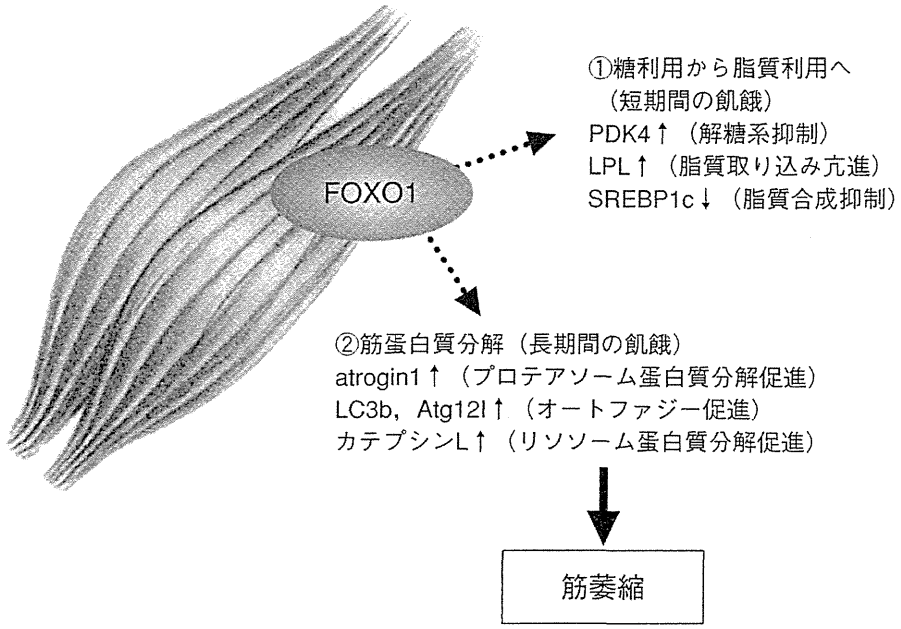

図3 飢餓時の骨格筋におけるFOXO1の役割

転写調節因子FOXO1は，飢餓に応答して骨格筋で多くの標的遺伝子を発現調節する．

6 FOXO1の細胞内作用メカニズム

FOXO1蛋白質は，生体の栄養変動時において，翻訳後修飾によりその細胞内局在や転写活性が調節されることが知られる．たとえば，摂食時にインスリンシグナルが増加し，PI3K/AktによりFOXO1のセリン・スレオニンがリン酸化され，核外に排出・ユビキチン化され蛋白質分解を受ける．絶食時にはNAD$^+$依存的にSirt1が活性化し，FOXO1は脱アセチル化され転写活性化能が亢進する（図2）[13]．絶食時にはインスリンやNAD以外にも様々なホルモンや代謝産物類（グルカゴン，グルココルチコイド，カテコールアミン，脂肪酸，ATP/AMP等）の量が変動するため[1]，これらの因子との関わりの解明は今後の課題である．

おわりに

本稿では，飢餓等の栄養条件変化時の生物個体における臓器間の代謝ネットワークおよび骨格筋量の調節分子として，フォークヘッド型転写調節因子FOXO1に着目し，概略を述べた．骨格筋は人体で最大の組織であり，エネルギー代謝・糖取り込み・運動において重要な役割を果たし，十分な骨格筋機能を保つことは肥満や糖尿病等の生活習慣病予防の観点からも重要である．FOXO1による骨格筋の量および糖・脂質・蛋白質代謝の分子機構に関する研究の進展により，これらをターゲットとした骨格筋不全予防法の提案が可能となることが期待される．

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筆者プロフィール

亀井 康富（かめい やすとみ）
1994年3月京都大学大学院農学研究科修了（農学博士）. 米国カリフォルニア大学、大阪バイオサイエンス研究所, 国立健康・栄養研究所を経て、2005年4月より現所属.
email : kamei.mmm@mri.tmd.ac.jp

Regulation of skeletal muscle metabolism in starvation

Yasutomi Kamei[1], Satoshi Sugita[1], Maki Hattori[1], Yoshihiro Ogawa[1,2]
[1] Department of Molecular Medicine and Metabolism, Medical Research Institute, Tokyo Medical and Dental University
[2] Global COE program, Tokyo Medical and Dental University

Animals adapt to nutritional environmental changes such as starvation by modifying energy metabolism in their organs. Skeletal muscle, liver and adipose tissue are the major organs for human body energy metabolism. Among them, the skeletal muscle is the largest organ in the human body, comprising about 40% of body weight. Sustaining skeletal muscle function is important to improve quality of life in an aging society as well as to prevent lifestyle-related diseases, including obesity and diabetes. Recent research suggests that a forkhead-type transcription factor, FOXO1, is the master regulator of gene regulation in the nutritional response, including reaction in the skeletal muscle. We will review the functional significance of FOXO1 in starvation and skeletal muscle metabolism.

Key words

skeletal muscle, FOX01, starvation, lifestyle-related diseases

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Corticotropin-releasing factor (CRF) receptor subtypes in mediating neuronal activation of brain areas involved in responses to intracerebroventricular CRF and stress in rats

Chiaki Takahashi*, Hisayuki Ohata, Tamotsu Shibasaki

Department of Physiology, Nippon Medical School, 1-1-5 Sendagi, Bunkyo-ku, Tokyo 113-8602, Japan

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ABSTRACT

Corticotropin-releasing factor (CRF) plays an important role in stress responses through activation of its receptor subtypes, CRF1 receptor (CRF_1) and CRF2 receptor (CRF_2). The parvocellular paraventricular nucleus of the hypothalamus (PVNp), the central nucleus of the amygdala (CeA), and the oval nucleus of the bed nucleus of the stria terminalis (BNSTov), which are rich in CRF neurons with equivocal expression of CRF_1 and CRF_2, are involved in stress-related responses. In these areas, Fos expression is induced by various stimuli, although the functions of CRF receptor subtypes in stimuli-induced Fos expression are unknown. To elucidate this issue and to examine whether Fos is expressed in CRF or non-CRF neurons in these areas, the effects of antalarmin and antisauvagine-30 (AS-30), CRF_1- and CRF_2-specific antagonists, respectively, on intracerebroventricular (ICV) CRF- or 60 min-restraint-induced Fos expression were examined in rats. ICV CRF increased the number of Fos-positive CRF and non-CRF neurons in the PVNp, with the increases being inhibited by antalarmin in CRF and non-CRF neurons and by AS-30 in CRF neurons. Restraint also increased Fos-positive CRF and non-CRF neurons in the PVNp, with the increases being inhibited by antalarmin in the CRF neurons. ICV CRF also increased Fos-positive non-CRF neurons in the CeA and the BNSTov, which was inhibited by AS-30 in both areas, and inhibited by antalarmin in the BNSTov only. Restraint increased Fos-positive non-CRF neurons in the CeA and BNSTov, with the increases being almost completely inhibited by either antagonist. These results indicate that both ICV CRF and restraint activate both CRF and non-CRF neurons in the PVNp and non-CRF neurons in the CeA and BNSTov, and that the activation is mediated by CRF_1 and/or CRF_2. However, the manner of involvement for CRF_1 and CRF_2 in ICV CRF- and restraint-induced activation of neurons differs with respect to the stimuli and brain areas; being roughly equivalent in the CeA and BNSTov, but different in the PVNp. Furthermore, the non-$CRF_{1\&2}$-mediated signals seem to primarily play a role in restraint-induced activation of non-CRF neurons in the PVNp since the activation was not inhibited by CRF receptor antagonists.

1. Introduction

Corticotropin releasing factor (CRF) induces a variety of changes in the endocrine system, autonomic nervous system, behavior, and mood, which resemble responses shown in stress [19,22,36,65,79]. The actions of CRF are induced through its receptor subtypes, CRF type 1 receptor (CRF_1) and type 2 receptor (CRF_2) [6,40,43,57]. CRF mainly binds to CRF_1, and with a low affinity to CRF_2. The CRF family peptides including urocortin (Ucn) 1, Ucn 2, and Ucn 3 and CRF receptor subtypes are widely distributed in the brain, with each peptide and CRF receptor subtype showing distinct distribution patterns, suggesting specific actions and roles in the central nervous system. The roles of CRF_1 and CRF_2 are known to be involved in the regulatory mechanisms of hypothalamic–pituitary–adrenal axis [64], gonadotropin secretion [35,52], gastrointestinal motility [47], feeding behavior [8,29,51,56], anxiety, and arousal [41,61,82]. CRF_1 is located in brain areas associated with emotionality, such as the cerebral cortex, amygdala, the bed nucleus of the stria terminalis (BNST) and brainstem, whereas CRF_2 shows a more restricted distribution, with the densest concentrations in the lateral septum, BNST, amygdala and the ventromedial hypothalamus [4,7,84].

The parvocellular paraventricular nucleus of the hypothalamus (PVN), where CRF neurons are present, is involved in the regulation of the hypothalamic–pituitary–adrenal axis [66,85], autonomic function [19,22], and feeding behavior [39,89]. The expression of CRF_1 and CRF_2 in the PVN is controversial [3,4,7,44,45,62,84]. The

* Corresponding author. Tel.: +81 3 3822 2131; fax: +81 3 3822 0766.
E-mail address: tsumoc@nms.ac.jp (C. Takahashi).

