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Expression of pro-opiomelanocortin and agouti-related protein in the hypothalamus of caffeine-administered rats

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In the present study, we examined the effects of caffeine on food intake and body weight, and pro-opiomelanocortin (POMC) and agouti-related protein (AgRP) expression in the hypothalamus. Rats were administered intraperitoneally with 100 mg/kg caffeine (a high, non-toxic dose) or saline during the light phase. Intraperitoneal administration of caffeine induced a significant reduction in food intake and body weight 12 hr after treatment. In addition, POMC expression was significantly increased and AgRP expression was decreased in the arcuate nucleus (Arc) after caffeine treatment. These results demonstrate that administration of caffeine up-regulates POMC expression and down-regulates AgRP expression in the Arc, suggesting that the activation of the hypothalamic POMC neurons and inhibition of the AgRP neurons might play a role in the regulation of food intake and body weight by caffeine.

Keywords: body weight; food intake; POMC; AgRP; caffeine

Introduction

Many studies have shown that neuroanatomical and neurophysiological control of food intake in the central nervous system (CNS) is mediated through specific neuropeptides, receptors, and neuronal circuits (Schwartz et al. 2000; Broberger and Hökfelt 2001). The hypothalamus is regarded as the major center for controlling feeding behavior in the brain (Hillebrand et al. 2002). In addition, hypothalamic neurons have been implicated in the control of various body functions such as the stress response, sexual behavior, and energy homeostasis (Belgardt et al. 2009).

The arcuate nucleus (Arc) is a principal hypothalamic nucleus involved in the regulation of feeding and contains two major neuronal populations. One population consists of agouti-related protein (AgRP)-expressing neurons and stimulates food intake. The second population consists of pro-opiomelanocortin (POMC)-expressing neurons and inhibits food intake. Neuronal projections from these two populations then innervate with other hypothalamic areas involved in food intake regulation, such as the paraventricular nucleus (PVN), dorsomedial nucleus (DMN), and lateral hypothalamic area (LHA) (Bouret et al. 2004; Simpson et al. 2009). POMC regulates energy homeostasis through α-melanocyte-stimulating hormone (α-MSH), one of the cleavage products of POMC. Alpha-MSH exerts an inhibitory control on food intake and energy storage in the CNS through activation of melanocortin-3 receptor (MC3R) and melanocortin-4 receptor (MC4R) (Cone 1999). Both MC3R and MC4R have been shown to be involved in the regulation of energy balance (Adan et al. 1997) and are widely distributed in the rodent brain (Mountjoy et al. 1994; Kishi et al. 2003). The functional basis of the role of POMC in feeding and obesity is mostly due to interactions between POMC-derived peptides in the brain and the specific receptors MC3R and MC4R (Pritchard et al. 2002). In contrast, AgRP acts as an endogenous antagonist at these receptors (Ollmann et al. 1997). AgRP is a potent stimulant of feeding behavior, which functions to control food intake and energy homeostasis in the hypothalamus (Wirth and Giraudo 2000; Haskell-Luevano and Monck 2001).

Caffeine is a commonly used CNS stimulant as well as a thermogenic agent, and has been studied for potential use in body weight reduction (Dulloo 1993; Murphy et al. 2003). Caffeine is a key component of many popular drinks such as coffee and tea (Ofluoglu et al. 2009). The stimulatory effect of caffeine on thermogenesis is well understood, and reduced food

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intake after caffeine consumption has also been shown. Thus, caffeine can affect both energy expenditure and energy intake (Svenningsson et al. 1999; Diepvens et al. 2007). In several studies, long-term consumption of caffeine, caffeinated cola, and caffeinated tea decreased body weight in rodents. Some studies have also found decreases in adipose tissue weight and the number of adipocytes, without a decrease in daily caloric intake (Greenberg et al. 2006). In addition, caffeine may induce significant weight loss in non-obese subjects because caffeine has been shown to cause greater thermogenesis, lipolysis, fat oxidation, and insulin secretion in non-obese persons than in obese (Astrup et al. 1992; Kovacs et al. 2004).

