPDH


Hypotahlamic MCH expression(3hr)
Relative percentage(%)
120
100
80
60
40
20
0
saline
α-MSH
NDP
α-MSH-ND
[Gln6] α-MSH-ND
[Lys6] α-MSH-ND
[Gln6] α-MSH-ND(6-10)
Peptides

(B)


Saline
α-MSH
NDP -MSH
α-MSH -ND
[Gln6] α-MSH -ND
[Lys6] α-MSH -ND
[Gln6] α-MSH -ND(6-10)
AGRP
GAPDH


Hypothalamic AGRP expression(3hr)
Relative percentage(%)
180
160
140
120
100
80
60
40
20
0
saline
α-MSH
NDP
α-MSH-ND
[Gln6] α-MSH-ND
[Lys6] α-MSH-ND
[Gln6] α-MSH-ND(6-10)
Peptide

(C)

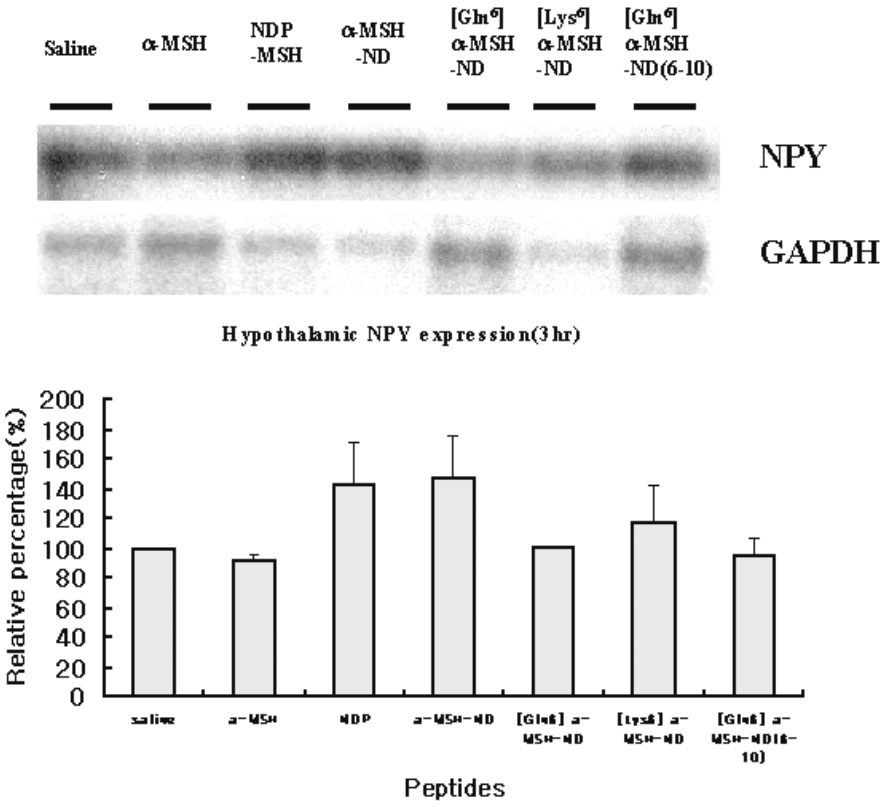
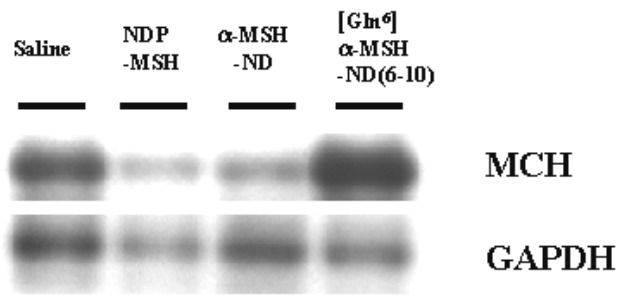
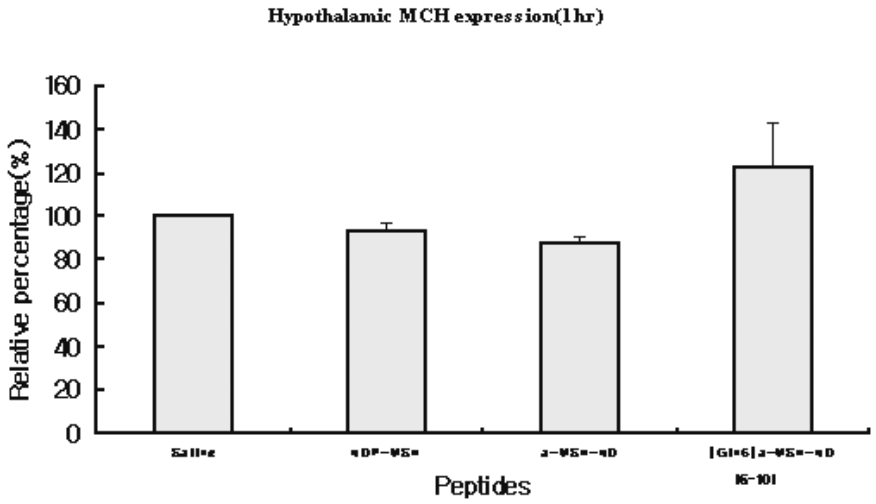


