Regulation and role of suppressor of cytokine signaling-3 in hypothalamic 4B cells

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Abstract

Corticotropin-releasing factor (CRF) plays a central role in regulating stress responses. In the hypothalamic paraventricular nucleus (PVN), CRF, produced in response to stress, stimulates the release of ACTH from the anterior pituitary. ACTH then stimulates the release of glucocorticoids from the adrenal glands; circulating glucocorticoids are critical for recovery from stress conditions. Cytokines are also implicated in the regulation of CRF expression. Among them, interleukin (IL)-6 plays a role in the regulation of CRF. Factors other than glucocorticoids are likely to be involved in limiting the stimulation of CRF during stress. Suppressor of cytokine signaling (SOCS)-3 acts as a potent negative regulator of cytokine signaling. Little is known about the ability of the inhibitory signaling pathways to limit activation of the CRF gene in parvocellular PVN neurons.

Hypothalamic 4B cells are useful for exploring the mechanisms, because these cells show characteristics of the parvocellular neurons of the PVN. In the present study, we examined whether SOCS-3 is regulated by IL-6 and cAMP in hypothalamic 4B cells. We also explored the involvement of SOCS-3 in the regulation of CRF gene expression. SOCS-3 was found to be regulated by IL-6 and via the cAMP/protein kinase A pathway in the hypothalamic cells. SOCS-3 knockdown increased IL-6- or forskolin-induced CRF gene transcription and mRNA levels. Therefore, SOCS-3, induced by a cAMP stimulant and IL-6, would be involved in the negative regulation of CRF gene expression in hypothalamic cells.

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Introduction

Corticotropin-releasing factor (CRF), a 41-amino acid polypeptide isolated originally from the ovine hypothalamus, plays a central role in regulating stress responses (Vale *et al.* 1981, Suda *et al.* 2004). CRF coordinates neuroendocrine, behavioral, autonomic, and immune responses, and controls the hypothalamic–pituitary–adrenal (HPA) axis during stressful periods. In the hypothalamic paraventricular nucleus (PVN) of the brain, CRF, produced in response to stress, stimulates the release of ACTH via CRF receptor type 1 (CRF₁ receptor) from the anterior pituitary (AP; Vale *et al.* 1981, Moriyama *et al.* 2005). ACTH then stimulates the release of glucocorticoids from the adrenal glands (Whitnall 1993). Circulating glucocorticoids are critical for recovery from stress conditions, because they inhibit hypothalamic PVN production of CRF and pituitary production of ACTH, thereby ensuring that serum levels of glucocorticoids are appropriate to the stress experienced (Whitnall 1993).

Stimulation of the protein kinase A (PKA) pathway increases expression of CRF in the hypothalamus (Suda *et al.* 1985). Other studies also indicate that cAMP-dependent

signaling activates CRF gene expression. A functional cAMP-response element (CRE) on the 5'-promoter region is important for increasing CRF gene expression (Seasholtz *et al.* 1988, Spengler *et al.* 1992). In addition, King *et al.* (2002) demonstrated a second response element by cAMP between bps –125 and –118, a caudal-type homeobox response element in the CRF promoter. Therefore, both the CRE and the caudal-type homeobox response element on the CRF promoter contribute to a cAMP-associated increase in the CRF gene (Cheng *et al.* 2000, King *et al.* 2002). In addition, pituitary adenylate cyclase-activating polypeptide (PACAP) induces cAMP, and stimulates the CRF gene via the cAMP/PKA signaling pathway (Agarwal *et al.* 2005).

Cytokines are also implicated in the regulation of CRF expression. Among them, interleukin (IL)-6 is an important mediator of the interaction between the neuroendocrine and immune systems, and plays a role in the regulation of CRF. For example, IL-6 increases CRF gene expression and secretion in the hypothalamus (Navarra *et al.* 1991, Vallieres & Rivest 1999). IL-6 stimulates the HPA axis (Venihaki *et al.* 2001), leading to an increase in glucocorticoid levels, which in turn suppresses IL-6 production (Karalis *et al.* 1997, Turnbull & Rivier 1999).