However, how caffeine regulates food intake-related neuronal mechanisms in the hypothalamus has not been well studied. Thus, this study aimed to elucidate the neuronal action of caffeine in food intake and body weight control.

Materials and methods

Animals

Male Sprague-Dawley rats (250–300 g) were purchased from KOATECH (Pyeongtaek, Korea) and housed individually in clear plastic cages in a temperature- and humidity-controlled environment under 12-hr light/dark cycle (light on at 6:00 a.m.) with free access to lab chow and water. The experiments were performed after the animals had been habituated to the experimental environment for 1 week. All animal procedures adhered to the Animal Care and Use Guidelines of Gyeongsang National University (Approval No.: GLA-090211-R0012, GLA-090311-R0020, GLA-090527-R0041 and GLA-090708-R0055).

Caffeine administration

To examine the effects of caffeine on the body weight and food intake, we administered caffeine through the drinking water (0.5 g/L and 1.0 g/L). We describe the precise protocol in the Results section.

To examine the effects of caffeine on the hypothalamic POMC and AgRP expression, rats were divided into two weight-matched groups: saline-treated rats (control or CTL) and caffeine-treated rats (caffeine or CAF). Caffeine (100 mg/mL/kg, in saline; Sigma, St. Louis, MO) or saline was injected intraperitoneally (i.p.) between 10:00 a.m. and 10:30 a.m. This caffeine dose is known as high but non-toxic (Deurveilher et al. 2006; Lage et al. 2006; Pettenuzzo et al. 2008; Ofluoglu et al. 2009; Westerterp-Plantenga 2010). After being injected, the rats were returned to their home cage and given food and water ad libitum. The food consumption and body weight of the rats were monitored each time point.

Tissue preparation

Rats were anesthetized and perfused through the left cardiac ventricle and ascending aorta with a fixative solution containing 4% paraformaldehyde. Brains were removed after perfusion and post-fixed in the same fixative overnight at 4°C and cryoprotected with 20% sucrose. Coronal sections of 30 μm thickness were cut on a freezing microtome (Leica, Tokyo, Japan) and sections were collected into anti-freeze solution and kept at −20°C in a freezer.

Immunohistochemistry

Immunohistochemistry was performed on free-floating sections using a standard avidin-biotin-horseradish peroxidase complex (ABC) method. The immunohistochemical procedure was performed in vials (antibody incubation) or tissue processing baskets (washings). Glass sticks were used for handling the delicate sections. The sections were sequentially treated with 1% sodium borohydride ($NaBH_4$) in 0.01 M PBS and 5% normal serum in 0.01 M PBS. They were next incubated with primary antibodies overnight at 4°C and subsequently exposed to secondary antibody (1:500; Vector Laboratories, Burlingame, CA) and ABC (1:100; Vectastain Elite ABC kit; Vector Laboratories). The sections were then visualized by staining with diaminobenzidine (DAB; Sigma) in 0.01 M PBS and mounted on gelatin-coated slides. Images were taken using a light microscope (BX50, Olympus, Tokyo, Japan). The POMC and AgRP immunoreactive cells were quantified by manual counting as a mean number per section. Equivalent results were obtained using sections from at least three different animals. The examiner (J.Y.J.) was blind to the treatment conditions. The following antibodies were used: rabbit anti-c-Fos antibody (1:10,000; Calbiochem, Darmstadt, Germany), goat anti-ACTH/CLIP antibody (1:100; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), goat anti-AgRP antibody (1:100; Santa Cruz Biotechnology, Inc.), rabbit anti-MC3R antibody (1:100; Santa Cruz Biotechnology, Inc.), and rabbit anti-MC4R antibody (1:100; Abcam, Cambridge, UK).

Statistical analysis

Two group comparisons were performed using Student's unpaired *t*-test. All data are shown as mean ± S.E. Statistical significance was considered at $P < 0.05$.

