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**Alpha and beta adrenoceptors activate interleukin-6 transcription through different pathways in cultured astrocytes from rat spinal cord**

Kohei Morimoto, Ryota Eguchi, Taisuke Kitano, and Ken-ichi Otsuguro

Laboratory of Pharmacology, Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan.

Corresponding author: K. Otsuguro

Laboratory of Pharmacology, Department of Basic Veterinary Sciences, Faculty of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan. Tel/Fax: +81-11-706-5220; E-mail: [otsuguro@vetmed.hokudai.ac.jp](mailto:otsuguro@vetmed.hokudai.ac.jp)

## Abstract

In brain astrocytes, noradrenaline (NA) has been shown to up-regulate IL-6 production via  $\beta$ -adrenoceptors (ARs). However, the underlying intracellular mechanisms for this regulation is not clear, and it remains unknown whether  $\alpha$ -ARs are involved. In this study, we investigated the AR-mediated regulation of IL-6 mRNA levels in the cultured astrocytes from rat spinal cord. NA, the  $\alpha_1$ -agonist phenylephrine, and the  $\beta$ -agonist isoproterenol increased IL-6 mRNA levels. The phenylephrine-induced IL-6 increase was accompanied by an increase in ERK phosphorylation, and these effects were blocked by inhibitors of PKC and ERK. The isoproterenol-induced IL-6 increase was accompanied by an increase in CREB phosphorylation, and these effects were blocked by a PKA inhibitor. Our results indicate that IL-6 increases by  $\alpha_1$ - and  $\beta$ -ARs are mediated via the PKC/ERK and cAMP/PKA/CREB pathways, respectively. Moreover, conditioned medium collected from astrocytes treated with the  $\alpha_2$ -AR agonist dexmedetomidine, increased IL-6 mRNA in other astrocytes. In this study, we elucidate that  $\alpha_1$ - and  $\alpha_2$ -ARs, in addition to  $\beta$ -ARs, promote IL-6 transcription through different pathways in spinal cord astrocytes.

## Keywords

astrocyte; noradrenaline; adrenoceptor; interleukin-6; MAPK

## 1. Introduction

Noradrenergic neurons project to most of regions of the central nervous system (CNS) [1]. Levels of noradrenaline (NA) in the CNS change under physiological and pathological conditions, such as ischemic stroke, neuropathic pain, and Alzheimer's disease [2–4]. Furthermore, inactivation of noradrenergic neurons enhances inflammation [5] and conversely, promotes recovery from ischemic brain injury [6], suggesting that NA is involved in the pathogenesis of the CNS diseases.

In the CNS, NA is released not only from synapses, but also from varicosities [7], and thus acts on astrocytes surrounding neurons. NA up-regulates the production of neurotrophins, growth factors, and proinflammatory cytokines such as interleukin-6 (IL-6) in astrocytes [8,9]. IL-6 has various roles besides enhancing inflammation, acts as a neurotrophic factor [10], and suppresses neuronal cell death [9]. Since the major source of IL-6 in the CNS is astrocytes [11], NA could affect physiological functions and disease pathogenesis by modulating IL-6 production via astrocytic adrenoceptors (ARs). There are two main groups of ARs, i.e.,  $\alpha$  and  $\beta$ , with 9 subtypes in total [12]; astrocytes express  $\alpha_1$ ,  $\alpha_2$  and  $\beta$ -ARs [13]. In cultured cerebral astrocytes, NA increases IL-6 production via activation of  $\beta$ -ARs [14]. However, the intracellular mechanisms that underlie NA-induced IL-6 production in astrocytes are not fully explained. In addition, it is not clear whether  $\alpha$ -ARs are involved in IL-6 production. It was reported that  $\alpha$ -ARs were not involved in

NA-activated IL-6 transcription in astrocytes [9], conversely, another study showed that NA-activated IL-6 production was inhibited by an  $\alpha$ -AR antagonist [15]. In both studies, only antagonists rather than both agonists and antagonists were used, and the expression of AR-subtypes was not confirmed. Astrocytic  $\alpha$ -ARs have functionally significant roles, such as regulation of neuronal activity and blood flow [16–18]. Since these functions may be related to astrocytic IL-6 production, it is important to elucidate the involvement of  $\alpha$ -ARs in IL-6 production.

In the spinal cord, the concentration of NA changes in response to ischemia, neuropathic pain, and aging [4,19,20]. *In vivo* administration of IL-6 promotes axonal sprouting and synapse formation, leading regeneration and functional recovery after spinal cord injury [21]. Furthermore, astrocytes show regional differences in receptor expression, transcriptional factor activity, and responses to inflammation [22–24]. Therefore, it is worth investigating the contribution of ARs to the regulation of IL-6 in spinal cord astrocytes.

In this study, we identified AR-subtypes that participate in the transcriptional regulation of IL-6 and investigated the intracellular signaling following the activation of each AR-subtypes in the cultured astrocytes from rat spinal cord.

## 2. Materials and methods

### 2.1. Materials

Antibodies against ERK1/2 (#4695S, 1:2500), phospho-ERK 1/2 (#9101S, 1:2500), p38 (#9212S, 1:2000), phospho-p38 (#9211S, 1:1000), SAPK/JNK (#9252S, 1:2500), phospho-SAPK/JNK (#9251S, 1:1500), STAT3 (#4904S, 1:4000), and phospho-STAT3 (#9145S, 1:2000) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody against CREB (#sc-377154, 1:500) and phospho-CREB (#sc-81486, 1:250) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A peroxidase-conjugated mouse anti-glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH; #G9295, 1:50000), dexmedetomidine hydrochloride, atipamezole hydrochloride, and isoproterenol hydrochloride were purchased from Sigma–Aldrich (St. Louis, MO, USA). Phenylephrine hydrochloride, propranolol hydrochloride, forskolin, U0126, and SP600125 were purchased from FUJIFILM Wako Pure Chemical (Osaka, Japan). L-noradrenaline bitartrate monohydrate and prazosin hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). H89 and bisindolylmaleimide II (BIM) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

## 2.2. Animals

All animal care and experimental protocols were approved by the Committee on Animal Experimentation, Graduate School of Veterinary Medicine, Hokkaido University (No. 19-0009), which has been awarded Accreditation Status by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. Wistar rats were obtained from CLEA Japan (Tokyo, Japan). Male and female pups aged 3–5 days were used for primary astrocyte cultures.

