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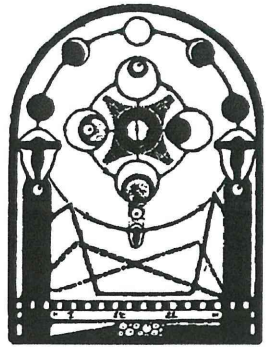

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Similar changes of hypothalamic feeding-regulating peptides mRNAs and plasma leptin levels in PTHrP-, LIF-secreting tumors-induced cachectic rats and adjuvant arthritic rats

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Parathyroid hormone-related protein (PTHrP) is a causative factor of humoral hypercalcemia in malignancy. However, it is difficult to explain the mechanism of anorexia/cachexia with PTHrP secretion in detail. Previously, we demonstrated that the expressions of orexigenic peptides increased and anorexigenic peptides decreased under cachectic conditions in rats carrying tumors secreting PTHrP. In this study, we investigated whether such changes in the expression of hypothalamic feeding-regulating peptides can be solely attributed to PTHrP or are a general response under cachectic conditions. Cachectic syndromes were induced in rats by: (*i*) inoculation of human lung cancer LC-6 cells that secreted PTHrP, (*ii*) inoculation of human melanoma SEKI cells that secrete not PTHrP but LIF1, (*iii*) injection of heat-killed *Mycobacterium* leading to arthritis (AA) and (*iv*) oral administration of a high dose of $1\alpha,25(OH)_2D_3$ that resulted in hypercalcemia. The LC-6-bearing rats and AA rats were treated with or without anti-PTHrP antibody and indomethacin, respectively, and the expression of the hypothalamic feeding-regulating peptide mRNAs were examined by *in situ* hybridization histochemistry. The orexigenic peptide mRNAs, such as neuropeptide Y and agouti-related protein, were significantly increased, and that of anorexigenic peptide mRNAs, such as proopiomelanocortin, cocaine- and amphetamine-regulated transcript and corticotropin-releasing hormone were significantly decreased when they developed cachectic syndromes and AA. A high dose of $1\alpha,25(OH)_2D_3$ caused hypercalcemia and body weight loss but did not affect the expression of hypothalamic feeding-regulating peptide mRNAs. The expressions of the hypothalamic feeding-regulating peptides change commonly in different chronic cachectic models without relating to serum calcium levels.

Cachexia is characterized by weight loss involving massive depletion of adipose tissue and lean body mass. Nutritional supplementation cannot replenish the loss of lean body mass.[1,2] The severity of cachexia in disease states such as cancer, end-stage renal disease, rheumatoid arthritis (RA) and acquired immunodeficiency syndrome may be the primary determining factor in both the quality of life and eventual mortality.[3,4] Hypercalcemia is also a frequent paraneoplastic syndrome and represents an important factor affecting the morbidity and mortality of cancer patients.[5] The main cause of humoral hypercalcemia in malignancy (HHM) is the tumor production of parathyroid hormone-related protein (PTHrP) that stimulates osteoclastic resorption and renal reabsorption of calcium.[6]

The homeostasis of food intake and body weight is controlled by complex mechanisms. The hypothalamus receives and integrates the neural and humoral signals that inform energy status from peripheral tissues.[7] Appetite and feeding behaviors are primarily controlled by feeding centers in the lateral hypothalamic area (LHA), the satiety center in the ventromedial hypothalamic nucleus, the arcuate nucleus (Arc) and the paraventricular nucleus (PVN) in the hypothalamus.[8,9] Among the hypothalamic feeding-regulating peptides, neuropeptide Y (NPY) and agouti-related protein (AgRP) in the Arc are potent orexigenic neuropeptides. Under physiological conditions, the orexigenic hormone ghrelin increases energy intake by increasing NPY and AgRP neurons.[10] Plasma ghrelin levels in anorexia nervosa patients

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are high and return to control levels after weight gain by renutrition.[11] On the other hand, anorexigenic hormone leptin, which derives mainly from fat tissue, decreases the activity of NPY and AgRP neurons and suppresses energy intake.[12] AgRP is an endogenous antagonist of the anorexigenic neuropeptide α-melanocyte-stimulating hormone (αMSH), which is derived from the proopiomelanocortin (POMC). AgRP promotes food intake *via* the inhibition of αMSH-stimulated signaling and antagonist of Type 4 central melanocortin receptors (MC4R).[13] Early studies in obese humans showed that leptin mRNA concentrations in adipose tissue and serum leptin concentrations correlated positively and closely with fat mass.[14] The leptin receptor is located in the hypothalamus as well as in some peripheral tissues. Injury to the hypothalamus can cause obesity, partly by destroying neurons that express the leptin receptor. In addition to NPY and AgRP, orexins in the LHA are also thought to participate in feeding regulation. Bolus injection of orexins to the rat lateral ventricle stimulated the food intake dose dependently, and orexin mRNA levels were upregulated on fasting.[15] Besides POMC, which blocks the autonomic, satiety and metabolic effects of leptin *via* the antagonism of MCRs,[16] cocaine- and amphetamine-regulated transcript (CART) in the Arc, which is also regulated by leptin, and corticotropin-releasing hormone (CRH) in the PVN function as anorexigenic neuropeptides.[8,17,18]

Previously, we showed that in animals carrying tumors secreting PTHrP, the levels of mRNA for orexigenic peptides were increased, whereas the levels of mRNA for anorexigenic peptides were decreased,[19–21] under cachectic conditions including HHM, reduced food intake and body weight loss. The administration of a humanized anti-PTHrP antibody raised against the NH_2-terminal 34 amino acids of the human PTHrP ($PTHrP_{1\text{-}34}$) rapidly improved the cachectic symptoms and also normalized the expression of NPY, AgRP, POMC, αMSH, CART and CRH mRNAs.[19–21] Although previous results suggested that HHM rats at least perceived starvation at the hypothalamus, there was no examination that those changes were observed especially in HHM rats secreting PTHrP or in other cachectic rats with or without tumors. There was also no examination of whether rats with hypercalcemia derive those changes.

In this study, we examined whether the increased mRNA expression of the hypothalamic orexigenic peptides and the decreased mRNA expression of the anorexigenic peptides in rats with cachexia are PTHrP-induced or rather general physiological responses under cachectic conditions. We also asked whether such expression of the hypothalamic feeding-regulating-peptides is related to hypercalcemia.

Material and Methods

Drugs

The humanized anti-PTHrP antibody raised against the NH_2-terminal 34 amino acids of the human PTHrP ($PTHrP_{1\text{-}34}$; Ref. 12) was dissolved in saline. Indomethacin and $1\alpha,25(OH)_2D_3$ were purchased from Sigma-Aldrich (St. Louis, MO) and Calbiochem (San Diego, CA), respectively.

Cells and animal experiments

Group 1. PTHrP-secreting human lung cancer cell line LC-6-JCK originating from human large cell lung cancer was purchased from the Central Institute for Experimental Animals (Kawasaki, Japan). The cells were maintained *in vivo* in nude mice (BALB/cAnN Crj-nu/nu). Small pieces of tumor tissues (~10 mm^3) were subcutaneously (s.c.) implanted into 5-week-old male F344/N Jcl-rnu nude rats. Rats that displayed blood ionized calcium (iCa) levels higher than 1.8 mmol/L and at least 0.5 mmol/L higher than normal (control) rats were used as the HHM rats.[20–23] Nude mice and nude rats were purchased from Charles River Japan (Yokohama, Japan) and Clea Japan (Tokyo, Japan), respectively, and kept in sterilized cages. For treatment with an anti-PTHrP mAb, the rats were given 3 mg/kg of anti-PTHrP antibody intravenously (i.v.) on days 42 and 49 (HHM + vehicle: $n = 6$, HHM + antibody: $n = 6$, and normal: $n = 6$). The body weight of the normal and HHM rats was measured once a week, and iCa was determined on day 51 after implantation of the tumor. Blood was collected from the tail vein, and the concentration of iCa was measured using a Ca^{2+}/pH electrolyte analyzer (Bayer 634, Bayer Diagnostics, Sunbury, UK).

Group 2. A SEKI melanoma cell line which does not express PTHrP was established at the National Cancer Center, Tokyo, Japan.[24] Five-week-old male F344/N Jcl-rnu nude rats were s.c. implanted in the right flank with 1×10^7 of SEKI cells; the rats displayed weight loss without PTHrP secretion or hypercalcemia after implantation of the cells.[25] Body weights (SEKI: $n = 6$, and nontumor-bearing rats: $n = 6$) were measured once a week, and iCa was measured on day 59.

Group 3. Adjuvant-induced arthritic rat (AA) were also used as a cachectic model.[26–29] To induce AA, 8-week-old male Wistar rats (Kyudo Co., Saga, Japan) were intracutaneously (i.c.) injected with 1 mg of heat-killed *Mycobacterium butyricum* (Difco Laboratories, Detroit, MI) in paraffin liquid at the base of the tail. The AA rats were divided into 2 groups: one was orally administered 1 mg/kg of indomethacin in a 0.5 mL suspension of 0.5% methylcellulose daily from day 15 to day 21, and the others was not treated (AA: $n = 6$, AA + indomethacin: $n = 6$, and control: $n = 6$). Body weight and arthritis index were measured every day. The arthritis index was scored by grading each paw from 0 to 4, based on erythema, swelling and deformity of the joints.[28,30]

Group 4. To create nontumor-bearing hypercalcemic rats, 13-week-old male F344/N Jcl-rnu nude rats were orally administered 10 μg/kg of $1\alpha,25(OH)_2D_3$ (active vitamin D3) for three consecutive days.

The animals used for *in situ* hybridization histochemistry were decapitated on day 51 (Group 1), day 59 (Group 2),

day 22 (Group 3) and day 3 (Group 4). The brains were rapidly removed, placed on a glass plate on dry ice and stored at −80°C until use. Trunk blood was collected and the plasma concentration of iCa was measured on the same as above and plasma concentrations of leptin were measured using an ELISA kit (YK051 Rat Leptin-HS, Yanaihara Institute, Shizuoka, Japan). The animals used in the experiment were treated in accordance with the ethical guidelines for animal care, handling and termination promulgated by the Chugai Pharmaceutical Co. (Tokyo, Japan).

In situ hybridization histochemistry

Frozen 12-μm-thick coronal brain sections were prepared in a cryostat at −20°C, thawed, and mounted onto gelatin/chrome alum-coated slides. The PVN, Arc and LHA were determined according to coordinates given by the atlas of Paxinos and Watson.[31] The localization of sections from each rat was checked by microscopic observation. Two sections containing the PVN (plate 24; Ref. 29) and four sections containing the Arc (plate 27 and 28; Ref. 29) and LHA (plate 28; Ref. 29) were used from each rat to measure the density of the autoradiography. *In situ* hybridization was performed as previously described.[32] Hybridization was carried out under a Nescofilm coverslip (Bando Chemical IMD, Osaka, Japan). [^{35}S]3′-end-labeled deoxyoligonucleotides complementary to transcripts coding for NPY (5′-GGA GTA GTA TCT GGC CAT GTC CTC TGC TGG CGC GTC-3′), AgRP (5′-CGA CGC GGA GAA CGA GAC TCG CGG TTC TGT GGA TCT AGC ACC TCT GCC-3′), POMC (5′-CTT CTT GCC CAG CGG CTT GCC CCA GCA GAA GTG CTC CAT GGA CTA GGA-3′), CART (5′-TGG GGA CTT GGC CGT ACT TCT TCT CAT AGA TCG GAA TGC-3′), orexin (5′-TTC GTA GAG ACG GCA GGA ACA CGT CTT CTG GCG ACA-3′) and CRH (5′-CAG TTT CCT GTT GCT GTG AGC TTG CTG AGC TAA CTG CTC TGC CCT GGC-3′) were used as the specific probes. The specificity of the probes was described previously.[19,33–36] Total counts of 6×10^5 cpm/slide for NPY, AgPR, POMC, CART and CRH and 4×10^5 cpm/slide for orexin were used. Hybridized sections containing the Arc, the LHA and the PVN were exposed to autoradiography film (Hyperfilm; Amersham, Buckinghamshire, UK) for 4 days for orexin and 7 days for NYP, AgRP, POMC, CART and CRH. The autoradiographic images were quantified using an MCID imaging analyzer (Imaging Research, St. Catherines, ON, Canada). The images were captured by a charge-coupled device camera (Dage-MTI, Michigan City, IN) at 40× magnification. The mean absorbance of the autoradiographs was measured and compared with simultaneously exposed ^{14}C microscale samples (Amersham). The standard curve was fitted by the absorbance of the ^{14}C microscale on the same film.