Results

Effects of caffeine on body weight and food intake

We studied the effects of caffeine consumption using caffeine-containing water. During 7 days, caffeine was given in drinking water (0.5 g/L and 1.0 g/L). The control group was given distilled water (Méndez et al. 2008; Ofluoglu et al. 2009), and both body weight and food intake were monitored every day. Caffeine consumption, at both doses, significantly reduced body weight over the course of the 7 days (Figure 1A).

The total food intake was also changed by caffeine treatment. The daily food intake of the caffeine groups (both doses) was dramatically decreased during the first few days. Subsequently, food intake of the 0.5 g/L caffeine group increased to almost that of control intake, whereas the 1.0 g/L caffeine group remained significantly lower than control intake (Figure 1B).

Since the effect of caffeine on the body weight and food intake was remarkable in early time points, we investigated the possible involvement of hypothalamic neurons using an acute caffeine injection model within 24 hr. Caffeine gradually decreased body weight, and a caffeine-induced significant reduction of body weight was seen at 12 and 24 hr after injection (Figure 1C). Caffeine also remarkably changed food intake. The cumulative food intake of the control group increased over time while the caffeine group showed no increase in cumulative food intake. The caffeine-induced reduction in feeding was still evident 24 hr after injection (Figure 1D).

Effects of caffeine on c-Fos immunoreactivity

Despite the fact that caffeine is a commonly used CNS stimulant, the neuronal mechanisms underlying its stimulatory effect are not fully understood, especially in the hypothalamic region of brain. By using c-Fos immunohistochemistry as a marker of neuronal activation, we examined the stimulatory effect of caffeine in the hypothalamus (Murphy et al. 2003; Singewald et al. 2003; Deurveilher et al. 2006). Caffeine injection significantly increased c-Fos immunoreactive neurons in the Arc and PVN 90 min after administration

