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Differential Regulation of Melanin-Concentrating Hormone and Orexin Genes in the Agouti-Related Protein/ Melanocortin-4 Receptor System

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Agouti protein and agouti-related protein (AGRP) antagonize α-melanocyte-stimulating hormone that binds to and activates the melanocortin-4 receptor (MC4-R) in the hypothalamus, thereby stimulating food intake. Melanin-concentrating hormone (MCH) and orexin are orexigenic peptides that specifically are synthesized in the lateral hypothalamus. MCH gene expression was augmented in A^y/a (*agouti*) mice which overexpress agouti protein, but orexin mRNA was not. AGRP administered intracerebroventricularly into wild-type rats augmented MCH but not orexin gene expression. Also, SHU9119, a peptidergic antagonist of MC4-R, increased only MCH mRNA. These findings indicate that interruption of signaling at MC4-R activates the MCH but not the orexin gene. The biosyntheses of MCH and orexin are regulated through different pathways. © 2000 Academic Press

The mammalian hypothalamus has a pivotal role in the integrated regulation of energy homeostasis and body weight (for review see Ref. 1). Alpha-melanocyte-stimulating hormone (α-MSH) synthesized in the hypothalamic arcuate nucleus binds to and activates the melanocortin-4 receptor (MC4-R), elevates the intracellular cAMP level, leading to reduced food intake (2). Agouti-related protein (AGRP), which also is synthesized in the arcuate nucleus as a 131-amino-acid protein, competes with the binding of α-MSH to MC4-R, thereby stimulating food intake (3–6). Murine agouti protein, a protein homologous to AGRP, normally is expressed in the hair follicles of wild type mice and reduces pigmentation by antagonizing the melanocortin-1 receptor (MC1-R) (7). *Agouti* mice (A^y/a) are genetically obese mice with a yellow coat color. These characteristics are caused by a dominantly inherited promoter rearrangement at the agouti gene which results in the constitutive and ubiquitous expression of agouti protein (8). MC4-R mRNA has been found in more than 100 nuclei in the cerebral cortex, thalamus, hypothalamus, brainstem, and spinal cord of the rats (9). It is highly expressed in the lateral hypothalamus; a classical "feeding" center. Recently, two orexigenic peptides, melanin-concentrating hormone (MCH) and orexin have been identified in this region (10, 11). These peptides are thought to be functionally related to α-MSH, AGRP, and neuropeptide Y because the MCH and orexin neurons receive innervations from these peptide neurons (12). The MCH gene is up-regulated in leptin-deficient *ob*/*ob* mice and leptin-insensitive *db*/*db* mice, whereas the orexin gene is down-regulated in these mice (10, 13), indicating that the MCH and orexin systems are regulated through different pathways. We studied the differences in gene expressions of MCH and orexin in the A^y/a mice and in wild type rats when AGRP and α-MSH were administered intracerebroventricularly (icv). We also compared changes in the amounts of these mRNAs in response to icv injections of SHU9119, a peptidergic antagonist of MC4-R (14).

MATERIALS AND METHODS

Animals. Fifteen-week-old male A^y/a and control C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) and 10-week-old male Wistar rats (Seac Yoshitomi Ltd., Fukuoka, Japan) were used. They were housed in independent plastic cages in an air-conditioned room (25–27°C) under a 12-h light (07:00–19:00)/12-h dark cycle and fed *ad libitum* on standard lab chow. Their plasma glucose concentrations were measured by the glucose oxidase method. Fifteen A^y/a mice were sacrificed after a 48 h fast. All procedures were done in accordance with the Japanese Physiological Society's guidelines for animal care.