## 2.3. Primary cultures of spinal cord astrocytes

Primary cultures of spinal cord astrocytes were obtained as previously described [25]. In brief, spinal cords were isolated from rat pups, minced, and incubated with papain (10 U/ml) and DNase (0.1 mg/ml). Dissociated cells were suspended in Dulbecco's Modified Eagle's Medium/Ham's F-12 containing 10% fetal bovine serum, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. The cell suspension was seeded onto a poly-l-lysine-coated T75 flask. After 7–8 days, the flask was shaken at 250 rpm at 37°C for at least 12 h to remove all cells except for astrocytes. Adherent cells were detached with trypsin and re-seeded onto poly-l-lysine-coated 6- and 12-well plates at a density of  $8.0 \times 10^3$  cells/cm<sup>2</sup>. After 3 days, the cell culture had reached confluence and the medium was changed to serum-free medium. Cell cultures were treated with NA or AR-agonists 1 h after the medium

exchange, and were treated with AR-antagonists or inhibitors immediately after the exchange. After the given time (detailed in the figure legends and results section), the cell culture was used for experiments.

#### **2.4. RNA extraction and real-time PCR analyses**

Total RNAs were extracted from cultured astrocytes using RNAiso Plus (Takara Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka, Japan). Real-time PCR was performed using Thunderbird SYBR qPCR Mix (TOYOBO), each primer, and the cDNA reaction solution. The primers used were as follows: 5'- GAT TGT ATG AAC AGC GAT GAT GC-3' (forward) and 5'- AGA AAC GGA ACT CCA GAA GAC C - 3' (reverse) for IL-6; 5'- GCA AGA GAG AGG CCC TCA G -3' (forward) and 5'- TGT GAG GGA GAT GCT CAG TG -3' (reverse) for GAPDH. Thermal cycles were performed using Eco Real Time PCR System (Illumina, CA, USA). Cycling conditions were 95°C for 1 min (for initial denaturation), followed by 40 cycles of denaturation (95°C, 15 s), annealing and extension (61°C, 45 s). RNAs without reverse transcription were used as a negative control to examine DNA contamination and these were not amplified by real-time PCR. Melt curve analysis confirmed that the obtained amplicon was only the one expected in each reaction. The expression levels of the IL-6 relative to GAPDH were calculated by the  $\Delta\Delta C_q$  method



and were expressed as relative to the control, which was arbitrarily set to a value of “1.0”.

## **2.5. Western blotting**

Astrocytes were lysed in RIPA buffer containing a protease inhibitor cocktail (nacalai tesque, Kyoto, Japan). The samples were separated by 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, CA, USA). The membranes were blocked with 5% skimmed milk and then incubated with a primary antibody at 4°C for at least 12 h. Thereafter, the membranes were incubated for 1 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont, UK). Antibody binding was visualized by ECL Prime (GE Healthcare). Band intensities were measured using ImageJ software (National Institutes of Health) and expressed as relative to the control, which was arbitrarily set to a value of “1.0”.

## **2.6. Preparation and treatments of astrocyte conditioned medium**

Agonists or antagonists were treated to the primary cultures of astrocytes in the manner described above. Following agonist treatments for 30 min, cells were refreshed with serum-free medium. After 24 h incubation, the medium was collected from each treated well. RNA extraction was performed at the same time as the medium was collected. The medium was centrifuged at  $300 \times g$  to remove dead cells and the supernatant was

used as astrocyte conditioned medium (ACM). The drug-treated astrocytes to produce ACM were defined as ACM-donor cells. The ACM was transferred to another primary culture of astrocytes, which was defined as ACM-recipient cells. The ACM-recipient cells were incubated for 3 h followed by RNA extraction.

## 2.7. Data analysis

Data are expressed as means  $\pm$  S.E.M (n = number of independent measurements). Statistical comparisons between two groups were made using the unpaired Student's t-test. For multiple comparisons, one-way ANOVA followed by the Dunnett's test was used. A value of  $p < 0.05$  was considered as a statistically significant level. All statistical analysis was performed with Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd., Tokyo, Japan).

### 3. Results

#### 3.1. Effects of AR-agonists and antagonists on IL-6 mRNA levels

We have previously confirmed the expression of  $\alpha_1$ -,  $\alpha_2$ - and  $\beta$ -ARs by RT-PCR analysis in the cultured astrocytes from rat spinal cord [26]. Treatment of astrocytes with NA (1 or 10  $\mu$ M) increased the IL-6 mRNA level (Fig. 1A), which reached a peak 1 h after treatment and remained significantly higher than the untreated control for up to 6 h. The treatment of either NA or the  $\beta$ -agonist isoproterenol for 3 h increased IL-6 mRNA level in a concentration-dependent manner (Fig. 1B). The maximum increase in the IL-6 mRNA level was higher in response to NA than that it was to isoproterenol.

In addition to isoproterenol (1  $\mu$ M), the IL-6 mRNA level was increased by the  $\alpha_1$ -agonist phenylephrine (1  $\mu$ M) but not by the  $\alpha_2$ -agonist dexmedetomidine (1  $\mu$ M) (Fig. 1C). The NA-induced increase in the IL-6 mRNA level was inhibited by the  $\alpha_1$ -antagonist prazosin (1  $\mu$ M), the  $\alpha_2$ -antagonist atipamezole (10  $\mu$ M), and the  $\beta$ -antagonist propranolol (10  $\mu$ M) (Fig. 1D). Co-treatment with prazosin and propranolol abolished the NA-induced increase in the IL-6 mRNA level. Additional treatment with atipamezole did not cause further inhibition. None of the antagonists alone had any effect on IL-6 mRNA levels (Fig. 1E).

### 3.2. Mechanisms of increase in IL-6 mRNA via $\beta$ -ARs

In general,  $\beta$ -ARs coupled to Gs proteins activate the cAMP/protein kinase A (PKA) pathway. The isoproterenol-induced increase in the IL-6 mRNA level was inhibited by the PKA inhibitor H89 (5  $\mu$ M), but not by the protein kinase C (PKC) inhibitor BIM (5  $\mu$ M) (Fig. 2A). The adenylyl cyclase activator forskolin (10  $\mu$ M) also increased the IL-6 mRNA level (Fig. 2B). PKA activates cAMP response element binding protein (CREB), which regulates the expression of a variety of genes [27]. Isoproterenol increased CREB phosphorylation 30 min after treatment (Fig. 2C). In the presence of propranolol or H89, isoproterenol did not increase CREB phosphorylation. Forskolin also increased CREB phosphorylation. These results suggest that the cAMP/PKA/CREB pathway is involved in the increase in IL-6 mRNA level following  $\beta$ -AR activation.