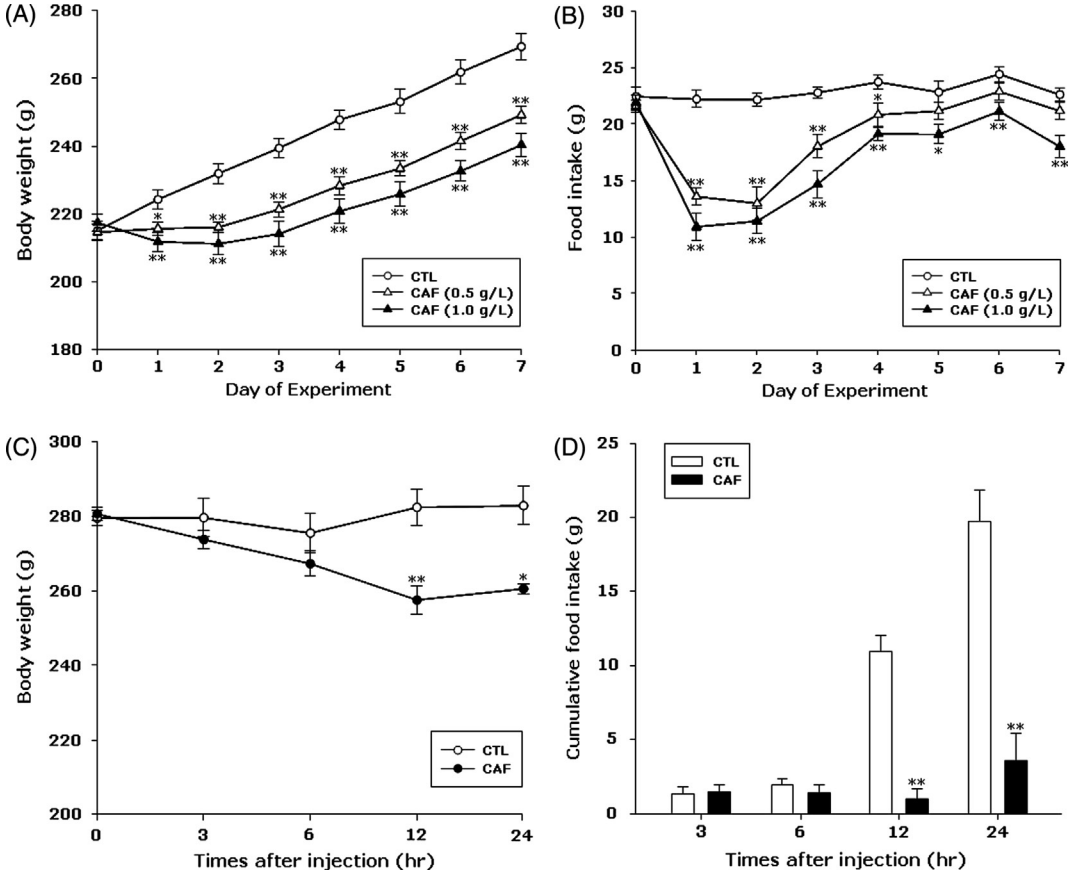

Figure 1. Effects of caffeine on body weight and food intake. Daily body weights (A) and food intake (B) of rats administered caffeine-containing water (0.5 g/L and 1.0 g/L) during 7 days. Acute effects of caffeine on body weight (C) and cumulative food intake (D). Caffeine was administered (i.p., 100 mg//kg) and animals were monitored for 24 hr. Data are presented as means ± S.E.; $*P < 0.05$, $**P < 0.01$ vs. control ($n = 7$ in each group).

(Figure 2A and B). These results indicate that the action sites of caffeine in the hypothalamus are mainly Arc and PVN.

Effects of caffeine on POMC and AgRP immunoreactivity

Since caffeine treatment increased the immunoreactivity of c-Fos in the Arc and PVN, we next examined the effects of caffeine on the expression of POMC and AgRP in these regions. In the Arc, the number of POMC immunoreactive neurons was significantly increased 6 hr after caffeine injection. Moreover, the caffeine-induced elevation of POMC immunoreactive neurons was still evident at 12 hr, and then recovered to control levels at 24 hr (Figure 3A). Several POMC immunoreactive fibers were also observed in the PVN. Caffeine injection slightly elevated the population of POMC immunoreactive fibers 6–12 hr after injection (Figure 3B). In contrast to POMC immunoreactivity, caffeine-induced AgRP immunoreactivity was similar to control levels until 6 hr after caffeine injection, and then decreased by 12 hr. Furthermore, the caffeine-induced reduction of AgRP immunoreactive neurons was significantly sustained until 24 hr after injection (Figure 4A). AgRP immunoreactive fibers were also observed in the PVN. AgRP immunoreactive fibers gradually decreased after caffeine injection (Figure 4B).

Effects of caffeine on MC3R and MC4R immunoreactivity

As the expression of POMC and AgRP was altered in the Arc and PVN after caffeine administration, we tested the effects of caffeine on MC3R (Figure 5A and B) and MC4R (Figure 5C and D) immunoreactivity in these regions 24 hr after injection. Caffeine significantly increased MC3R (Figure 5A(a) and (b)) and MC4R (Figure 5C(e) and (f)) immunoreactivity in the Arc. In addition, a slight increase in MC3R immunoreactivity was observed in the PVN after caffeine injection (Figure 5A(c) and (d)). However, differences in MC4R immunoreactivity between the two groups were not observed in the PVN (Figure 5C(g) and (h)).

Discussion

In previous studies, the effects of caffeine on body weight and food intake were well examined. However, the neuronal mechanisms underlying these caffeine-induced changes in body weight and food intake remain to be elucidated.

In this study, we revealed the neuronal stimulatory effects of caffeine on body weight and food intake regulation by an acute injection model. Caffeine significantly decreased body weight 12 hr after administration, and this weight loss was sustained 24 hr post-injection. At this time point, food intake of the control group was significantly greater than that of the caffeine group. These results are consistent with other studies in which caffeine reduced body weight and food intake (Greenberg et al. 2006; Diepvens et al. 2007). It has been reported that systemically administered caffeine widely activated neurons in the brain (Svenningsson et al. 1999; Murphy et al. 2003). Thus, we examined caffeine-induced neuronal activation by measuring c-Fos immunoreactivity in hypothalamic regions related to food intake regulation. Caffeine injection stimulated c-Fos expression in both the Arc and PVN.

