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Bidirectional modulation of TNF- α transcription via α - and β -adrenoceptors in cultured astrocytes from rat spinal cord

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

*Corresponding author

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

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Abstract

Noradrenaline (NA) suppresses TNF- α production via β -adrenoceptors (ARs) in brain astrocytes. However, the downstream pathways from β -ARs, and the involvement of α -ARs, remains unknown. In this study, we investigated the AR-mediated regulation of TNF- α mRNA levels in cultured astrocytes from rat spinal cord. NA, the α_1 -agonist phenylephrine, and the β -agonist isoproterenol decreased the TNF- α mRNA level, while the α_2 -agonist dexmedetomidine increased it. The isoproterenol-induced TNF- α mRNA decrease was accompanied by a decrease in ERK phosphorylation. An adenylyl cyclase activator and an ERK inhibitor mimicked these effects. These results indicate that the transcriptional regulation of TNF- α by β -ARs is mediated via cAMP pathways followed by the ERK pathway inhibition. The dexmedetomidine-induced TNF- α mRNA increase was accompanied by phosphorylation of JNK and ERK, which was blocked by a JNK inhibitor. Furthermore, the LPS-induced increase in the TNF- α mRNA level was accompanied by NF- κ B nuclear translocation, and both these effects were blocked by phenylephrine. An NF- κ B inhibitor suppressed the LPS-induced increase in the TNF- α mRNA level. These results suggest that α_1 -ARs suppress the LPS-induced increase in the TNF- α mRNA level via inhibition of NF- κ B nuclear translocation. Taken together, our study reveals that both α - and β -ARs are involved in the transcriptional regulation of TNF- α in astrocytes.

1. Introduction

The projections of locus coeruleus, the principal site for synthesis of noradrenaline (NA) in the central nervous system (CNS), reach far and wide including the spinal cord [1]. Levels of NA in the CNS change under pathological conditions, such as depression, neuropathic pain, and Parkinson's disease [2,3]. Moreover, inactivation of noradrenergic neurons increases inflammatory responses [4] and decreases infarct size in cerebral ischemia [5], suggesting that noradrenergic system participates in the pathogenesis of the CNS diseases.

In the CNS, NA regulates the production of various bioactive substances, such as inflammatory cytokines, neurotrophins, and growth factors [6,7]. Inflammatory cytokines affect neuronal functions besides enhancing inflammation. Excessive production of TNF- α causes neuronal excitotoxicity by increasing glutamate release, which contributes to depression and memory impairment [8]. NA is released through not only synaptic transmission, but also volume transmission [9], and thus acts on the glial cells including astrocytes via α - and β -ARs. NA could affect physiological functions and disease pathogenesis by regulating TNF- α production via astrocytic ARs. In cultured hippocampal astrocytes, NA decreases TNF- α production via β -ARs [10]. Also, a β -agonist inhibit release of TNF- α evoked by LPS [11]. However, the intracellular signaling pathways that underlie a NA-induced decrease in TNF- α production in astrocytes are not fully explained.

Furthermore, it remains unknown whether α -ARs are involved in it, although α -ARs in astrocytes play important roles in regulation of astrocyte functions such as gliotransmitter release and Ca^{2+} signaling [12,13]. Moreover, astrocytes show regional differences in receptor expression, gene expression, and morphology [14–16]. Levels of NA in the spinal cord changes in response to neuropathic pain and ischemia [2,17]. Therefore, it is worth investigating the contribution of not only β -ARs but also α -ARs to the regulation of TNF- α production in spinal cord astrocytes.

In this study, we investigated the transcriptional regulation of TNF- α by ARs in cultured astrocytes from rat spinal cord. We found that α -AR subtypes were also involved in the transcriptional regulation of TNF- α mRNA and investigated intracellular signaling pathways following the activation of each AR subtype.

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), and p65 (#8242S, 1:100) were purchased from Cell Signaling Technology (Danvers, MA, USA). Dexmedetomidine hydrochloride, atipamezole hydrochloride, isoproterenol hydrochloride, and lipopolysaccharides (LPS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phenylephrine hydrochloride, propranolol hydrochloride, forskolin, U0126, and SP600125 were purchased from Wako Pure Chemical (Osaka, Japan). L-noradrenaline bitartrate monohydrate and prazosin hydrochloride were purchased from Tokyo Chemical Industry (Tokyo, Japan). BAY-11-7082 and 1-oleoyl lysophosphatidic acid (LPA) 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. Culture of spinal cord astrocytes

Primary cultures of spinal cord astrocytes were obtained as previously described [18]. 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. Adherent cells were detached with trypsin and re-seeded onto poly-l-lysine-coated 6- and 12-well plates or coverslips at a density of 8.0×10^3 cells/cm². After 3 days, the cell culture had reached confluence and the medium was changed to serum-free medium. Cell cultures were treated with antagonists or inhibitors immediately after the medium exchange, and were treated with noradrenaline or agonists 1 h after the medium exchange. After the given time (detailed in the figure legends and results section), the cell culture was used for experiments.

2.4. Real-time PCR

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 primer sequence and product size can be found in Table S1 (Supplementary data). 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 for GAPDH or 63°C for TNF- α , 45 s). Melt curve analysis confirmed that the obtained amplicon was the only one expected in each reaction. The expression levels of the target genes relative to GAPDH were calculated by the $\Delta\Delta C_q$ method and were expressed as relative to the control.

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 overnight at 4°C with a primary antibody. 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.

2.6. Immunocytochemistry

Astrocytes were fixed with 4% paraformaldehyde for 20 min at room temperature, permeabilized with 0.3% Triton X-100 in PBS at room temperature for 10 min, and then blocked with 10% normal goat serum in PBS for 30 min. Cells were then incubated with a rabbit anti-p65 antibody in PBS containing 1% normal goat serum at 4°C for at least 12 h. After washing in PBS, cells were incubated with an Alexa Fluor 488-conjugated goat anti-rabbit antibody (1:500; Thermo Fisher Scientific, MA, USA) in the dark at room temperature for 30 min. Coverslips were mounted on glass slides with Dapi-Fluoromount G (SouthernBiotech, Birmingham, AL, USA). The number of cells with p65 nuclear translocation was determined using a fluorescence microscope (LSM 700, Carl Zeiss, Oberkochen, Germany) and ImageJ software. More than 150 cells in three random fields were counted.