It has been suggested that factors other than glucocorticoids are also involved in limiting the stimulation of CRF during stress (Liu *et al.* 2006). Suppressor of cytokine signaling (SOCS)-3 acts as a potent negative regulator of cytokine signaling (Krebs & Hilton 2000), and is known to suppress cytokine-induced proopiomelanocortin (POMC) gene transcription and ACTH secretion in corticotrophs (Auernhammer *et al.* 1999). IL-6 stimulates the Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, while IL-6-induced SOCS-3 acts as a negative regulator and inhibits STAT phosphorylation by JAK at the receptor complex (Ram & Waxman 1999, Schmitz *et al.* 2000).

Little is known about the ability of the inhibitory signaling pathways to limit activation of the CRF gene in parvocellular PVN neurons. Hypothalamic 4B cells are useful for exploring the mechanisms, because these cells express CRF and vasopressin along with CRF₁ and glucocorticoid receptors, and show characteristics of the parvocellular neurons of the PVN. In 4B cells transfected with a CRF promoter-luciferase construct, forskolin increased the activity of the CRF promoter in parallel with an increase in intracellular cAMP (Nikodemova *et al.* 2003).

In the present study, we examined whether SOCS-3 is regulated by IL-6 and cAMP in hypothalamic 4B cells. We also explored the involvement of SOCS-3 in the regulation of CRF gene expression in hypothalamic 4B cells.

Materials and Methods

Materials

Forskolin was purchased from Calbiochem (San Diego, CA, USA). Rat IL-6 was purchased from PeproTech (London, UK). H89 was purchased from Seikagaku Corporation (Tokyo, Japan), and JAK Inhibitor I was purchased from Calbiochem.

Cell culture

4B cells were incubated in DMEM, supplemented with 10% fetal bovine serum (FBS), 100 mg/ml streptomycin, and 100 U/ml penicillin at 37 °C, in a humidified atmosphere of 5% CO₂ and 95% air. Cells were plated at 10⁴ cells/cm² for 4 days before each experiment and the medium was changed every 48 h. One day before each experiment, cells were washed and then serum-starved overnight in DMEM supplemented with 0.2% BSA.

RNA extraction

Cellular total RNA was extracted using the RNeasy Mini Kit (Qiagen), according to the manufacturer's protocol. cDNAs were then synthesized from total RNA (0.5 µg) using random hexamers as primers with the SuperScript

First Strand Synthesis System for reverse transcriptase-PCR (RT-PCR; Invitrogen Corp.), according to the manufacturer's instructions.

Real-time RT-PCR

The resulting cDNAs were subjected to real-time PCR as follows. The expression level of rat SOCS-3 mRNA was evaluated using quantitative real-time PCR based on specific sets of primers and probes (Assays-on-Demand Gene Expression Products, Applied Biosystems, Foster City, CA, USA). The expression level of rat CRF mRNA was evaluated using quantitative real-time PCR based on the following specific sets of primers and probes: CRF-F (5'-TGGATCTCACCTTCCACCTTCTG-3'); CRF-R (5'-CCGATAATCTCCATCAGTTTCCTG-3'); probe (5'-FAM-GCCAGGGCAGAGCAGTTAGC-TAMRA-3'). β 2-microglobulin (B2MG), which was not changed by any treatments, was used as a housekeeping gene to normalize values. Each reaction consisted of 1× TaqMan Universal PCR Master Mix (Applied Biosystems), 1× Assays-on-Demand Gene Expression Product (Rn00585674 s1 for rat SOCS-3; Rn00560865 m1 for rat B2MG) or a set of CRF primers and probes, and 2 µl cDNA in a total volume of 50 µl, using the following parameters with an ABI PRISM 7000 Sequence Detection System (Applied Biosystems): 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 1 min. Data were collected and recorded by ABI PRISM 7000 SDS Software (Applied Biosystems) and expressed as a function of the threshold cycle (C_t). Using diluted samples, the amplification efficacies for each gene of interest and the housekeeping gene amplimers were found to be identical.