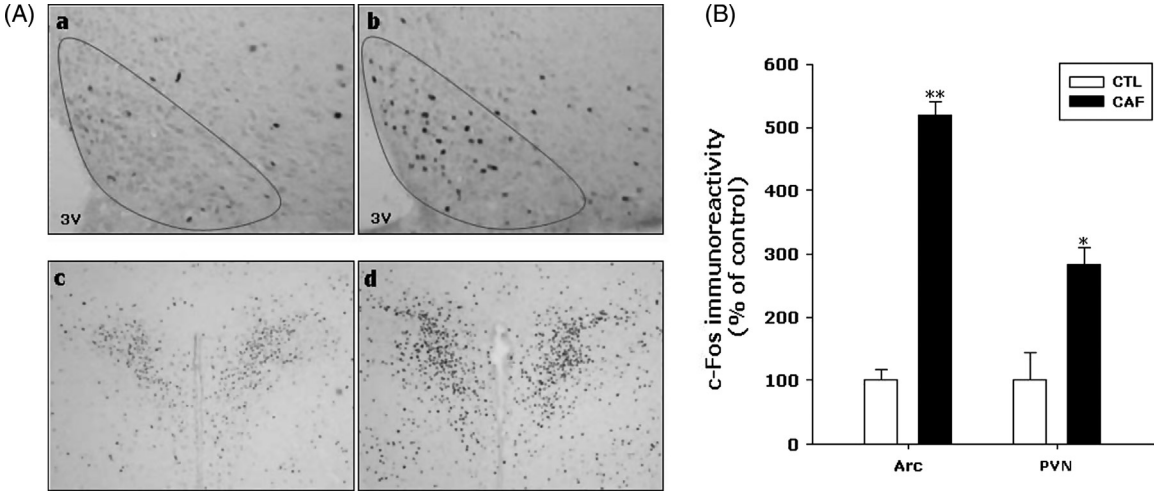

Figure 2. Effects of caffeine injection on c-Fos expression in the hypothalamus. Expression of c-Fos was increased in the Arc (a, b) and PVN (c, d). Data are presented as means ± S.E.; $*P < 0.05$, $**P < 0.01$ vs. control (a, c: control; b, d: caffeine). Original magnification: ×100 (a, b), ×40 (c, d).

