



Title	Bidirectional modulation of TNF-alpha transcription via alpha- and beta-adrenoceptors in cultured astrocytes from rat spinal cord
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Citation	Biochemical and biophysical research communications, 528(1), 78-84 https://doi.org/10.1016/j.bbrc.2020.05.011
Issue Date	2020-07-12
Doc URL	http://hdl.handle.net/2115/82212
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Rights(URL)	http://creativecommons.org/licenses/by-nc-nd/4.0/
Type	article (author version)
File Information	Biochemical and biophysical research communications528(1)_78_84.pdf



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

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10

11 Keywords: astrocyte, noradrenaline, adrenoceptors, TNF- α , transcription factor

12

13 **Abstract**

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

31

32 1. Introduction

33 The projections of locus coeruleus, the principal site for synthesis of noradrenaline
34 (NA) in the central nervous system (CNS), reach far and wide including the spinal cord
35 [1]. Levels of NA in the CNS change under pathological conditions, such as depression,
36 neuropathic pain, and Parkinson's disease [2,3]. Moreover, inactivation of noradrenergic
37 neurons increases inflammatory responses [4] and decreases infarct size in cerebral
38 ischemia [5], suggesting that noradrenergic system participates in the pathogenesis of the
39 CNS diseases.

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

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

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

62

63 2. Materials and methods

64 2.1. Materials

65 Antibodies against ERK1/2 (#4695S, 1:2500), phospho-ERK 1/2 (#9101S, 1:2500),
66 p38 (#9212S, 1:2000), phospho-p38 (#9211S, 1:1000), SAPK/JNK (#9252S, 1:2500),
67 phospho-SAPK/JNK (#9251S, 1:1500), and p65 (#8242S, 1:100) were purchased from Cell
68 Signaling Technology (Danvers, MA, USA). Dexmedetomidine hydrochloride, atipamezole
69 hydrochloride, isoproterenol hydrochloride, and lipopolysaccharides (LPS) were purchased
70 from Sigma-Aldrich (St. Louis, MO, USA). Phenylephrine hydrochloride, propranolol
71 hydrochloride, forskolin, U0126, and SP600125 were purchased from Wako Pure Chemical
72 (Osaka, Japan). L-noradrenaline bitartrate monohydrate and prazosin hydrochloride were
73 purchased from Tokyo Chemical Industry (Tokyo, Japan). BAY-11-7082 and 1-oleoyl
74 lysophosphatidic acid (LPA) were purchased from Cayman Chemical (Ann Arbor, MI, USA).

75

76 2.2. Animals

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

82 3-5 days were used for primary astrocyte cultures.

83

84 **2.3. Culture of spinal cord astrocytes**

85 Primary cultures of spinal cord astrocytes were obtained as previously described
86 [18]. In brief, spinal cords were isolated from rat pups, minced, and incubated with papain
87 (10 U/ml) and DNase (0.1 mg/ml). Dissociated cells were suspended in Dulbecco's Modified
88 Eagle's Medium/Ham's F-12 containing 10% fetal bovine serum, 100 U/ml penicillin, and
89 0.1 mg/ml streptomycin. The cell suspension was seeded onto a poly-l-lysine-coated T75
90 flask. After 7-8 days, the flask was shaken at 250 rpm at 37°C for at least 12 h. Adherent
91 cells were detached with trypsin and re-seeded onto poly-l-lysine-coated 6- and 12-well
92 plates or coverslips at a density of 8.0×10^3 cells/cm². After 3 days, the cell culture had
93 reached confluence and the medium was changed to serum-free medium. Cell cultures
94 were treated with antagonists or inhibitors immediately after the medium exchange, and
95 were treated with noradrenaline or agonists 1 h after the medium exchange. After the
96 given time (detailed in the figure legends and results section), the cell culture was used
97 for experiments.

98

99 **2.4. Real-time PCR**

100 Total RNAs were extracted from cultured astrocytes using RNAiso Plus (Takara

101 Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was
102 then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka Japan).
103 Real-time PCR was performed using Thunderbird SYBR qPCR Mix (TOYOBO), each
104 primer, and the cDNA reaction solution. The primer sequence and product size can be
105 found in Table S1 (Supplementary data). Thermal cycles were performed using Eco Real
106 Time PCR System (Illumina, CA, USA). Cycling conditions were 95°C for 1 min (for initial
107 denaturation), followed by 40 cycles of denaturation (95°C, 15 s), annealing and extension
108 (61°C for GAPDH or 63°C for TNF- α , 45 s). Melt curve analysis confirmed that the
109 obtained amplicon was the only one expected in each reaction. The expression levels of the
110 target genes relative to GAPDH were calculated by the $\Delta\Delta Cq$ method and were expressed
111 as relative to the control.