Relative quantitative gene expression

Relative quantitative gene expression was calculated by the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen 2001). In brief, for each sample assayed, the C_t for the reactions amplifying the gene of interest (rat SOCS-3) and a housekeeping gene (rat B2MG) was determined. The C_t for the gene of interest of each sample was corrected by subtracting the C_t for the housekeeping gene (ΔC_t). Untreated controls were chosen as reference samples, and the ΔC_t for all experimental samples was reduced by the average ΔC_t for the control samples ($\Delta\Delta C_t$). Finally, the abundance of the experimental mRNA relative to that of the control mRNA was calculated with use of the formula $2^{-\Delta\Delta C_t}$.

Western-blot analysis

After each treatment, cells were washed twice with PBS and lysed with Laemmli sample buffer. Cell debris was pelleted by centrifugation, and the supernatant was recovered. Samples were boiled and subjected to electrophoresis on a gradient (4–20%) polyacrylamide gel. Proteins were transferred to a PVDF membrane (Daiichi Kagaku, Tokyo, Japan).

After blocking by Detector Block blocking buffer (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, MD, USA), the membrane was incubated for 1 h with a rabbit anti-CRE-binding protein (CREB) antibody (Cell Signaling Technology, Beverly, MA, USA), anti-phosphorylated (p)-CREB antibody (Cell Signaling Technology), anti-STAT3 antibody (Cell Signaling Technology), anti-p-STAT3 antibody (Cell Signaling Technology), anti-SOCS-3 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), or anti-actin antibody (Santa Cruz Biotechnology), washed with PBS containing 0.05% Tween 20, and incubated with HRP-labeled anti-rabbit IgG (Daiichi Kagaku). Detection was performed using a chemiluminescent substrate Super-signal WestPico (Pierce Chemical Co., Rockford, IL, USA), and the membrane was exposed to BioMax film (Eastman Kodak Co.), and followed by quantitative analysis using the NIH image software 1.61. The results are expressed as corrected arbitrary units.

Constructs and transfection

A 1077 bp restriction fragment containing the human CRF promoter (−907 to +170 relative to the proximal transcription start point) was obtained by PCR. The PCR products were then confirmed by sequencing. This DNA fragment was used to produce the CRF promoter-driven luciferase reporter construct, CRF−907luc, by a two-step cloning method. First, the DNA fragment was cloned into pGEM-T Easy vector (Promega Corp.), then digested with Kpn I and Hind III, and subcloned into Kpn I- and Hind III-cloning sites of the pA3-Luc plasmid. SOCS-3−2757luc containing the promoter region −2757 to +929 of the murine SOCS-3 gene fused to the luciferase-encoding sequence was provided by Dr S Melmed (Sinai Research Center, Los Angeles, CA, USA). SOCS-3−159luc and SOCS-3−107luc were generated by Dr C Ehrling (Heinrich-Heine University, Dusseldorf, Germany).

For the luciferase activity assay, cells were placed in 12-well (22-mm diameter) culture dishes at 60% confluency. The following day, the cells were transiently transfected using the FuGENE 6 Transfection Reagent Kit (Roche Diagnostics) in accordance with the manufacturer's instructions. A FuGENE to DNA ratio of 3 µl:1 µg was used. The total amount of DNA in each well was 0.5 µg. The culture medium was then replaced with DMEM supplemented with 10% FBS. Three days after transfection, the cells were washed and serum-starved overnight in DMEM supplemented with 0.2% BSA. Each experiment was then performed.

Luciferase activity

A luciferase assay was performed according to the manufacturer's protocol. At the end of each experiment, cells were washed twice with PBS without Ca^{2+} and Mg^{2+} , harvested with PicaGene lysis buffer (Toyo Inki, Tokyo, Japan), and centrifuged at 9660 g for 2 min. Twenty microliters of each supernatant was used for the luciferase assay.

The reactions were initiated by the injection of 100 µl of luciferin solution, PicaGene buffer. Light output was measured for 20 s at room temperature using a luminometer (Berthold Lumat LB9501, Postfach, Germany). The activity of β -galactosidase was used as an internal control.