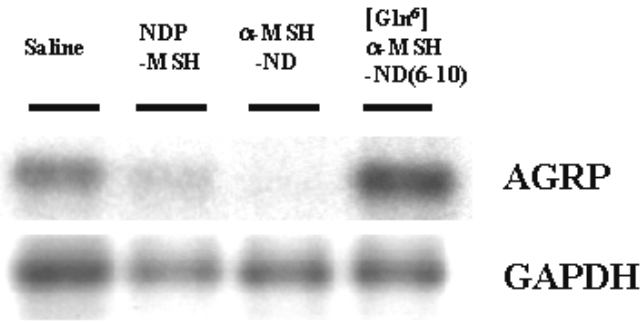
Fig. 6 RT-PCR analysis for hypothalamic mRNA expression levels of MCH, AGRP and NPY after 3hr of administration of melanocortin analogues. Expression of hypothalamic mRNA levels is quantified by RT-PCR and southern blot anaysis. Expression level of MCH(A), AGRP(B) and NPY(C) was standardized by co-amplification with GAPDH primers

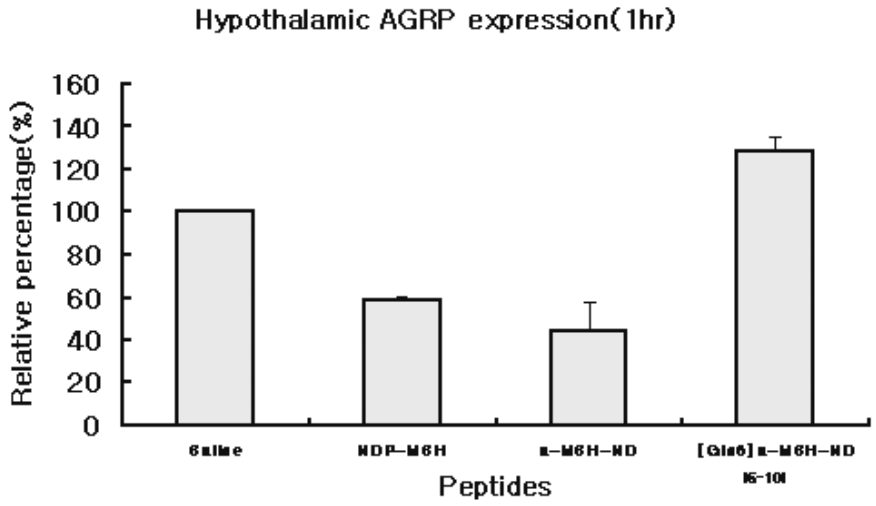
(A)


Saline
NDP
-MSH
α-MSH
-ND
[Gln6]
α-MSH
-ND(6-10)
MCH
GAPDH

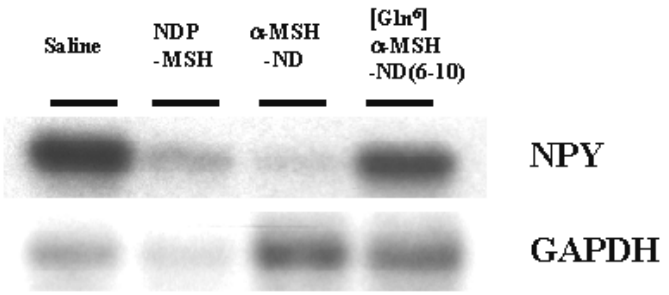

Hypothalamic MCH expression(1hr)
Relative percentage(%)
160
140
120
100
80
60
40
20
0
Peptides