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central nucleus of the amygdala (CeA) and BNST mediate stress-induced changes in behavior and emotion [25], and are rich in CRF neurons [13,21,32,48,54,71,81]. The expression of CRF_1 and CRF_2 mRNA in the CeA is absent or low, if even present [3,67,84]. In the oval nucleus of the BNST (BNSTov), the expression of either CRF_1 or CRF_2 mRNA is absent or barely detectable [3,84].

Physiological and pharmacological stimuli including stress induce the expression of proto-oncogenes such as c-fos in discrete brain regions. Intracerebroventricular (ICV) administration of CRF has been used as tactics to study stress response mechanism in the brain because it induces various changes in the endocrine system, autonomic nervous system, behavior, and mood which resemble stress responses as stated earlier [19,22,36,65,79], and also induces the expression of c-fos in brain regions. Since Fos plays an important role in signal transmission and transcriptional regulation in cells [27,69,73], c-fos mRNA and Fos protein have been used as markers of neuronal activation in various studies [5,8,29,30]. Therefore, this study was aimed at clarifying the functions of CRF_1 and CRF_2 in the activation of neurons by ICV administration of CRF and stress in brain regions that participate in stress responses, namely the parvocellular PVN (PVNp), CeA, and BNSTov. We examined the effects of intraperitoneal (IP) administration of antalarmin, a CRF_1 specific antagonist, and ICV administration of antisauvagine-30 (AS-30), a CRF_2 specific antagonist, on ICV CRF- or restraint-induced changes in Fos-ir in the aforementioned brain areas in rats. Furthermore, since both CRF and non-CRF neurons are present in the PVNp, CeA, and BNSTov, a dual immuno-staining method for CRF and Fos was performed to explore whether CRF and/or non-CRF neurons are activated by ICV CRF and restraint, and to examine whether the manner of involvement for CRF_1 and CRF_2 in the activation of CRF neurons differs from that of non-CRF neurons in the nuclei.

2. Materials and methods

2.1. Animals

Male Wistar rats weighing 180–200 g were used in all experiments. The animals were individually housed under conditions of controlled temperature and illumination (08.00–20.00 h) and allowed *ad libitum* access to food and water. Five days before the experiment, a cannula (SP-45 polyethylene tube, Natsume, Tokyo, Japan) was implanted into the right lateral ventricle under sodium pentobarbital anesthesia (50 mg/kg IP), as described previously [74]. The stereotaxic coordinates for implantation of the cannula were 1.6 mm lateral to bregma and 4.5 mm below the surface of the skull. During the recovery period after the cannula implantation, the rats were handled once a day. All the experiments were performed between 12.00 and 17.00 h. All experimental procedures were conducted in accordance with the guidelines on the use and care of laboratory animals approved by the Local Animal Ethics Committee of Nippon Medical School.

2.2. Drugs

Antalarmin hydrochloride (Sigma–Aldrich Inc., St. Louis, MO) was dissolved in 0.9% saline containing 10% cremophore (Sigma–Aldrich Inc.) and 5% ethanol. We had determined an effective dose of antalarmin to reverse restraint-induced inhibition of food intake using the same procedure as in our previous study [72], and found that 5 mg/kg antalarmin, but not 1, 10 or 20 mg/kg, significantly reversed restraint-induced inhibition of food intake ($p < 0.05$ Fisher's Protected Least Significant Difference (PLSD) test). Therefore, 5 mg/kg antalarmin was used for this study. Twenty micrograms of AS-30 (Tocris Bioscience, Bristol, UK) dissolved in 5 μl 0.9% saline was also used. The dose of AS-30 used in the present study was derived from our previous study in which restraint-induced inhibition of food intake was significantly reversed by 20 μg of AS-30 [72].

3. Experimental protocol

3.1. Effect of CRF receptor antagonists on CRF-/or restraint-induced Fos immunoreactivity

Antalarmin at a dose of 5 mg/ml/kg, or the same volume of vehicle, was injected IP 15 min before exposure to restraint or ICV injection of CRF. Then, 20 μg of AS-30, dissolved in 5 μl of 0.9% saline, or 5 μl of saline as control was administered ICV via SP-10 (polyethylene tube, Natsume, Tokyo, Japan) tubing connected to a 10-μl microsyringe (Hamilton, Reno, NV) over a period of approximately 1 min. After injection, the tube was kept in the guide cannula for 3 min. The animals were then administered ICV with 1 μg of CRF dissolved in 3 μl 0.9% saline or the same volume of 0.9% saline as vehicle. ICV administration of CRF or vehicle was performed in the same way as that of AS-30 administration, as described above. Rats were returned to their home cages until perfusion.

For experimental restraint, rats were injected IP with antalarmin or vehicle, and ICV with AS-30 or vehicle as described above. The entire body of half of the animals was wrapped with a flexible wire mesh for 60 min. The remaining animals were returned to their home cages without wrapping. After 60-min restraint, the animals were returned to their home cages.

3.2. Perfusion and tissue processing

Sixty minutes after the 60-min restraint period, or 120 min after ICV administration of CRF or vehicle, the rats were deeply anesthetized with sodium pentobarbital (50 mg/kg, IP) and perfused via an intracardiac cannula with 0.1 M phosphate-buffer saline (PBS) followed by 4% formaldehyde prepared from paraformaldehyde. The brain was removed, left overnight in 4% formaldehyde in PBS, and then transferred to 20% sucrose in PBS. The frozen tissue was cut coronally using a cryostat (Microm HM 500 OM, Microm, Heidelberg, Germany) into 40 μm thick sections. The atlas of the rat brain by Paxinos and Watson was used for determination of section coordinates [55]. The sections were taken from the following sites: PVN, −1.50 to −2.10 mm; CeA, −1.70 to −2.80 mm; BNST, 0.00 to −0.40 mm posterior from the bregma shown in the atlas.

3.3. Immunohistochemistry for Fos and CRF

Immunohistochemistry was performed with the avidin–biotin–peroxidase method using specific anti-Fos polyclonal antiserum (1:20,000, Oncogene, San Diego, CA) and specific anti-rat CRF antiserum (1:10,000). The specificity of anti-CRF antiserum has been demonstrated in previous reports [11,52,70,71]. Each antiserum was diluted in 0.01 M PBS containing 3% normal goat serum (NGS) and 0.25% Triton X-100. Sections were washed in PBS and placed into a solution of 3% NGS in PBS for 2 h. They were then transferred into vials containing anti-Fos antiserum and incubated overnight at room temperature. The sections were rinsed in PBS and incubated in biotinylated goat anti-rabbit IgG solution (1:200, Vector Laboratories, Burlingame, CA) for 1 h at room temperature. This was followed by another 1-h incubation in an avidin–biotin complex solution (Vectorstain ABC Elite Kit, Vector Laboratories) at room temperature. The reaction product was visualized using diaminobenzidine (Vector DAB Kit, Vector Laboratories), which produces a brown precipitate.

The sections that had been stained with Fos, as described above, were washed in PBS and further incubated with anti-CRF antiserum (1:10,000) overnight at room temperature. The sections were