RNA interference experiments

SOCS-3 and control siRNAs were designed and purchased from Ambion (Austin, TX, USA). The 4B cells were transfected with siRNA and siPORT NeoFX transfection reagent (Ambion) according to the manufacturer's protocol. The 4B cells, seeded into 12-well plates at a density of 2×10^4 cells/well, were incubated for 24 h in 1 ml of culture medium containing siRNA for either control (siControl) or SOCS-3 (siSOCS-3). For the luciferase assay, after transfection the cells were re-transfected with CRF-promoter constructs, and then incubated with forskolin or IL-6. For a measurement of CRF mRNA levels, the cells were incubated with 1 mM forskolin or IL-6 following transfection. The expression of CRF and B2MG mRNA was examined by quantitative RT-PCR.

Statistical analysis

Each value is expressed as mean $\pm$ S.E.M. Statistical analyses of the data were performed using either unpaired *t*-test or ANOVA, followed by the Bonferroni/Dunn *post hoc* test. The level of statistical significance was set at $P < 0.05$.

Results

Effects of IL-6 or forskolin on SOCS-3 levels in 4B cells

We examined time-dependent and dose-dependent changes in SOCS-3 mRNA levels by the stimulation of IL-6. The time-course study showed that IL-6 significantly increased SOCS-3 mRNA levels (Fig. 1A-1). The maximal effect of IL-6 (100 ng/ml) occurred 24 h after treatment, with an approximate fourfold increase in SOCS-3 mRNA levels. The dose-response study showed that IL-6 induced significant stimulatory effects at both 10 and 100 ng/ml (Fig. 1A-2). We also examined time-dependent and dose-dependent changes in SOCS-3 mRNA levels by the stimulation of forskolin. The time-course study showed that the most significant effect of forskolin (10 µM) was observed at 2 h, with approximately a twofold increase compared with the basal level (Fig. 1B-1). The dose-response study showed that forskolin induced a significant stimulatory effect at 10 µM in 4B cells (Fig. 1B-2).

Forskolin stimulates adenylate cyclase, and then intracellular cAMP production. We examined cAMP-dependent changes in SOCS-3 protein levels through stimulation by forskolin. The time-course study showed that the significant effect of forskolin (10 µM) was observed at 6 and 24 h, with an ~ 2.5 -fold increase compared with the basal level

Figure 1 Effects of IL-6 and forskolin on SOCS-3 levels in 4B cells. Control cells treated with medium alone are indicated as (C). Experiments were conducted in triplicate, and the means of three independent experiments are shown. Statistical analyses were performed using one-way ANOVA, followed by *post hoc* tests. * $P < 0.05$ (compared with control (C)). (A-1) Time-dependent changes in IL-6-induced SOCS-3 mRNA levels. Cells were incubated with medium containing 100 ng/ml IL-6 for the durations shown. (A-2) Dose-dependent changes in IL-6-induced SOCS-3 mRNA levels. Cells were incubated for 24 h with medium containing 0.1–100 ng/ml IL-6. (B-1) Time-dependent changes in forskolin-induced SOCS-3 mRNA levels. Cells were incubated with medium containing 10 µM forskolin (Fsk) for the durations shown. (B-2) Dose-dependent changes in forskolin-induced SOCS-3 mRNA levels. Cells were incubated for 2 h with medium containing 0.1–10 µM forskolin (Fsk). (C-1) Effects of forskolin or IL-6 on SOCS-3 protein in 4B cells. Cells were incubated with medium containing 10 µM forskolin (Fsk) or 100 ng/ml IL-6 for the durations shown. A representative image of western blot for SOCS-3 and actin proteins in 4B cells. (C-2) Effects of forskolin or IL-6 on SOCS-3 protein in 4B cells. Experiments were conducted in triplicate, and the means of three independent experiments are shown. Statistical analyses were performed using two-way ANOVA, followed by *post hoc* tests. * $P < 0.05$ (compared with each control).

(Fig. 1C-1 and C-2). We also examined a time-dependent change in SOCS-3 protein levels by stimulation with IL-6. The time-course study showed that IL-6 significantly increased SOCS-3 protein levels. The maximal effect of IL-6 (100 ng/ml) occurred 6 h after treatment, with an approximate 3.5-fold increase in SOCS-3 protein levels (Fig. 1C-1 and C-2).