### 3.3. Mechanisms of increase in IL-6 mRNA via $\alpha_1$ -ARs

$\alpha_1$ -ARs coupled to Gq proteins activate the  $\text{Ca}^{2+}$ /PKC pathway, which increases mitogen-activated protein kinases (MAPKs) phosphorylation [28]. Therefore, we investigated whether PKC and/or MAPKs participates in the transcriptional activation of IL-6 following  $\alpha_1$ -AR activation. The phenylephrine-induced increase in the IL-6 mRNA level was inhibited by BIM, but not by H89 (Fig. 3A). Phenylephrine did not affect CREB phosphorylation (Fig. 3B). The MAPK-ERK (extracellular signal-regulated kinase) kinase

(MEK)/ERK inhibitor U0126 (10  $\mu$ M) abolished the phenylephrine-induced increase in the IL-6 mRNA level (Fig. 3C). Phenylephrine increased ERK phosphorylation (Fig. 3D). In the presence of prazosin, BIM, or U0126, phenylephrine did not increase ERK phosphorylation. On the other hand, phenylephrine did not affect c-jun N-terminal kinase (JNK) or p38 phosphorylation (Fig. 3E, F). These results suggest that the PKC/ERK pathway is involved in the increase in IL-6 mRNA level following  $\alpha_1$ -AR activation.

#### **3.4. Effects of MAPKs or PKA inhibitor on NA-induced transcriptional activation of IL-6**

NA increased ERK and JNK phosphorylation but not p38, and these increases were inhibited by prazosin (Fig. 3D, E, F). Therefore, we investigated whether ERK or JNK phosphorylation affects the NA-induced increase in the IL-6 mRNA levels. U0126 and the JNK inhibitor SP600125 (10  $\mu$ M) suppressed the NA-induced ERK and JNK phosphorylation (Fig. 4A, B). Furthermore, U0126 and SP600125 abolished the NA-induced IL-6 increase (Fig. 4C). SP600125 alone did not have any effect on IL-6 mRNA level (Fig. 4D). Moreover, H89 abolished the NA-induced IL-6 increase (Fig. 4E). Since IL-6 transcription is activated by STAT3 phosphorylation [29], we investigated the effect of AR-agonists on it. NA, phenylephrine, and isoproterenol had no effect on STAT3 phosphorylation (Fig. 4F). These results suggest that both cAMP/PKA and ERK and/or JNK pathways are involved in the NA-induced increase in the IL-6 mRNA level.

217

218 **3.5. Effects of AR-agonists on IL-6 mRNA levels via astrocyte conditioned medium.**

219           We tested the long-term or indirect effects on IL-6 transcription following AR-  
220 activation. The treatment of NA or AR-agonists for 24 h did not have any effect on IL-6  
221 mRNA levels (Fig. 5A). Then, we examined whether some factors released by AR-  
222 activation are involved in IL-6 transcription. The 3 h treatment of astrocyte conditioned  
223 medium (ACM), derived from the ACM-donor cells treated with NA or dexmedetomidine  
224 but not phenylephrine and isoproterenol, increased IL-6 mRNA levels in the ACM-  
225 recipient cells (Fig. 5B, C). The effect of dexmedetomidine was abolished by the treatment  
226 of atipamezole for the ACM-donor cells (Fig 5D). However, there were no changes in IL-6  
227 mRNA levels in the ACM-donor cells (Fig. 5E). These results suggest that  $\alpha_2$ -ARs increase  
228 IL-6 mRNA level via some factors released into the extracellular medium.

229

#### 4. Discussion

In this study, we elucidate that  $\alpha_1$ - and  $\alpha_2$ -ARs, in addition to  $\beta$ -ARs, are involved in the transcriptional activation of IL-6 in astrocytes. Moreover, we showed that AR-mediated activation of IL-6 transcription involves CREB and MAPKs. Astrocytic ARs are likely to play a role in physiological and/or pathophysiological functions in the CNS by regulating the production of IL-6.

NA activated the transcription of IL-6 in spinal cord astrocytes. In this study, NA concentration-dependently increased IL-6 transcription at concentrations of 10 nM to 1  $\mu$ M. The physiological concentration of NA in cerebrospinal fluid is 1 nM to 100 nM [2,30]. Therefore, physiological concentrations of NA are sufficient to induce IL-6 production by astrocytes. In addition, since NA levels increase in ischemia and neuropathic pain [2,4] and decrease in Alzheimer's disease [3], IL-6 production by NA in astrocytes is likely to change under pathological conditions.

The maximum effect of NA on IL-6 mRNA transcription was higher than that of isoproterenol. NA has a higher affinity for  $\alpha$ -ARs than  $\beta$ -ARs [31]. The effect of NA on IL-6 transcription was suppressed by both  $\alpha_1$ - and  $\beta$ -antagonists, and the  $\alpha_1$ -agonist phenylephrine also activated the IL-6 transcription. These results indicate that  $\alpha_1$ -ARs are involved in the transcriptional activation of IL-6 in spinal cord astrocytes. On the other hand, in cultured cerebral astrocytes, NA activates IL-6 transcription, which is inhibited

249 by antagonists for  $\beta$ -ARs, but not for  $\alpha$ -ARs [9]. Moreover,  $\alpha_1$ -AR activation stimulates  
250 ERK phosphorylation in cultured spinal cord astrocytes [32], but not in cerebral astrocytes  
251 [33]. Astrocytes show regional differences in receptor expression, transcriptional factor  
252 activity, and responses to inflammation [22–24]. Therefore, these astrocyte heterogeneities  
253 may be involved in the discrepancy in the involvement of  $\alpha_1$ -ARs between spinal and  
254 cerebral astrocytes.

255         The  $\alpha_2$ -antagonist atipamezole suppressed the effect of NA on IL-6, whereas the  
256 treatment of the  $\alpha_2$ -agonist dexmedetomidine for 3 h showed no effect. Furthermore, the  
257 effect of NA on IL-6 transcription was completely suppressed by co-treatment with  $\alpha_1$ - and  
258  $\beta$ -AR antagonists, indicating that NA activates IL-6 transcription mainly via  $\alpha_1$ - and  $\beta$ -  
259 ARs. One of the possible explanations for this discrepancy between the agonist's and  
260 antagonist's effects is that the mechanisms involved in the activation of one receptor  
261 subtype by a specific agonist are different from the mechanisms involved in the activation  
262 of multiple receptor subtypes by NA. For example,  $\beta_1$ - and  $\alpha_{2A}$ , or  $\alpha_{1A}$ - and  $\alpha_{1B}$ -ARs form  
263 receptor-heterodimers [34,35]. When only one receptor from the heterodimer is activated,  
264 the intracellular signaling of the heterodimer is different to the signaling seen when both  
265 receptors in the heterodimer are activated [36,37]. This could provide an explanation for  
266 why NA-increased JNK phosphorylation was suppressed by prazosin, while exerting no  
267 effect of phenylephrine on the JNK phosphorylation. Further investigations are needed to



268 reveal these mechanisms.