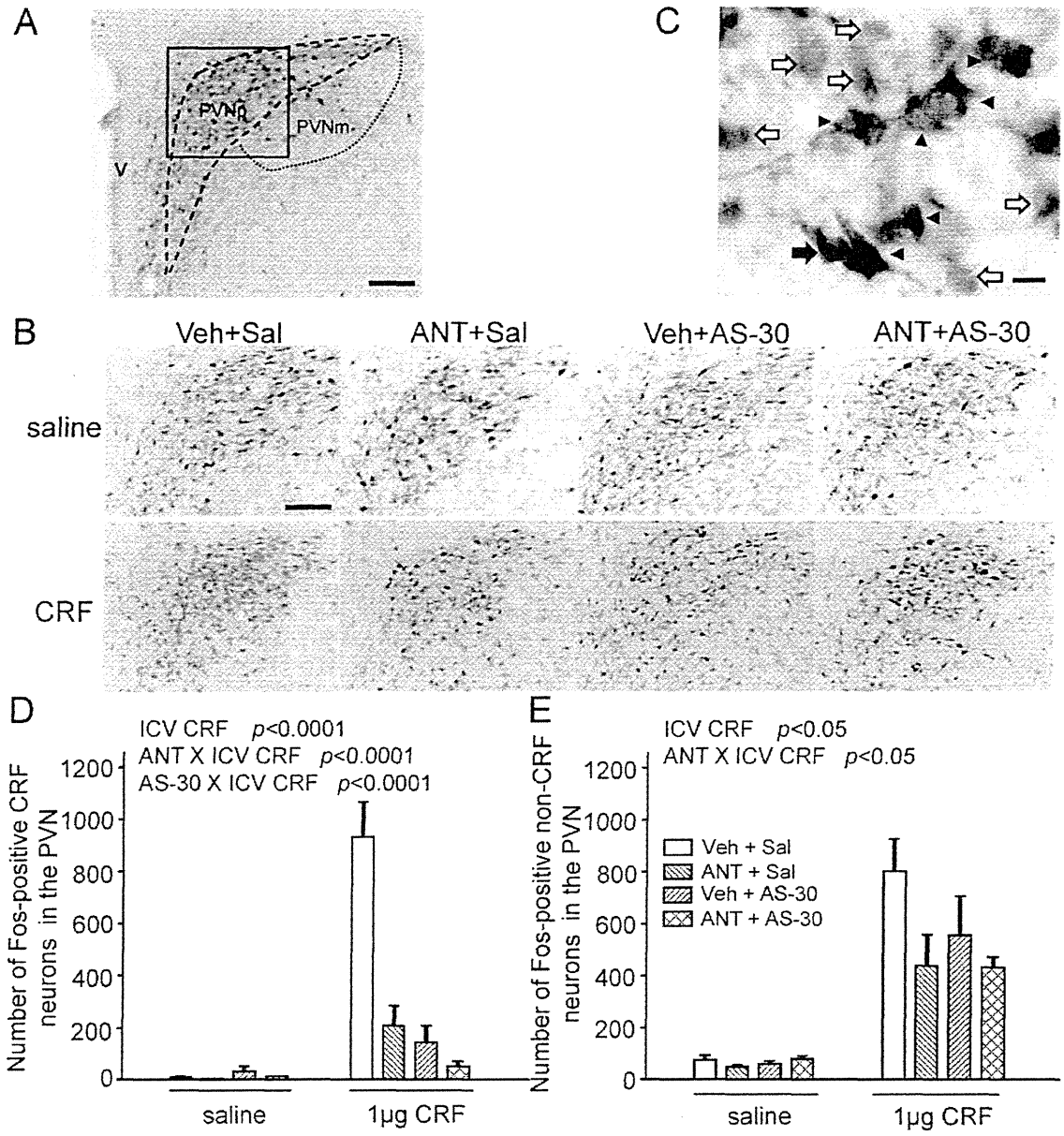

Fig. 1. Effects of antalarmin and AS-30 on ICV CRF-induced increases in Fos-positive CRF and non-CRF neurons in the PVN. The number of Fos-positive CRF and non-CRF neurons was counted in the PVNp, which is indicated by the dotted line (A), and their representative distribution patterns in each experimental group within a rectangle in (A) are shown in (B). PVNp, parvocellular PVN; PVNm, magnocellular PVN; V, third ventricle; Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. The Fos-positive nuclei were labeled in brown, and cytoplasmic CRF in blue. A representative high magnification picture from a rat that was treated with Veh + Sal followed by ICV CRF is shown in (C). Black and white arrows indicate CRF- and Fos-positive neurons, respectively, and arrowheads indicate neurons positive for both Fos and CRF. Scale bars: 100 μm (A) and 10 μm (B and C). Effects of antalarmin and/or AS-30 on ICV CRF-induced increases in the number of Fos-positive CRF neurons (D) and non-CRF neurons (E). Main effect of ICV CRF (D and E), ANT × ICV CRF interaction (D and E), and AS-30 × ICV CRF (D) interaction is significant. The number of rats in each experimental group was 5–8. Data are presented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

rinsed in PBS and incubated in biotinylated goat anti-rabbit IgG solution (1:200; Vector Laboratories) for 1 h at room temperature. This was followed by another 1-h incubation in an avidin–biotin complex solution (Vectorstain ABC Elite Kit, Vector Laboratories) at room temperature. The reaction product was visualized using the Vector SG Substrate Kit (Vector Laboratories), which produces a blue-gray precipitate.

The sections were mounted on gelatin-coated slides and dried by an air-drier. The slides were then washed in water and dehydrated through graded alcohols, cleared in xylenes, and then coverslipped with malinol (Muto Pure Chemicals, Tokyo, Japan). The number of neurons positive for Fos and/or CRF in all the sections of the PVN, CeA, and BSTNov was counted under light microscopy at ×400 magnification. Sections were viewed and photographed with an Olympus BX-51 microscope (Olympus Co., Tokyo, Japan).

3.4. Statistical analysis

All data are represented as mean ± SEM. The data were analyzed using analysis of variance (ANOVA). Statistical significance was established at the $p < 0.05$ level. The numbers of rats in each group are shown in the figure legends.

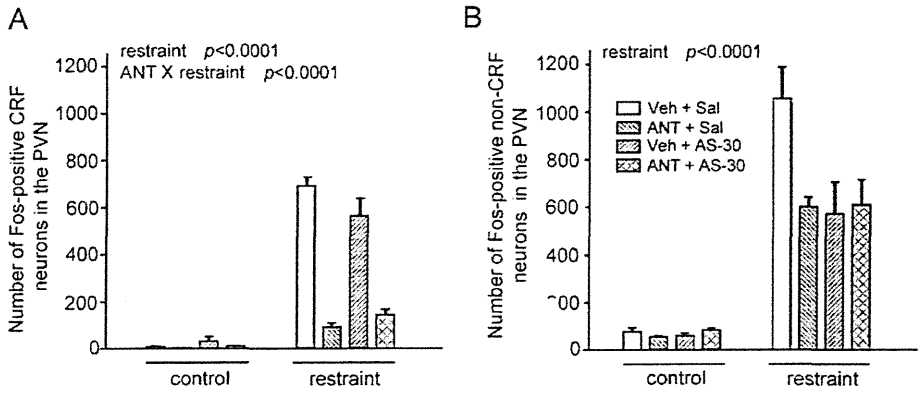

Fig. 2. Effects of antalarmin and AS-30 on restraint-induced increases in the number of Fos-positive CRF (A) and non-CRF (B) neurons in the PVN. The number of Fos-positive CRF and non-CRF neurons was counted in the PVNp. Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. Main effect of restraint (A and B), ANT × restraint interaction (A) is significant. The number of rats in each experimental group was 5–8. Data are presented as mean ± SEM.

4. Results

4.1. Effect of antalarmin and/or AS-30 on stimuli-induced Fos immunoreactivity in the PVN

In the PVN, most CRF neurons were observed in the PVNp in accordance with our and other reports [11,71] (Fig. 1A and B). Three-way ANOVA showed that the number of CRF neurons in the PVNp was not affected by any of the treatments, ICV injection of 1 μg CRF, or CRF receptor antagonists. In control rats that were administered ICV with 0.9% saline, very low levels of Fos-immunoreactivity (ir) were sparsely detected, and neither antalarmin nor AS-30 affected Fos-ir or CRF-ir (Fig. 1B). Fig. 1C shows a high-power magnification picture of Fos-ir shown in brown and CRF-ir shown in blue in the PVNp obtained from a rat administered ICV with CRF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.) Most Fos-positive neurons were present in the PVNp with a few in the magnocellular PVN (PVNm). Fos-positive neurons were divided into two groups, CRF neurons (Fig. 1D) and non-CRF neurons (Fig. 1E). ICV administration of 1 μg CRF significantly increased the number of Fos-positive CRF neurons in the PVN [$F(1,44) = 63.41$, $p < 0.0001$] (Fig. 1D) as well as the number of the Fos-positive non-CRF neurons in the PVN [$F(1,44) = 4.58$, $p < 0.05$] (Fig. 1E). The percentage of CRF neurons that showed Fos-ir after ICV CRF was 84.7 ± 3.1%. The ratio of CRF to non-CRF neurons in the Fos-positive neuronal population after ICV CRF was 11:10. There were significant main effects on the number of the Fos-positive CRF neurons in the PVNp (Fig. 1D) for antalarmin [$F(1,44) = 27.21$, $p < 0.0001$] and interactions between antalarmin and CRF administration [$F(1,44) = 24.03$, $p < 0.0001$]. Furthermore, the main effect of AS-30 and interactions between AS-30 and CRF injection were also significant [$F(1,44) = 32.47$, $p < 0.0001$ for main effect, $F(1,44) = 36.83$, $p < 0.0001$ for interaction], suggesting that pretreatment of 5 mg/kg antalarmin as well as 20 μg AS-30 inhibits CRF-stimulated Fos activation in CRF neurons of the PVNp (Fig. 1D). Interactions between antalarmin, AS-30 and CRF treatments had a significant effect on the number of the Fos-positive CRF neurons, [$F(1,44) = 15.80$, $p < 0.001$]. Three-way ANOVA on the number of Fos-positive non-CRF neurons in the PVN revealed a significant main effect of antalarmin [$F(1,44) = 4.77$, $p < 0.05$] as well as a significant interaction between antalarmin and CRF injection [$F(1,44) = 4.58$, $p < 0.05$], suggesting that antalarmin significantly inhibited CRF-induced Fos expression (Fig. 1E).

One hour period of restraint significantly increased Fos expression in both CRF neurons [$F(1,42) = 218.40$, $p < 0.0001$] and non-CRF [$F(1,42) = 100.35$, $p < 0.0001$] in the PVNp (Fig. 2), although it did not significantly change the number of CRF neurons in the PVNp (data not shown), as reported in a previous study [29]. The percentage of CRF neurons that showed Fos-ir after restraint was 86.7 ± 2.8%. The ratio of CRF to non-CRF neurons in the population of neurons with Fos-ir induction after restraint was 5:8. On Fos-positive CRF neurons, there were significant main effects of antalarmin [$F(1,42) = 114.69$, $p < 0.0001$] and the interaction between antalarmin and restraint [$F(1,42) = 103.918$, $p < 0.0001$] (Fig. 2A), suggesting that antalarmin significantly attenuated stress-induced Fos expression in CRF neurons within the PVNp. With respect to the Fos-positive non-CRF neurons, neither interaction between antalarmin and restraint, nor interaction between AS-30 and restraint was significant although a significant interaction between antalarmin and AS-30 treatment [$F(1,42) = 4.39$, $p < 0.05$] was detected (Fig. 2B).