Statistical analysis

All data are given as mean ± SE calculated from the results of the *in situ* hybridization histochemistry. The results of each experimental animal group were compared with those of the control group. The data were analyzed using a one-way factorial ANOVA followed by a Bonferroni correction for multiple comparisons. The changes in body weight, iCa and arthritis index were also statistically analyzed using one-way ANOVA followed by a Bonferroni correction for multiple comparisons. Statistical significance was defined as $p < 0.05$.

Results

Body weight, arthritis index, iCa and plasma leptin in Normal rats, HHM rats, SEKI rats, AA rats and $1\alpha,25(OH)_2D_3$ rats

Consistent with previous results,[19,20,22,23] the body weight of the HHM rats bearing PTHrP secreting LC-6 significantly decreased after day 30, but significantly increased after the HHM rats were given the anti-PTHrP antibody (Fig. 1*a*). The body weights of the SEKI rats and the AA rats also decreased after day 30 and after day 13, respectively, but significantly increased in the AA rats after they were given indomethacin (Figs. 1*b* and 1*c*). The arthritis index in the AA rats increased sharply from day 10 to 19 and remained the same until day 22, but significantly decreased after they were given indomethacin (Fig. 1*d*). The rats treated with $1\alpha,25(OH)_2D_3$ had a significant decrease in body weight accompanied by an elevation of iCa (Figs. 1*e* and 1*f*). The levels of iCa in the HHM rats were significantly higher than in the normal rats, but it decreased when the HHM rats received the anti-PTHrP antibody (Fig. 1*f*). On the other hand, the SEKI rats had no change in the levels of iCa, though they experienced body weight loss (Figs. 1*b* and 1*f*), and neither did the AA rats (Figs. 1*c* and 1*f*).

Concentration in plasma leptin in HHM rats were significantly lower than that in nontumor-bearing rats and also lower in SEKI and AA rats. Administration of anti-PTHrP antibody to HHM rats increased the plasma leptin level, but it was still lower than that of the nontumor-bearing rats. On the other hand, vitamin D treated rats did not change the plasma leptin concentration (Fig. 2).

Expression of hypothalamic peptides: orexigenic peptides genes in HHM, SEKI, AA and $1\alpha,25(OH)_2D_3$ rats

After the HHM rats developed cachexia, the levels of orexigenic peptide mRNAs, NPY and AgRP in the Arc were significantly higher than in normal rats. Administration of the anti-PTHrP antibody to the HHM rats showed reduced levels of NPY and AgRP mRNAs compared with levels in the untreated HHM rats (Figs. 3*a*–3*d*). Although neither the SEKI rats nor the AA rats showed elevated levels of blood iCa (Fig. 1*f*), similar changes in the mRNA expression of orexigenic peptides were observed in both rat models; the orexigenic peptide mRNAs, such as NPY and AgRP in the Arc, became higher (Figs. 3*a*–3*d*). Administration of indomethacin to the AA rats not only improved their body weight and arthritis index but also restored the mRNA levels

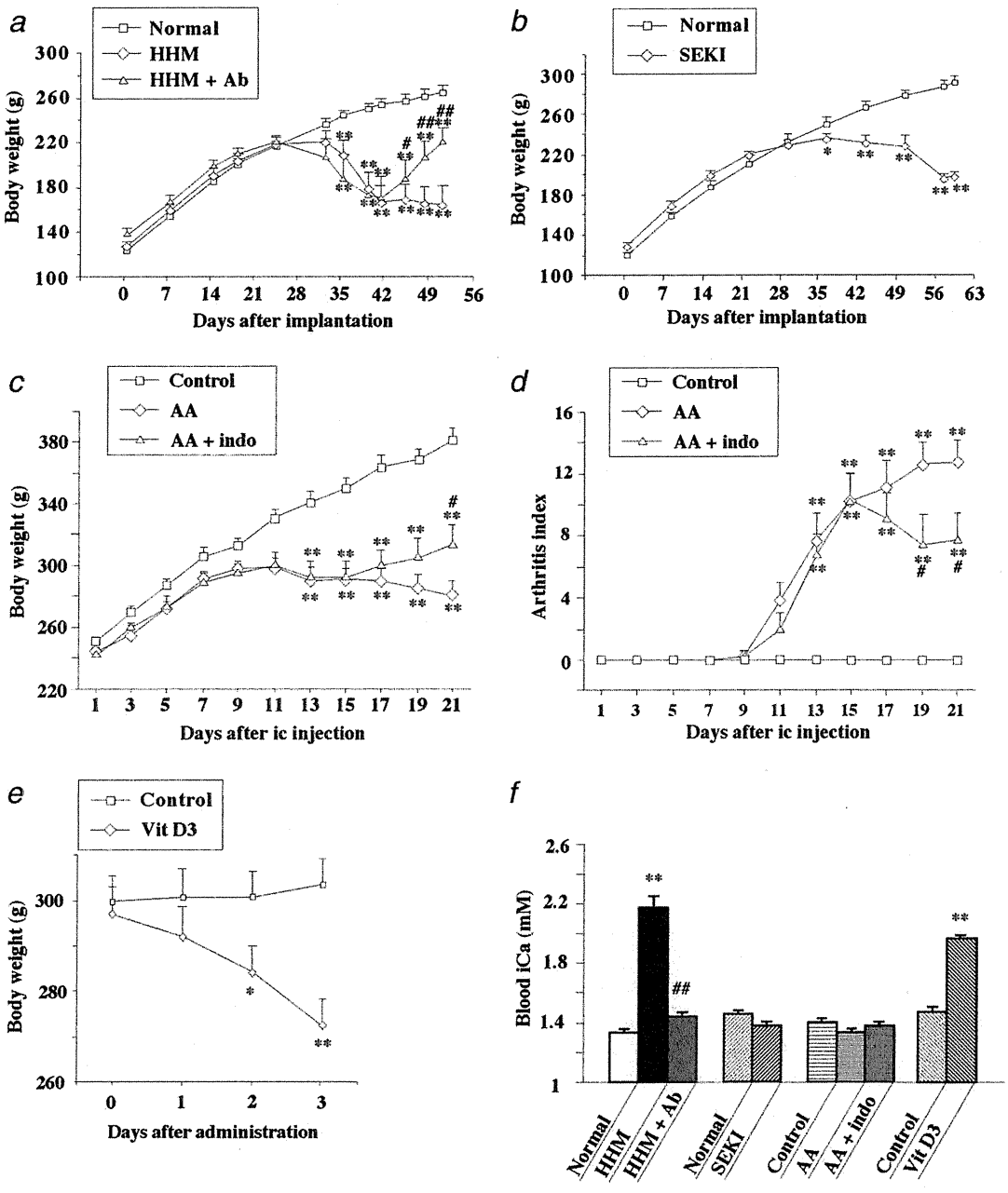

Figure 1. Time course of changes in body weight in Group 1 containing nontumor-bearing rats (Normal), humoral hypercalcemia of malignancy rats (HHM) and HHM rats intravenously (i.v.) injected with anti-PTHrP antibody (HHM + Ab) (*a*), Group 2 containing nontumor-bearing rats (Normal) and SEKI rats (SEKI) (*b*) and Group 3 containing rats injected with vehicle (Control), adjuvant-induced arthritis rats treated with vehicle (AA), and AA rats perorally (po) treated with indomethacin (AA + indo) (*c*). Changes in the arthritis index in Group 3 (*d*). Time course of changes in body weight in Group 4 containing rats treated with vehicle (Control) and rats orally treated with 10 μg/kg of $1\alpha,25(OH)_2D_3$ (Vit. D3) daily from day 0 to 3 (*e*), and changes in iCa of the rats in Group 1 to Group 4 (*f*). For treatment with or without an anti-PTHrP antibody, the rats were given 3 mg/kg of anti-PTHrP antibody or saline i.v. on days 42 and 49 in Group 1. AA rats in Group 3 were not treated or orally administered 1 mg/kg of indomethacin daily from days 15–21. Data points, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ compared with HHM rats or AA rats.

of the hypothalamic feeding peptides. The level of orexin mRNA in the LHA did not change even under cachectic conditions in the HHM rats, but decreased in the SEKI rats and AA rats when they developed cachexia and, in the AA rats, was not increased by administration of indomethacin (Figs. 3*e* and 3*f*).

Because HHM rats bearing LC-6 concurrently develop cachexia and hypercalcemia, there is a possibility that serum

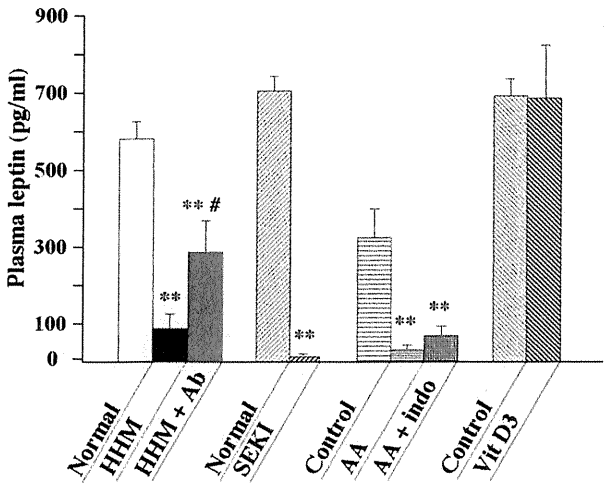

Figure 2. The changes of plasma leptin concentration in Group 1 containing nontumor-bearing rats (Normal), humoral hypercalcemia of malignancy rats (HHM) and HHM rats intravenously (i.v.) injected with anti-PTHrP antibody (HHM + Ab), Group 2 containing nontumor-bearing rats (Normal) and SEKI rats (SEKI), Group 3 containing rats injected with vehicle (Control), adjuvant-induced arthritis rats treated with vehicle (AA) and AA rats perorally (po) treated with indomethacin (AA + indo) and Group 4 containing rats treated with vehicle (Control) and rats orally treated with $1\alpha,25(OH)_2D_3$ (Vit. D3). Data points, mean ($n = 6$); bars, SE. $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats.

calcium is also involved in the changes in the expression of hypothalamic feeding-regulating peptides. The SEKI and AA rats showed an increased expression of the orexygenic peptide without an increase in serum iCa. This indicates that changes in the expression of hypothalamic feeding-regulating peptides are not related to an increase in serum calcium. To further confirm the relationship between hypercalcemia and the expression of hypothalamic feeding-regulating peptides, rats were administered a high dose of $1\alpha,25(OH)_2D_3$ to induce hypercalcemia. In the $1\alpha,25(OH)_2D_3$ rats, the serum levels of iCa increased as body weight decreased, but the levels of NPY, AgRP and orexin mRNA did not change significantly.

Expression of hypothalamic peptides: anorexigenic peptides genes in HHM, SEKI, AA and $1\alpha,25(OH)_2D_3$ rats

POMC and CART in the Arc and CRH in the PVN became lower after the HHM rats developed cachexia (Fig. 4). The administration of the anti-PTHrP antibody restored not only their body weight but also restored the mRNA expression of the POMC, CART and CRH.