269 Transcription of IL-6 is promoted by binding of transcription factors, such as AP1,  
270 CREB, and NF- $\kappa$ B, to specific DNA sequences and p300/CBP [38,39]. In addition,  
271 activation of MAPKs signaling can lead to AP1 activation [40]. In this study,  
272 phenylephrine activated ERK and IL-6 transcription, both of which were suppressed by  
273 the PKC and ERK inhibitor. Phenylephrine also did not affect CREB, JNK, and p38  
274 phosphorylation. These results indicate that  $\alpha_1$ -ARs activate IL-6 transcription via the  
275 PKC/ERK pathway in spinal cord astrocytes. Meanwhile, isoproterenol activated CREB  
276 and IL-6 transcription, both of which were suppressed by the PKA inhibitor. The adenylate  
277 cyclase activator forskolin mimicked the action of isoproterenol. These results indicate  
278 that  $\beta$ -ARs activate IL-6 transcription via the cAMP/PKA/CREB pathway in spinal cord  
279 astrocytes. As in this study, the effects of  $\beta$ -ARs on IL-6 secretion or transcription are  
280 mediated via the cAMP/PKA pathway in cerebral astrocytes [9,14]. Moreover, the NA-  
281 activated transcription of IL-6 was inhibited by both the ERK inhibitor and the JNK  
282 inhibitor. The phosphorylation of ERK or JNK activates common factors, such as c-jun and  
283 Elk [41], which may be also involved in IL-6 transcription. NA-activated transcription of  
284 IL-6 was also inhibited by the PKA inhibitor. Therefore, the pathway of NA-activated IL-  
285 6 transcription is likely to include the pathways activated by phenylephrine and  
286 isoproterenol. In addition to these pathways, STAT3 phosphorylation activates IL-6

transcription in astrocytes [29], but NA, phenylephrine, and isoproterenol had no effect on STAT3 phosphorylation in this study. Therefore, the STAT3 pathway is likely not involved in AR-induced IL-6 transcriptional activation in astrocytes. Furthermore, in contrast to this study, it has been shown that isoproterenol suppresses lipopolysaccharide (LPS)-induced IL-6 promoter activities via  $\beta_2$ -ARs in astrocytes [42]. LPS-induced IL-6 transcriptional activation is mediated via the TLR4-NF- $\kappa$ B pathway [43,44], and the activation of  $\beta$ -ARs attenuates NF- $\kappa$ B activity by increasing I $\kappa$ B $\alpha$  gene expression and protein levels [45]. In the presence of LPS,  $\beta$ -ARs may suppress IL-6 transcription by potent suppressing NF- $\kappa$ B. Therefore, it is likely that the activation of  $\beta$ -ARs shows bidirectional effects on IL-6 transcription via the CREB and NF- $\kappa$ B pathways.

ACM derived from NA- or dexmedetomidine-treated astrocytes increased IL-6 mRNA levels in the ACM-recipient cells. It is possible that factor(s), released into the extracellular medium by activation of  $\alpha_2$ -ARs, may promote IL-6 transcription in neighboring and distant astrocytes. Activation of astrocytic  $\alpha_2$ -ARs increases accumulation of glutamine, as a precursor of neurotoxic glutamate [46], and activation of metabotropic glutamate receptors enhances the release or transcription of IL-6 in astrocytes [47,48]. In addition, the effect of released factor(s) may disappear within a few hours, because there were no changes in IL-6 mRNA levels in the ACM-donor cells. Further studies are needed to elucidate the indirect pathway of IL-6 transcriptional

activation via the  $\alpha_2$ -ARs.

Intraperitoneal injection of lipopolysaccharide to mice increases IL-6 mRNA levels several-fold in brain astrocytes *in vivo* [49]. The IL-6 increases in responses to NA or AR-agonists in this study were similar in magnitude to these *in vivo* studies. Besides immune responses in the CNS, IL-6 acts as a neurotrophic factor [10], induces the differentiation of neural stem cells [50], and promotes vasculogenesis during the brain development [51]. Therefore, under physiological conditions, NA may play a beneficial role in the CNS functions by promoting IL-6 release from astrocytes. Conversely, treatment of spinal cord-injured rats with anti-IL-6 neutralizing antibodies reduces the area of injury [52]. Under pathological conditions, NA may act on astrocytes to promote IL-6 production and delay recovery. Further studies are needed to reveal whether astrocytic ARs regulate IL-6 production *in vivo* and how astrocytic IL-6 acts under physiological and pathological conditions.

## 5. Conclusion

In this study, we elucidated that NA promotes the transcriptional activation of IL-6 in spinal cord astrocytes, and  $\alpha_1$ - and  $\alpha_2$ -ARs, in addition to  $\beta$ -ARs, are involved. IL-6 increase by  $\alpha_1$ - and  $\beta$ -ARs are mediated via the PKC/ERK and cAMP/PKA/CREB pathways, respectively. Moreover, astrocyte conditioned medium collected from cells treated with the  $\alpha_2$ -AR agonist dexmedetomidine, increased IL-6 mRNA in other astrocytes. Based on these

326 results, we suggest a role for NA in the CNS homeostasis and pathogenesis via modulation

327 of cytokine production by astrocytic ARs.

328

**CRedit authorship contribution statement**

**Kohei Morimoto:** Conceptualization, Data curation, Formal analysis, Investigation, Visualization, Writing – original draft. **Ryota Eguchi:** Funding acquisition, Writing - review & editing. **Taisuke Kitano:** Investigation, Writing - review & editing. **Ken-ich Otsuguro:** Conceptualization, Supervision, Funding acquisition, Writing - review & editing.

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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534

## Figure legends

### Figure 1. Effects of NA, AR-agonists, and AR-antagonists on IL-6 mRNA levels in cultured astrocytes

(A) IL-6 mRNA levels in astrocytes treated with NA (1 and 10  $\mu$ M) for 1, 3, 6, and 12 h. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. time-matched control (Dunnett's test),  $n$  = 6. (B) IL-6 mRNA levels in astrocytes treated with NA or the  $\beta$ -agonist isoproterenol (ISO) (1 nM-10  $\mu$ M) for 3 h. \* $p$  < 0.05, \*\* $p$  < 0.01 vs. control (Dunnett's test),  $n$  = 6. (C) IL-6 mRNA levels in astrocytes treated with the  $\alpha_1$ -agonist phenylephrine (PHE, 1  $\mu$ M), the  $\alpha_2$ -agonist dexmedetomidine (DEX, 1  $\mu$ M), and ISO (1  $\mu$ M) for 3 h. \*\* $p$  < 0.01 vs. control (Dunnett's test),  $n$  = 6. (D) IL-6 mRNA levels in astrocytes treated with NA (1  $\mu$ M) in the presence or absence of the  $\alpha_1$ -antagonist prazosin (PRAZ, 1  $\mu$ M), the  $\alpha_2$ -antagonist atipamezole (ATIP, 10  $\mu$ M), and the  $\beta$ -antagonist propranolol (PROP, 10  $\mu$ M) for 3 h. ## $p$  < 0.01 vs. NA alone (Dunnett's test),  $n$  = 6. (E) IL-6 mRNA levels in astrocytes treated with PRAZ, ATIP, and PROP for 4 h.  $n$  = 6. Data are presented as means  $\pm$  S.E.M. The mRNA levels of cytokines were expressed as relative to the control, which was arbitrarily set to a value of "1.0".