4.2. Effect of antalarmin and/or AS-30 on stimuli-induced Fos-immunoreactivity in the CeA

Dense CRF neurons and CRF fibers were observed in the CeA (Fig. 3A–C). ICV injection of CRF significantly increased the number of Fos-positive neurons [$F(1,48) = 73.37$, $p < 0.0001$] (Fig. 3D), but it should be noted that none of the CRF neurons showed Fos-ir in the CeA (Fig. 3C). The main effect of AS-30 [$F(1,48) = 55.98$, $p < 0.0001$] and the interaction between AS-30 and CRF treatment [$F(1,48) = 52.40$, $p < 0.0001$] was significant, suggesting that AS-30 significantly inhibited CRF-induced Fos expression (Fig. 3D).

Restraint significantly increased Fos-positive non-CRF neurons [$F(1,41) = 19.16$, $p < 0.0001$], while Fos-positive CRF neurons were not increased (Fig. 4). Main effects of antalarmin [$F(1,41) = 14.88$, $p < 0.001$], AS-30 [$F(1,41) = 30.41$, $p < 0.0001$] and the interactions between antalarmin and AS-30 [$F(1,41) = 15.85$, $p < 0.001$], antalarmin and restraint [$F(1,41) = 11.42$, $p < 0.05$], AS-30 and restraint [$F(1,41) = 18.48$, $p < 0.0001$], and antalarmin, AS-30 and restraint [$F(1,41) = 9.92$, $p < 0.01$] were all significant. These results indicate that antalarmin as well as AS-30 strongly inhibited restraint-induced Fos expression in non-CRF neurons in the CeA (Fig. 4).

4.3. Effect of antalarmin and/or AS-30 on stimuli-induced Fos-immunoreactivity in the BNSTov

In the BNSTov, dense CRF neurons and CRF fibers were observed (Fig. 5A–C). ICV injection of CRF significantly increased the number of Fos-positive neurons [$F(1,46) = 254.18$, $p < 0.0001$] (Fig. 5D); however, the Fos-ir was not observed in CRF neurons in the BNSTov (Fig. 5C). There were significant main effects of AS-30 [$F(1,46) = 191.51$, $p < 0.0001$] and interactions between

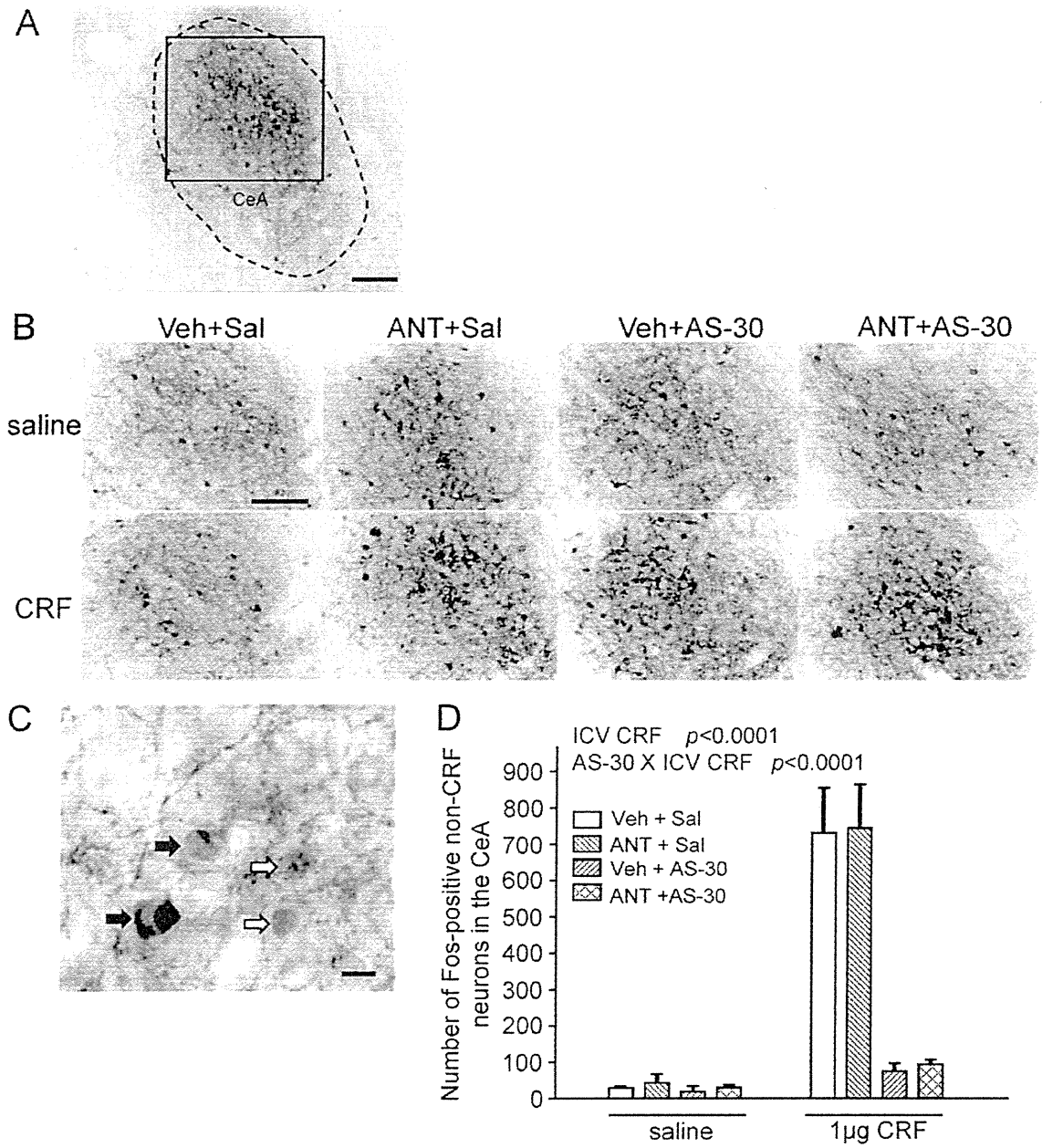

Fig. 3. Effects of antalarmin and AS-30 on ICV CRF-induced increases in Fos-positive neurons in the CeA. The number of Fos-positive CRF and non-CRF neurons was counted in the CeA, which is indicated by the dotted line (A), and their representative distribution patterns in each experimental group within a rectangle in A are shown in (B). CeA, central nucleus of the amygdala; Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. The Fos-positive nuclei were labeled in brown, and cytoplasmic CRF-ir in blue. A representative high magnification picture from a rat that was treated with Veh + Sal followed by ICV CRF is shown in (C). Black and white arrows indicate CRF- and Fos-positive neurons, respectively. Neurons positive for both CRF and Fos were not detected. Scale bars: 100 μm (A) and 10 μm (B and C). Effects of antalarmin and/or AS-30 on ICV CRF-induced increases in the number of Fos-positive non-CRF neurons (D). Main effect of ICV CRF and AS-30 × ICV CRF interaction is significant (D). The number of rats in each experimental group was 5–9. Data are presented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

AS-30 and CRF treatment [$F(1,46) = 198.52$, $p < 0.0001$]. The main effects of antalarmin [$F(1,46) = 5.38$, $p < 0.05$] and the interaction between antalarmin and CRF injection [$F(1,46) = 5.21$, $p < 0.05$] were also significant, suggesting that both AS-30 and antalarmin significantly attenuated CRF-induced Fos-ir in the BNSTov (Fig. 5D).

Restraint significantly increased Fos-positive non-CRF neurons [$F(1,40) = 8.75$, $p < 0.01$], but not CRF neurons (Fig. 6), which is consistent with a previous report [10]. In the BNSTov, the main effects of antalarmin [$F(1,40) = 6.45$, $p < 0.05$] and AS-30 [$F(1,40) = 4.41$, $p < 0.05$], as well as the interactions between antalarmin and stress [$F(1,40) = 5.94$ $p < 0.05$], AS-30 and stress [$F(1,40) = 5.34$, $p < 0.05$], antalarmin and AS-30 [$F(1,40) = 8.28$, $p < 0.01$], and antalarmin, AS-30 and restraint [$F(1,40) = 5.02$, $p < 0.05$] were all significant. Similar to the CeA, these results indicate that antalarmin as well as AS-30 significantly inhibited stress-induced Fos-ir in non-CRF neurons in the BNSTov (Fig. 6).