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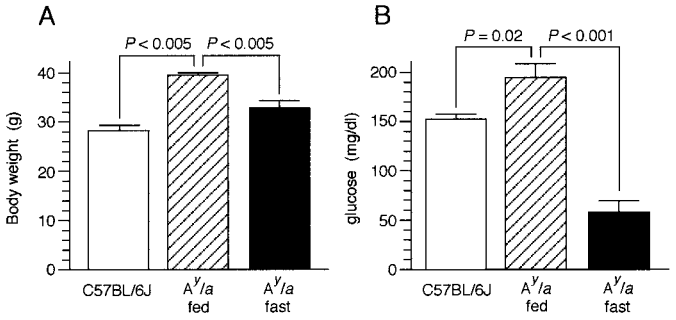

FIG. 1. (A) Body weights and (B) plasma glucose concentrations of C57BL/6J mice, A^y/a mice fed *ad libitum*, and A^y/a mice that had fasted 48 h.

ICV experiment. Wistar male rats weighing 330–350 g ($n = 90$) were anesthetized with sodium pentobarbital (45 mg/kg body wt), after which each was mounted in a brain stereotaxic device (Narishige, Tokyo, Japan). A stainless steel cannula (guide steel cannula, 23-gauge; insert cannula, 27-gauge) was implanted in the left lateral ventricle. Four days after surgery, the insert cannula was replaced with a 27-gauge injection cannula. Cumulative food intake was measured over a period of 2 h after an icv injection of 1 or 3 nmol AGRP (Phoenix Pharmaceuticals Inc., Mountain View, CA) in 10 μl saline, 0.5 or 1 nmol of SHU9119 (Phoenix Pharmaceuticals Inc.) in 10 μl saline, 1 nmol of α-MSH (Sigma Chemical Co., St. Louis, MO), or the same volume of saline, to free-moving rats. One nanomole AGRP also was injected to rats that had fasted 12 h. The rats then were decapitated, and their brains removed.

Northern blot analysis. The lateral hypothalamus was punched out from the frozen brain slices (2.1–3.6 mm rostal to the interaural line in the mouse atlas (15) and 5.2–7.7 mm rostal to the interaural line in the rat brain atlas (16)), as reported previously (17), the punched-out diameter being 1.5 mm in the mice and 1.6 mm in the rats. Total RNA was extracted from the lateral hypothalamus with Trizol (Life Technologies Inc., Grand Island, NY). One microgram of the total RNA was denatured with 16 μl of 1 M glyoxal and 50% dimethyl sulfoxide then electrophoresed on a 0.8% agarose gel (FMC Bio Products, Rockland, ME) in 10 mM sodium phosphate buffer (pH 7.0), after which the sample was transferred to a Zeta Probe membrane (Bio-Rad Laboratories, Richmond, CA) and fixed by ultraviolet irradiation. The probes used for Northern blot analyses were a 287-bp cDNA fragment of rat orexin (sense primer 5′-TGTCGCCAGAAGACGTGTTCCTG-3′ and antisense primer 5′-AAGACGGGTTCAGACTCTGGATC-3′); a 320-bp cDNA fragment of rat MCH (sense primer 5′-AACAGGGTCGGTAGACTCGT-3′ and antisense primer 5′-ATCGGTTGTTGCTCCTTCTC-3′); and a 203-bp cDNA of mouse GAPDH (sense primer 5′-GTTTGTGATGGGTGTGAACC-3′ and antisense primer 5′-TCACGCCACAGCTTTCCAGA-3′). The membrane first was treated for 2 h at 42°C in 6× SSPE (900 mM NaCl, 60 mM $NaH_2PO_4 \cdot H_2O$, 7 mM EDTA, pH 7.4) containing 40% formamide, 5× Denhardt's solution, 0.5% SDS, and 0.1 mg/ml denatured salmon sperm DNA, after which it was hybridized for 18 h at 42°C in an identical solution that contained the ^{32}P-labeled orexin and GAPDH cDNA probes. The RNA blot was washed with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0)/0.1% SDS solution at 55°C, then with 1× SSC/0.1% SDS solution at 55°C and exposed to film. The membrane then was boiled for 15 min at 70°C in 0.1× SSC solution to strip it of the orexin and GAPDH probes, after which it was used for sequential hybridization with the MCH and GAPDH probes. Hybridization signals were measured in a Fujix Bio-image analyzer, BAS 2000 (Fuji Photo Film Co., Tokyo, Japan). The amounts of the orexin and MCH mRNAs were calculated relative to the radioactivity of GAPDH.