### Figure 2. Intracellular mechanisms of transcriptional activation of IL-6 via $\beta$ -ARs

(A, B) IL-6 mRNA levels in astrocytes treated with the  $\beta$ -agonist isoproterenol (1  $\mu$ M), the



554 adenylyl cyclase activator forskolin (FSK, 10  $\mu$ M) in the presence or absence of the PKA  
555 inhibitor H89 (5  $\mu$ M) and the PKC inhibitor BIM (5  $\mu$ M) for 3 h. #p < 0.05 (Dunnett's test),  
556 \*\*p < 0.01 (unpaired Student's t-test), n.s.: not significant, n = 6. (C) The protein expression  
557 levels of phosphorylated and total CREB were quantified and representative blots are  
558 shown. GAPDH was used as a loading control. Astrocytes were treated with isoproterenol  
559 and FSK in the presence or absence of the  $\beta$ -antagonist propranolol (PROP, 10  $\mu$ M) and  
560 H89 for 30 min. \*\*p < 0.01 vs. control (Dunnett's test), n = 6. Data are presented as means  
561  $\pm$  S.E.M.

562

563 **Figure 3. Intracellular mechanisms of transcriptional activation of IL-6 via  $\alpha_1$ -ARs**

564 (A, C) IL-6 mRNA levels in astrocytes treated with the  $\alpha_1$ -agonist phenylephrine (1  $\mu$ M) in  
565 the presence or absence of the PKC inhibitor BIM (5  $\mu$ M), the PKA inhibitor H89 (5  $\mu$ M),  
566 and the MEK/ERK inhibitor U0126 (10  $\mu$ M) for 3 h. ##p < 0.01 (Dunnett's test), \*\*p < 0.01  
567 (unpaired Student's t-test), n.s.: not significant, n = 6. (B, D-F) The protein expression  
568 levels of phosphorylated and total CREB (B), ERK (D), JNK (E), and p38 (F) were  
569 quantified and representative blots are shown. GAPDH was used as a loading control.  
570 Astrocytes were treated with phenylephrine (PHE), or NA (1  $\mu$ M) in the presence or  
571 absence of the  $\alpha_1$ -antagonist prazosin (PRAZ, 1  $\mu$ M), BIM and U0126 for 30 min (B) or 10  
572 min (D-F). \*p < 0.05, \*\*p < 0.01 vs. control (Dunnett's test), n = 6. Data are presented as

573 means  $\pm$  S.E.M.

574

575 **Figure 4. Effects of ERK, JNK, and PKA inhibitor on NA-induced transcriptional**  
576 **activation of IL-6.**

577 (A, B, F) The protein expression levels of phosphorylated and total ERK (A), JNK (B), and  
578 STAT3 (F) were quantified and representative blots are shown. GAPDH was used as a  
579 loading control. Astrocytes were treated with NA (1  $\mu$ M), the  $\alpha_1$ -agonist phenylephrine  
580 (PHE, 1  $\mu$ M), and the  $\beta$ -agonist isoproterenol (ISO, 1  $\mu$ M) in the presence or absence of the  
581 MEK/ERK inhibitor U0126 (10  $\mu$ M) and the JNK inhibitor SP600125 (SP, 10  $\mu$ M) for 10  
582 min (A, B) or 30 min (F). # $p$  < 0.05, ## $p$  < 0.01 vs. NA alone (Dunnett's test),  $n$  = 6. (C, E)  
583 IL-6 mRNA levels in astrocytes treated with NA in the presence or absence of U0126, SP  
584 and the PKA inhibitor H89 (5  $\mu$ M) for 3 h. ## $p$  < 0.01 vs. NA alone (Dunnett's test),  $n$  = 6.  
585 (D) IL-6 mRNA levels in astrocytes treated with SP600125 for 4 h.  $n$  = 6. Data are  
586 presented as means  $\pm$  S.E.M.

587

588 **Figure 5. Effects of AR-agonists on IL-6 mRNA levels via astrocyte conditioned medium.**

589 (A) IL-6 mRNA levels in astrocytes treated with NA (1  $\mu$ M), the  $\alpha_1$ -agonist phenylephrine  
590 (PHE, 1  $\mu$ M), the  $\alpha_2$ -agonist dexmedetomidine (DEX, 1 $\mu$ M), and the  $\beta$ -agonist isoproterenol  
591 (1  $\mu$ M) for 24 h,  $n$  = 6. (B) Schematic depiction of astrocyte conditioned medium (ACM)

592 transfer experiment. (C, D) IL-6 mRNA levels in astrocytes incubated with ACM derived  
593 from astrocytes treated with NA, PHE, DEX and ISO in the presence or absence of  $\alpha_2$ -  
594 antagonist atipamezole (ATIP, 1  $\mu$ M) for 3 h. \* $p < 0.05$ , \*\* $p < 0.01$  vs. control (C, Dunnett's  
595 test), # $p < 0.05$ , ## $p < 0.01$  vs. DEX alone (D, Dunnett's test),  $n = 6$ . (E) IL-6 mRNA levels  
596 in astrocytes treated with DEX in the presence or absence of ATIP for 30 min and  
597 incubated for 24 h following medium exchange.  $n = 6$ . Data are presented as means  $\pm$   
598 S.E.M.