5. Discussion

In the present study, ICV CRF- or restraint-induced Fos-ir was observed in the PVNp, CeA and BNSTov in agreement with previous reports [3,8,29,53]. Since there are both CRF and non-CRF neurons in each of the target areas, the present study has examined

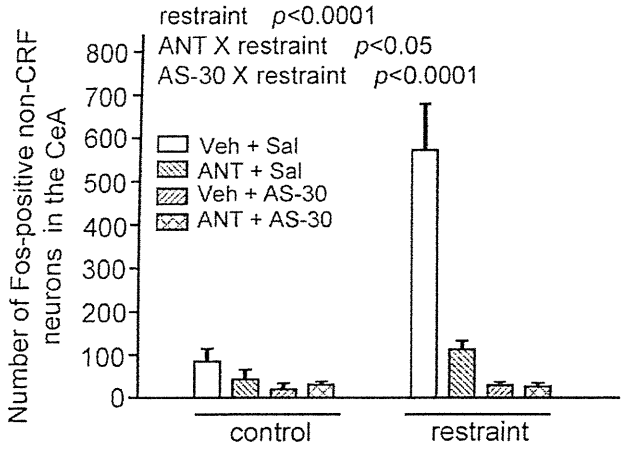

Fig. 4. Effects of antalarmin and/or AS-30 on restraint-induced increases in the number of Fos-ir-positive neurons in the CeA. The number of Fos-positive CRF and non-CRF neurons was counted in the CeA. Neurons positive for both CRF and Fos were not detected. Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. Main effect of restraint, ANT × restraint interaction, and AS-30 × restraint interaction are significant. The number of rats in each experimental group was 5–8. Data are presented as mean ± SEM.

whether Fos-ir is induced by ICV CRF and restraint in CRF and/or non-CRF neurons by double staining for CRF and Fos. Increased Fos-ir induced by various stress and stimuli, such as restraint, noise, forced swim, hemorrhage, ICV CRF and peripheral administration of IL-1β, are reportedly shown in CRF neurons of the PVNp [3,14,68] and non-CRF neurons of the CeA and BNSTov [3,10,13,14,28]. However, the percentage of activated CRF neurons in the PVNp that respond to ICV CRF and restraint has not been reported. The results of the present study have shown that Fos-ir is expressed in response to ICV CRF and restraint in both CRF and non-CRF neurons in the PVNp, and that approximately 85 and 87% of the CRF neurons in this area expressed Fos-ir in response to ICV CRF and restraint, respectively. This suggests that all of the CRF neurons in the PVNp do not necessarily respond to each stimulus. Since the number of non-CRF neurons without Fos-ir could not be assessed with the dual immuno-staining method used here, the percentage of activated non-CRF neurons in the PVNp remains unclear. Fos-ir was found to be increased in non-CRF neurons, but not CRF neurons, in the CeA and BNSTov in the present study. This observation is consistent with previous reports that several stimuli, such as hemorrhage, hypotension, restraint, and amphetamine, induce Fos-ir or c-fos mRNA in only non-CRF neurons, or mostly non-CRF neurons with a minority of CRF neurons in the CeA or BNST [10,12,14,28,68].

ICV CRF-induced c-fos mRNA in the PVN has been shown to be blocked by a non-selective CRF receptor antagonist [46]. However, there has been no study in which specific antagonists for CRF_1 and CRF_2 are used to elucidate how CRF receptor subtypes are involved in ICV CRF- and stress-induced Fos-ir in the neurons of the PVN, CeA, and BNST. Therefore, in the present study, antalarmin, a CRF_1 specific antagonist, and AS-30, a CRF_2 specific antagonist, were administered prior to ICV CRF or restraint, and the effects of each antagonist on induced Fos-ir were examined in the neurons of the PVNp, CeA, and BNSTov.

The results of the present study showed that, in CRF neurons of the PVNp, the CRF_1 and CRF_2 antagonists decreased ICV CRF-induction of Fos-ir by 77% and 85%, respectively and that co-administration of both antagonists almost completely blocked the induction, suggesting both CRF_1 and CRF_2 play major roles in the ICV CRF-induced activation of CRF neurons. The restraint-induced Fos-ir was almost completely inhibited by CRF_1 antagonist by 87%, but not influenced by CRF_2 antagonist. In contrast, the CRF_1 antagonist decreased the number of ICV CRF-induced Fos-positive non-CRF neurons of the PVNp by 48%. The CRF_1 and CRF_2 antagonists did not significantly decrease restraint-induced Fos-positive non-CRF neurons of the PVNp. In non-CRF neurons, each antagonist showed similar inhibitory patterns in ICV CRF- and restraint-induced Fos-ir, suggesting the involvement of similar signaling systems in response to ICV CRF and restraint in non-CRF neurons of the PVNp. These findings suggest that participation patterns of CRF_1 and CRF_2 in the ICV CRF- and restraint-induced activation differ between non-CRF and CRF-neurons in the PVN.

CRF mainly binds to CRF_1, and with a moderate affinity to CRF_2. The results of the present study indicate that CRF_2 participates in the activation of CRF neurons by ICV CRF. Therefore, it is suggested that ICV CRF might induce Fos through direct binding to CRF_2 or ICV CRF may directly or indirectly stimulate neurons that express CRF family peptides, such as Ucn 1, Ucn 2, and Ucn 3, which are endogenous ligands for CRF_2. Importantly, the levels of Ucn 1, Ucn 2, and Ucn 3 mRNA expression increase in response to stressful stimuli [24,31,38]. In addition to CRF family peptides, other signals seem to participate especially in restraint-induced activation of non-CRF neurons in the PVNp because no significant inhibition of this effect could be achieved with either CRF_1 or CRF_2 antagonist. This possibility is supported by the findings that nerve fibers ascend to the PVNp that contains norepinephrine, serotonin, and GABA, as well as various peptides [9,16,49,54,59].

The results of the present study have shown that ICV CRF induced Fos-ir in the CeA and BNSTov in consistence with the results of a previous report [1]. In the CeA, the CRF_2 antagonist almost completely decreased ICV CRF-induction of Fos-ir by 90%, while the CRF_1 antagonist did not influence it. In contrast, restraint-induced activation of neurons was markedly inhibited by 80% and blocked completely by the CRF_1 and CRF_2 antagonists, respectively. These results suggest that both CRF_1 and CRF_2 participate in restraint-induced activation of neurons in the CeA and that only CRF_2 is involved in ICV CRF-induced activation of these neurons. In the BNSTov, the CRF_1 and CRF_2 antagonists decreased ICV CRF-induction of Fos-ir by 20% and 87%, respectively, and co-administration of both antagonists almost completely blocked the effect. Restraint-induced activation of these neurons was almost completely blocked by either CRF_1 or CRF_2. Therefore, this indicates that ICV CRF and restraint seem to activate BNSTov neurons in different manners, where CRF_2 is mainly involved in the former, while both CRF_1 and CRF_2 participate in the latter activation. Since CRF_1 and CRF_2 antagonists showed a similar degree of inhibition on restraint-induced activation of neurons in the CeA and BNSTov, it is suggested that a common signaling pathway may be present for the restraint signal to reach the CeA and BNSTov. In the present study, the dose of each CRF receptor antagonist was determined based on our preliminary and previous studies [72]. The specificity of each CRF_1 and CRF_2 antagonist seems to be preserved at doses used in the present study because completely opposite results were found in the effects of each antagonist between the restraint-induced Fos-positive CRF neurons of the PVNp and ICV CRF-induced Fos-positive non-CRF neurons of the CeA: CRF_1 antagonist was active with no activity of CRF_2 antagonist in the former while CRF_2 antagonist was active with no activity of CRF_1 antagonist in the latter.

The ligand–receptor mismatch in the PVN, CeA, and BNST has been discussed in several studies [3,34]. Although CRF_1 protein is present in the PVNp or the PVN [7,62], no significant ^{125}I-Tyr–CRF binding was detected in the PVNp under basal conditions [44], suggesting that the production CRF_1 protein is extremely low, if present at all, in the neurons of the PVNp. Furthermore, the expression of CRF_1 mRNA is reported to be marginal or not detected [3,4,84]. There are no reports concerning the production of CRF_2 in the PVN because specific anti-CRF_2 antiserum is not available. It is reported that the expression of CRF_2 mRNA is absent in the