2.7. Data analysis

Data are expressed as means $\pm$ S.E.M (n = number of independent measurements).

139 Statistical comparisons between two group were made using the unpaired Student's t-test.
140 For multiple comparisons, one-way ANOVA followed by the Dunnett's test was used. A
141 value of $p < 0.05$ was considered as a statistically significant level. All statistical analysis
142 was performed with Ekuseru-Toukei 2008 (Social Survey Research Information Co., Ltd.,
143 Tokyo, Japan).
144

3. Results

3.1. Effects of adrenoceptor agonists and antagonists on TNF- α mRNA levels

RT-PCR analysis revealed that the cultured astrocytes from rat spinal cord expressed α_1 -, α_2 - and β -ARs (Fig. S1). We investigated time- and concentration-dependent effects of NA and the β -agonist isoproterenol (ISO) on TNF- α mRNA levels in cultured astrocytes. Treatment of astrocytes with NA (1 or 10 μ M) decreased the TNF- α mRNA level, which reached a trough 3 h after treatment before gradually recovering (Fig. 1A). Treatment of astrocytes with either NA or ISO for 3 h decreased the TNF- α mRNA level in a concentration-dependent manner (Fig. 1B).

Next, we investigated which AR subtypes are involved in the transcriptional regulation of TNF- α . The TNF- α mRNA level was decreased by the α_1 -agonist phenylephrine (PHE, 1 μ M) or ISO (1 μ M) but increased by the α_2 -agonist dexmedetomidine (DEX, 1 μ M) (Fig. 1C). The β -antagonist propranolol (10 μ M), but not the α_1 -antagonist prazosin (1 μ M) and the α_2 -antagonist atipamezole (10 μ M), blocked the NA-induced decrease in the TNF- α mRNA level (Fig. 1D). In the presence of prazosin and propranolol, NA increased the TNF- α mRNA level, which was blocked by additional treatment with atipamezole (Fig. 1E). None of the antagonists alone had any effect on TNF- α mRNA levels (Fig. 1F). These results indicate that α -ARs, as well as β -ARs, participate in transcriptional regulation of TNF- α .

Under pathological conditions, lipopolysaccharide (LPS) increases in the CNS

[19], and excessive LPS evoke inflammatory cytokine release from glial cells [20]. LPS (100 ng/ml) increased the TNF- α mRNA levels, which was suppressed by NA, PHE, and ISO (Fig. 1G). These results suggest that both α - and β -ARs also regulate TNF- α transcription under pathological conditions.

3.2. Transcriptional regulation of TNF- α via β -adrenoceptors

In general, β -ARs coupled to Gs proteins activate the cAMP-PKA pathway. However, we could not investigate the effects of PKA inhibitors, because PKA inhibitors, such as H89, KT5720, Rp-cAMPS, themselves changed the TNF- α mRNA levels (data not shown). Increased cAMP levels either activate or inactivate extracellular signal-regulated kinase (ERK), one of a family of mitogen-activated protein kinases (MAPK) [21,22]. ISO decreased ERK phosphorylation, which was blocked by propranolol (Fig. 2A). On the other hand, ISO did not affect phosphorylation of other MAPKs, c-jun N-terminal kinase (JNK) or p38 (Fig. S2). Like ISO, the adenylyl cyclase activator forskolin (10 μ M) and the MEK/ERK inhibitor U0126 (10 μ M) also decreased the TNF- α mRNA level (Fig. 2B and C) and ERK phosphorylation (Fig. 2A). These results suggest that ISO decreases the TNF- α mRNA level via cAMP-induced ERK inactivation. Unexpectedly, NA increased the phosphorylation of ERK and JNK, but not p38, and these increases were not affected by propranolol (Fig. 2A and S2).

184

185 **3.3. Transcriptional regulation of TNF- α via α_1 -adrenoceptors**

186 Next, we investigated the mechanisms by which PHE suppresses the LPS-induced
187 increases in the TNF- α mRNA level. Nuclear translocation of NF- κ B up-regulates TNF- α
188 gene transcription [23]. The NF- κ B inhibitor BAY-11-7082 (BAY, 1 μ M) suppressed the
189 LPS-induced increases in the TNF- α mRNA level (Fig. 3A). BAY alone did not have any
190 effect on the TNF- α mRNA level (Fig. 3B). LPS stimulation for 60 min increased the p65
191 nuclear translocation (Fig. 3C and D). PHE suppressed the LPS-induced p65 translocation,
192 which was abolished by prazosin. These results suggest that α_1 -ARs suppress the LPS-
193 induced increase in the TNF- α mRNA level via inhibition of NF- κ B nuclear translocation.
194 Although we also tried to investigate the effect of NA and ISO on the LPS-induced p65
195 translocation, these agonists caused marked morphological change with a reduction of
196 soma size, and thus we could not precisely evaluate the fluorescent intensity in the nuclear
197 in comparison with that in the soma (data not shown).

198

199 **3.4. Transcriptional regulation of TNF- α via α_2 -adrenoceptors**

200 Next, we elucidated the mechanisms of the DEX-induced increase in the TNF- α
201 mRNA level. The JNK inhibitor SP600125 (10 μ M) inhibited the DEX-induced TNF- α
202 mRNA increase (Fig 4A). SP600125 alone did not have any effect on TNF- α mRNA level

(Fig. 4C). DEX increased JNK phosphorylation, which was blocked by atipamezole or SP600125 (Fig. 4 D). DEX also increased ERK phosphorylation, which was blocked by atipamezole or SP600125 (Fig 4E). On the other hand, DEX did not affect p38 phosphorylation (Fig. S3). Lysophosphatidic acid (LPA, 10 μ g/ml), which increases JNK phosphorylation [24], also increased the TNF- α mRNA level, and JNK and ERK phosphorylation (Fig. 4B,D and E). These increases were abolished by SP600125. These results suggest that the activation of α_2 -ARs increases the TNF- α mRNA level by activating JNK and/or ERK. Like DEX, NA increased JNK and ERK phosphorylation, and these effects were abolished by atipamezole (Fig 4D and E).