(B)


Saline
NDP -MSH
α-MSH -ND
[Gln6] α-MSH -ND(6-10)
AGRP
GAPDH


Hypothalamic AGRP expression(1hr)
Relative percentage(%)
160
140
120
100
80
60
40
20
0
Saline
NDP-MSH
α-MSH-ND
[Gln6]α-MSH-ND
6-10
Peptides

(C)



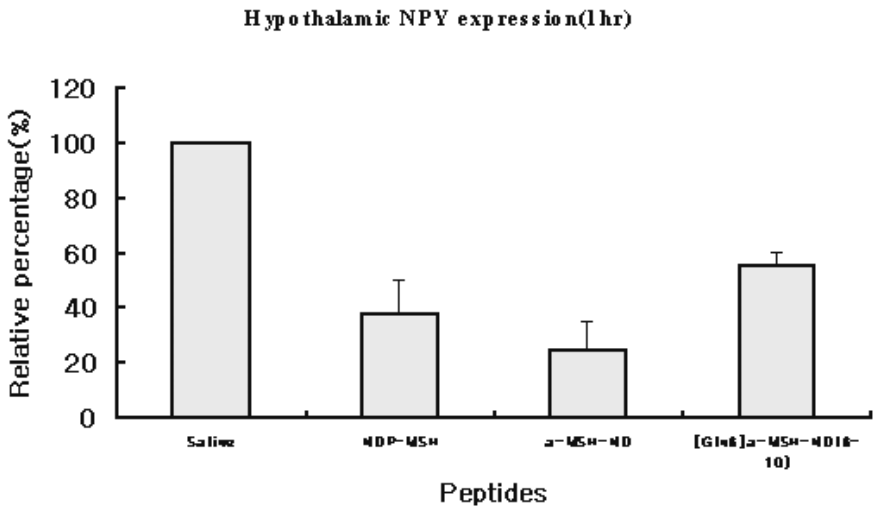


Fig. 7 RT-PCR analysis for hypothalamic mRNA expression levels of MCH, AGRP and NPY after 1hr of administration of melanocortin analogues. Expression of hypothalamic mRNA levels is quantified by RT-PCR and southern blot anaysis. All expression level of MCH(A), AGRP(B) and NPY(C) was standardized by co-amplification with GAPDH primers

Ⅳ. Discussion

Several melanocortin receptors were cloned and identified and it has been thought that these melanocortin receptors may be

involved in mediating the diverse effects of melanocortins. Recently, brain specific melanocortin receptor MC3R and MC4R were identified and it has been reported that these two subtypes are important mediators in feeding behavior and energy expenditure. Many analogues of a-MSH which is endogenous agonist of MC3R and MC4R have been constructed and analyzed in the view of receptor selectivity and biological activity to evolve as an anti-obesity medicine.[23]

We previously reported the differential regulation of cAMP-mediated gene transcription and ligand selectivity by two hypothalamic melanocortin receptor subtypes, MC3R and MC4R with several melanocortin analogues[15]. Here, in the present study, we analyzed the effect of these analogues(Table.1) on food intake *in vivo*. Intracerebroventricular administration of different melanocortin analogues to C57/BL6 mice showed that NDP-MSH and a-MSH-ND were the most efficient peptides in food-intake inhibition and other peptides, such as a-MSH , [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND and cyclic 16k-MSH, also significantly inhibited food-intake whereas truncated form of [Gln^6] a-MSH-ND had no effect. These observations *in vivo* showed a strong consistency with

our previous results. Indeed, in previous our assay of CRE-mediated gene transcription activity, a-MSH-ND was the most efficient a-MSH analogue for MC4R whereas NDP-MSH was the most efficient for MC3R. However, truncated form of [Gln^6] a-MSH-ND showed low CRE-mediated gene transcription activity upon stimulation of MC3R and MC4R.