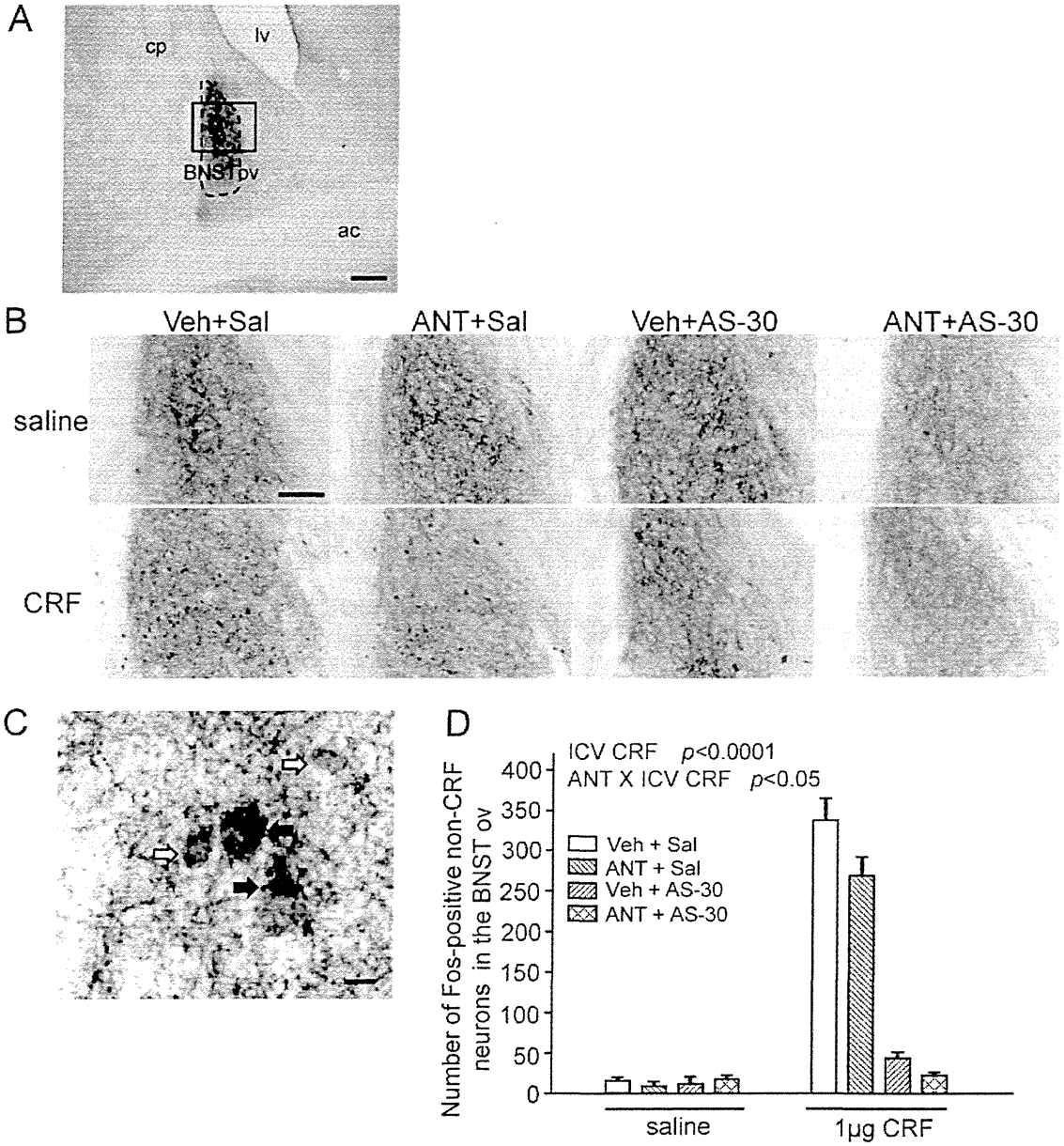

Fig. 5. Effects of antalarmin and AS-30 on ICV CRF-induced increases in Fos-positive neurons in the BNSTov. The number of Fos-positive CRF and non-CRF neurons was counted in the BNSTov which is indicated by the dotted line (A), and their representative distribution patterns in each experimental group within a rectangle in (A) are shown in (B). BNSTov, oval nucleus of the bed nucleus of stria terminalis; ac, anterior commissure; cp, caudate putamen; lv, lateral ventricle; Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. The Fos-positive nuclei were labeled in brown, and cytoplasmic CRF in blue. A representative high magnification picture from a rat that was treated with Veh + Sal followed by ICV CRF is shown in (C). Black and white arrows indicate CRF- and Fos-positive neurons, respectively. Neurons positive for both CRF and Fos were not detected. Scale bars: 100 μm (A) and 10 μm (B and C). Effects of antalarmin and/or AS-30 on ICV CRF-induced increases in the number of Fos-positive non-CRF neurons (D). Main effect of ICV CRF, ANT × ICV CRF interaction, and AS-30 × ICV CRF interaction are significant (D). The number of rats in each experimental group was 5–9. Data are presented as mean ± SEM. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

PVNp or the PVN [3,84], while its expression has been reported in the PVNp or the PVN by several other groups [4,45]. A dual labeling study for Fos-ir and CRF_1 or CRF_2 mRNA has shown that neurons activated by ICV CRF in the PVNp are negative for both messages [3]. Very recently, transgenic mice that co-express CRF_1 with green fluorescent protein (GFP) have been generated [36]. In these animals, a substantial population of GFP-stained cells did not express CRF, with only a small minority of the CRF_1 population expressing CRF in the PVNp. Furthermore, Fos-ir induced by ICV CRF was found essentially in the GFP (CRF_1)-negative neurons in the PVN [34]. Taken together, these reports suggest that either CRF_1 or CRF_2 is absent in the neurons of the PVNp, or their expression is extremely low. Anatomical evidence in the PVN suggests the presence of homotypic and heterotypic CRF synaptic connections [75,80]. It has been shown that approximately 90% of the synaptic connections are symmetrical and inhibitory, with the remainder being asymmetrical and excitatory. This suggests that CRF receptors are involved in the modulation of CRF neuronal function in the PVN. Taken together with the results of the present study, CRF_1 and CRF_2 at presynaptic sites may be involved in the ICV CRF- or restraint-induced activation of CRF and non-CRF neurons in the PVNp. However, the presence of both CRF_1 and CRF_2 at presynaptic sites remains to be verified.

Extrahypothalamic CRF has been heavily implicated in mediating many of the behavioral responses to stress [19,37]. In the present study, the CeA and BNSTov have been chosen to examine

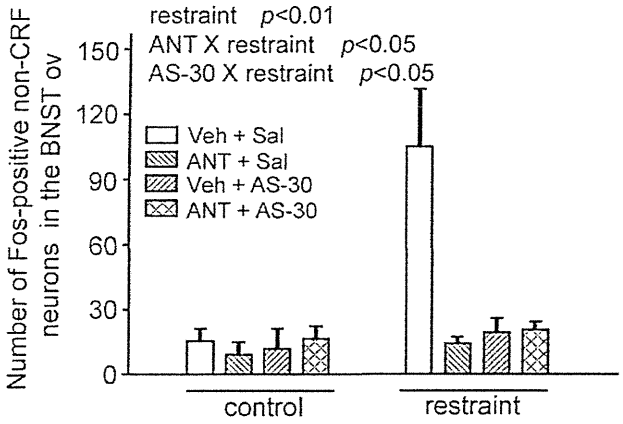

Fig. 6. Effects of antalarmin and/or AS-30 on restraint-induced increases of the number of Fos-positive neurons in the BNSTov. The number of Fos-positive CRF and non-CRF neurons was counted in the BNSTov. Neurons positive for both CRF and Fos were not detected. Veh, vehicle; Sal, saline; ANT, antalarmin; AS-30, antisauvagine-30. Main effect of restraint, ANT × restraint interaction, and AS-30 × restraint interaction is significant. The number of rats in each experimental group was 5–7. Data are presented as mean ± SEM.

the role of CRF neurons in stress since these two areas are rich in CRF neurons [13,33,54,70,88]. Based on the neuroanatomical evidence, it has been suggested that the BNST and CeA form a functional unit [15,50]. The CeA can be divided into two subnuclei, the lateral (CeAl) and medial nucleus (CeAm). The CeAm has many descending projections to areas involved in the control of autonomic and behavioral responses to emotional stimuli [17,86], while the CeAl has projections only to the CeAm, BNSTov and parabrachial nucleus [17,50,58]. The BNST is also an important relay area in the control of the hypothalamic and brain stem centers responsible for behavioral, endocrine and physiological changes during stress responses [20,26,77,87]. The BNST are divided into the anterior and posterior regions based on anatomical evidence [32,33]. The BNSTov is within the lateral area of the anterior BNST [18,63], and contains GABAergic neurons co-expressing CRF [13,33,76,88]. The BNSTov and CeAl have reciprocal connections [18,58,78]. CRF-containing projections from the CeA to the BNST have been identified [71]. However, neither CRF_1 nor CRF_2 mRNA was detected in the CeA or BNSTov [3,84], although very low levels of CRF_1 protein were detected in the CeA [7]. Expression of CRF_1 or CRF_2 mRNA was either absent or minimal in Fos-positive neurons in the CeA or BNSTov following ICV CRF [3]. Although there is a difference between rats and mice in the expression of CRF receptor mRNA and protein in the brain [7,84], a significant amount of GFP in the CeA and BNST was found in transgenic mice co-expressing CRF_1 with GFP; however, ICV CRF-induced Fos-ir was not detected in GFP (CRF_1)-positive neurons in the CeA and BNSTov [34].

Groups of cells in the CeA that are lacking or are limited in their cellular CRF_1 expression are found to be innervated with CRF_1 expressing axons terminals, suggesting possible presynaptic CRF_1 localization [34]. Furthermore, an electron microscopic study has shown both postsynaptic and presynaptic CRF receptor-ir at excitatory synapses of the CeA, although the former is predominant [83]. In accordance with these morphological findings, there exists electrophysiological evidence supporting presynaptic CRF_2 mechanisms in modulating glutamatergic transmission in the CeA [23,42]. Taken together with these reports, the results of the present study therefore suggest that the neurons exhibiting Fos-ir in response to ICV CRF and restraint appear to be activated through presynaptic CRF_2 and CRF_1 plus CRF_2, respectively, at excitatory synapses of the CeA. Since the vast majority of the neurons in the CeA is GABAergic [60,76,88], the excitatory type terminals containing CRF_1-ir are suggested to be extrinsic in origin [83]. Since CRF receptor-ir was also observed in terminals that form inhibitory synapses [83] and a recent study has shown that activation of amygdala CRF_1 increases the release of GABA [2], CRF_1 might also play a role at inhibitory synapses in modulating stress signals by increasing GABA release. The significance of the interaction between presynaptic CRF_1 at excitatory synapses and presynaptic CRF_2 at inhibitory synapses, in the regulatory mechanism of the CeA function, remains to be elucidated. Such presynaptic CRF_1 and CRF_2 would also play a role in the activation of neurons in the BNSTov, as well as in the CeA, in response to ICV CRF and restraint. However, the presence of presynaptic CRF receptors in the BNST remains unverified.

In summary, these results indicate that ICV CRF and restraint increased both Fos-positive CRF and non-CRF neurons in the PVNp, and non-CRF neurons in the CeA and BNSTov through activation of CRF_1 and/or CRF_2. It has also been shown that the manner of involvement of both CRF receptor subtypes in the ICV CRF- and restraint-induced activation of neurons are almost same in the CeA and BNSTov, while distinct from that in the PVNp. Additional signals, other than CRF_1- and CRF_2-mediated signals, also seem to be involved in the stimuli-induced activation of neurons, especially in the restraint-induced activation of non-CRF neurons in the PVNp, since CRF antagonists did not inhibit their activation.