The SEKI rats and the AA rats also showed similar changes in the mRNA expression of anorexigenic peptides; the mRNA levels of POMC and CART in the Arc and CRH in the PVN were lower after they developed cachexia. On the other hand, $1\alpha,25(OH)_2D_3$ rats did not experience a change in the levels of POMC, CART and CRH mRNAs similar to orexigenic peptides such as NPY, AgRP and orexin mRNAs. Taken together, the results demonstrate that the upregulation of orexigenic peptides and downregulation of anorexigenic peptides are not specific to PTHrP-induced cachexia but rather to other physiological responses under cachectic conditions. Furthermore, such changes in the expression of hypothalamic feeding-regulating peptides occur independently of hypercalcemia.

Discussion

Tumor and host tissues containing macrophages often secrete proinflammatory cytokines and elevated levels of cytokines have been thought to directly or indirectly transmit signals to the hypothalumus, repressing the feeding center and activating the satiety center. In fact, it was reported that macrophage inhibitory cytokine-1 (MIC-1), which causes cachexia in cancer and renal disorders, binds to TGFβ Type II receptors and downregulates NPY and upregulates POMC.[37] In addition, MIC-1 and leptin have similar effects on the expression of hypothalamic feeding peptides that act at different sites of the hypothalamus.

However, in this study, the expression of orexigenic peptide mRNAs was upregulated and anorexigenic peptide mRNAs expression was downregulated in several cachectic models. Particularly, nontumor-bearing cachectic AA rats revealed the same mRNA changes in the hypothalamus as did HHM and SEKI rats. In addition to the observation that those mRNA changes were restored in HHM rats after they were treated with an anti-PTHrP antibody, the AA rats treated with indomethacin, which suppressed the synthesis of prostaglandins, had a partial restoration of those mRNA changes accompanied by a decrease of the arthritis index. It is possible that under cachectic conditions, the feeding center is activated and the satiety center is repressed, and yet, inflammatory cytokines, hormones and bioactive substances affect the orexigenic and anorexigenic peptide mRNA expression downstream of the feeding and satiety centers. In addition, we have previously reported that the body weight loss in HHM rats was accompanied by reduced amounts of muscle as well as fat.[23] Indeed, the concentration of plasma leptin in the HHM rats was significantly lower and administration of the anti-PTHrP antibody increased the plasma leptin level. A recessive mutation in the mouse *ob* genes results in obesity, and the *ob* gene encodes a hormone leptin that is expressed in adipose tissue.[38] Leptin regulates energy balance in part by suppressing NPY neurons and activating POMC neurons in the Arc[39] and it would be possible that leptin deficiency partly affects the changes of the gene expression in this study.

Although the mechanisms that explain the differences in the expression of hypothalamic feeding-regulating peptides between MIC-1-induced cachexia and our models remain to be elucidated, one possibility is that downstream processes of

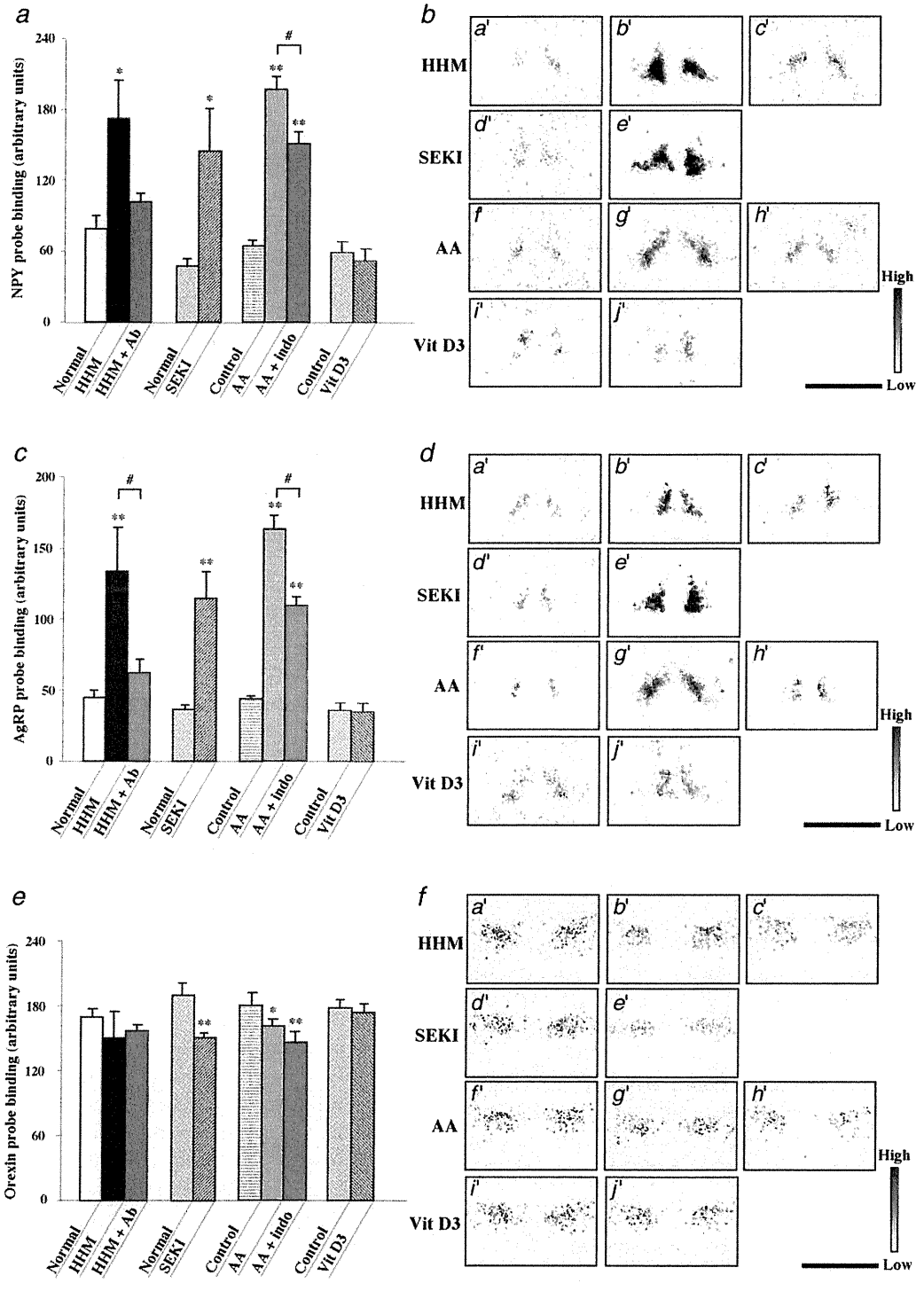

Figure 3. Expression of mRNA for neuropeptide Y (NPY) (*a* and *b*), agouti-related protein (AgRP) (*c* and *d*) in the arcuate nucleus (Arc) and orexin (*e* and *f*) in the lateral hypothalamic area (LHA) of Group 1 containing nontumor-bearing rats (Normal; *b-a′*, *d-a′*, *f-a′*), HHM rats (HHM; *b-b′*, *d-b′*, *f-b′*) and HHM rats injected with anti-PTHrP antibody (HHM + Ab; *b-c′*, *d-c′*, *f-c′*), Group 2 containing nontumor-bearing rats (Normal; *b-d′*, *d-d′*, *f-d′*) and SEKI rats (SEKI; *b-e′*, *d-e′*, *f-e′*), Group 3 containing rats injected with vehicle (Control; *b-f′*, *d-f′*, *f-f′*), adjuvant-induced arthritis rats treated with vehicle (AA; *b-g′*, *d-g′*, *f-g′*), and AA rats treated with indomethacin (AA + indo; *b-h′*, *d-h′*, *f-h′*) and Group 4 containing rats treated with vehicle (Control; *b-i′*, *d-i′*, *f-i′*) and rats treated with $1\alpha,25(OH)_2D_3$ (Vit. D3; *b-j′*, *d-j′*, *f-j′*). Representative autoradiographs of sections hybridized by a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for NPY (*b-a′-j′*), AgRP (*d-a′-j′*) and orexin (*f-a′-j′*). Signal intensity ranges from high (black) to low (white). Bar, 1 mm. Columns, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats or AA rats.