It is reported that structure of melanocortin analogue and receptor selectivity is strongly relevant and important. It has previously been demonstrated that the core sequence of a-MSH analogues (Asp-His-D-Phe-Arg) is critically important for ligand-receptor interaction and selectivity and we previously reported that type I b turn structure in a-MSH-ND is important for receptor binding and ligand selectivity[24]. Indeed, analogues which contain core amino-acid sequence or modified core sequence (Asp-Gln-D-Phe-Arg and Asp-Lys-D-Phe-Arg) displayed remarkable efficiency for MC3R and MC4R in our previous report and these peptides also showed the significant inhibition effect on food intake. However, truncated form of [Gln^6] a-MSH-ND(6-10) which is lack of core sequence did not showed significant effect *in vitro* or *in vivo*.

These data strongly indicate that these core-residues are important for receptor binding and activation.

Many orexigenic and anorexigenic genes in CNS are reported with gene expression regulation by leptin and reported that hypothalamus is a major center in the control of food and body mass. However, the interaction and the mechanisms of regulation between hypothalamic neuropeptides are not much defined. In this study, we analyzed gene expression of feeding-related hypothalamic neuropeptides after administration of melanocortin analogues by *in situ* hybridization and RT-PCR. First, we analyzed the expression of MCH in hypothalamus after 1 and 3 hr of melanocortin analogue administration . MCH expression in VMH and LH was significantly decreased in association with food-intake inhibition results by administration of melanocortin analogues. It was previously reported that MCH is a critical regulator of feeding and energy balance which probably acts downstream of leptin ad melanocortin system[2]. Recently, it is reported that hypothalamic expression of MCH, which are normally up-regulated in fasted animals leading to an increase in food intake, are significantly reduced in muscarinic acetylcholine 3 receptor knock-out mice[25].

Thus, MCH seems to play a regulatory role in interaction with several receptors as downstream components of feeding behavior. On the basis of our data, it is expected that MCH play as a mediator for adiposity signal in downstream of melanocortin system.

Expression of AGRP and NPY in hypothalamus was significantly decreased by a-MSH, [Gln^6] a-MSH, [Lys^6] a-MSH and Cyclic 16k-MSH after 3hr of administration. Interestingly, expression of AGRP and NPY was decreased by administration of NDP-MSH and a-MSH-ND after 1hr and their expression was increased lately after 3hr. These data demonstrated that NDP-MSH and a-MSH-ND which showed the strongest effects on food intake inhibition, induced a biphasic regulation of AGRP and NPY expression. It is possible that this up-regulation might be a feed back regulation upon the stimulation of melanocortin receptors by NDP-MSH and a-MSH-ND. Taken together, our data suggest that melanocortin system and AGRP/NPY system are balanced and harmonized for the regulation of feeding and energy expenditure in hypothalamus.

AGRP and NPY are highly expressed in ARC where is co-localized with expression region of melanocortin and its receptors, MC3R and MC4R with high density. Indeed, AGRP is the endogenous antagonist of MC3R/4R[26] and a-MSH and AGRP are synthesized within adjacent, but distinct subgroup of hypothalamic neurons (POMC and AGRP neuron) that are sensitive to input from adiposity related signal, leptin[27]. The AGRP and NPY are co-expressed in ARC neuron and it is suggested that leptin stimulates POMC neurons and increase signaling of MC3R and MC4R which have an opposite effect on NPY/AGRP neuron. POMC and NPY/AGRP neurons are co-expressed with leptin receptor indicating that ARC is a principal site for transducing adiposity signal from leptin[28]. It was also reported that neuropeptide Y (NPY) mRNA levels was increased in ARC during fasting, whereas POMC mRNA levels was decreased[30]. NPY expression was significantly attenuated by leptin in ARC suggesting that ARC is an important region for expression and regulation of AGRP, NPY and melanocortin system. Thus, our data clearly demonstrated that expression of NPY and AGRP is regulated by melanocortin analogue administration which is mediated by MC3R and MC4R.

Furthermore, in our experiments, regulation of expression for AGRP and NPY was different by NDP-MSH and a-MSH-ND as compared to other melanocortin analogues. These data raise a possibility that the specific melanocortin analogues for MC3R or MC4R might induce the distinct gene expression regulation of hypothalamic neuropeptides with differential adiposity signaling mediated by MC3R and MC4R.