Acknowledgments

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Increased expression of miR-325-3p by urocortin 2 and its involvement in stress-induced suppression of LH secretion in rat pituitary

Takahiro Nemoto*, Asuka Mano and Tamotsu Shibasaki
Department of Physiology, Nippon Medical School, Tokyo, Japan

Running title: miR-325-3p is involved in LH suppression

*Address all correspondence and requests for reprints to: Takahiro Nemoto
Department of Physiology, Nippon Medical School
1-1-5 Sendagi, Bunkyo-ku, Tokyo, 113-8602 Japan
TEL: 81-3-3822-2131
FAX: 81-3-3822-0766
E-mail: taknemo@nms.ac.jp

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Abbreviations: CRF, corticotropin-releasing factor; CRF-R, CRF receptor; FSH, follicle-stimulating hormone; LH, luteinizing hormone; miRNA, micro RNA; NRS, normal rabbit serum; PKA, protein kinase A; POMC, proopiomelanocortin; Ucn 2, urocortin 2; UTR, untraslated region

Urocortin 2 (Ucn 2) is a member of the CRF peptide family, which binds to corticotropin releasing factor (CRF) type 2 receptor. We previously reported on expression of Ucn 2 in proopiomelanocortin cells of rat pituitary and its inhibitory action on luteinizing hormone (LH) secretion. We also demonstrated that Ucn 2 is involved in the mechanism underlying immobilization-induced suppression of LH secretion; the details remain unclear. Here, we found that Ucn 2 increased the expression of miR-325-3p, one of three microRNAs with predicted sequence for binding to LH β-subunit 3'-untranslated region (UTR) in monolayer cultured rat anterior pituitary cells, and that miR-325-3p was expressed in LH cells of the anterior pituitary. Immobilization also increased miR-325-3p expression in the anterior pituitary and its increase was blocked by pretreatment with anti-Ucn 2 IgG. Overexpression of miR-325-3p in cultured pituitary cells significantly suppressed intracellular contents and secretion of LH, while miR-325-3p knockdown blocked Ucn 2-induced suppression of intracellular contents and secretion of LH. Co-expression of miR-325-3p with LH β-subunit 3'-UTR-fused luciferase vector significantly suppressed luciferase activity compared with that of mock transfectants. These results suggest that miR-325-3p is involved in immobilization-induced suppression of LH translation and secretion, and that Ucn 2 plays a role in the increase in miR-325-3p expression.

Introduction

Reproductive function is suppressed by corticotropin-releasing factor (CRF), adrenocorticotropin, β-endorphin and glucocorticoids, composing the hypothalamic-pituitary-adrenal axis under stress exposure (28, 29). We previously demonstrated that Urocortin 2 (Ucn 2), a member of the CRF peptide family (27), is expressed in proopiomelanocortin (POMC) cells of the anterior and intermediate lobes of the rat pituitary (32), and that in both sites CRF increases its mRNA expression (20). We also reported that Ucn 2 suppresses luteinizing hormone (LH) secretion without influencing LH β-subunit mRNA expression, whereas a selective CRF type 2 receptor (CRF-R2) antagonist and a specific siRNA against CRF-R2 significantly increase LH secretion and LH β-subunit mRNA expression in monolayer cultured anterior pituitary cells of rats (23). A possible explanation for the inconsistency of effects between Ucn 2 and its blockades on LH β-subunit mRNA expression is proposed; the expression level of LH β-subunit mRNA is already suppressed by endogenous Ucn 2 secreted by POMC cells into the culture media and exogenous Ucn 2 could not induce further suppression of LH β-subunit mRNA expression (6). Furthermore, we have shown that anti-Ucn 2 IgG partially blocks immobilization-induced suppression of LH secretion and LH β-subunit mRNA expression (21). Considered together, these findings suggest that pituitary Ucn 2 inhibits LH secretion and LH β-subunit mRNA expression and is involved in immobilization-induced suppression of reproductive function although the details of the

mechanism remain unclear.

Glycoprotein hormones such as LH, follicle-stimulating hormone (FSH), thyroid stimulating hormone, and human chorionic gonadotropin are members of a family of cysteine rich proteins, each consisting of α- and β-subunits; the α-subunit is common to all and the β-subunit confers specificity (26). Expression of gonadtropins is regulated by positive and negative feedback mechanisms (25). Sex steroid hormones control synthesis of each subunit separately but in a coordinated fashion (10). Regulation of transcription and translation differs for LH and FSH; inhibin-induced inhibition of SMAD signaling prevents activation of the FSH but not the LH β-subunit promoter (8). Regulatory mechanisms involved in the post-transcriptional and translational processes during LH β-subunit synthesis, especially their suppressive mechanisms, are unclear at present.

Micro RNAs (miRNAs) are short RNA molecules, approximately 22 nucleotides long on average, that are found in most types of eukaryotic cells (3). They are post-transcriptional regulators that usually induce translational repression and gene silencing in animals after binding to complementary sequences on target mRNAs. To the best of our knowledge there have been no previous reports of the involvement of miRNAs in the regulation of pituitary hormone secretion. We hypothesized that Ucn 2 may induce expression of miRNAs, which may then be involved in stress-induced suppression of LH secretion. To test this hypothesis, through database searches we identified miRNAs predicted to bind to the LH

β-subunit 3'-UTR, determined their expression levels in the Ucn 2-treated monolayer cultured anterior pituitary cells of rats and pituitaries of immobilization-exposed rats, and then assessed the effects of miRNA overexpression and knockdown on LH β-subunit mRNA expression, intracellular LH content and LH secretion.

Materials and Methods

Animals

Six- and seven-week-old male Wistar rats were maintained at 23 ± 2 °C in a 12:12-h light-dark cycle (lights on at 0800, off at 2000). They were allowed *ad libitum* access to laboratory chow and distilled water. All experimental procedures were reviewed and approved by the Laboratory Animals Ethics Review Committee of Nippon Medical School.

Primary culture of pituitary cells

Thirty male rats (6 wk old) were killed by decapitation and their pituitary glands were removed under sterile conditions. The anterior pituitary lobes were collected, pooled together and then mechano-enzymatically dispersed as previously described (20), but with several modifications. Briefly, lobes were washed twice in PBS and then incubated at room temperature in the PBS containing 0.01% dispase (Godoshusei, Tokyo, Japan) with constant stirring for 30 min. After washing with PBS three times, cells were plated in 24-well plates or 60 mm dishes and cultured with 10% FBS containing DMEM/F10 HAM culture medium (Sigma-Aldrich Co., St. Louis, MO) supplemented with an antibiotics/antimyotic solution (GIBCO, Auckland, NZ). The cells were subsequently allowed to attach to the plating surfaces at 37 °C in a humidified 5% CO_2/95% air incubator for 4 d. On the day of

experiment the culture media were changed. Cells were treated with Ucn 2 at concentrations of 1, 10, 30 and 100 pM for 1 h, or 100 pM Ucn 2 for 0.5, 1 and 2 h. After incubations, the cells were assayed for miRNA expression.

Passive immunization

We previously generated antisera against mouse Ucn 2 (32). The IgG fraction was purified from serum obtained after the fifth booster using a protein A-sepharose column. The specificity of this antiserum to Ucn 2 was described in our previous reports (32). Cross-reactivity with rat Ucn 2, rat CRF, Ucn 1, and Ucn 3 were 83.3%, 0.0%, 0.014% and 0.023%, respectively. Twenty male rats were administrated antiserum IgG to Ucn 2 or normal rabbit serum (NRS) IgG intraperitoneally (1 mg/kg b.w. dissolved in 1 ml normal saline). Two hours later, they were exposed to 90 min of immobilization stress (21). Rats were then sacrificed immediately; their trunk blood and pituitaries were collected. Trunk blood was placed into tubes containing EDTA 2Na (1 mg/ml blood) and centrifuged at 3,000 rpm for 20 min at 4 °C. One-milliliter aliquots were transferred into 1.5-ml Eppendorf tubes and store at -80 °C until use.

Stress exposure

Twenty male rats (7 wk old) were wrapped in a flexible wire mesh, and kept for 90 min between 1300 and 1500 in an isolated room (1, 21). Rats were sacrificed in the adjacent room immediately after immobilization and their pituitaries were collected for RNA expression analysis. Non-stressed control rats were housed in a separate room from the stressed rats, and were otherwise treated the same way.

miRNA identification

The miRBase (www.mirbase.org) and TargetScan (www.targetscan.org) database were searched for miRNAs predicted to bind to the LH β-subunit 3'-UTR.

In situ hybridization and immunohistochemistry

Digoxigenin labeled LNA probe against mmu-miR-325-3p was purchased from Exiqon (Woburn, MA). Pituitaries were cryosectioned, dried for 30 min, and then fixed in 4% paraformaldehyde. Sections were permeabilized by proteinase K (10 μg/ml) for 10 min at 37 °C, and postfixed with 4% paraformaldehyde at 4 °C. Prior to prehybridization, sections were acetylated in 0.25% acetic anhydride, 1.16% triethanolamine (Wako Pure Chemicals Inc., Tokyo, Japan). Sections were pre-hybridized 60 min in 50% formamide hybridization buffer, and then the sections were hybridized with denatured probe in 50% formamide hybridization buffer at 55 C overnight. The slides were washed in decreasing

salt solutions (4 X, 2 X, and 0.5 X saline sodium citrate). Signals were detected by anti-DIG-alkaline phosphatase conjugate (Roche Diagnistics GmbH, Mannheim, Germany) followed by detection with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnistics GmbH).