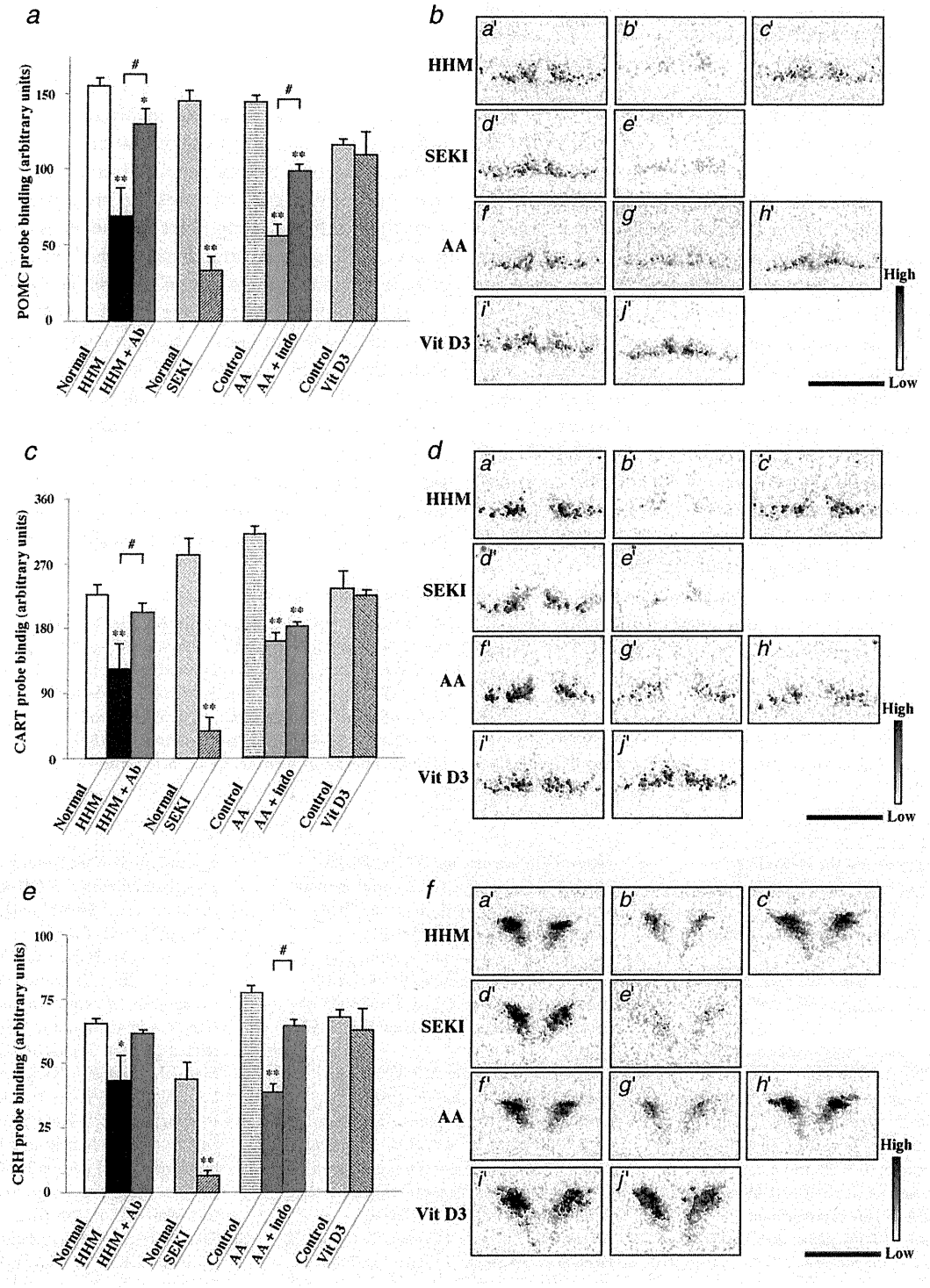

Figure 4. Expression of mRNA for proopiomelanocortin (POMC) (*a* and *b*) and cocaine-and amphetamine-regulated transcript (CART) (*c* and *d*) in the arcuate nucleus (Arc) and corticotropin-releasing hormone (CRH) (*e* and *f*) in the paraventricular nucleus (PVN) of Group 1 containing nontumor-bearing rats (Normal; *b-a′*, *d-a′*, *f-a′*), HHM rats (HHM; *b-b′*, *d-b′*, *f-b′*) and HHM rats injected with anti-PTHrP antibody (HHM + Ab; *b-c′*, *d-c′*, *f-c′*), Group 2 containing nontumor-bearing rats (Normal; *b-d′*, *d-d′*, *f-d′*) and SEKI rats (SEKI; *b-e′*, *d-e′*, *f-e′*), Group 3 containing rats injected with vehicle (Control; *b-f′*, *d-f′*, *f-f′*), adjuvant-induced arthritis rats treated with vehicle (AA; *b-g′*, *d-g′*, *f-g′*) and AA rats treated with indomethacin (AA + indo; *b-h′*, *d-h′*, *f-h′*) and Group 4 containing rats treated with vehicle (Control; *b-i′*, *d-i′*, *f-i′*) and rats treated with $1\alpha,25(OH)_2D_3$ (Vit. D3; *b-j′*, *d-j′*, *f-j′*). Representative autoradiographs of sections hybridized by a ^{35}S-labeled oligodeoxynucleotide probe complementary to mRNA for POMC (*b-a′-j′*), CART (*d-a′-j′*) and CRH (*f-a′-j′*). Bar, 1 mm. Columns, mean ($n = 6$); bars, SE. $^{*}p < 0.05$ and $^{**}p < 0.01$ compared with each control. $^{\#}p < 0.05$ compared with HHM rats or AA rats.

NPY action are affected by humoral factors such as cytokines and tumor-derived factors.[40] In fact, intracerebroventricular injection of TNF-α reportedly increased the NPY mRNA level in the hypothalamus but reduced food intake,[41] and proinflammatory signals decreased the secretion of AgRP but increased the transcription of the AgRP gene.[42]

On the other hand, the rats treated with $1\alpha,25(OH)_2D_3$ had reduced body weight without changes in feeding-regulating peptide gene expression. One possible explanation is that changes of metabolic rates and locomotor activity related to $1\alpha,25(OH)_2D_3$-induced hypercalcemia may be involved in the decrease of body weight without affecting feeding. Another possible explanation is that body fluid balance related to drinking and urine volume will change and cause dehydration. The reason why body weight was reduced after $1\alpha,25(OH)_2D_3$ treatment without affecting the feeding-regulating peptide genes should be clarified by further study.

Previously, using HHM rats treated with anti-PTHrP antibody, we have demonstrated: (*i*) body weight gain accompanied by restoration of locomotor activity and food and water intake, (*ii*) restoration of plasma calcium levels and (*iii*) restoration of feeding-regulating peptide genes.[19,23] It could be possible that proinflammatory cytokines such as IL-1, IL-6 and TNFα are responsible for the changes in feeding-regulating peptide gene expression, to be sure, but PTHrP might also be responsible for those changes. In this study, there were no effects of hypercalcemia induced by $1\alpha,25(OH)_2D_3$ treatment on the hypothalamic feeding-regulating peptide gene expression. Consequently, not PTHrP-induced hypercalcemia but hormonal effects of PTHrP might have brought about the changes in feeding-regulating peptide gene expression.

In the HHM rats, the level of orexin mRNA that enhanced feeding was not significantly increased but rather decreased in the LHA. Because orexin is involved not only in feeding behavior but also in sleep regulation and narcolepsy,[43–45] orexin expression may be regulated in a more complex manner. Especially, orexin increases the proportion of time spent awake through projecting fibers for the locus coeruleus that is a key modulator of attentional state.[46] Previously, Onuma *et al.* reported that there was an approximately double increase in the locomotor activity of the HHM rats after they received the anti-PTHrP antibody.[23] Consequently, in tumor-bearing cachextic rats and AA rats it can be presumed that locomotor activity and waking state are reduced and result in reduced orexin gene expression. Further studies are necessary both to clearly understand the mechanisms of orexigenic and anorexigenic peptide regulations in response to cachectic conditions and the mechanisms by which orexigenic and anorexigenic peptide regulations could cause the cachectic conditions.

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Neuropharmacology and Analgesia

Allyl isothiocyanates and cinnamaldehyde potentiate miniature excitatory postsynaptic inputs in the supraoptic nucleus in rats

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ABSTRACT

Allyl isothiocyanates (AITC) and cinnamaldehyde are pungent compounds present in mustard oil and cinnamon oil, respectively. These compounds are well known as transient receptor potential ankyrin 1 (TRPA1) agonists. TRPA1 is activated by low temperature stimuli, mechanosensation and pungent irritants such as AITC and cinnamaldehyde. TRPA1 is often co-expressed in TRPV1. Recent study showed that hypertonic solution activated TRPA1 as well as TRPV1. TRPV1 is involved in excitatory synaptic inputs to the magnocellular neurosecretory cells (MNCs) that produce vasopressin in the supraoptic nucleus (SON). However, it remains unclear whether TRPA1 may be involved in this activation. In the present study, we examined the role of TRPA1 on the synaptic inputs to the MNCs in *in vitro* rat brain slice preparations, using whole-cell patch-clamp recordings. In the presence of tetrodotoxin, AITC (50 μM) and cinnamaldehyde (30 μM) increased the frequency of miniature excitatory postsynaptic currents without affecting the amplitude. This effect was significantly attenuated by previous exposure to ruthenium red (10 μM), non-specific TRP channels blocker, high concentration of menthol (300 μM) and HC-030031 (10 μM), which are known to antagonize the effects of TRPA1 agonists. These results suggest that TRPA1 may exist at presynaptic terminals to the MNCs and enhance glutamate release in the SON.

1. Introduction

Allyl isothiocyanates (AITC) and cinnamaldehyde are pungent compounds present in mustard oil and cinnamon oil, respectively (Jordt et al., 2004; Bandell et al., 2004). Transient receptor potential ankyrin 1 (TRPA1) (formerly ANKTM1) is the only mammalian member of the TRPA subfamily, and belongs to the TRP superfamily (Story et al., 2003). TRPA1 is a non-selective cation channel expressed widely, encompassing 20–35% of sensory neurones (Jordt et al., 2004; Nagata et al., 2005). TRPA1 is activated by a variety of noxious stimuli, including cold temperatures (below 17 °C), alkaline pH (Story et al., 2003; Fujita et al., 2008) and mechanosensation (Kwan et al., 2006, 2009). Surprisingly, a recent study demonstrated that hypertonic solution activates TRPA1 channels in human embryonic kidney 293 cells transiently expressing rat TRPA1 (Zhang et al., 2008).

TRPA1 is found in a subset of primary sensory neurons of dorsal root ganglia (DRG) that coexpressed with noxious heat-sensing TRPV1 (Story et al., 2003; Kobayashi et al., 2005). A recent finding has indicated that an N-terminal variant of the TRPV1 channel is required for osmosensory transduction in mouse supraoptic nucleus (SON) neurones (Sharif Naeini et al., 2006). The release of arginine vasopressin (AVP) from the magnocellular neurosecretory cells (MNCs) in the SON is crucial for body fluid homeostasis. The neuronal activity of the MNCs and AVP release is modulated by excitatory and inhibitory synaptic inputs and humoral factors such as osmotic change in plasma and many different endogenous factors (Mason, 1980; Leng et al., 1982; Bourque, 1989; Nagatomo et al., 1995). Excitatory synaptic inputs to the SON in rats are activated by hyperosmotic stimulation (Inenaga et al., 1997). To our knowledge, it is unknown whether TRPA1 is involved in modulating excitatory and inhibitory synaptic inputs to the SON or whether TPPA1 expresses in the SON. Therefore, we examined the effects of AITC and cinnamaldehyde on excitatory and inhibitory synaptic inputs in the SON in rats.

2. Materials and methods

2.1. Animals

Experiments were performed on male Wistar rats weighting 100–200 g. All procedures described in the present study were carried out

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in accordance with the guidelines on the use and care of laboratory animals as set out by the Physiological Society of Japan and under the control of the Ethics Committee of Animal Care and Experimentation, University of Occupational and Environmental Health, Japan.

2.2. Slice preparations

Rats were sacrificed by decapitation. We ensured the absence of gross contusion and hemorrhage after removal of each brain from the skull. The brains were rapidly removed and cooled in a perfusion medium at 4 °C for 1 min. A block containing the hypothalamus was cut and glued to the stage of a vibratome-type slicer (DSK Linearslicer™ PRO7; DSK, Kyoto, Japan). After careful removal of the meninges, coronal slices (150-μm thick) containing the SON were cut as described previously (Nagatomo et al., 1995). The slices were carefully trimmed with a circular punch (inner diameter 1.8 mm) and preincubated in the perfusion medium at room temperature for at least 1 h, after which they were transferred to the recording chamber.

2.3. Solutions and drugs

The perfusion medium contained (in mM): NaCl 124; KCl 5; KH_2PO_4 1.24; $CaCl_2$ 2; $NaHCO_3$ 25.9; and glucose 10. For Ca^{2+}-free solution, Ca^{2+} was replaced by Mg^{2+} and the osmolality was adjusted by lowering the Na^+ concentration. The pH was adjusted to 7.3, and the osmolality of all the solutions ranged between 298 and 303 mOsmol/kg. AITC was purchased from Wako (Osaka, Japan). Cinnamaldehyde, HC-030031 and ruthenium red were purchased from Sigma (St. Louis, MO, USA). Tetrodotoxin (TTX) was obtained from Sankyo Co. (Tokyo, Japan). Menthol was purchased from Nacalai (Kyoto, Japan). For stock solution, AITC, cinnamaldehyde and HC-030031 were dissolved in dimethyl sulfoxide (DMSO). Menthol was dissolved in ethanol. TTX and ruthenium red were dissolved in distilled water, and then all the drugs were dissolved into a working solution, with the final concentration of the solvents not being more than 0.1%. All the solutions used in this experiment were bubbled with a mixture of 95% O_2–5% CO_2. The pipette solution used in the recording electrodes contained (in mM): K-gluconate 140; $MgCl_2$ 1, $CaCl_2$ 1; EGTA 10; and Mg-ATP 2 (pH 7.3 with Tris base). TTX was present in all experiments except for Ca^{2+}-free solution.

2.4. Whole-cell recordings and data analyses

The slices were fixed in a recording chamber as described previously (Kabashima et al., 1997). Briefly, the slices were placed onto a glass-bottomed chamber and fixed with a grid of parallel nylon threads supported by a U-shaped stainless steel weight. The volume of the recording chamber was 1 ml, and the perfusion rate was 1.4 ml/min. The solution level was kept constant by a low-pressure aspiration system. To identify magnocellular neurones in the SON, we used an upright microscope (BX-50, Olympus, Japan) with Nomarski optics (×400). The drugs were applied to the slice preparation by switching the perfusion solution using a two-way valve (HV 4-4, Hamilton, Reno, NV, USA). The dead space washing time was excluded from the calculations.

The electrodes used in this study were triple-pulled with a puller (P-87, Sutter Instrument Co., Novoto, CA, USA) from a glass capillary, and had a final resistance of 5–9 MΩ when filled with the electrode solution. Electrophysiological recordings were carried out at 32–33 °C. Whole-cell recordings were made from microscopically identified SON neurones in the upper surface layers of the slices. Recordings of postsynaptic currents began 5 min after membrane rupture when the current reached a steady state. Currents and voltages were recorded with an EPC-10 amplifier (HEKA, Lambrecht, Germany). Signals were filtered at 3 kHz, digitised at 1 kHz with an analogue-to-digital converter (Mac lab/v. 3.5, Castle Hill, NSW, Australia), and stored on the hard disk of a personal computer. For quantitative analysis of the synaptic currents, only the AC components (using a 1-Hz high pass filter) were used for analysis with software (AxoGraph V.3.6.1, Axon Instruments, Foster Hill, CA, USA). Spontaneous events were automatically screened using an amplitude threshold of 15 pA and then were visually accepted or rejected based on the rise time and decay time. Recordings included for data analysis were collected during periods of stable series resistance (10–20 MΩ with no compensation).