In conclusion, our results suggest that the effect of melanocortin analogues on food intake inhibition related to the structure of peptides and this structure is important for physiological role of melanocortin receptor. And we also suggest that hypophagic effect of brain melanocortin receptor MC3R and MC4R is associated with differential regulation on expression of hypothalamic neuropeptides. Identification of potential target genes in this regulation are currently undertaking to understand the signaling mediated by MC3R and MC4R.

V. Conclusion

Intracerebroventricular administration of different melanocortin analogues to C57/BL6 mice showed that NDP-MSH and a-MSH-ND were the most efficient peptides in food-intake inhibition and other peptides, such as a-MSH , [Gln6] a-MSH-ND, [Lys6] a-MSH-ND and cyclic 16k-MSH, also significantly inhibited food-intake whereas truncated form of [Gln6] a-MSH-ND had no effect. Expression of hypothalamic neuropeptides such as MCH, AGRP and NPY after administration of melanocortin analogues was analyzed by *in situ* hybridization and RT-PCR. MCH expression was significantly decreased by administration of a-MSH, NDP-MSH, a-MSH-ND, [Gln6] a-MSH, [Lys6] a-MSH and Cyclic 16k-MSH after 1 and 3hr. Expression of AGRP and NPY in ARC was significantly decreased by a-MSH, [Gln6] a-MSH, [Lys6] a-MSH and Cyclic 16k-MSH after 3hr of administration. Administration of NDP-MSH and a-MSH-ND induced a biphasic regulation in expression of AGRP and NPY, showing a decrease after 1hr and an increase after 3hr. Our results suggest that MC3R and MC4R melanocortin receptors mediate hypophagic signaling in association

with differential and harmonized regulation of other hypothalamic neuropeptides.

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국문요약

멜라노코틴 유도체에 의한 섭식행동 조절 및 시상하부 신경펩타이드의 조절

멜라노코틴은 시상하부의 멜라노코틴 3 수용체 (MC3R)와 4 (MC4R) 수용체에 결합하여 식욕조절 및 에너지 대사를 조절하는 비만 조절인자로 알려져 있다. 본 연구에서는 멜라노코틴과 그 구조적 변형을 꾀한 NDP-MSH, a-MSH-ND 와, a-MSH-ND 의 6 번째 His^6 을 Gln 과 Lys 으로 치환한 [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND 그리고 Cyclic16k-MSH 등의 유도체들이 마우스의 섭식에 미치는 영향을 연구하였으며 Intracerebro-ventricular (I.C.V) administration 으로 이 유도체들을 마우스에 주입한 결과 이들에 의해 마우스의 섭식이 감소함을 관찰하였다. 반면 [Gln^6] a-MSH-ND 의 절단된 형인 [Gln^6] a-MSH-ND (6-10) 에 의해서는 섭식이 크게 영향을 받지 않았다. 이와 함께 멜라노코틴 유도체들의 주입이 시상하부의 비만조절 인자들의 발현조절에 미치는 영향을 In situ hybridization 과 RT-PCR 로 분석하였다. Melanin-concentrating hormone (MCH)은 멜라노코틴

유도체의 주입 1 시간 및 3 시간 후에 그 발현이 감소하였다. 또 다른 시상하부 신경 펩타이드인 agouti-related protein (AGRP)과 nueropeptide Y (NPY)의 경우 a-MSH, [Gln^6] a-MSH-ND, [Lys^6] a-MSH-ND 그리고 Cyclic 16k-MSH 의 주입 3 시간 후에 그 발현이 감소하였다. NDP-MSH 와 a-MSH-ND 의 주입에 의한 AGRP 및 NPY 의 발현은 주입 1 시간 후에 감소하며 3 시간 후에는 증가하였다. 이러한 결과들은 멜라노코틴 3 과 4 수용체의 섭식 저해 작용이 다른 신경 펩타이드 들의 다양한 조절작용과 연관되어 있음을 제시한다. 그러므로 멜라노코틴 및 그 수용체의 작용에 대해 표적이 될 가능성을 가진 유전자를 규명한다면 섭식 및 에너지 대사 조절에 있어서의 멜라노코틴 3, 4 수용체의 신호전달기전의 이해는 물론 비만치료제 개발에 대한 기초가 될 것이다.

핵심되는 말 : 멜라노코틴 수용체, 유도체, 섭식, 유전자발현, 시상하부.