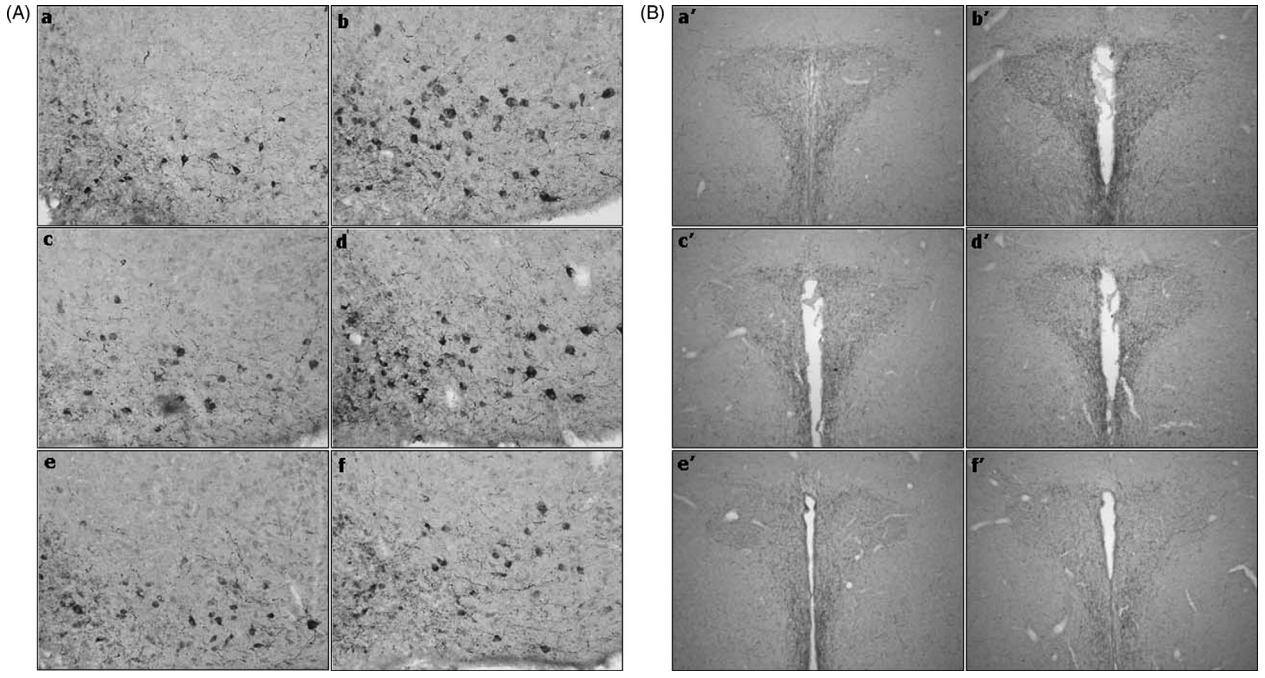

Figure 3. Effects of caffeine injection on POMC expression in the hypothalamus. POMC immunoreactivity was increased in the Arc (A) and PVN (B) after caffeine injection (a, a′, control 6 hr; c, c′, control 12 hr; e, e′, control 24 hr; b, b′, caffeine 6 hr; d, d′, caffeine 12 hr; f, f′, caffeine 24 hr). Original magnification: ×200 (A), ×40 (B).

The Arc and PVN are main targets for studies concerning the control of food intake and body weight (Simpson et al. 2009). The Arc contains the anorexigenic peptide POMC and the orexigenic peptide AgRP, which are the primary regulators of diverse nutritional signals (Lenard and Berthoud 2008). Considering these reports, we hypothesized that caffeine may participate in regulation of feeding behavior through POMC and AgRP