Astrocyte

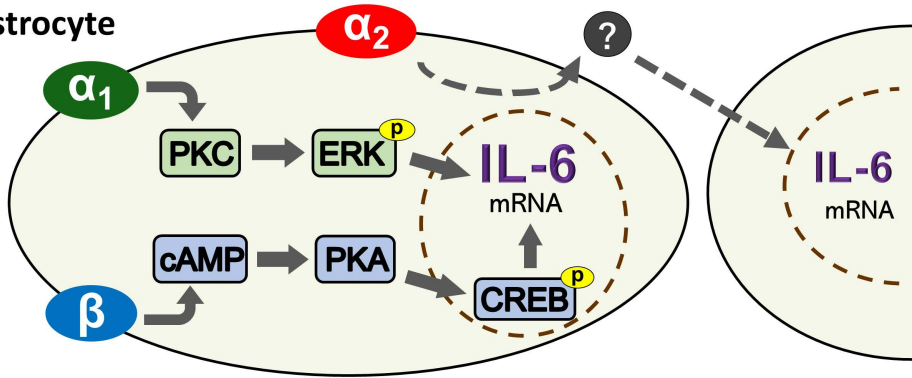
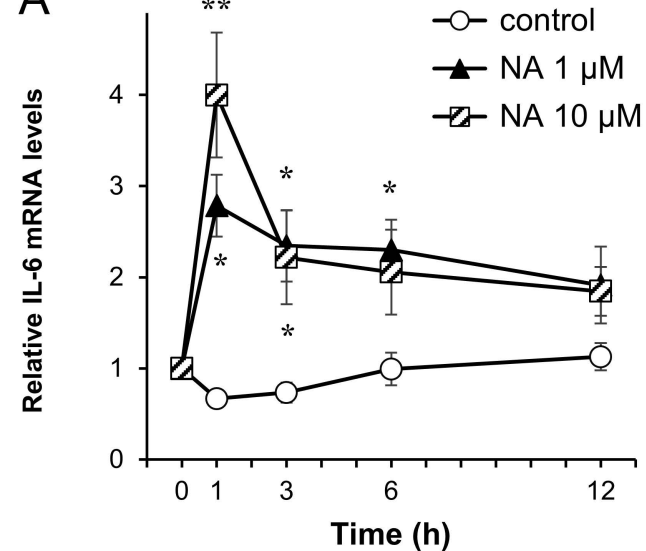
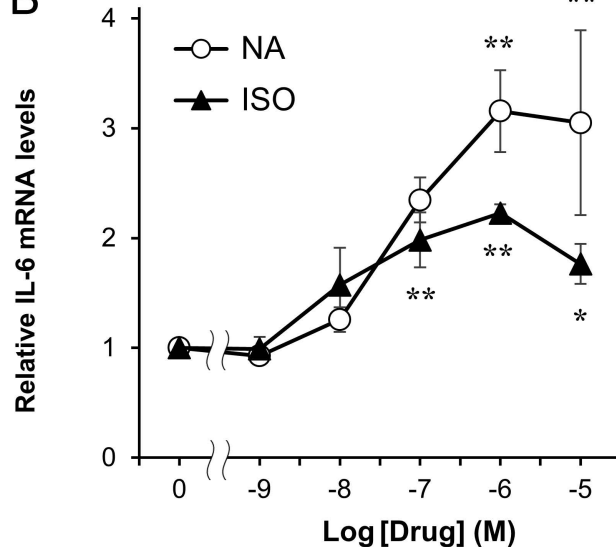


Fig.1

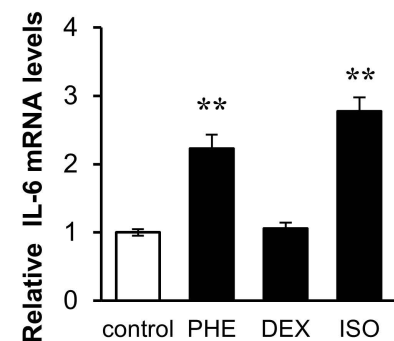
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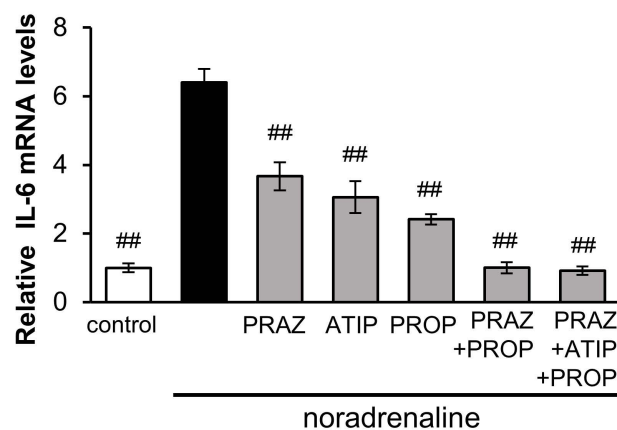
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D



E

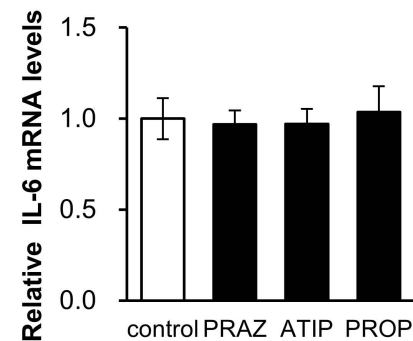
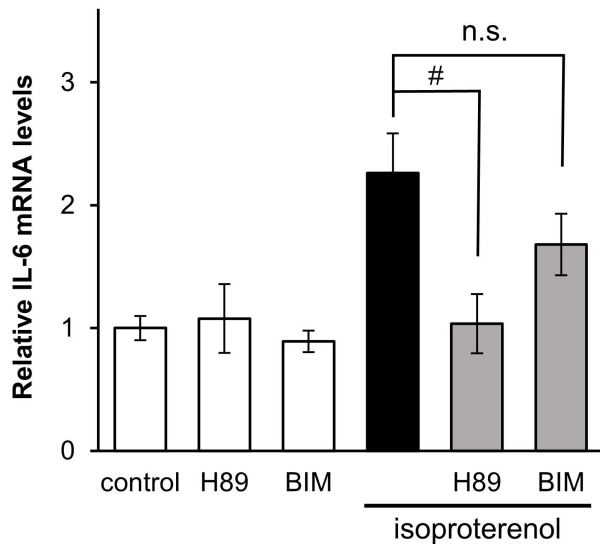
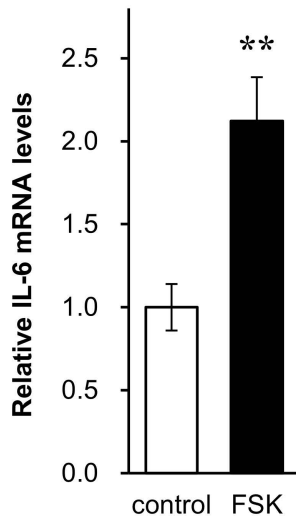