4. Discussion

This study indicates that NA suppresses TNF- α transcription in cultured astrocytes from rat spinal cord. We found that α -ARs, in addition to β -ARs, participated in the transcriptional regulation of TNF- α in astrocytes. We showed that α_1 - and β -ARs suppresses LPS-activated TNF- α transcription. MAPK and NF- κ B pathways are involved in the intracellular signaling of AR-regulated TNF- α transcription. Regulation of the TNF- α production by astrocytic α - and β -ARs are likely to participate in physiological and/or pathophysiological functions in the CNS.

NA concentration-dependently suppressed TNF- α transcription and significantly suppressed at concentration of 1 μ M. The concentration of NA in normal cerebrospinal fluid is 1 nM to 100 nM [25,26]. Transient ischemia increases extracellular NA levels more than 10-fold [25]. Therefore, it is likely that pathological concentrations of NA are sufficient to affect TNF- α production in astrocytes. In this study, the effect of NA on TNF- α transcription was transient, and was not significantly different from the control after 12 h treatment. In control cultures, the TNF- α mRNA levels tended to increase over time. Although the reason for this is not clear, we speculate that it is due to the cellular stress response to the exposure to serum-free medium. Under pathological conditions, such as neuropathic pain or Alzheimer's disease, NA levels increase or decrease chronically [2,27]. Further investigation is necessary to elucidate how the longer-term changes in NA

concentrations that are observed during chronic disease modulate TNF- α production by astrocytes *in vivo*.

The β -antagonist propranolol abolished the NA-induced decrease in the TNF- α mRNA level, indicating that NA-induced transcriptional suppression of TNF- α is mainly mediated via β -ARs. On the other hand, an α_1 -agonist suppressed TNF- α transcription and an α_2 -agonist activated TNF- α transcription. Moreover, in the presence of both β - and α_1 -AR antagonists, NA activated TNF- α transcription, and this effect was blocked by the additional treatment of the α_2 -antagonist. These results suggest that α_1 - and α_2 -ARs are also involved in NA-mediated TNF- α transcription, but their effects seem to be masked by the potent effect of β -ARs under normal conditions.

A β_2 -agonist suppresses TNF- α transcription with upregulation of cAMP levels in mouse cortical astrocytes [10]. In this study, ISO suppressed ERK phosphorylation. In addition, the adenylyl cyclase activator forskolin and the ERK inhibitor U0126 mimicked the effects by ISO on TNF- α transcription and ERK phosphorylation. Our results indicate that the suppression of ERK phosphorylation is related to the transcriptional suppression of TNF- α via β -ARs-cAMP pathway. The possibility that the kinase inhibitors used in this study change receptor expression cannot be ruled out. However, we thought that the influence of the inhibitors on the expression level of adrenoceptors was little, if any, because the treatment time for the kinase inhibitors was 4 hours at the longest. The

251 pattern of ERK phosphorylation by β -agonists is cell-type dependent. β -agonists promote
252 ERK phosphorylation in neurons or microglia [28–30], but suppress ERK phosphorylation
253 in cultured cerebral astrocytes [31]. In this study, unlike ISO, NA activated ERK and JNK
254 phosphorylation. This raise the question whether NA and ISO mainly regulate TNF- α
255 transcription via same pathways. Further investigation is needed to determine the
256 intracellular pathways that are involved in the NA-mediated suppression of TNF- α
257 transcription.

258 The α_2 -agonist DEX, contrary to PHE and ISO, activated the TNF- α transcription.
259 DEX also increased JNK and ERK phosphorylation. The JNK activator LPA showed the
260 similar effects and these effects were abolished by the JNK inhibitor SP600125. These
261 results indicate that α_2 -ARs activate TNF- α transcription by activating JNK and/or ERK.
262 Moreover, the α_2 -antagonist atipamezole abolished the NA-induced JNK and ERK
263 phosphorylation, supporting the involvement of α -ARs in the astrocytic response to NA.
264 NA is likely to modulate TNF- α transcription by bidirectional effects mediated via α - and
265 β -ARs.

266 In rat cortical astrocytes, NA suppresses a LPS-induced increase in TNF- α mRNA
267 level via β_2 -ARs [11]. We found that the α_1 -agonist PHE also suppressed the LPS-induced
268 increase in the TNF- α mRNA level. PHE decreased the LPS-induced NF- κ B nuclear
269 translocation, which was abolished by the α_1 -antagonist prazosin. Under pathological

270 conditions, NF- κ B activation promotes TNF- α transcription in glial cells [32]. Excess TNF-
271 α induces excitotoxicity via astrocytic TNF- α receptors, which results in memory
272 impairment [8], and inhibition of myelination in hypoxia [33]. Further studies are needed
273 to reveal whether astrocytic ARs regulate cytokine production *in vivo* and how astrocytic
274 cytokines act under physiological and pathological conditions.

275

276 **Declaration of competing interest**

277 **None.**

278

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406

Figure legends

Figure 1. Effects of adrenoceptor agonists and antagonists on TNF- α mRNA levels in cultured astrocytes from rat spinal cord.