2.5. Statistical analysis

Data are expressed as mean ± S.E.M. with n representing the number of neurones tested. Differences between two groups were examined for statistical significance using the paired t-test and between multiple groups by one-way ANOVA. A P value less than 0.05 denoted the presence of a statistically significant difference.

3. Results

Spontaneous synaptic currents were recorded from a total of 97 MNCs that were identified microscopically in thin punch-out SON slice preparations from 42 rats. As reported previously (Kabashima et al., 1997), excitatory postsynaptic currents (EPSCs) and inhibitory postsynaptic currents (IPSCs) were observed under basal conditions (without any stimulus). The EPSCs and IPSCs could be recorded selectively by setting the holding potential at −70 mV for the EPSCs and at −20 mV for the IPSCs. The EPSCs were abolished by the application of 6-cyano-7-nitroquin-oxaline-2,3-dione (CNQX; a blocker of non-NMDA receptors), and the IPSCs were abolished by the application of picrotoxin (a blocker of $GABA_A$ receptor-gated Cl^- channels), indicating that EPSCs and IPSCs reflect glutamate and GABA release, respectively. Spontaneous EPSCs and IPSCs (sEPSCs and sIPSCs) were insensitive to the Na^+ channel blocker TTX (1 μM), indicating that the sEPSCs and sIPSCs recorded from the thin punch-out slice preparations were miniature EPSCs and miniature IPSCs (mEPSCs and mIPSCs) that reflected the spontaneous quantal release of glutamate and GABA, respectively.

3.1. The effects of AITC and cinnamaldehyde on the mEPSCs

For this analysis, the mEPSCs recorded during 3-min periods under and after the application of AITC and cinnamaldehyde were compared with the mEPSCs recorded during 3-min periods before AITC and cinnamaldehyde application. AITC (50 μM) potentiated the mEPSCs frequency significantly (149 ± 4.6% of control, $P<0.01$, $n=9$). The effects of AITC were selective on the frequency of the mEPSCs, and the amplitude remained virtually unaffected (99.6 ± 1.4% of control, $P>0.05$, $n=9$) (Fig. 1). AITC significantly potentiated the frequency of the mEPSCs in a dose-dependent manner when tested at three concentrations (10, 30 and 50 μM) (Fig. 3A). AITC increased the mEPSC frequency to 106 ± 4.7%, 133 ± 7.8% and 149 ± 4.6% of the control values (n = 5–9). The increases of mEPSC were significant at 30 μM and 50 μM. The application of cinnamaldehyde (30 μM) significantly increased the frequency of the mEPSCs without affecting the amplitude, like AITC (frequency 143 ± 9.1% of control, $P<0.01$, $n=9$, amplitude 100 ± 1.5% of control, $P>0.05$, $n=9$) (Fig. 2). Like AITC, cinnamaldehyde also significantly potentiated the frequency of the mEPSCs in a dose-dependent manner when tested at three concentrations (10, 20 and 30 μM) (Fig. 2B). Cinnamaldehyde increased the mEPSC frequency to 102 ± 4.1%, 127 ± 8.2% and 143 ± 9.1% of the control values (n = 5–9). The increases of mEPSC were significant at 20 μM and 30 μM.

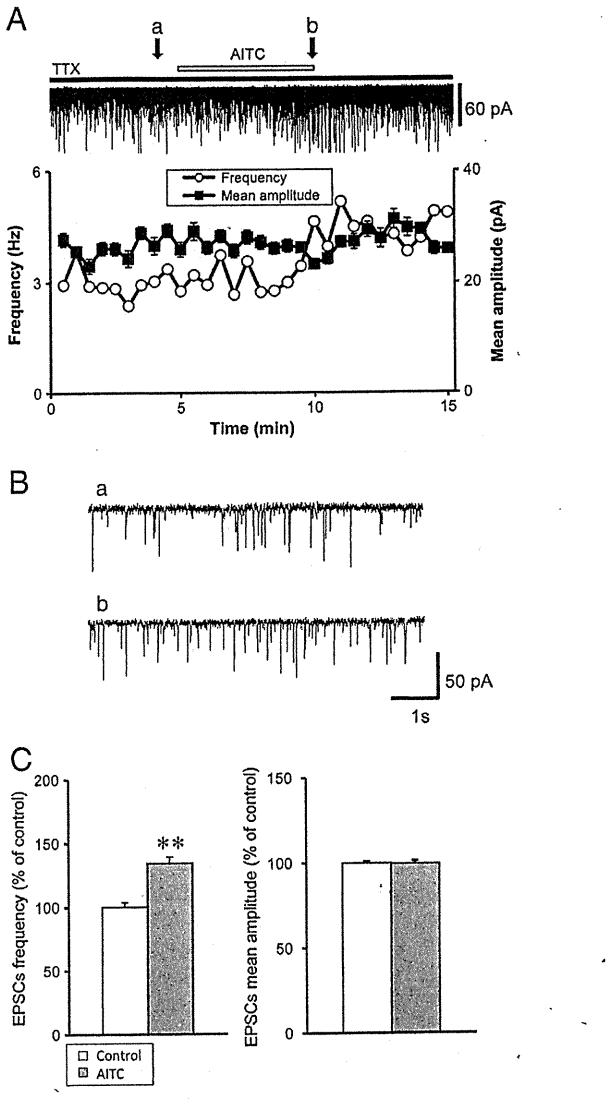

Fig. 1. Effect of AITC and related substances on miniature excitatory postsynaptic currents (mEPSCs) in the SON. (A) Representative example of AITC (50 μM) on mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B) Consecutive trace of mEPSCs is shown in an expanded scale in time, (a) before and (b) during the action of AITC. (C) Summary of the effect of AITC on the frequency and amplitude of mEPSCs ($n=9$). The values are percentage changes (±S.E.M.) from control values obtained during a 3-min period at the beginning of the experiments (before adding AITC). **$P<0.01$ versus control.

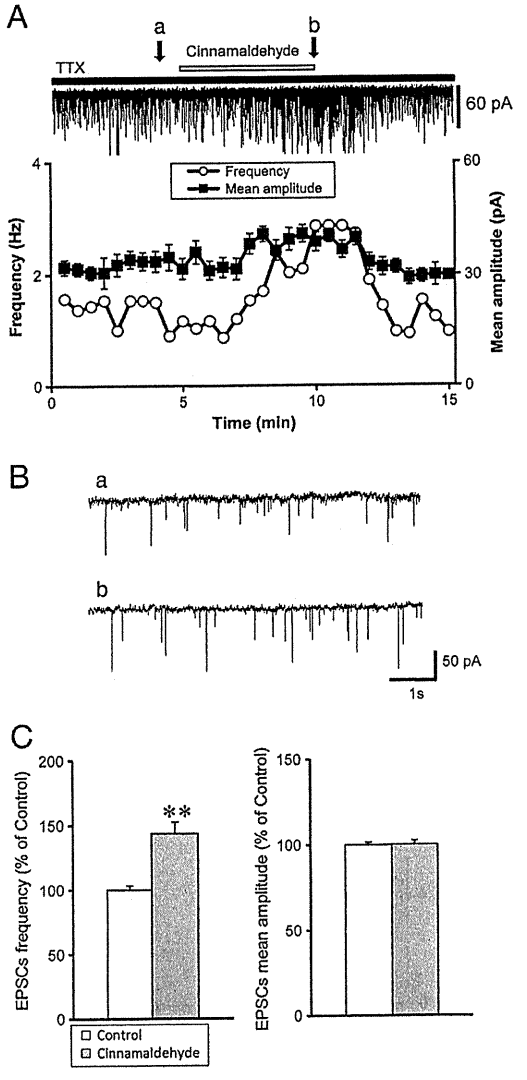

Fig. 2. Effect of cinnamaldehyde and related substances on miniature excitatory postsynaptic currents (mEPSCs) in the SON. (A) Representative example of cinnamaldehyde (30 μM) on mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B) Consecutive trace of mEPSCs is shown in an expanded scale in time, (a) before and (b) during the action of cinnamaldehyde. (C) Summary of the effect of cinnamaldehyde on the frequency and amplitude of mEPSCs ($n=9$). The values are percentage changes (±S.E.M.) from control values obtained during a 3-min period at the beginning of the experiments (before adding cinnamaldehyde). **$P<0.01$ versus control.

3.2. The effects of AITC and cinnamaldehyde on the mIPSCs

In contrast to the effect on the mEPSCs, the application of AITC (50 μM) did not have significant effects (frequency 102 ± 3.6%, amplitude 106 ± 3.6% of control, $P>0.05$, $n=7$) on mIPSCs. In the same way as AITC, cinnamaldehyde (30 μM) did not have significant effects on the mIPSCs (frequency 100 ± 2.3%, amplitude 101 ± 1.3% of control, $P>0.05$, $n=6$).

3.3. Effects of TRP blocker on AITC- and cinnamaldehyde-induced potentiation of mEPSCs

To examine whether the effects of AITC and cinnamaldehyde are mediated by TRP channels, we used 10 μM ruthenium red, a non-specific TRP channel blocker. Figs. 4A and 5A show the representative examples of the effects of ruthenium red. Pre-exposure to ruthenium red attenuated the potentiation of mEPSCs by AITC (50 μM) and cinnamaldehyde (30 μM). Figs. 4B and 5B show the summary data for the effects of ruthenium red on the amplitude and frequency. Ruthenium red almost completely abolished the AITC- and cinnamaldehyde-induced increase in mEPSCs frequency, but had no effect on the amplitude of mEPSCs (AITC: frequency 102 ± 8.1%, amplitude 103 ± 1.8% of control, $n=6$; cinnamaldehyde: frequency 103 ± 6.3%, amplitude 104 ± 1.9% of control, $n=6$). These results suggest the possible involvement of TRP channels in both AITC- and cinnamaldehyde-induced potentiation of the mEPSCs.