Effects of JAK and PKA inhibitors on SOCS-3 mRNA levels

To determine whether a JAK pathway is involved in IL-6-induced SOCS-3 mRNA levels, 4B cells were incubated with a potent JAK inhibitor. SOCS-3 mRNA levels were not affected by treatment with 1 µM JAK Inhibitor I. However, the stimulatory effects of IL-6 on SOCS-3 mRNA levels were completely inhibited by treatment with 1 µM JAK Inhibitor I (Fig. 2A).

To determine whether the PKA pathway is involved in forskolin-induced SOCS-3 mRNA levels, 4B cells were incubated with 1 µM H89, a potent and selective PKA inhibitor ($IC_{50} = 48$ nM). SOCS-3 mRNA levels were not affected by treatment with 1 µM H89; however, the stimulatory effects of forskolin on SOCS-3 mRNA levels were completely inhibited by treatment with 1 µM H89 (Fig. 2B).

Effects of forskolin or IL-6 on CREB or STAT3 phosphorylation in 4B cells

We studied 4B cells to determine whether treatment with IL-6 had an effect on STAT3 phosphorylation. Incubation with 100 ng/ml of IL-6 increased STAT3 phosphorylation from between 5 min and 2 h after the addition (Fig. 3A).

Figure 2 Effects of JAK and PKA inhibitors on SOCS-3 mRNA levels. Control cells treated with medium alone are indicated as (C). Experiments were conducted in triplicate, and the means of three independent experiments are shown. Statistical analyses were performed using one-way ANOVA, followed by *post hoc* tests. * $P < 0.05$ (compared with control (C)). + $P < 0.05$ (compared with forskolin (Fsk)). (A) Effects of JAK inhibitor I on IL-6-stimulated SOCS-3 mRNA levels. Cells were pre-incubated for 30 min with medium containing 1 µM JAK inhibitor I (JAK I) or vehicle, and then incubated for 6 h with medium containing 100 ng/ml IL-6 or vehicle. (B) Effects of H89 on forskolin-stimulated SOCS-3 mRNA levels. Cells were pre-incubated for 30 min with medium containing 1 µM H89 or vehicle, and then incubated for 2 h with medium containing 10 µM forskolin (Fsk) or vehicle.

Figure 3 Effects of forskolin or IL-6 on CREB or STAT3 phosphorylation in 4B cells. Independent experiments were repeated three times, and a representative blot is shown. (A) Time-dependent changes in IL-6-induced STAT3 phosphorylation. Cells were incubated with medium containing 100 ng/ml IL-6 for the durations shown. (B) Time-dependent changes in forskolin-induced CREB phosphorylation. Cells were incubated with medium containing 10 μ M forskolin for the durations shown. (C) Effects of JAK inhibitor I on IL-6-stimulated STAT3 phosphorylation. Cells were pre-incubated for 30 min with medium containing 1 μ M JAK inhibitor I (JAK I) or vehicle, and then incubated for 15 min with medium containing 100 ng/ml IL-6 or vehicle. (D) Effects of H89 on forskolin-stimulated CREB phosphorylation. Cells were pre-incubated for 30 min with medium containing 1 μ M H89 or vehicle, and then incubated for 15 min with medium containing 10 μ M forskolin (Fsk) or vehicle.

We then examined 4B cells to determine whether treatment with forskolin affected CREB phosphorylation. Incubation with 10 μ M of forskolin increased CREB phosphorylation at 5 and 15 min after the addition (Fig. 3B).

To determine whether the JAK pathway was involved in IL-6-induced phosphorylation of STAT3, 4B cells were pre-incubated for 30 min with JAK I inhibitor, prior to the addition of IL-6. JAK I inhibitor inhibited an IL-6-mediated increase in STAT3 phosphorylation (Fig. 3C). To determine whether PKA was involved in

forskolin-induced phosphorylation of CREB, 4B cells were pre-incubated for 30 min with H89 prior to the addition of forskolin. H89 inhibited a forskolin-mediated increase in CREB phosphorylation (Fig. 3D).