Statistical analysis. The means ± SEM were analyzed by a one-way analysis of variance (ANOVA), followed by a Bonferroni-type adjustment for multiple comparison. Differences were considered significant when the P value was less than 0.05.

RESULTS AND DISCUSSION

MCH and orexin mRNA contents in the A^y/a mice. Body weights and plasma glucose concentrations of the A^y/a mice fed *ad libitum* were significantly higher than

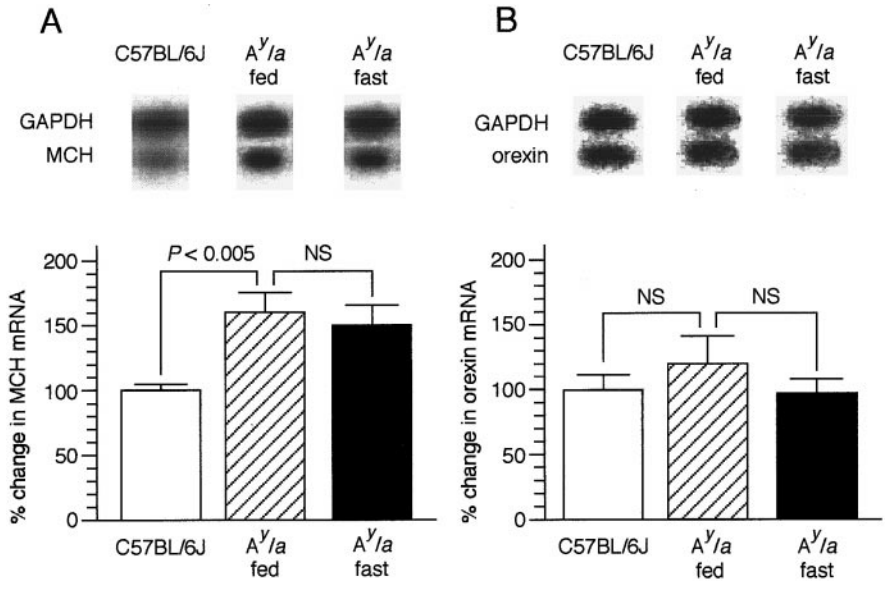

FIG. 2. (Upper) Representative Northern blot analysis patterns of (A) MCH and (B) orexin mRNAs in the lateral hypothalamus of C57BL/6J mice, A^y/a mice fed *ad libitum*, and A^y/a mice that had fasted 48 h ($n = 15$ per group). (Lower) MCH and orexin mRNA percentages relative to an arbitrary GAPDH unit. Values are means ± SEM. NS, no significant difference.

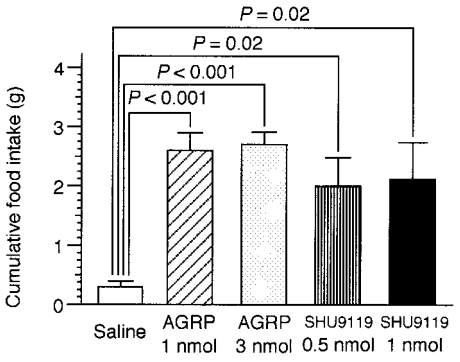

FIG. 3. Stimulation of food intake caused by icv injections of AGRP and SHU9119 to freely fed rats. Data show cumulative food intake for the 2 h period after injection. Values are means ± SEM (n = 15 per group).

those of the controls (Fig. 1). MCH mRNA in the lateral hypothalamus of the A^y/a mice was 1.6-fold that of the controls ($P < 0.005$, Fig. 2), whereas orexin mRNA on the same hybridization membrane used in the MCH mRNA quantification did not differ between the A^y/a mice and controls. Although the body weights and plasma glucose concentrations of the fasted A^y/a mice were significantly lower than those of the fed A^y/a mice (Fig. 1), the mRNA amounts of MCH and orexin did not differ between two groups (Fig. 2).