112

113 **2.5. Western blotting**

114 Astrocytes were lysed in RIPA buffer containing a protease inhibitor cocktail
115 (nacalai tesque, Kyoto, Japan). The samples were separated by 10% SDS-PAGE and
116 transferred to polyvinylidene difluoride membranes (Millipore, CA, USA). The membranes
117 were blocked with 5% skimmed milk and then incubated overnight at 4°C with a primary
118 antibody. Thereafter, the membranes were incubated for 1 h at room temperature with a
119 horseradish peroxidase-conjugated secondary antibody (GE Healthcare, Little Chalfont,

120 UK). Antibody binding was visualized by ECL Prime (GE Healthcare). Band intensities
121 were measured using ImageJ software (National Institutes of Health) and expressed as
122 relative to the control.

123

124 **2.6. Immunocytochemistry**

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

136

137 **2.7. Data analysis**

138 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

145 **3. Results**

146 **3.1. Effects of adrenoceptor agonists and antagonists on TNF- α mRNA levels**

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

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

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

165 [19], and excessive LPS evoke inflammatory cytokine release from glial cells [20]. LPS (100
166 ng/ml) increased the TNF- α mRNA levels, which was suppressed by NA, PHE, and ISO
167 (Fig. 1G). These results suggest that both α - and β -ARs also regulate TNF- α transcription
168 under pathological conditions.

169

170 **3.2. Transcriptional regulation of TNF- α via β -adrenoceptors**

171 In general, β -ARs coupled to Gs proteins activate the cAMP-PKA pathway.
172 However, we could not investigate the effects of PKA inhibitors, because PKA inhibitors,
173 such as H89, KT5720, Rp-cAMPS, themselves changed the TNF- α mRNA levels (data not
174 shown). Increased cAMP levels either activate or inactivate extracellular signal-regulated
175 kinase (ERK), one of a family of mitogen-activated protein kinases (MAPK) [21,22]. ISO
176 decreased ERK phosphorylation, which was blocked by propranolol (Fig. 2A). On the other
177 hand, ISO did not affect phosphorylation of other MAPKs, c-jun N-terminal kinase (JNK)
178 or p38 (Fig. S2). Like ISO, the adenylyl cyclase activator forskolin (10 μ M) and the
179 MEK/ERK inhibitor U0126 (10 μ M) also decreased the TNF- α mRNA level (Fig. 2B and C)
180 and ERK phosphorylation (Fig. 2A). These results suggest that ISO decreases the TNF- α
181 mRNA level via cAMP-induced ERK inactivation. Unexpectedly, NA increased the
182 phosphorylation of ERK and JNK, but not p38, and these increases were not affected by
183 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

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

212

213 **4. Discussion**

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

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

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

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

242 A β_2 -agonist suppresses TNF- α transcription with upregulation of cAMP levels in
243 mouse cortical astrocytes [10]. In this study, ISO suppressed ERK phosphorylation. In
244 addition, the adenylyl cyclase activator forskolin and the ERK inhibitor U0126 mimicked
245 the effects by ISO on TNF- α transcription and ERK phosphorylation. Our results indicate
246 that the suppression of ERK phosphorylation is related to the transcriptional suppression
247 of TNF- α via β -ARs-cAMP pathway. The possibility that the kinase inhibitors used in this
248 study change receptor expression cannot be ruled out. However, we thought that the
249 influence of the inhibitors on the expression level of adrenoceptors was little, if any,
250 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