(A) TNF- α mRNA levels in astrocytes treated with noradrenaline (NA, 1 and 10 μ M) for 1, 3, 6, and 12 h. * p < 0.05, ** p < 0.01 vs. time-matched control (Dunnett's test). (B) TNF- α mRNA levels in astrocytes treated with NA (1 nM-10 μ M) or the β -agonist isoproterenol (ISO, 1 nM-10 μ M) for 3 h. * p < 0.05, ** p < 0.01 vs. control (Dunnett's test). (C) TNF- α mRNA levels in astrocytes treated with the α_1 -agonist phenylephrine (PHE, 1 μ M), the α_2 -agonist dexmedetomidine (DEX, 1 μ M), and ISO (1 μ M) for 3 h. * p < 0.05, ** p < 0.01 vs. control (Dunnett's test). (D, E) TNF- α mRNA levels in astrocytes treated with NA (1 μ M) in the presence or absence of the α_1 -antagonist prazosin (PRAZ, 1 μ M), the α_2 -antagonist atipamezole (ATIP, 10 μ M), and the β -antagonist propranolol (PROP, 10 μ M) for 3 h. ** p < 0.01 vs. NA alone (Dunnett's test), ### p < 0.01 vs. NA+PRAZ+PROP (Dunnett's test). (F) TNF- α mRNA levels in astrocytes treated with PRAZ, ATIP, and PROP for 4 h. n.s. = not significant (Dunnett's test). (G) TNF- α mRNA levels in astrocytes treated with LPS (100 ng/ml) in the presence or absence of NA, PHE, DEX, and ISO for 3 h. * p < 0.05, ** p < 0.01 vs. LPS alone (Dunnett's test). Data are presented as means $\pm$ S.E.M. n = 6.

Figure 2. Intracellular mechanisms of transcriptional regulation of TNF- α via β -

adrenoceptors.

(A) The protein expression levels of phosphorylated or total ERK were quantified and representative blots are shown. Astrocytes were treated with isoproterenol (1 μ M), noradrenaline (1 μ M), the MEK/ERK inhibitor U0126 (10 μ M), or the adenylyl cyclase activator forskolin (FSK, 10 μ M) in the presence or absence of the β -antagonist propranolol (PROP, 10 μ M) for 10 min. * p < 0.05, ** p < 0.01 vs. control (Dunnett's test). (B,C) TNF- α mRNA levels in astrocytes treated with FSK (B) or U0126 (C). ** p < 0.01 (unpaired Student's t -test). Data are presented as means $\pm$ S.E.M. n = 6.

Figure 3. Effects of α_1 -agonist on LPS-induced increases in TNF- α mRNA level and p65 nuclear translocation.

(A) TNF- α mRNA levels in astrocytes treated with LPS (100 ng/ml) in the presence or absence of the NF- κ B inhibitor BAY-11-7082 (BAY, 1 μ M) for 3 h. ** p < 0.01 vs. LPS alone (Dunnett's test). (B) TNF- α mRNA levels in astrocytes treated with BAY for 4h. n.s. = not significant (unpaired Student's t -test). (C) Representative confocal microscopy images of p65 (green) and DAPI (blue). Scale bar = 100 μ m. Astrocytes were treated with LPS in the presence or absence of the α_1 -agonist phenylephrine (PHE, 1 μ M) and the α_1 -antagonist prazosin (PRAZ, 1 μ M) for 1 h. (D) The percentage of p65 nuclear translocation cells. More than 150 cells in three random fields were counted. ** p < 0.01 vs. LPS alone (Dunnett's

445 test). Data are presented as means $\pm$ S.E.M. n = 6.

446

447 **Figure 4. Transcriptional regulation of TNF- α via α_2 -adrenoceptors.**

448 (A, B) TNF- α mRNA levels in astrocytes treated with the α_2 -agonist dexmedetomidine (1

449 μ M, A) and LPA (10 μ g/ml, B) in the presence or absence of the JNK inhibitor SP600125

450 (SP, 10 μ M) for 3 h. **p < 0.01 vs. dexmedetomidine or LPA alone (Dunnett's test). (C)

451 TNF- α mRNA levels in astrocytes treated with SP for 4h. n.s. = not significant (unpaired

452 Student's t-test). (D,E) The protein expression levels of phosphorylated or total JNK (D)

453 and ERK (E) was quantified and representative blots are shown. Astrocytes were treated

454 with dexmedetomidine, noradrenaline (NA, 1 μ M), or LPA in the presence or absence of

455 the α_2 -antagonist atipamezole (ATIP, 10 μ M) and SP for 10 min. **p < 0.01 vs. control

456 (Dunnett's test). Data are presented as means $\pm$ S.E.M. n = 6.

Highlights

- Noradrenaline decreased TNF- α mRNA levels in cultured astrocytes from spinal cord.
- β -Adrenoceptors mediated TNF- α mRNA decrease via the ERK pathway inhibition.
- LPS-induced TNF- α mRNA increase were suppressed by α_1 - and β -agonists.
- α_1 -Adrenoceptors suppress LPS-induced TNF- α mRNA increase via inhibition of NF- κ B.
- α_2 -Adrenoceptors mediated TNF- α mRNA increase via the JNK/ERK pathway.