ICV injections of AGRP, SHU9119, and α-MSH. One and three nanomole AGRP significantly increased food intake 9.5- and 9.8-fold, respectively, in freely fed Wistar rats compared to the value for saline injection (Fig. 3). Also 0.5 and 1 nmol SHU9119 increased it 7.1- and 7.5-fold, respectively, compared to the control group (Fig. 3). The contents of MCH mRNA in the three AGRP groups, of which two were fed *ad libitum* and one was fasted, were 1.4-fold the control values (Fig. 4A). In contrast, there was no significant difference in the orexin mRNA contents of these three groups (Fig. 4B). The MCH mRNA content in SHU9119-injected rats was 1.8-fold that of the controls ($P < 0.005$, Fig. 4A), but the orexin mRNA contents did not differ. The content of MCH mRNA in α-MSH-injected rats was not different from that of the controls (Fig. 4A), whereas the orexin mRNA content in the α-MSH group was 60% the controls ($P = 0.013$, Fig. 4B).

Our study showed that MCH gene expression is augmented in the A^y/a mice that overexpress agouti protein, whereas orexin gene expression is not. We also showed that AGRP, a protein homologous to the agouti gene product, markedly increased food intake and augmented MCH gene expression, but not orexin gene expression. Moreover, SHU9119 enhanced only MCH gene expression. Agouti protein, AGRP, and SHU9119 antagonize α-MSH which binds to and activates MC4-R, thereby stimulating food intake (3, 4, 14, 18). MC4-R knockout mice and AGRP-overexpressing transgenic mice developed maturity-onset obesity similar to the A^y/a mice (3, 4). Although MCH and orexin neurons are in contact with AGRP neurons, the above