Fig. 2

A



B



C

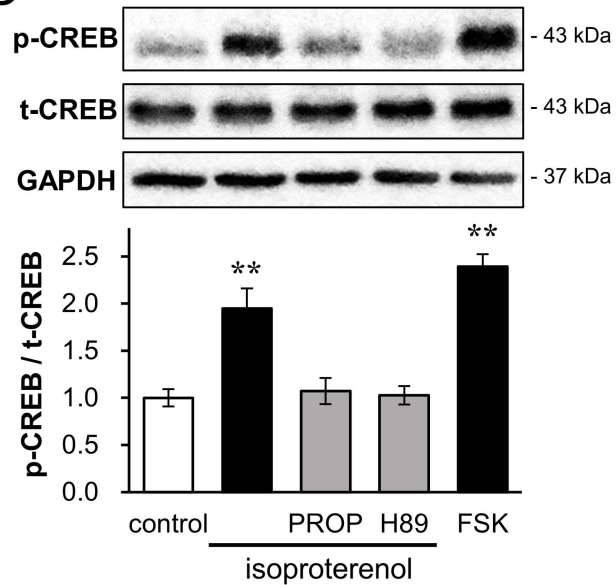
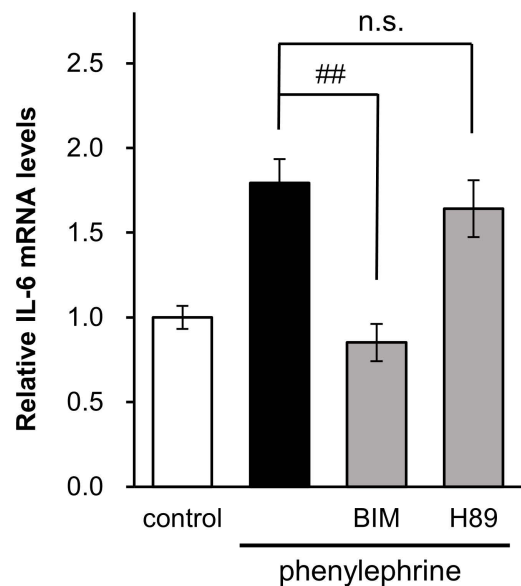
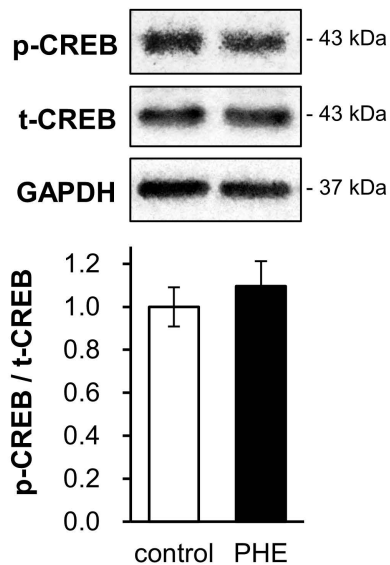


Fig. 3

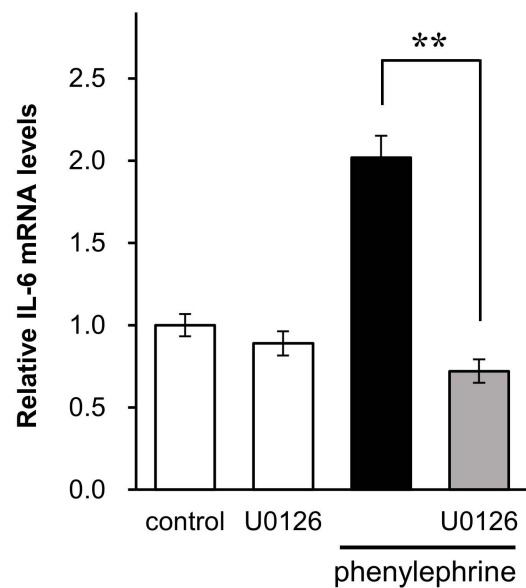
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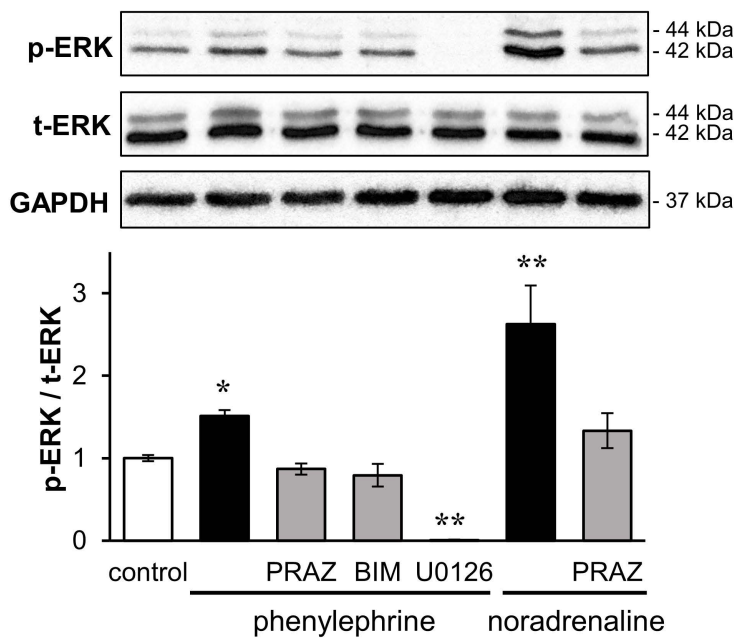
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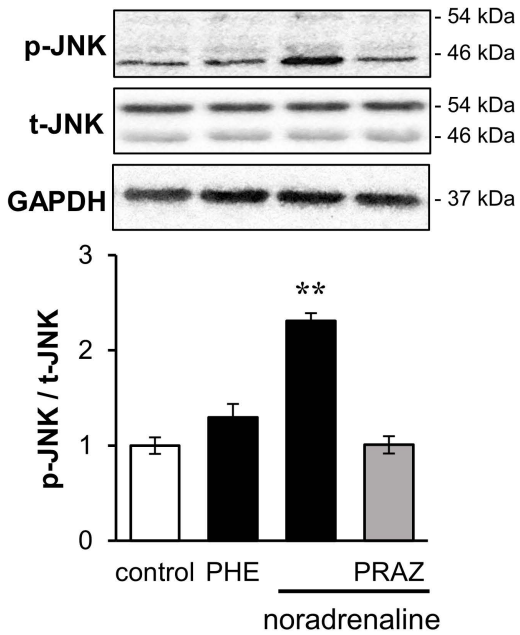
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E