3.4. Effects of TRPA1 antagonists on AITC- and cinnamaldehyde-induced potentiation of mEPSCs

To determine whether the effects of AITC and cinnamaldehyde on the mEPSCs are involved in TRPA1, we examined the pre-exposure high concentration of menthol and HC-030031 on AITC- and cinnamaldehyde-induced potentiation of mEPSCs. A previous study demonstrated that a low concentration of menthol activates TRPA1

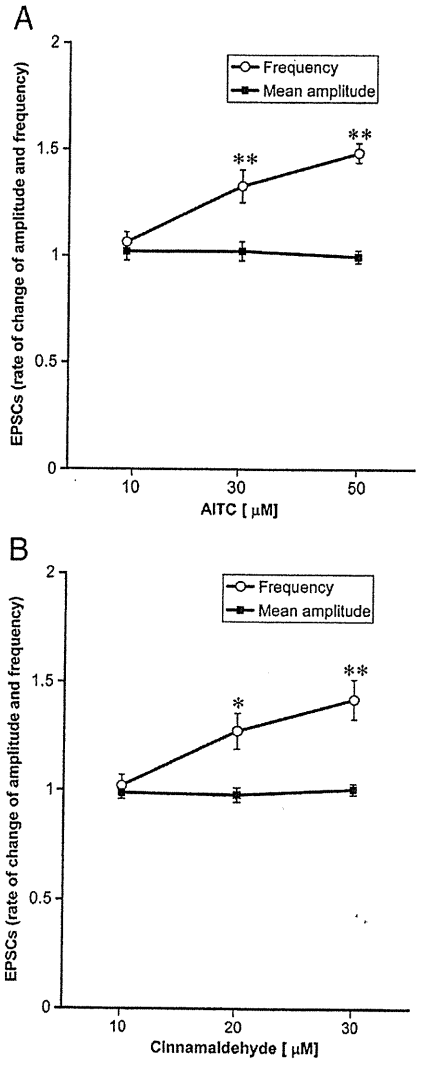

Fig. 3. Concentration–response relationship of AITC- and cinnamaldehyde-induced potentiation of miniature excitatory postsynaptic currents (mEPSCs). Supraoptic neurones were exposed to AITC at three different concentrations (10 μM, 30 μM and 50 μM; $n=5$–9) (A) or cinnamaldehyde at three different concentrations (10 μM, 20 μM and 30 μM; $n=5$–9), and the effects were expressed as rate of change (±S.E.M.) of frequency and amplitude of mEPSCs recorded for 3-min periods (after the start of AITC or cinnamaldehyde application) from value recorded in control periods before AITC application. Open circle, rate change of frequency; closed square, rate change of amplitude. *$P<0.05$ versus control and **$P<0.01$ versus control.

and a high concentration of menthol attenuates the effect of activation of TRPA1 (Karashima et al., 2007; Macpherson et al., 2006). Exposure to a high concentration of menthol (300 μM) did not affect the mEPSCs (frequency 99.0±4.9%, amplitude 99.1±1.9% of control. $n=6$). Pre-exposure to a high concentration of menthol attenuated the potentiation of mEPSCs by AITC (50 μM) and cinnamaldehyde (30 μM). High concentration of menthol almost abolished the AITC- and cinnamaldehyde-induced increase in the mEPSCs frequency, but had no effect on the amplitude of the mEPSCs (AITC: frequency 106± 8.6%, amplitude 103±3.4% of control, $n=6$; cinnamaldehyde: frequency 103±4.9%, amplitude 103±1.6% of control, $n=3$) (Figs. 4C, D and 5C, D). HC-030031$_{(10\ \mu M)}$ also attenuated the AITC- and cinnamaldehyde-induced potentiation of mEPSCs (AITC: frequency 107±4.1%, amplitude 102±1.6% of control, $n=6$; cinnamaldehyde: frequency 107±4.9%, amplitude 96.9±1.6% of control, $n=6$) (Figs. 4E, F and 5E, F). These results suggest the possible involvement of TRPA1 channels in both AITC- and cinnamaldehyde-induced potentiation of the mEPSCs.

3.5. Effect of AITC on Ca^{2+}-free perfusion medium

To examine whether the potentiation of mEPSC by AITC is dependent on extracellular Ca^{2+}, we used Ca^{2+}-free solution. The frequency and amplitude of mEPSC in the Ca^{2+}-free solution were significantly smaller than that in normal perfusion solution (normal versus Ca^{2+}-free: frequency 1.67±0.2 Hz versus 0.97±0.1 Hz, $n=8$–9, $P<0.01$, amplitude 24.4±0.4 pA versus 22.9±0.3 pA, $n=8$–9, $P<0.05$). Under this condition, AITC did not increase the frequency and amplitude of mEPSC (frequency 105±6.1% of control, amplitude 104±1.8% of control, $n=8$) (Fig. 6). Thus, the AITC-induced potentiation of mEPSC was extracellular Ca^{2+}-dependent.

4. Discussion

In the present study, we provided the first evidence that AITC and cinnamaldehyde are well known as TRPA1 agonists potentiate excitatory synaptic inputs to the supraoptic MNCs in rats using a whole-cell patch-clamp technique. Because glutamate and GABA are two major synaptic inputs into the SON neurones (Meeker et al., 1993; Wuarin and Dudek, 1993), the potentiation of mEPSCs by TRPA1 agonists, AITC and cinnamaldehyde may, at least in part, account for the excitatory action on electrical activity. The mEPSCs recorded in the SON slice preparations that we employed in this study were virtually insensitive to TTX. This result suggests that TRPA1 modulates glutamate release from the presynaptic terminal, and that increases of EPSCs do not depend upon action potential. The neurones were recorded in thin SON slices containing only the SON and the perinuclear zone, and the mEPSCs and mIPSCs probably reflect spontaneous transmitter release from the terminals of the cut axons, disconnected from their cell origin.

TRPA1 is a non-selective cation channel and is activated by noxious cold, pungent natural compounds such as AITC and cinnamaldehyde, mechanosensation and alkaline pH (Story et al., 2003; Bandell et al., 2004; Fujita et al., 2008; Jordt et al., 2004; Kwan et al., 2006, 2009). Activation of the TRPA1 channel is reversibly blocked by a high concentration of menthol (Macpherson et al., 2006; Karashima et al., 2007; Xiao et al., 2008). Moreover, a recent study demonstrated that hypertonic solution activates TRPA1 channels in human embryonic kidney 293 cells transiently expressing rat TRPA1 (Zhang et al., 2008).

Plasma osmolality is well known to regulate the activity of MNCs (Mason, 1980; Leng et al., 1982; Bourque, 1989). The supraoptic MNCs receive synaptic inputs from the organum vasculosum lamina terminals, the median nucleus of the preoptic area and the subfronical organ (Chaudhry et al., 1989; Honda et al., 1990; Richard and Bourque, 1992; 1995). These areas are very sensitive to osmotic changes and regulate body fluid and drinking behaviour (Bourque et al., 1994). In addition to integrative information from the osmosensitive areas, the MNCs are themselves also osmosensitive (Mason, 1980; Oliet and Bourque, 1992, 1993). Hyperosmotic stimuli also directly modulate glutamatergic inputs to the supraoptic MNCs by acting on the presynaptic terminals (Inenaga et al., 1997). A recent finding has indicated that an N-terminal variant of TRPV1 channel is required for osmosensory transduction in mouse SON neurones (Sharif Naeini et al., 2006). TRPA1 is present in TRPV1-expressing sensory neurones (Story et al., 2003; Kobayashi et al., 2005). However, it is unknown whether TRPA1 expresses in the SON. Our present study demonstrated that TRPA1 agonists potentiate excitatory synaptic inputs and these effects were attenuated by a high concentration of menthol and HC-030031. The results of our studies did not contradict previous reports which provide the relationship with TRPA1 channel and menthol (Karashima et al., 2007; Macpherson et al., 2007a; Xiao et al., 2008). These results suggest that activation of the TRPA1 channel possibly potentiates the excitatory synaptic inputs to the supraoptic MNCs neurones. Formalin, known as TRPA1 agonist-induced nociceptive stimulation, causes a rapid elevation of AVP levels (Suzuki

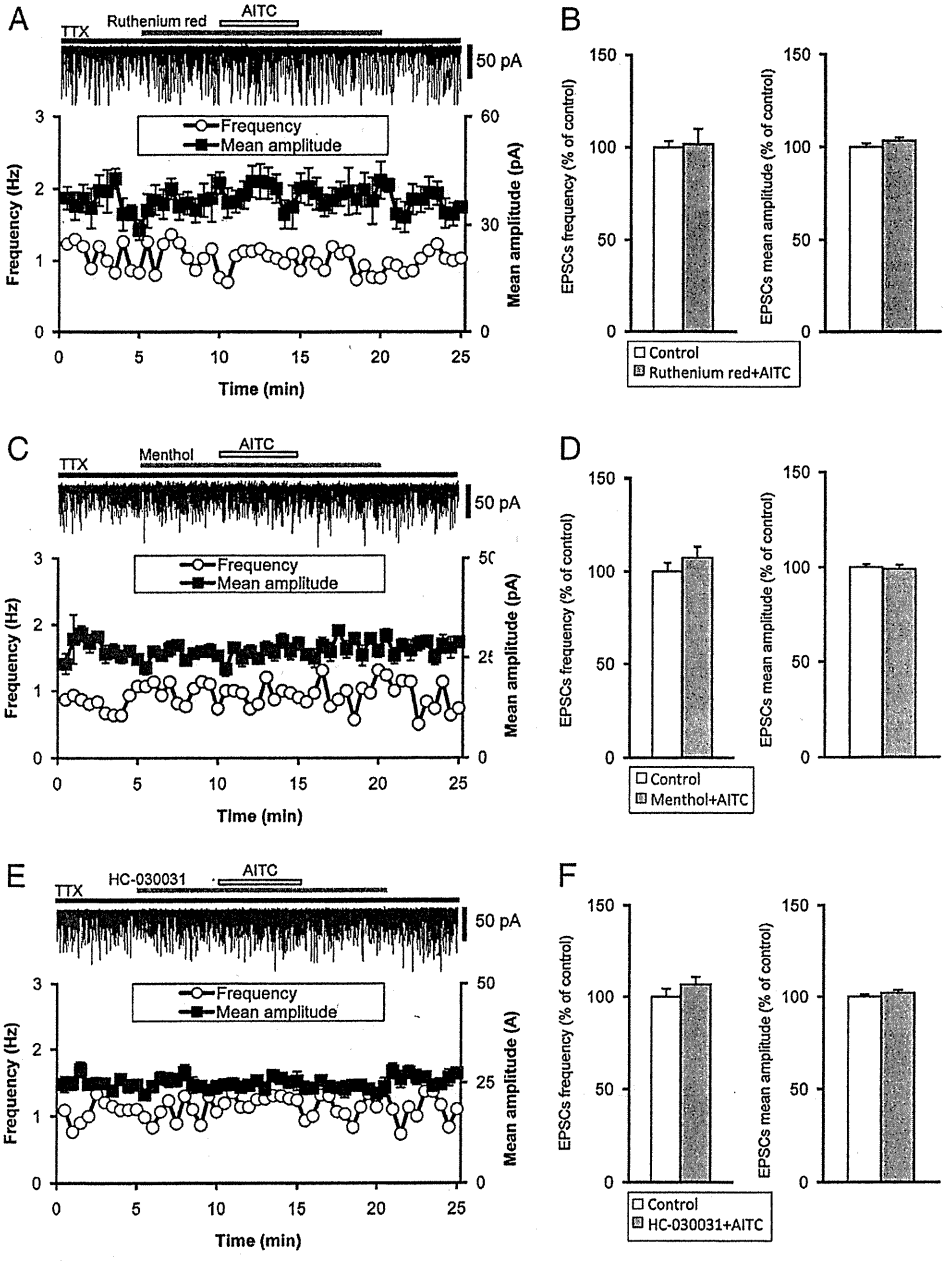

Fig. 4. Characterization of AITC-induced potentiation of mEPSCs. (A, C and E) Representative examples of the effects of ruthenium red (10 μM), non-specific TRP channels blocker (A), high concentration of menthol (300 μM) (C) and HC-030031 (10 μM) (E), TRPA1 selective channel blockers, on AITC-induced potentiation of mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B, D and F) Summary data for characterization of mEPSCs under AITC (50 μM) application. Frequency (left) and amplitude (right) of mEPSCs. Ruthenium red plus AITC ($n=6$) (B), high concentration of menthol plus AITC ($n=6$) (D) and HC-030031 plus AITC ($n=6$) (F), respectively. Data are mean ± S.E.M.

et al., 2009). TRPA1 recognizes temperature and a chemical sense in the brain of the snake (Gracheva et al., 2010). The potentiation of excitatory synaptic transmission by the activation of presynaptic terminals TRPA1 may have an important role in neuronal activity and the secretion of neurohypophysial hormones (AVP and oxytocin (OXT)). It cannot be denied that extracellular matrixes around the MNCs, such as protein, glial cell and other mechano-channels, may participate in the regulation of glutamatergic release by TRPA1 agonists.

Previous studies have reported that noxious compounds such as AITC are activated through covalent modification of cysteine residues in the intercellular N-terminal domain (Hinman et al., 2006; Macpherson et al., 2007b), whereas activation by intercellular calcium appears to be dependent on the N-terminal EF-hand calcium-binding domain (Doerner et al., 2007; Zurborg et al., 2007). In the present study, AITC-induced potentiation of mEPSCs was attenuated under extracellular Ca^{2+} free solution. A recent study demonstrated that transmembrane domain 5 is a critical molecular determinant of menthol sensitivity in mammalian TRPA1 channels (Xiao et al., 2008). These results indicate that TRPA1 in the SON may be activated through covalent modification of cysteine residues in the N-terminal domain and by extracellular Ca^{2+}-dependent. However, it remains obscure what kind of physiological role TRPA1 mediates.