Effects of IL-6 on SOCS-3 5'-promoter activity in 4B cells

We examined time-dependent changes in SOCS-3 5'-promoter activity. The time-course study showed an increasing effect of IL-6 in a time-dependent manner (Fig. 4). To determine whether a proximal part of the SOCS-3 5' promoter was involved in the regulation of the IL-6 activity, 4B cells transfected with a series of promoter deletion constructs, such as SOCS-3-2757luc, SOCS-3-159luc, and SOCS-3-107luc, were stimulated with IL-6. 4B cells transfected with SOCS-3-2757luc showed similar potent SOCS-3-promoter activity to the cells transfected with SOCS-3-159luc. Cells transfected with SOCS-3-107luc showed enhanced activity compared with the longer constructs.

Effects of SOCS-3 on the regulation of CRF gene in 4B cells

We then examined the functional role of SOCS-3 in 4B cells, since SOCS-3 might be involved in the regulation of CRF 5'-promoter activity. SOCS-3 mRNA levels were reduced by 48% in cells transfected with siRNA against the SOCS-3 gene. SOCS-3 knockdown did not have any significant effect on basal CRF 5'-promoter activity compared with the control (Fig. 5A). However, there was a significant increase in forskolin- or IL-6-induced CRF 5'-promoter activity (Fig. 5B and C).

Figure 4 Effects of IL-6 on SOCS-3 5'-promoter activity in 4B cells. Cells transfected with a full-length (SOCS-3-2757luc) or shorter (SOCS-3-159luc or SOCS-3-107luc) promoter constructs were incubated with medium containing 100 ng/ml IL-6 for the durations shown. Experiments were conducted in triplicate, and the means of three independent experiments are shown. Statistical analyses were performed using two-way ANOVA, followed by *post hoc* tests. * $P < 0.05$ (compared with SOCS-3-107luc).

Figure 5 Effects of SOCS-3 on the regulation of CRF gene in 4B cells. Experiments were conducted in triplicate, and the means of three independent experiments are shown. Statistical analyses were performed using unpaired *t*-test. **P* < 0.05 (compared with control (siControl)). The 4B cells, seeded into 12-well plates at a density of 2×10^4 cells/well, were incubated for 24 h in 1 ml of culture medium containing siRNA for either control (siControl) or SOCS (siSOCS). (A–C) Effects of SOCS-3 on the regulation of CRF 5'-promoter activity. After transfection, the cells were re-transfected with a CRF-promoter construct, and then incubated with vehicle (A), 10 μ M forskolin (Fsk) for 2 h (B), or 100 ng/ml IL-6 for 24 h (C). (D and E) Effects of SOCS-3 on the regulation of CRF mRNA levels. The cells were incubated with vehicle (D), 10 μ M forskolin (Fsk) for 2 h (E), or 100 ng/ml IL-6 for 24 h (F).

We next examined the expression levels of CRF mRNA by quantitative RT-PCR. SOCS-3 knockdown did not have any significant effect on basal CRF mRNA levels compared with the control (Fig. 5D). CRF mRNA levels were induced by incubation with forskolin or IL-6 (Fig. 5E and F). SOCS-3 knockdown significantly increased forskolin- or IL-6-induced CRF mRNA levels compared with the control (Fig. 5E and F).

Discussion

SOCS-3 is known to be a potent negative regulator of cytokine signaling (Krebs & Hilton 2000), and is induced by IL-6-type cytokines in corticotrophs (Chesnokova & Melmed 2002). In this study, we found that SOCS-3 was regulated by IL-6 in hypothalamic cells. Thus, it is suggested that SOCS-3 would be regulated by IL-6 both in the hypothalamus and in the pituitary corticotrophs. In fact, in the hypothalamic cells, IL-6-induced STAT3 tyrosine phosphorylation depended on the JAK pathway. IL-6-induced SOCS-3 mRNA levels were also regulated via the JAK pathway. Therefore, cytokine-induced expression of SOCS-3 molecules would be important for the negative

regulatory control of STAT-dependent cytokine signaling through the JAK/STAT cascade. STAT activation would be critical for the activation of SOCS-3 gene expression. Furthermore, promoter analysis in AtT-20 has shown that the proximal STAT3-response element and GC-rich element, –107 to –48 bp on the 5'-upstream region of the POMC promoter, are implicated for its transcription (Ehltling *et al.* 2005). In addition, our data suggest that some inhibitory elements might exist on the region between –159 and –107 bp of the promoter, because cells transfected with SOCS-3–107luc showed enhanced activity compared with the longer constructs.