Upon completion of the chromogen reaction, slides were treated in 0.3% H_2O_2 for 30 min at room temperature, and the sections were incubated in 10% normal goat serum for 20 min and then incubated for 60 min at room temperature with an antibody to LH (1:5,000 dilution; CHEMICON, Inc., Tamecula, CA). Sections were incubated for 30 min with a secondary biotinylated goat anti-rabbit IgG (1:200 dilution; Vector Laboratories, Inc., Burlingame, CA). Sections were then incubated with an avidin-biotin-peroxidase complex (Vector Labolatories) for 30 min at room temperature and the antibody-peroxidase complex was visualized using Vector DAB kit (Vector Laboratories). When the staining had reached appropriate intensity, the tissue was rinsed in PBS, dehydrated through graded alcohols, cleared in xylenes, and coverslipped with VectaMount (Vector Laboratories). Images were captured with a microscope (AX-80; Olympus, Tokyo, Japan) using a color digital camera (DP72; Olympus) and digitized by an image analysis system (cellSens® software, Olympus).

Transfection and Ucn 2 treatment

miRNA overexpression vectors pBA-miR-325-3p and pBA-mock, and miRNA knockdown vectors

pDecoy-miR-325-3p and pDecoy-mock were purchased from Takara Bio (Shiga, Japan). Each plasmid (2.5 μg) was co-transfected into primary pituitary cells with pAcGFP vector (2.5 μg) to validate the success of the transfection using Multifectam (Promega, Madison, WI) according to manufacturer's instructions. After 72 h, culture media were changed to fresh DMEM/F10 HAM medium containing Ucn 2 at a concentration of 100 pM and then incubated for 1 h. To check whether the plasmids were successfully transfected into the cells, GFP fluorescence was assessed 48 to 72 h after transfection under fluorescent microscope.

Hormone assay

Frozen 1-ml aliquots of plasma or culture media were thawed and LH concentrations were measured using a rodent LH ELISA test (ERKR7010, Endocrine Technologies, Inc, Newark, CA), according to the manufacturers' instructions. Literature supplied with the kit indicated 0.5 ng/ml as the assay sensitivity.

RNA extraction and real-time RT-PCR analysis

Total RNA was extracted from cultured pituitary cells and rat pituitaries using RNAiso Plus (Takara). For miRNA expression analysis, first strand cDNA was synthesized using 1 μg of denatured

total RNA at 37 °C for 1 h and then terminated at 85 °C for 5 min using Mir-X® miRNA First-Strand Synthesis and SYBR® qRT-PCR kit (Clontech Laboratories Inc., Mountain View, CA). For mRNA expression analysis, first strand cDNA was generated using 0.5 μg of denatured total RNA at 37 °C for 15 min, 84 °C for 5 s, and 4 °C for 5 min using a PrimeScript® RT reagent kit with gDNA Eraser (Takara). PCR was performed by denaturation at 94 °C for 5 s and annealing-extension at 60 °C for 30 s for 40 cycles, using SYBR premix Ex Taq (Takara) and specific primers for rat LH β-subunit and GAPDH as previously reported (21). To normalize each sample for RNA content, GAPDH, a house-keeping gene, or U6 small nuclear RNA (Clontech) were used for mRNA and miRNA expression analyses, respectively. Diluted normal rat pituitary cDNA and the 2nd derivative method were used as the standard and for calculating Ct values, respectively (24).

Northern blotting of miRNA

Total RNA from transfectants was separated by electrophoresis in 15% acrylamide gel containing 8 M urea. RNA were then electrically transferred to nylon membranes and fixed by UV cross-linking, prehybridized with DIG Easy Hyb solution (Roche Diagnostics GmbH) containing 10 μg/ml yeast tRNA at 45 °C for 1 h, and then hybridized with 3' DIG-labeled miRCURY LNA miRNA detection probe against mmu-miR-325-3p (Exiqon) at 45 °C for 14 h. Signals were detected with a DIG

luminescent detection kit according to the manufacturer's instructions (Roche Diagnostics GmbH).

3'-UTR assay

Synthetic oligonucleotide for LH β-subunit 3'-UTR, positioned from translational termination codon together with Nhe I and Xho I linker at its 5'- and 3'-ends respectively, and its antisense oligonucleotide were synthesized by Invitrogen (Carlsbad, CA). Oligonucleotides were annealed, digested with *Nhe* I and *Xho* I, and then *Nhe* I-*Xho* I fragment was subcloned into *Nhe* I and *Xho* I sites of pmir-Glo plasmid (Promega, Madison, WI) (pmir-Glo-LHβ 3'UTR (WT)) (Fig. 5A) (30). We also generated a 3'-UTR variant which was not recognized by miR-325-3p (pmir-Glo-LHβ 3' UTR (SC)) (Fig. 5A). pmir-Glo-LHβ 3'UTR (WT), pmir-Glo-LHβ 3'UTR (SC) or pmir-Glo-mock plasmid and pBA-miR-325-3p or pBA-mock plasmid (2.5 μg of each plasmid amount) were co-transfected with Multifectam (Promega) into the HEK293 cells, seeded in 60 mm dishes. Cells were collected after 72 h and assayed using the dual-luciferase reporter assay system according to the manufacturer's instruction (Promega).

Statistical analysis.

Statistical analysis was performed by ANOVA followed by turkey *post hoc* test using Prism 5.0

software (GraphPad Software, Inc., La Jolla, CA). Pituitary cell culture using the same experimental protocol was performed twice. For real-time RT-PCR data, all results were expressed as percent of control values. Statistical significance was defined as $p < 0.05$ level.

Results

Ucn 2 increases miRNA expression in monolayer cultured rat pituitary cells

A search of miRNA databases revealed 3 miRNAs, rno-miR-325-3p (MI0000596), rno-miR-370 (MI0003486), and rno-miR-742 (MI0006161), with sequences predicted for binding to positions 59-65, 34-40, and 66-71 of LH β-subunit 3'-UTR, respectively (Fig. 1A). miR-325-3p appears to be present in essentially all pituitary cells including those that are immunopostive for LH by a double labeling technique for combined *in situ* hybridization and immunohistochemical analysis (Fig. 1B). Treatment of monolayer cultured rat anterior pituitary cells with Ucn 2 significantly increased miR-325-3p expression, while there was no change in miR-370 and miR-742 levels (data not shown). Treatment with Ucn 2 for 1 h significantly increased miR-325-3p expression at concentrations of 30 pM (1.75 ± 0.16-fold compared to control) and 100 pM (2.41 ± 0.15-fold compared to control) (Fig. 2A). A time course study revealed that miR-325-3p expression was significantly increased 0.5 h, 1 h and 2 h (1.79 ± 0.18-fold, 2.41 ± 0.16-fold and 1.59 ± 0.12-fold compared to control, respectively) after treatment with Ucn 2 at a concentration of 100 pM (Fig. 2B).

Immobilization stress increases miR-325-3p expression and anti-Ucn 2 IgG blocks the increase in the pituitary

Ninety-minute immobilization stress significantly increased miR-325-3p expression in the anterior pituitary of rats pretreated with NRS IgG (1.42 ± 0.12-fold compared to non-stressed rats pretreated with NRS IgG) (Fig. 3), and pretreatment with anti-Ucn 2 IgG blocked immobilization stress-induced increase in miR-325-3p expression in the anterior pituitary (Fig. 3). There was a significant interaction between IgG injection and immobilization exposure ($F_{1, 16}$=5.01, p=0.040, n=5).

Overexpression and suppression of miR-325-3p affect intracellular contents and secretion of LH in vitro

miR-325-3p was overexpressed and suppressed by transfection with pBA-miR-325-3p and pDecoy-miR-325-3p, respectively (Fig. 4A). LH β-subunit mRNA expression was unaffected by miR-325-3p overexpression or knockdown (Fig. 4B). Overexpression of miR-325-3p significantly decreased intracellular LH (60.5 ± 4.7% of mock transfectants) and LH secretion (46.5 ± 11.4% of mock transfectants) (Fig. 4C). Ucn 2 significantly decreased intracellular LH contents (60.4 ± 4.7% of control) (Fig. 4C) and LH secretion (33.7 ± 8.9% of control) (Fig. 4E) in pBA-mock transfectants, while there was no significant difference in intracellular contents and secretion of LH between control and Ucn 2 treatment in pBA-miR-325-3p transfectants (Fig. 4C and E). There was a significant interaction between transfectants and treatment ($F_{1, 28}$=8.06, p=0.008, n=8) in intracellular LH content,

but not in LH secretion. Knockdown of miR-325-3p expression by pDecoy vector blocked Ucn 2-induced suppression of intracellular contents and secretion of LH (Fig. 4D and F). There was no significant interaction between transfectants and treatment in intracellular LH content and LH secretion.

LH β-subunit 3'-UTR activity assay

When intact LH β-subunit 3'-UTR-fused luciferase vector [pmir-Glo LHβ 3'UTR (WT)] was co-transfected with miR-325-3p, its luciferase activity was significantly lower than that of co-transfected with pBA-mock vector (75.9 ± 3.5% of mock transfectants) (Fig. 5B). When miR-325-3p binding site of LH β-subunit 3'-UTR was replaced with scramble mutation [pmir-Glo LHβ 3'UTR (SC)], there was no difference in luciferase activity between pBA-325-3p co-expression and pBA-mock co-expression (Fig. 5B).