F

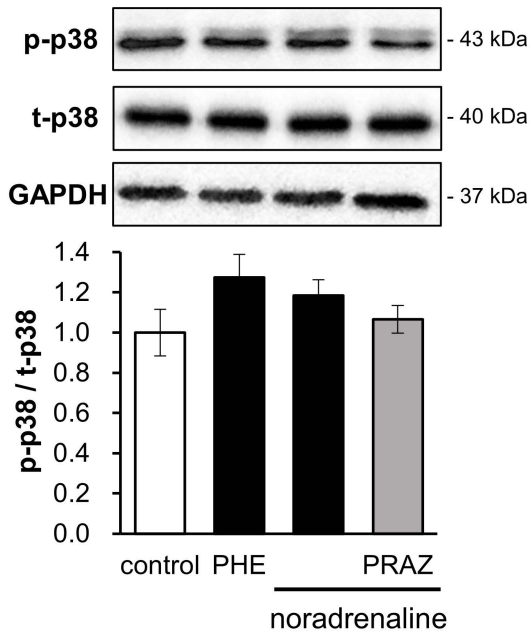


Fig. 4

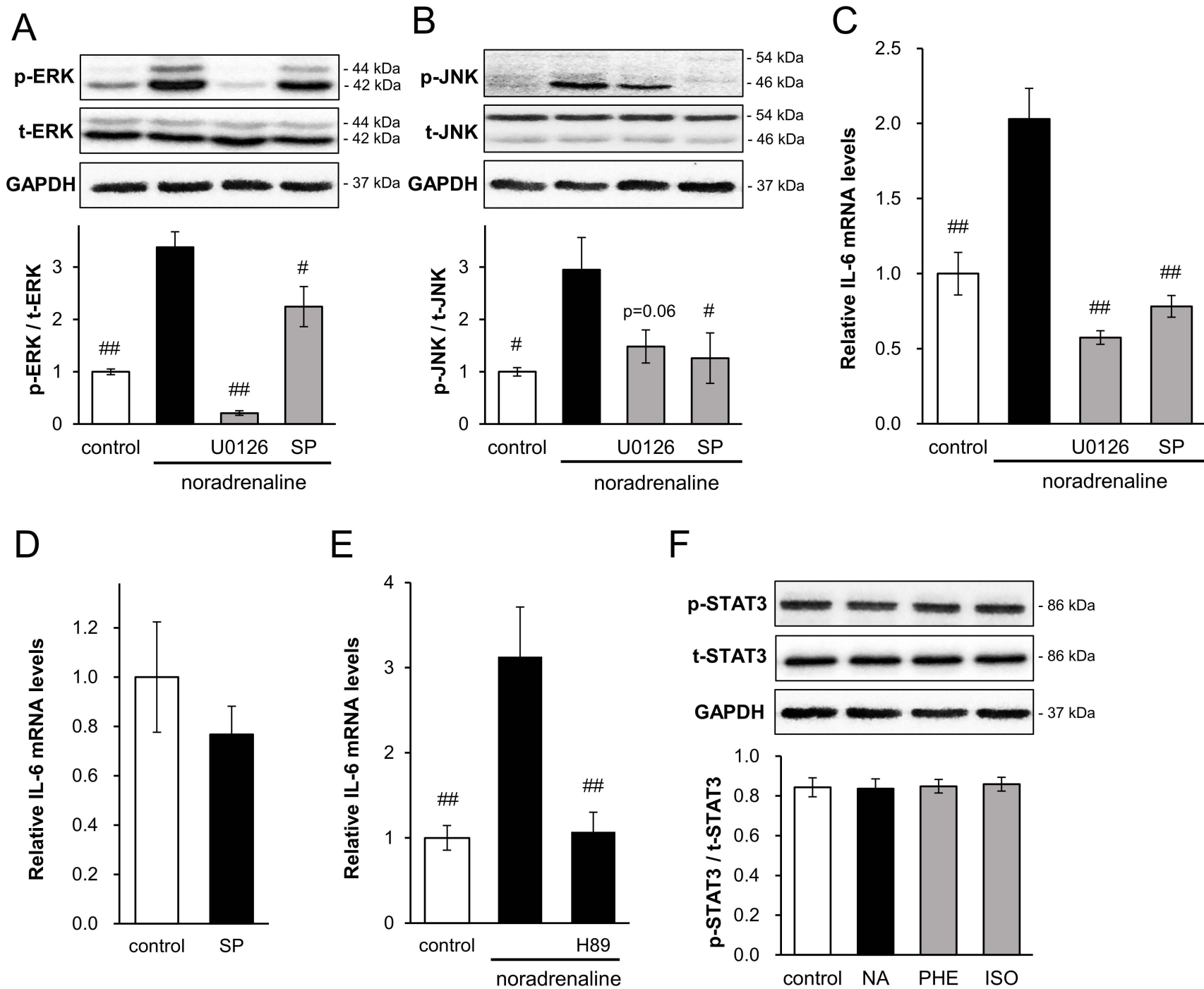
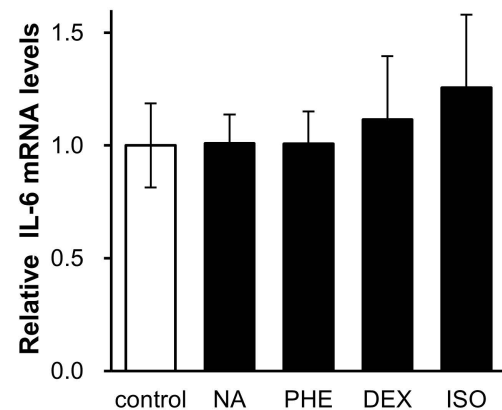


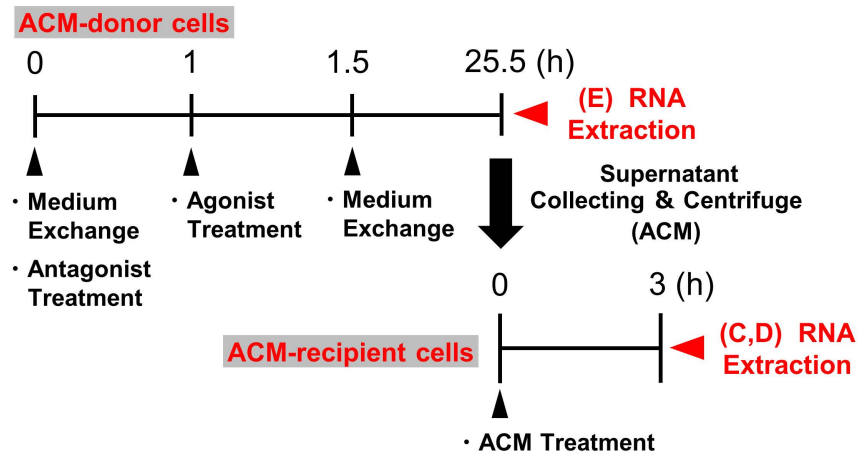


Fig. 5

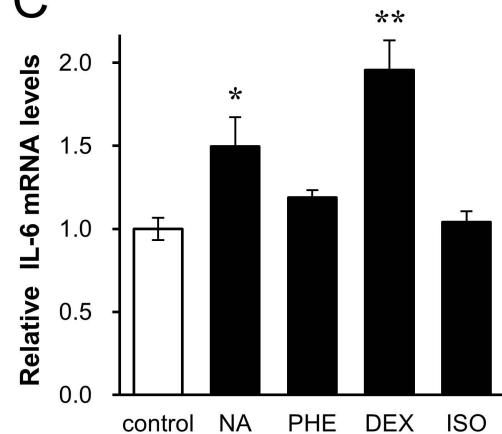
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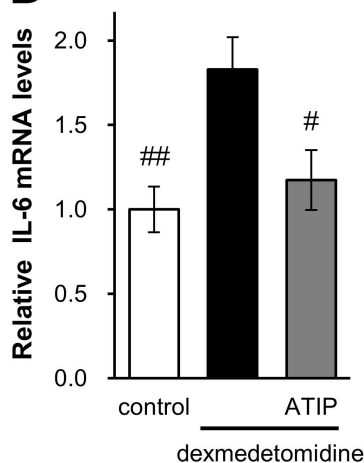
B



C



D



E