astrocyte

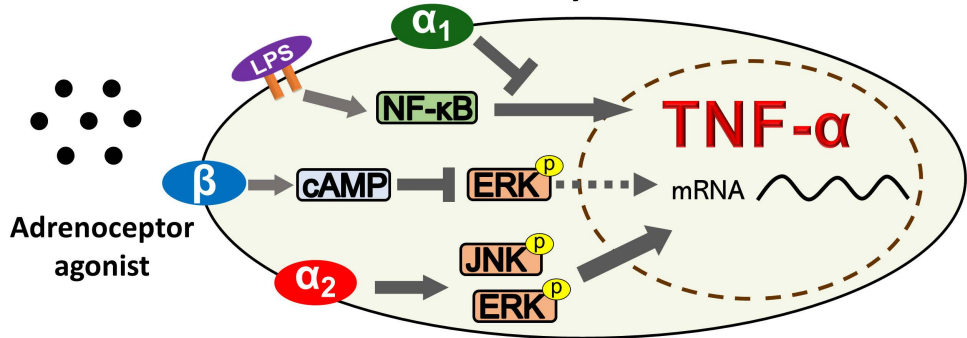


Fig.1

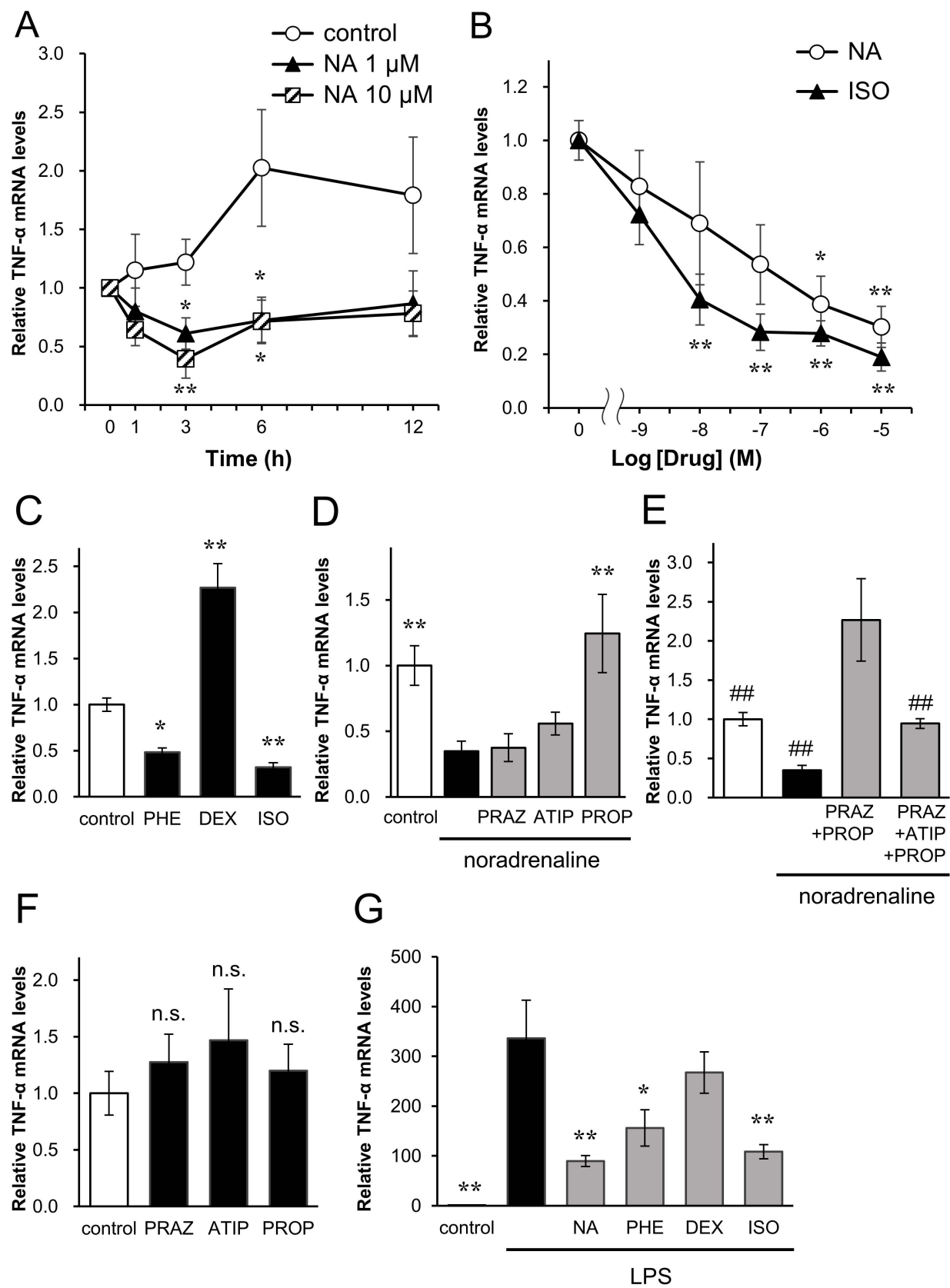
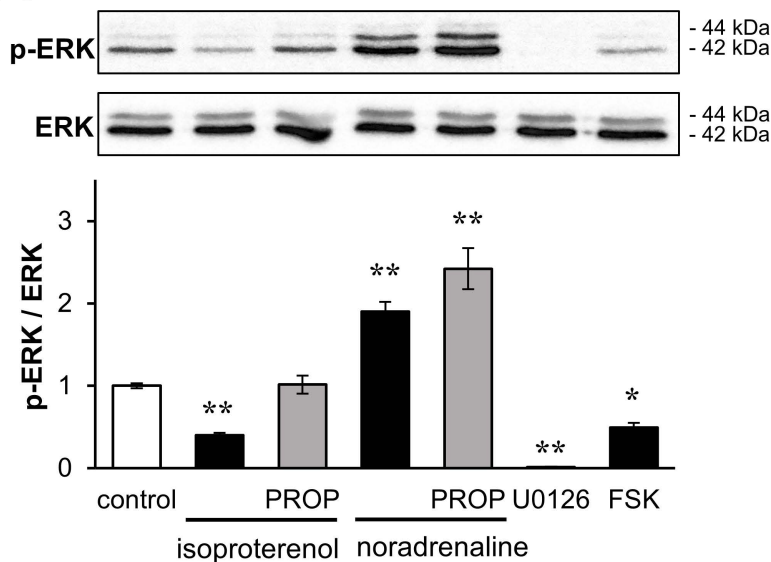
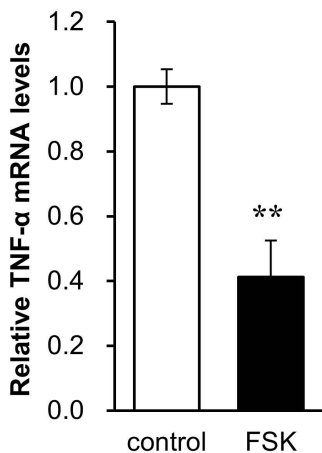