The MNCs (AVP- or OXT-producing neurones) in the SON can be divided into two groups based on their firing pattern. By combining immunohistochemical and electrophysiological techniques, most of the phasically firing neurones contain AVP (phasic neurones), whereas the other neurones that do not fire phasically (nonphasic neurones) contain OXT (Yamashita et al., 1983; Cobbett et al., 1986; Armstrong et al., 1994). A previous immunohistochemical study

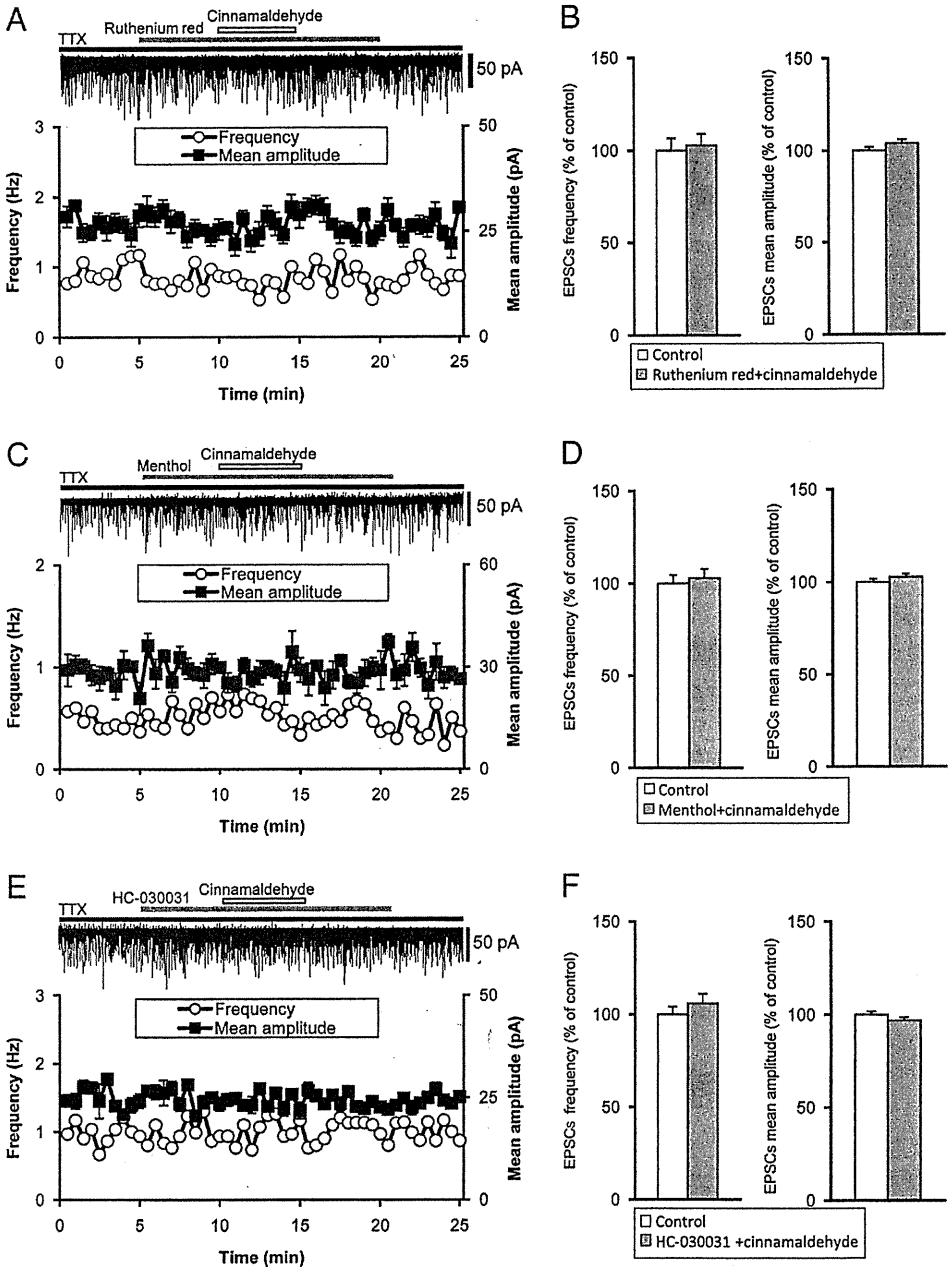

Fig. 5. Characterization of cinnamaldehyde-induced potentiation of mEPSCs. (A, C and E) Representative examples of the effects of ruthenium red (10 μM), non-specific TRP channels blocker (A), high concentration of menthol (300 μM) (C) and HC-030031 (10 μM) (E), TRPA1 selective channel blockers, on cinnamaldehyde-induced potentiation of mEPSCs. EPSCs were recorded in the presence of TTX (1 μM). The holding potential was −70 mV. Plots of frequency are single measurement, whereas plots of amplitude are mean ± S.E.M. over 30 s. (B, D and F) Summary data for characterization of mEPSCs under cinnamaldehyde (30 μM) application. Frequency (left) and amplitude (right) of mEPSCs. Ruthenium red plus cinnamaldehyde ($n=6$) (B), high concentration of menthol plus cinnamaldehyde ($n=3$) (D) and HC-030031 plus cinnamaldehyde ($n=6$) (F), respectively. Data are mean ± S.E.M.

demonstrated that AVP neurones are more common in the caudal and ventral parts of the SON, while OXT neurones tend to be found rostrally and dorsally (Rhodes et al., 1981). Subsequent topographic analysis revealed the majority of Fos-expressing AVP neurones occupy the ventral part of the SON, while Fos-OXT neurons are mainly in the dorsal part on hyperosmotic stimulation (Pirnik et al., 2004). We recorded mEPSCs in the ventral part of the SON. In the present study, approximately 75% of the tested supraoptic MNCs were sensitive to AITC and cinnamaldehyde. Taken together, and although we identified the cell types electrophysiologically, the possibility that the action of AITC and cinnamaldehyde is restricted to a single cell type (AVP- or OXT-producing neurones) is unlikely.

In conclusion, AITC and cinnamaldehyde potentiate excitatory synaptic inputs to the MNCs in the SON on electrophysiolgy. Additional investigations will be required to clarify the physiological role of TRPA1 in glutamatergic excitatory synaptic transmission in supraoptic MNCs.

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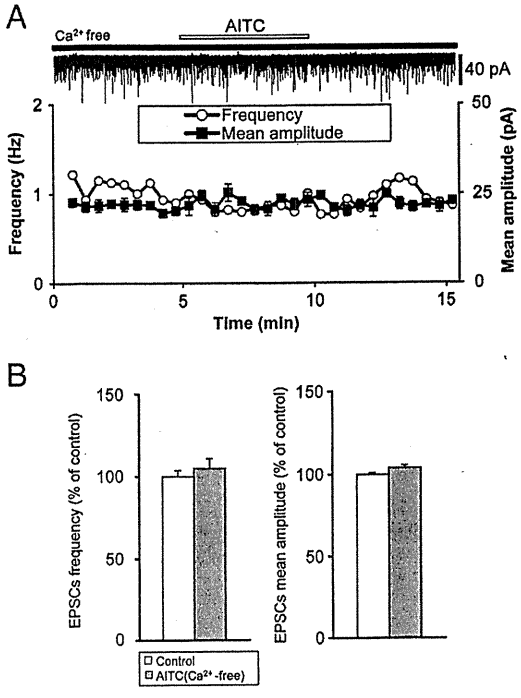

Fig. 6. AITC-induced potentiation of mEPSCs is extracellular Ca^{2+} dependent. (A) A representative example of the effect of AITC (50 μM) on AITC-induced potentiation of mEPSCs in the Ca^{2+}-free perfusion medium. (B) Summary data for the effects of AITC on frequency and amplitude of mEPSCs in Ca^{2+}-free solution ($n=8$). Data are mean ± S.E.M.

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Central Effects of Ghrelin, a Unique Peptide, on Appetite and Fluid/Water Drinking Behavior

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Abstract: Ghrelin is a stomach-derived peptide discovered as a ligand of the orphan G-protein coupled receptor. Ghrelin is now recognized as a major orexigenic neuropeptide. Immunohistochemical studies demonstrated that centrally administered ghrelin induced c-*fos* protein expression in many areas in the brain. Indeed, centrally administered ghrelin has various effects such as stimulating feeding, arousal, increasing gastric acid secretion, release of hormones from the pituitary, and inhibition of water intake. In particular, we recently showed that ghrelin was an antidipsogenic peptide with a simultaneous orexigenic effect. This may be of important, because most spontaneous daily water intake is temporally associated with feeding. Here, we summarise recent findings on the integration of central effects of ghrelin that regulate feeding, release hormones from the pituitary and inhibit fluid/water intake.

Keywords: Angiotensin II, feeding, hypothalamus, intracerebroventricular, neuroendocrine, pituitary, polyethylene glycol, fluid/ water intake.

INTRODUCTION

Ghrelin, consisting of 28 amino acids, is a peptide discovered in the stomach as a ligand of the orphan G-protein coupled receptor and participates in the regulation of growth hormone (GH) release via interactions with the GH secretagougue (GHS) type 1a receptor (GHS-R1a), the functionally active form of the GHS-R [1]. Ghrelin is also found in the brain and is now recognized as a neuropeptide released not only from the periphery but also locally in the brain. Ghrelin-immunoreactive neurons are present in the hypothalamus, especially in the arcuate hypothalamic nucleus (Arc), the paraventricular nucleus (PVN), the dorsomedial hypothalamus (DMH), and lateral hypothalamus [1, 2]. Intracerebroventricular (icv) administration of ghrelin induced c-*fos* protein (Fos) expression in various areas in the central nervous system (CNS) in rats, including the organum vasculosum of the lamina terminalis (OVLT), the median preoptic nucleus (MnPO), the subfornical organ (SFO), the supraoptic nucleus (SON), the PVN, the Arc, the area postrema (AP) and the nucleus of the tractus solitarius (NTS) [3-5]. The expression of the c-*fos* gene has been widely used to detect neuronal activity in the CNS [6]. Indeed, central administration of ghrelin has various effects (Table **1**), including 1) stimulation of not only GH secretion, but also secretion of prolactin (PRL) and adrenocorticotrophic hormone (ACTH), 2) an increase in appetite and energy intake, 3) influences on sleep and behavior, and 4) stimulation of gastric motility and gastric acid secretion. Moreover, recently, we found inhibitory effects of centrally administered ghrelin in dehydration-, angiotensin II (AII) and isotonic hypovolemia-induced water intake in rats [3, 7]. Centrally administered ghrelin also inhibited hypertonic-induced water intake [8].

In this review, we focus on the representative roles of ghrelin in the CNS in 1) the physiological, 2) neuroendocrinological, 3) hypothalamo-neurohypophysial, and 4) body fluid homeostatic effects.

CENTRAL EFFECTS OF GHRELIN

Although ghrelin was initially identified by virtue of its ability to elicit GH secretion from stomach [1, 9], central administration of ghrelin strongly stimulates feeding and an increase in body weight in mammals [10, 11] and non-mammals [12], except for bird [13-15]. Chronic icv administration of ghrelin strongly stimulated feeding and increased body weight gain in rats [5, 16]. Therefore, ghrelin has been established as a major orexigenic hormone acting not only from the periphery but also locally in the brain [1]. In addition, centrally administered ghrelin has various physiological effects (Table **1**), including regulation of sleeping, gastric acid secretion and body temperature. In this context, we describe recent finding on the central physiological effects of ghrelin.