We also found that SOCS-3 was regulated via the cAMP/PKA pathway in the hypothalamic cells. Generally, stimulation of G-protein-coupled receptors produces intracellular cAMP, which then activates the PKA pathway. In 4B cells, stimulation of the adenylate cyclase pathway by forskolin induces an accumulation of intracellular cAMP and activation of the PKA pathway (Kasckow *et al.* 2003, Nikodemova *et al.* 2003). Activation of this pathway leads to the binding of CREB to the CRE on the CRF promoter (Cheng *et al.* 2000). Bousquet *et al.* (2001) reported that activation of the cAMP/PKA pathway is involved in the regulation of SOCS-3 in AtT-20 cells. In our study, forskolin-induced CREB

phosphorylation depended on the PKA pathway in the hypothalamic cells. Forskolin-induced SOCS-3 mRNA levels were also regulated via the PKA pathway. Bousquet *et al.* (2001) reported that CREB transduces cAMP-mediated induction of c-fos and JunB, and that multiple cAMP-responsive DNA-binding elements or CRE/AP-1 sites would be present on the SOCS-3 promoter. c-fos and JunB bound to an AP-1 site were identified as potential mediators of cAMP-induced SOCS-3-promoter activity. Stimulation with forskolin was less effective at inducing both SOCS-3 mRNA and protein levels than with IL-6. We previously found that an interaction between cAMP-PKA and IL-6 pathways stimulates the CRF gene in 4B cells (Kageyama *et al.* 2007). However, such an interaction was not observed to stimulate SOCS-3, because IL-6- and forskolin-induced SOCS-3 mRNA levels were not blocked by a PKA inhibitor and a JAK inhibitor respectively (data not shown). Different signaling pathways might cause differential potencies between cAMP and IL-6.

In this study, SOCS-3 was regulated in the hypothalamic cells, suggesting that SOCS-3 would be involved in the negative regulation of CRF gene expression. We previously found that PACAP stimulates CRF-promoter activity via the PKA pathway in 4B cells (Kageyama *et al.* 2007). The previous study also showed that CRF-promoter activity declines within 6 h of treatment with a cAMP stimulant, PACAP (Kageyama *et al.* 2007), further suggesting this effect of SOCS-3. Our present study demonstrated that SOCS-3 knockdown increased forskolin-induced CRF gene transcription and mRNA levels. These results may suggest that cAMP-induced SOCS-3 would regulate the HPA axis by suppression of CRF gene expression in the hypothalamus. IL-6 also stimulated CRF-promoter activity and SOCS-3 protein in 4B cells (Kageyama *et al.* 2007). SOCS-3 knockdown increased IL-6-induced CRF gene transcription and mRNA levels. However, CRF-promoter activity still gradually increased 24 h after incubation with IL-6 (Kageyama *et al.* 2007). Therefore, the extent of involvement of IL-6-induced SOCS-3 in suppressing the increased levels of CRF gene expression in the hypothalamus needs to be established.

Hypothalamic 4B cells have been useful for exploring the stress responses in the PVN, because they show characteristics of the parvocellular neurons of the PVN. While the basal expression levels of CRF mRNA were weak in 4B cells, they were induced by forskolin and IL-6. We previously reported that the cAMP-dependent PKA pathway plays important roles in the activation of CRF neurons *in vivo* under physiological conditions (Itoi *et al.* 1996). IL-6 is required to prolong or maintain CRF expression during the later phases of inflammation *in vivo* (Venihaki *et al.* 2001). Therefore, the mechanisms regulating CRF gene expression would remain in 4B cells, reflecting the hypothalamus *in vivo*.

In conclusion, this study demonstrated that SOCS-3 was regulated by IL-6 and by the cAMP/PKA pathway in hypothalamic cells. SOCS-3 knockdown increased IL-6- or

forskolin-induced CRF gene transcription and mRNA levels. Therefore, SOCS-3, induced by a cAMP stimulant and IL-6, would be involved in the negative regulation of CRF gene expression in the hypothalamic cells.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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