Fig. 2

A



B



C

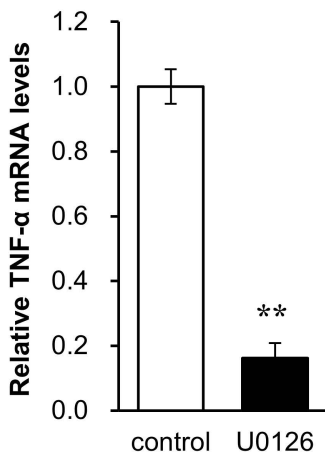


Fig. 3

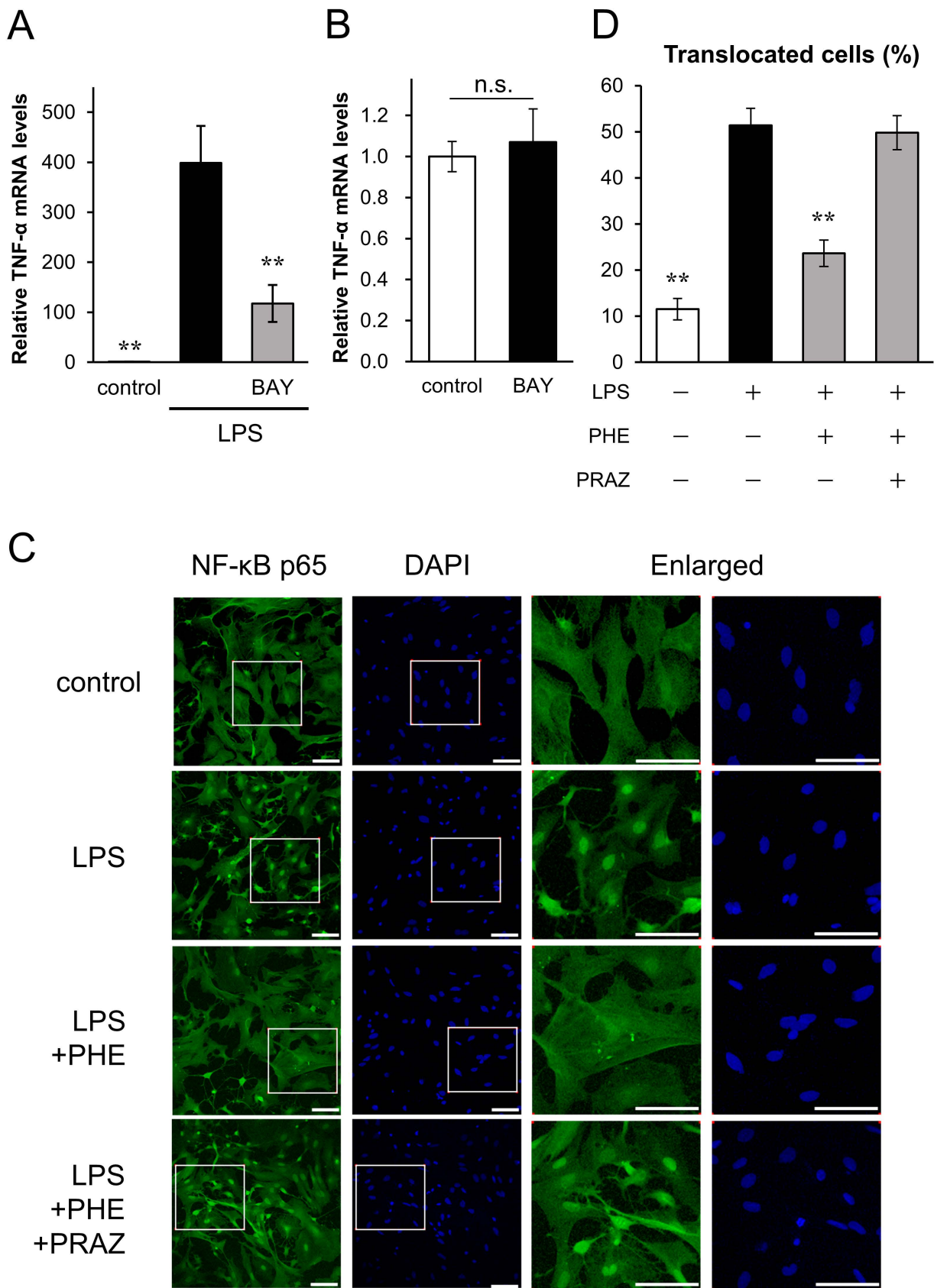
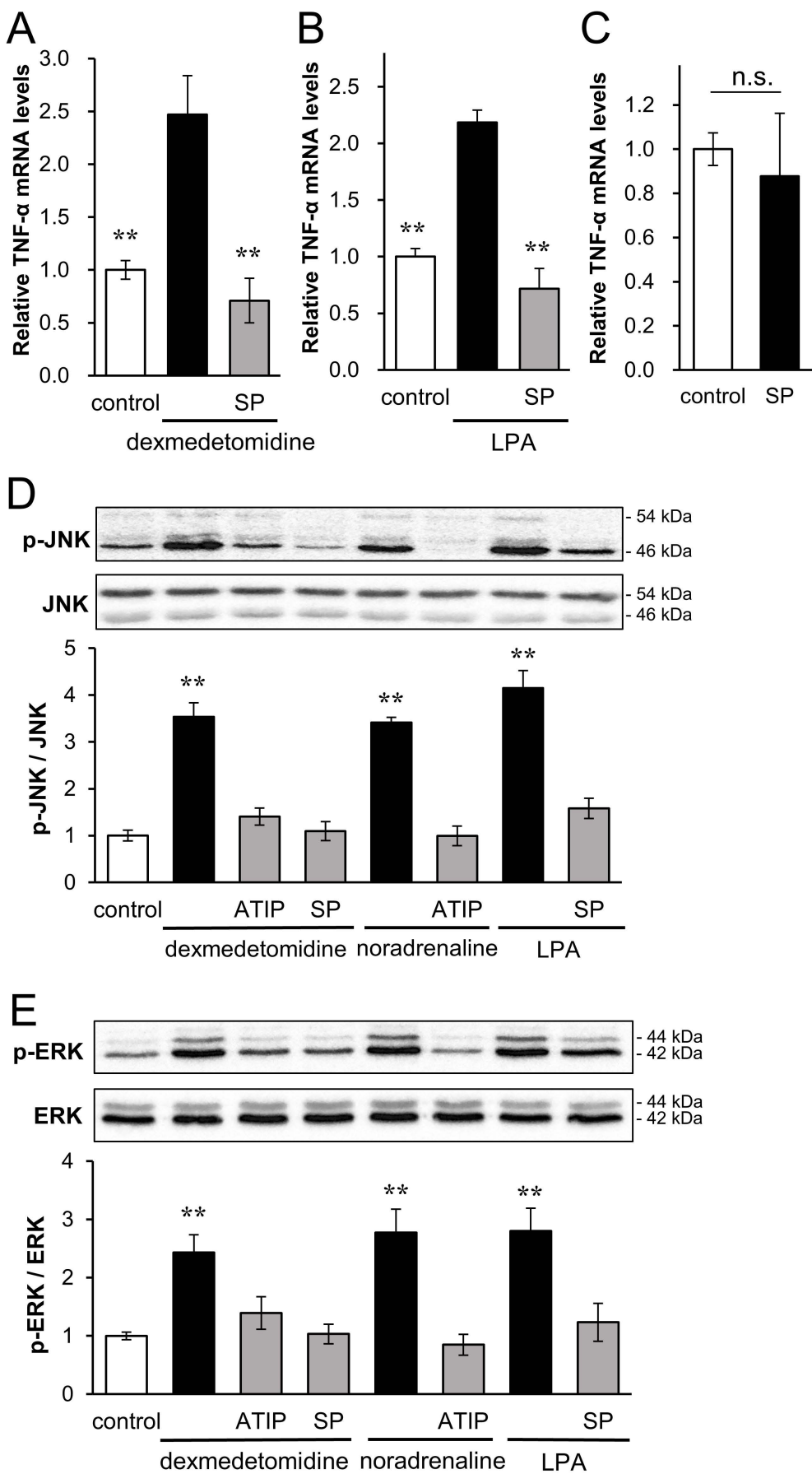