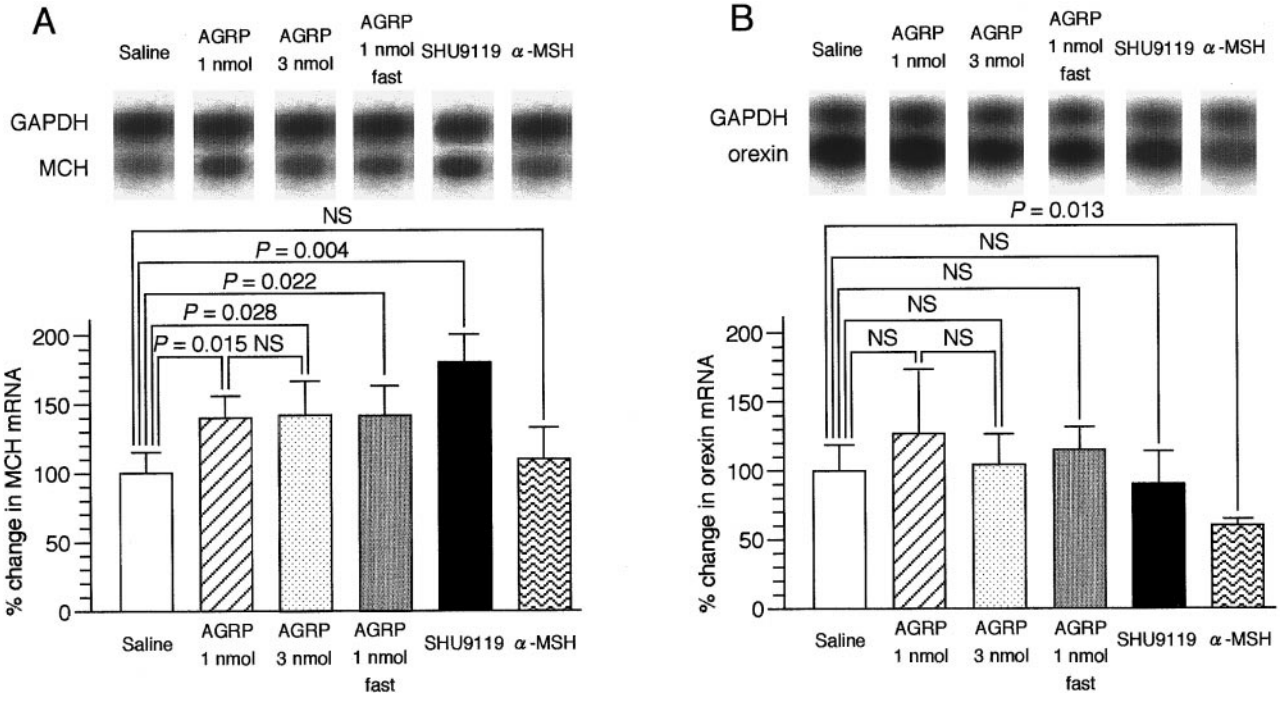

FIG. 4. (Upper) Representative Northern blot analysis patterns of (A) MCH and (B) orexin mRNAs in the lateral hypothalamus of rats that were fed *ad libitum* and given saline, AGRP, SHU9119, and α-MSH (n = 15 per group). One nanomole AGRP also was injected to the rats that had fasted 12 h. (Lower) Quantification of MCH and orexin mRNA contents. Values are means ± SEM. NS, no significant difference.

findings indicate that the interruption of signaling at MC4-R caused by these antagonists affects only MCH. Overexpression of the MCH gene in the A^y/a mice may be involved in the etiology of their hyperphagia.

MCH and orexin mRNAs in normal rat hypothalamus are up-regulated upon fasting (10, 11), but these two mRNA levels did not change in the fasting A^y/a mice. MCH gene expression might be fully up-regulated in the A^y/a mice. Only 13% of orexin neurons was activated by 53 mg/dl plasma glucose (19) which was seen in the fasting A^y/a mice in this study. Moreover, persistent hyperglycemia induced by streptozotocin injection and chronic hypoglycemia by insulin injection did not change orexin gene expression (20). The regulatory mechanisms that govern the biosyntheses of MCH and orexin in the A^y/a mice that have persistent hyperglycemia may differ from those in normoglycemic mice.

MCH mRNA is up-regulated in leptin-deficient *ob*/*ob* mice and leptin-insensitive *db*/*db* mice, whereas orexin mRNA is down-regulated in these mice (10, 13). We showed that icv injection of α-MSH did not change MCH gene expression, but suppressed orexin gene expression. We investigated changes in MCH and orexin gene expressions in rats exposed to cold stress; only the orexin mRNA content increased (unpublished observations). Furthermore, a very recent neurophysiological study of orexin knockout mice found that they have a phenotype of narcolepsy and that orexin has an important role in the regulation of sleep/wakefulness states (21). Bolus icv injections of orexin-A and -B dose-dependently increased heart rate, blood pressure, and renal sympathetic nerve activity in conscious unrestrained rats, suggesting that the orexin system modulates the autonomic functions (22). No similar physiological roles have been found for MCH. MCH and orexin neurons are coextensive but not colocalized in the lateral hypothalamus (12). Accumulated evidence suggests that the two peptides function independently but complementarily in energy homeostasis. Our study showed that the MCH and orexin systems most probably are regulated by different pathways. Feeding behavior and energy homeostasis are controlled by redundant and complicated neuronal networks. Further investigation of gene expressions of MCH and orexin in physiological experiments, as in this study, should provide information as to which neuronal networks regulate the biosyntheses of these orexigenic hypothalamic peptides.

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