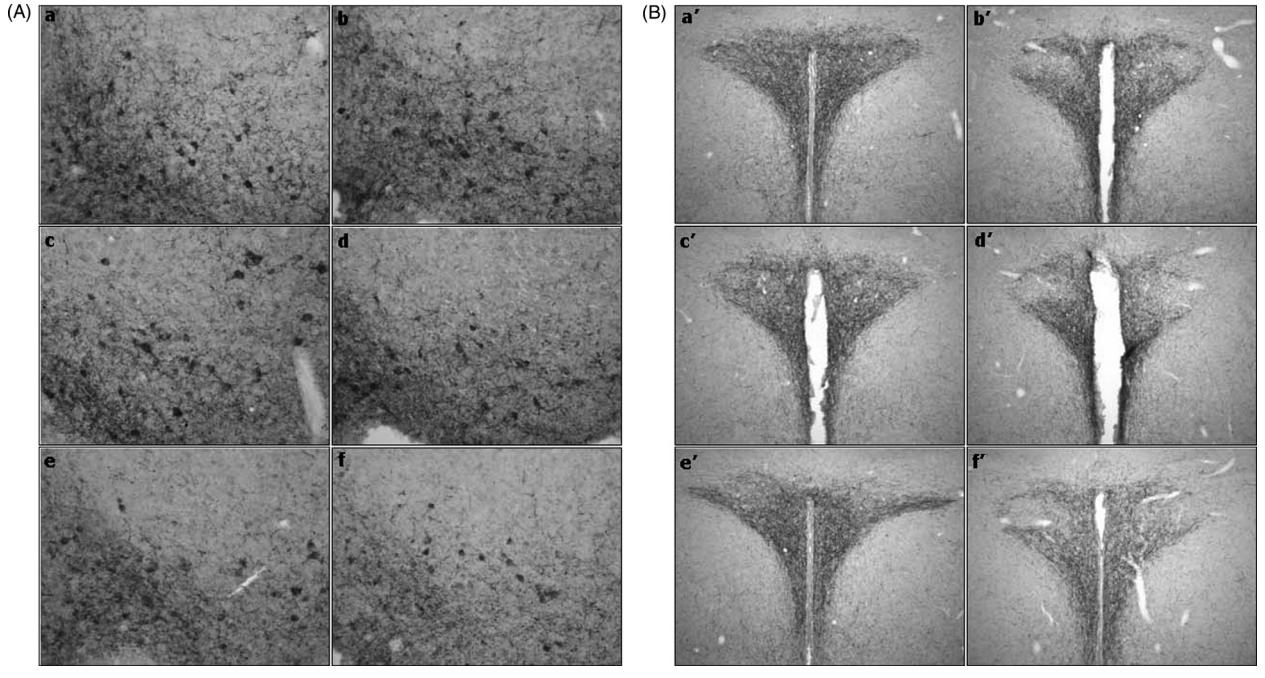

Figure 4. Effects of caffeine injection on AgRP expression in the hypothalamus. AgRP immunoreactivity was decreased in the Arc (A) and PVN (B) after caffeine injection (a, a′, control 6 hr; c, c′, control 12 hr; e, e′, control 24 hr; b, b′, caffeine 6 hr; d, d′, caffeine 12 hr; f, f′, caffeine 24 hr). Original magnification: ×200 (A), ×40 (B).