Fig. 4



Supplementary data

1. Materials and methods

1.1. RT-PCR

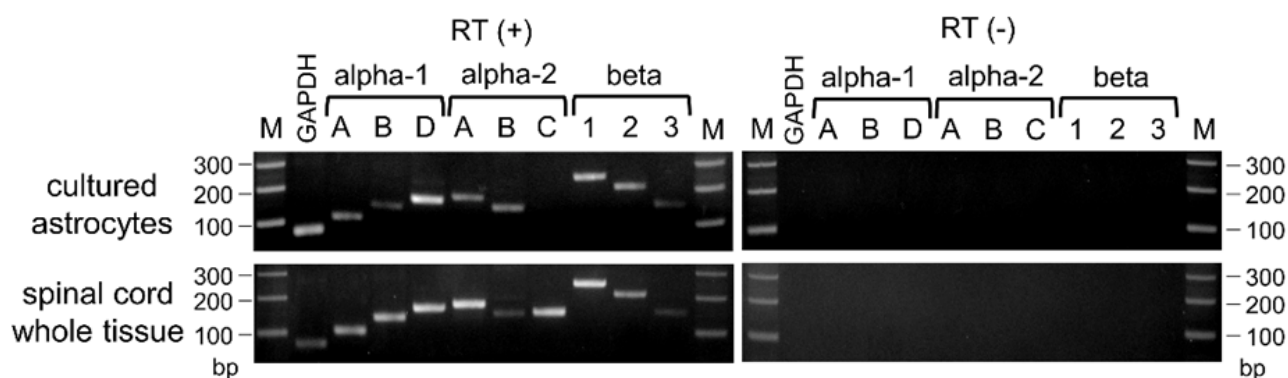
Total RNAs were extracted from cultured astrocytes and spinal cord whole tissue using RNAiso Plus (Takara Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka Japan). PCR was performed using KOD FX Neo (TOYOBO), each primer, and the cDNA reaction solution. The primers for adrenoceptor subtypes were used as previously reported (Koppel et al., 2018). The primer sequence and product size can be found in Table S2. Thermal cycles were performed using a PC320 system (ASTEC, Fukuoka, Japan). Cycling conditions were 94°C for 1 min (for initial denaturation), followed by 35 cycles of denaturation (98°C, 10 s), annealing (60°C, 10 s), and extension (68°C, 30 s). RNAs without RT were used as a negative control to examine DNA contamination. PCR products and a 100 bp DNA Ladder (Takara Bio) were separated on a 3% agarose gel and visualized with ethidium bromide under UV illumination (Mupid-Scope WD, Mupid, Tokyo, Japan).

Table S1. Primers used to determine TNF- α mRNA levels by real-time PCR.

Target gene	Product size (bp)	Sequence (upper; sense, lower; antisense)
<i>tumor necrosis factor</i>	125	5'-CATGAGCACGGAAAGCATGA-3' 5'-CCACGAGCAGGAATGAGAAGA-3'
<i>glyceraldehyde -3-phosphate dehydrogenase</i>	74	5'-GCAAGAGAGAGGGCCCTCAG-3' 5'-TGTGAGGGAGATGCTCAGTG-3'