It is well known that brain controls appetite and that feeding behavior is regulated by the hypothalamus [17]. Ghrelin is synthesized in the brain as the peptide was detected by immunohistochemistry in the Arc in colchicine-treated rats [1]. Icv administration of ghrelin induced Fos expression in the Arc [3-5]. The Arc is a critical site for feeding and body weight control because its neurons express the leptin-regulated orexigenic peptides, neuropeptide Y (NPY) and agouti-related protein (AGRP), and leptin-dependent anorexic pro-opiomelanocortin (POMC), and cocaine- and amphetamine-regulated transcript (CART) [18-22]. Icv administration of ghrelin increased both NPY and AGRP gene

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expression in the Arc [5]. Antibodies against and antagonists of NPY and AGRP abolished ghrelin-induced feeding [5, 23]. Moreover, in the Arc ablated rats, icv administration of ghrelin stimulated GH secretion, but did not stimulate food intake [24]. These results indicate that ghrelin may stimulate feeding behavior by exciting NPY and AGRP neurons in the Arc.

Other hypothalamic peptides, apart from ghrelin, are involved with feeding behavior. Leptin is the product of the *ob* gene, and an anorexia-mediated molecule produced from adipose tissue [17]. Most Arc NPY, AGRP, POMC and CART neurons also express leptin receptors and are regulated by leptin [17]. Icv administration of ghrelin blocked leptin-induced feeding reduction [5, 25, 26]. Ghrelin may conflict with leptin in regulating the NPY and AGRP neurons.

Orexin, an orexigenic hypothalamic neuropeptide, is also involved in the regulation of food intake [27]. Icv administration of ghrelin induced Fos expression in orexin-producing neurons [28]. The ghrelin-induced increase of food intake was reduced in orexin knockout mice [28]. The feeding is regulated by ghrelin in co-operating with orexin.

Interestingly, centrally administered ghrelin inhibits food intake in birds [13-15]. In the Japanese quail, Shousha *et al.* showed that high dose peripherally administered ghrelin inhibited food intake whereas a low dose of ghrelin stimulated food intake [15]. Shousha *et al.* also showed that centrally administered ghrelin inhibited food intake [15]. In chicks, centrally administered ghrelin inhibit food intake [13, 14]. Although it is unclear why the effect of ghrelin is opposite in mammals and birds, the role of ghrelin on feeding may have altered during evolution.

The interactions between feeding and sleep are well-known. For example, the starvation-induced sleep loss in rats [29]. Icv administration or microinjection of ghrelin in the CNS increased arousal in rats [30, 31]. Moreover, ghrelin knockout mice have reduced duration of non-rapid-eye movement sleep and increase amount of wakefulness and rapid-eye-movement sleep compared with wild-type mice [31]. Therefore, although the mechanism of ghrelin at sleep and waking rhythm is unclear, ghrelin may have an important role in sleep regulation.

Ghrelin is a rational orexigenic peptide, because of stimulating not only feeding but also gastric acid secretion. Centrally administered ghrelin stimulates gastric acid secretion in urethane-anesthetized rats [32]. Ghrelin-induced gastric acid secretion is completely abolished in both the vagotomized and atropine-treated rats [32]. As icv administration of ghrelin induced Fos in the NTS [3-5, 32], this effect of ghrelin relates to the central regulation of gastric acid secretion by the vagus nerve.

Icv administered ghrelin decrease body temperature in rats [23]. Recently, Mano-Otagiri *et al.* demonstrated that icv administered ghrelin inhibited noradrenaline release in brown adipose tissue of rats [33]. Although the mechanism of ghrelin-induced hypothermia is unclear, it may be related to the metabolic change after icv administration of ghrelin in rodents [10, 34]. Further study will clarify the role of ghrelin on the regulating body temperature.

Table 1. Central Effects of Ghrelin

Hormone Release	
Growth hormone (GH) release	↑[1, 9]
Prolactin (PRL) release	→[37] ? ↓[52]
Adrenocorticotrophic hormone (ACTH) release	↑[37, 40, 41]
Cortisol release	↑[37, 40, 41]
Thyroid stimulating hormone (TSH) release	→[49] ? ↓[41, 48]
Arginine vasopressin (AVP) release	↑[37, 59]
Luteinizing hormone (LH) release	↓[57, 58]
Appetite	↑[10 11] (↓[13-15], in birds)
Adiposity	↑[10]
Gastric Functions	
Gastric acid secretion	↑[32]
Gastric motility	↑[32]
Body Temperature	↓[23]
Water Intake	
Dehydration-induced water intake	↓[3]
Angiotensin II-induced water intake	↓[7, 8]
Hypovolemia induced water intake	↓[7]
Hypertonia-induced water intake	↓[8]
Alcohol Intake	↑[35]
Sleep	↓[30, 31]
Anxiety	↑[38, 39]

↑ increase, ↓ decrease, → no change

Recently, there are some noteworthy evidence that ghrelin may have an important role in alcoholism [35, 36]. Icv administered ghrelin increased and ghrelin receptor antagonists reduced alcohol intake in mice [35]. They suggested that central ghrelin signalling was required for alcohol reward. Ghrelin may be a target for treatment on alcohol-related disorder.

NEUROENDOCRINE EFFECTS OF GHRELIN

Ghrelin is a multi-functional peptide (Table **1**). It is well known that ghrelin was discovered in the stomach and stimulates GH release via interactions with the GHS-R1a in human and rat [1, 9]. However, the effects of ghrelin on pituitary hormones are not restricted to GH. Ghrelin stimulates the release of various kinds of hormones, including corticotropin-releasing hormone (CRH), NPY, and arginine vasopressin (AVP), from the rat pituitary *in vitro* [37]. In this context, we describe the neuroendocrine effects of ghrelin on adenohypophyseal hormone release.

The effects of centrally administered ghrelin have been examined on anxiety-like behavior in rodents. Icv administration of ghrelin induced anxious-like behavior in mice and rats [38, 39]. These effects of ghrelin were inhibited by treatment with CRH receptor antagonist [38]. Icv administration of ghrelin stimulated ACTH and corticosterone secretion [37, 40, 41]. Kristensen *et al.* reported that acute psychological stress increased plasma ghrelin level in rats (2). In *in vitro* electrophysiological studies, ghrelin decreased inhibitory postsynaptic currents (IPSCs) in CRH neuron in the PVN [42]. Various kinds of stress cause neuroendocrine response such as CRH or AVP release from the PVN and activation of the hypothalamus-pituitary adrenal (HPA) axis. These data indicate that centrally administered ghrelin may have an important role in the regulation of stress response.

The thyroid hormones are key in regulating metabolism and energy homeostasis [43, 44]. Ghrelin is thought to be a link with metabolism and energy homeostasis [45]. Plasma ghrelin level are reduced in hyperthyroidism and normalized by medical antithyroid treatment [46]. On the other hand, hypothyroidism increases plasma ghrelin level in rat [47]. Icv administered ghrelin suppressed TSH secretion in rats [41, 48]. Ghrelin also suppressed plasma T_4 level, not T_3 level in rats [48]. However, chronic icv administration of ghrelin does not change plasma TSH and T_4 level in rats [49]. The discrepancy between these studies may be explained by different experimental protocols, duration of administration and dose of ghrelin.

Ghrelin may be involved with the lactotrophic axis. Intravenous (iv) administration of ghrelin stimulates PRL secretion in human [50, 51]. Wren *et al.* reported that icv administration of ghrelin did not any effect PRL secretion in rats [37]. However, icv and ip administration of ghrelin inhibited PRL secretion in prepubertal rats [52]. Tana-Sempere *et al.* demonstrated that icv administration of ghrelin inhibited serum PRL level in 23-day-old male and female rats [52]. Moreover, the inhibitory effects of ghrelin were observed in aged hyperprolactinaemic female rats [52]. Interestingly, ghrelin stimulates PRL secretion in 23-day-old male and female rats *in vitro* [52]. Although the mechanism of ghrelin-induced PRL secretion is not clear, the discrepancy between these studies may be explained by different species (human and rat) and ages and dose of ghrelin. Ghrelin appears to have an important role on regulation of PRL secretion in human and rat at least.

Ghrelin may also participate in the modulation of the hypothalamic-pituitary-gonadal (HPG) axis. Previous studies have demonstrated the expression of the ghrelin gene and protein in the testis and ovary in both humans and rodents [53-56]. Icv administration of ghrelin suppressed luteinizing hormone (LH) secretion in ovariectomized rats [57, 58]. Ghrelin increased LH and follicle-stimulating hormone (FSH) secretion and decreased LH responsiveness to gonadotropin-releasing hormone (GnRH) in prepubertal male rats *in vitro* [58]. With limited evidence it is very difficult to explain fully the effects of ghrelin on the HPG axis, but ghrelin may be important in the regulation of LH and FSH secretion.

CENTRAL EFFECTS OF GHRELIN ON THE HYPOTHALAMO-NEUROHYPOPHYSIAL SYSTEM

Ghrelin has effects not only on the anterior lobe but also on the posterior lobe of the pituitary. Some investigations have been demonstrated excitatory effects of ghrelin on AVP secretion. Firstly, Ishizaki *et al.* reported that icv and iv injection of ghrelin increased plasma AVP levels in conscious rats [59]. GHSs stimulated AVP release from acute hypothalamic explants *in vitro* [60]. The release of AVP from the magnocellular neurosecretory cells (MNCs) in the SON is crucial for body fluid homeostasis. The MNCs project their axons to the posterior pituitary and secrete AVP and oxytocin (OXT) into the systemic blood flow. Thus, there is a possibility that ghrelin may have a potent effect on drinking behavior and body fluid balance in mammals.

Recently, Yokoyama *et al.* examined that the effect of ghrelin on the excitatory synaptic inputs to the MNCs in the SON using whole-cell patch-clamp recordings in rat brain slice preparations *in vitro* (61). Ghrelin (1 μM) increased the firing rate and depolarized the membrane. Application of CNQX (10 μM), a blocker of non-NMDA receptors, significantly decreased the firing rate, and membrane potential reversed the resting potential. The application of ghrelin (1 μM) caused a significant increase in the frequency of the miniature excitatory postsynaptic currents (mEPSCs) without affecting the amplitude. The increased frequency of the sEPSCs persisted in the presence of tetrodtoxin (1 μM). In contrast to the effect on the mEPSCs, the application of ghrelin (1 μM) did not have significant effects on miniature inhibitory postsynaptic currents (mIPSCs). As glutamate and GABA are two major synaptic inputs into the SON neurones [62, 63], the potentiation of mEPSCs by ghrelin may, at least in part, account for the excitatory action of ghrelin on electrical activity.

Two major molecular forms of ghrelin are found in the stomach and plasma; acylated ghrelin, which has n-octanoylated serine in position 3; and desacyl ghrelin [64]. Acylation is essential for the binding of ghrelin to the GHS-R, and although desacyl ghrelin does not bind to the GHS-R, it may be biologically active [64-66]. Desacyl ghrelin (1 μM) did not have a significant effect on mEPSCs in the experiments described above [61]. In addition, ghrelin-induced potentiation of mEPSCs was attenuated by pre-exposure to BIM28163, GHS-R1a receptor antagonist [61]. The peptidergic excitation and AVP response are absent in the MNCs of transient receptor potential vanilloid 1 (trpv1)-/- mice [67]. The ghrelin-induced potentiation of the mEPSCs was significantly suppressed by previous exposure to ruthenium red (10 μM), a TRPV blocker, and BIM28163 (10 μM), a GHS type 1a receptor selective antagonist [61]. The effects of ghrelin on the supraoptic MNCs in *trpv1*-/- mice were significantly attenuated compared with those in wild-type mice counterparts [61]. These results suggest that ghrelin participates in the regulation of synaptic inputs to the MNCs in the SON via interaction with the GH secretagogue type 1a receptor, and that the TRPV1 channel may be involved in ghrelin-induced potentiation of mEPSCs to the MNCs in the SON.