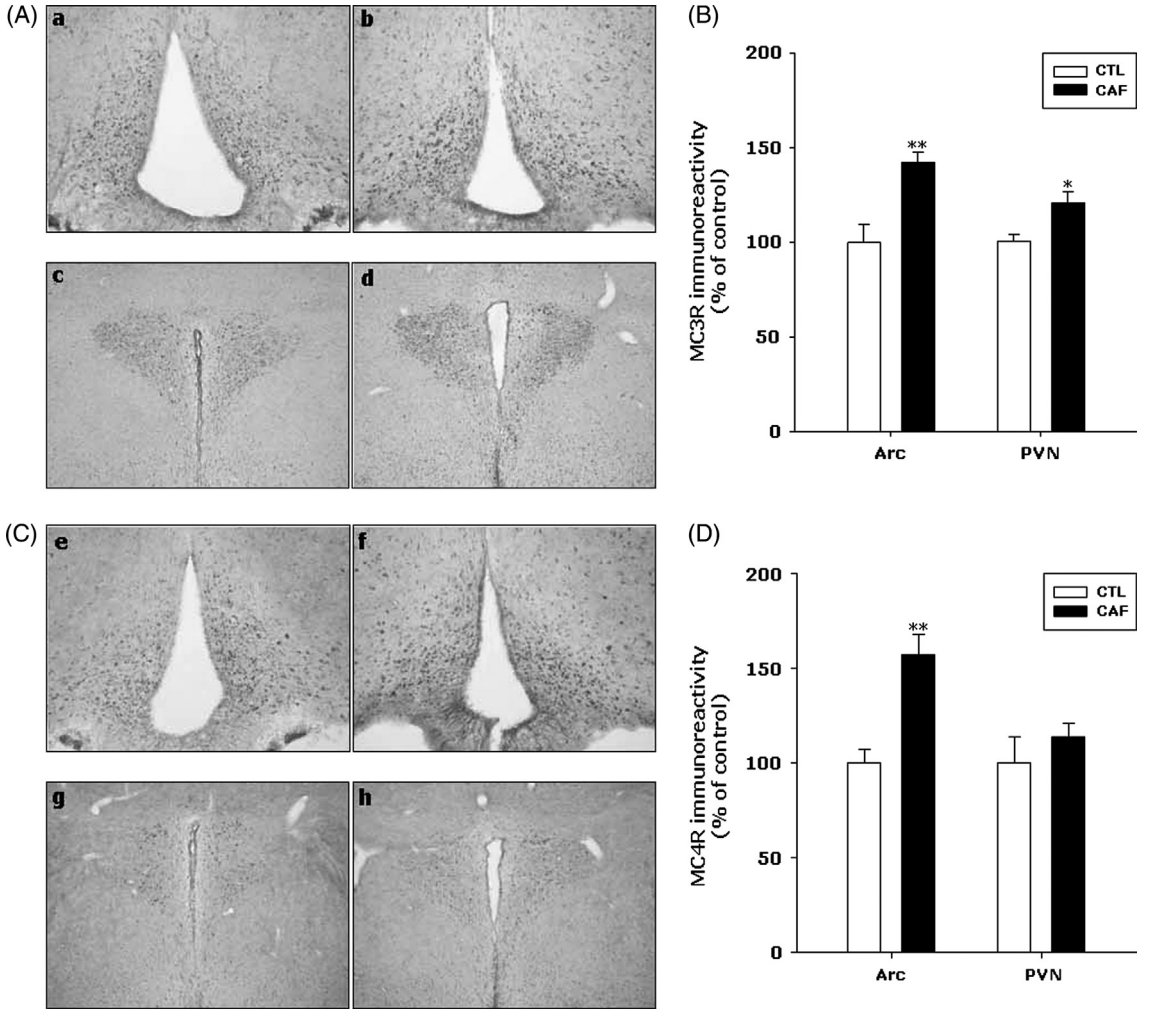

Figure 5. Effects of caffeine injection on MC3R (A, B) and MC4R (C, D) expression in the hypothalamus. MC3R (a, b) and MC4R (e, f) immunoreactivity were increased in the Arc. In the PVN, MC3R immunoreactivity (c, d) was increased but MC4R immunoreactivity (g, h) was not changed. Data are presented as means $\pm$ S.E.; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control (a, c, e, g, control 24 hr; b, d, f, h, caffeine 24 hr). Original magnification: $\times 100$ (a, b, e, f), $\times 40$ (c, d, g, h).

expression in the Arc. Therefore, we identified the POMC- and AgRP-expressing neurons known to regulate food intake in the hypothalamus. This study showed that caffeine significantly increased POMC immunoreactivity and decreased AgRP immunoreactivity in the Arc and PVN. These results suggest that the 6–12 hr increase in POMC expression contributes to the initial reduction of food intake and body weight, and delayed recovery of food intake may be due to decreased AgRP expression 12 hr after caffeine treatment.

It was reported that the α-MSH is released by POMC neurons and activates MC3R and MC4R, both of which are expressed by Arc neurons (Jégou et al. 1987; Mountjoy et al. 1994). Indeed, central administration of α-MSH decreased body weight and food intake through activation of hypothalamic MC3R and MC4R (Cummings and Schwartz 2000). On the other hand, central administration of AgRP potently increased feeding behavior and body weight in rats (Rossi et al. 1998; Wirth and Giraudo 2000), and AgRP acts as a specific competitive antagonist of both MC3R and MC4R (Ollmann et al. 1997). It has been reported that MC3R and MC4R are involved in homeostatic regulation of food intake and energy expenditure (Cone 2005). Other reports showed that mutation of the MC4R gene in mice causes obesity, hyperinsulinemia, and hyperphagia (Huszar et al. 1997), while MC3R gene-deficient mice have normal feeding behavior but increased fat mass (Chen et al. 2000). Obesity is more severe in mice deficient in both MC3R and MC4R than in MC4R-deficient animals, suggesting that these two receptors play complementary roles in the control of energy homeostasis (Chen et al. 2000; Mounien et al. 2005).

Here we show that caffeine markedly increased MC3R and MC4R immunoreactivity in the Arc. However, in the PVN, only MC3R immunoreactivity was increased. These results indicate that intervention of orexigenic and anorexigenic neuropeptides and their receptors plays an important role in the regulation of food intake and body weight in the hypothalamus.

In summary, caffeine affects body weight and food intake through neuronal regulation. Caffeine-induced reductions in body weight may be caused by a reduction of food intake and this feeding behavior may be regulated by collaboration between POMC/AgRP and MC3R/MC4R. However, the specific mechanisms underlying these caffeine-induced changes in body weight and food intake remain to be elucidated. Further study will be necessary to determine whether caffeine acts directly or indirectly on the hypothalamus and food intake.

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