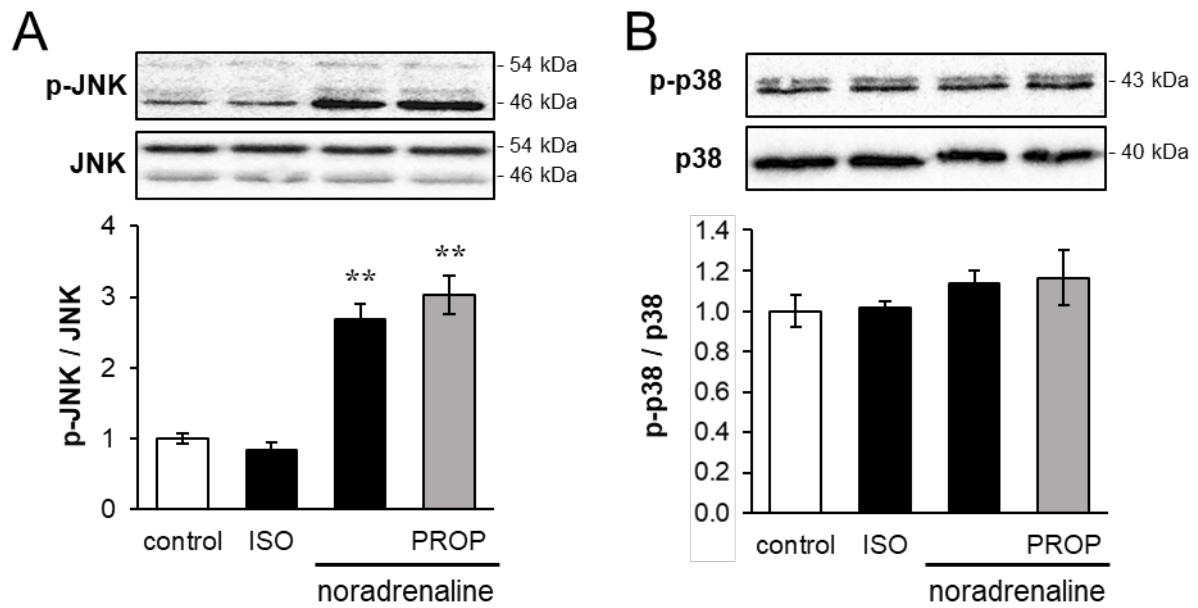
Table S2. Primers used to determine adrenoceptor subtype by RT-PCR.

Target gene	Product size (bp)	Sequence (upper; sense, lower; antisense)
<i>adrenoceptor alpha 1A</i>	104	5'-AGAAGAAAGCTGCCAAGACG-3' 5'-GAAATCCGGAAGAAAGACC-3'
<i>adrenoceptor alpha 1B</i>	138	5'-TCTTATGTTGGCTCCCCTTC-3' 5'-ACGGGTAGATGATGGGATTG-3'
<i>adrenoceptor alpha 1D</i>	165	5'-TGAGGCTGCTCAAGTTTTCC-3' 5'-GCCAGAAGATGACCTTGAAGAC-3'
<i>adrenoceptor alpha 2A</i>	176	5'-TTCCTGAGAGGGAAGGGATT-3' 5'-AGTTACTGGGGCAAGTGGTG-3'
<i>adrenoceptor alpha 2B</i>	147	5'-AATTCTCTGAACCCCCAAGC-3' 5'-CAAGTTGGGAAGACAACCAG-3'
<i>adrenoceptor alpha 2C</i>	150	5'-GGTTTCCTCATCGTTTTCA-3' 5'-GAAAAGGGCATGACCAGTGT-3'
<i>adrenoceptor beta 1</i>	248	5'-GCTCTGGACTTCGGTAGACG-3' 5'-ACTTGGGGTCGTTGTAGCAG-3'
<i>adrenoceptor beta 2</i>	208	5'-AGCCACCTACGGTCTCTGAA-3' 5'-GTCCCGTTCCTGAGTGATGT-3'
<i>adrenoceptor beta 3</i>	150	5'-TCGTCTTCTGTGCAGCTACG-3' 5'-ATGGTCCTTCATGTGGGAAA-3'
<i>glyceraldehyde -3-phosphate dehydrogenase</i>	74	5'-GCAAGAGAGAGGCCCTCAG-3' 5'-TGTGAGGGAGATGCTCAGTG-3'



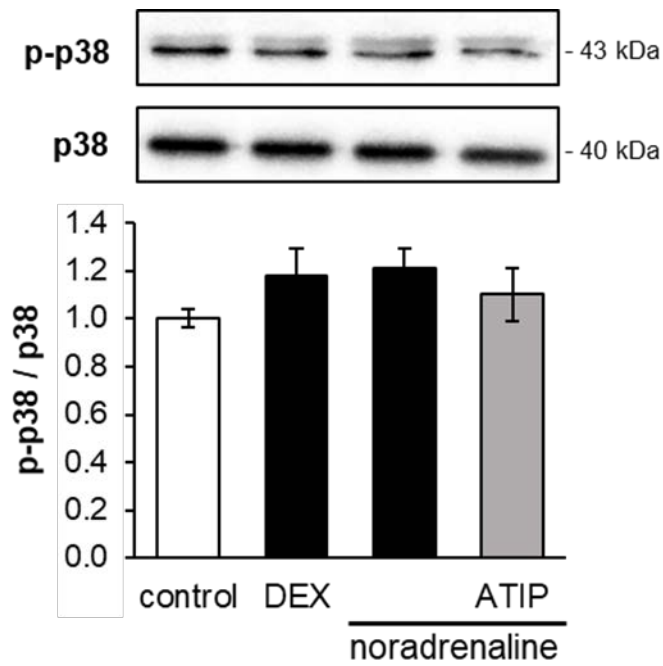
Supplementary Figure S1. Adrenoceptor subtype mRNA expression in the cultured astrocytes

RT (+) and (-) indicates samples reverse-transcribed (+) and not (-), respectively. Bands for all adrenoceptor subtypes except α_2c -adrenoceptors were detected in the cultured astrocytes (upper). The spinal cord whole tissue, used as a positive control, expressed all adrenoceptor subtypes (lower).



Supplementary Figure S2. Effects of isoproterenol on JNK and p-38 phosphorylation

(A,B) The protein expression levels of phosphorylated or total JNK (A) and p38 (B) were quantified and representative blots are shown. Astrocytes were treated with the β -agonist isoproterenol (ISO, 1 μ M), or noradrenaline (1 μ M) in the presence or absence of propranolol (PROP, 10 μ M) for 10 min. ** $p < 0.01$ vs. control (Dunnett's test). Data are presented as means $\pm$ S.E.M. $n = 6$.



Supplementary Figure S3. Effects of dexmedetomidine on p-38 phosphorylation

The protein expression levels of phosphorylated or total p38 were quantified and representative blots are shown. Astrocytes were treated with the α_2 -agonist dexmedetomidine (DEX, 1 μ M), or noradrenaline (1 μ M) in the presence or absence of atipamezole (ATIP, 10 μ M) for 10 min. n = 6. Data are presented as means $\pm$ S.E.M.