279 **Acknowledgments**

280 This work was supported by a Grant-in-Aid for Scientific Research from the Japan

281 Society for the Promotion of Science (No.19K23701).

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406

407 **Figure legends**

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

410 (A) TNF- α mRNA levels in astrocytes treated with noradrenaline (NA, 1 and 10 μ M) for 1,
411 3, 6, and 12 h. * $p < 0.05$, ** $p < 0.01$ vs. time-matched control (Dunnett's test). (B) TNF- α
412 mRNA levels in astrocytes treated with NA (1 nM-10 μ M) or the β -agonist isoproterenol
413 (ISO, 1 nM-10 μ M) for 3 h. * $p < 0.05$, ** $p < 0.01$ vs. control (Dunnett's test). (C) TNF- α
414 mRNA levels in astrocytes treated with the α_1 -agonist phenylephrine (PHE, 1 μ M), the α_2 -
415 agonist dexmedetomidine (DEX, 1 μ M), and ISO (1 μ M) for 3 h. * $p < 0.05$, ** $p < 0.01$ vs.
416 control (Dunnett's test). (D, E) TNF- α mRNA levels in astrocytes treated with NA (1 μ M)
417 in the presence or absence of the α_1 -antagonist prazosin (PRAZ, 1 μ M), the α_2 -antagonist
418 atipamezole (ATIP, 10 μ M), and the β -antagonist propranolol (PROP, 10 μ M) for 3 h. ** $p <$
419 0.01 vs. NA alone (Dunnett's test), ### $p < 0.01$ vs. NA+PRAZ+PROP (Dunnett's test). (F)
420 TNF- α mRNA levels in astrocytes treated with PRAZ, ATIP, and PROP for 4 h. n.s. = not
421 significant (Dunnett's test). (G) TNF- α mRNA levels in astrocytes treated with LPS (100
422 ng/ml) in the presence or absence of NA, PHE, DEX, and ISO for 3 h. * $p < 0.05$, ** $p < 0.01$
423 vs. LPS alone (Dunnett's test). Data are presented as means \pm S.E.M. n = 6.

424

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

426 **adrenoceptors.**

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

434

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

437 (A) TNF- α mRNA levels in astrocytes treated with LPS (100 ng/ml) in the presence or
438 absence of the NF- κ B inhibitor BAY-11-7082 (BAY, 1 μ M) for 3 h. ** p < 0.01 vs. LPS alone
439 (Dunnett's test). (B) TNF- α mRNA levels in astrocytes treated with BAY for 4h. n.s. = not
440 significant (unpaired Student's t -test). (C) Representative confocal microscopy images of
441 p65 (green) and DAPI (blue). Scale bar = 100 μ m. Astrocytes were treated with LPS in the
442 presence or absence of the α_1 -agonist phenylephrine (PHE, 1 μ M) and the α_1 -antagonist
443 prazosin (PRAZ, 1 μ M) for 1 h. (D) The percentage of p65 nuclear translocation cells. More
444 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

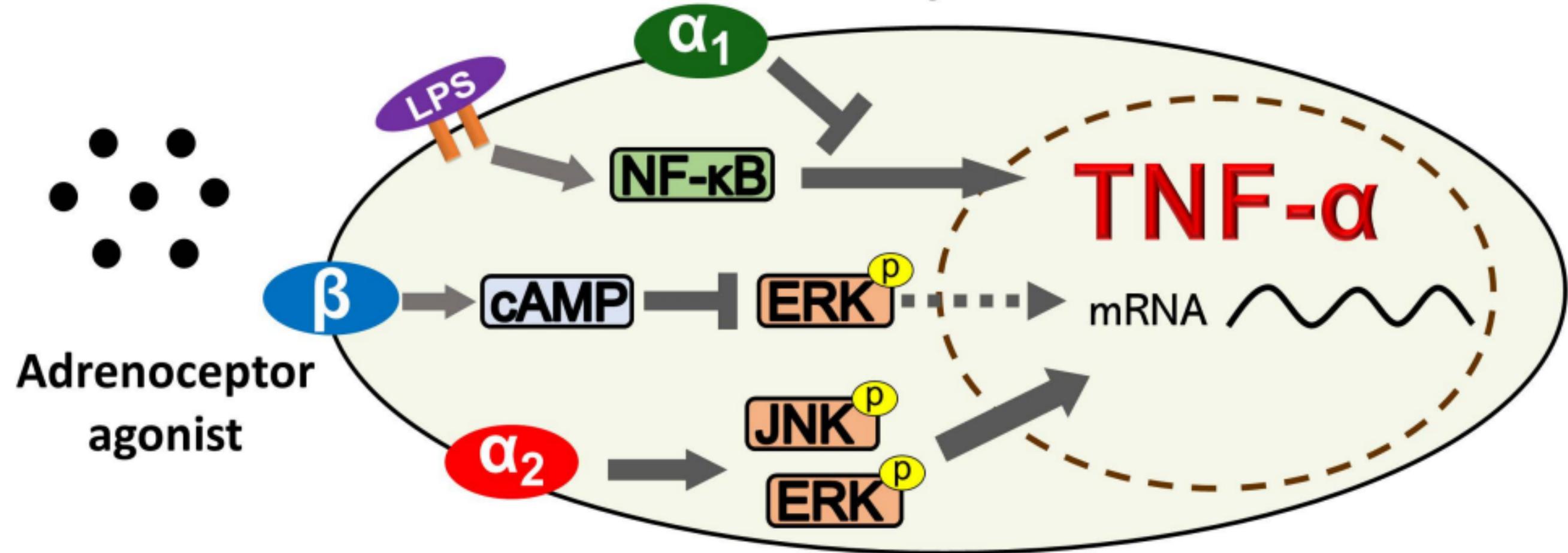


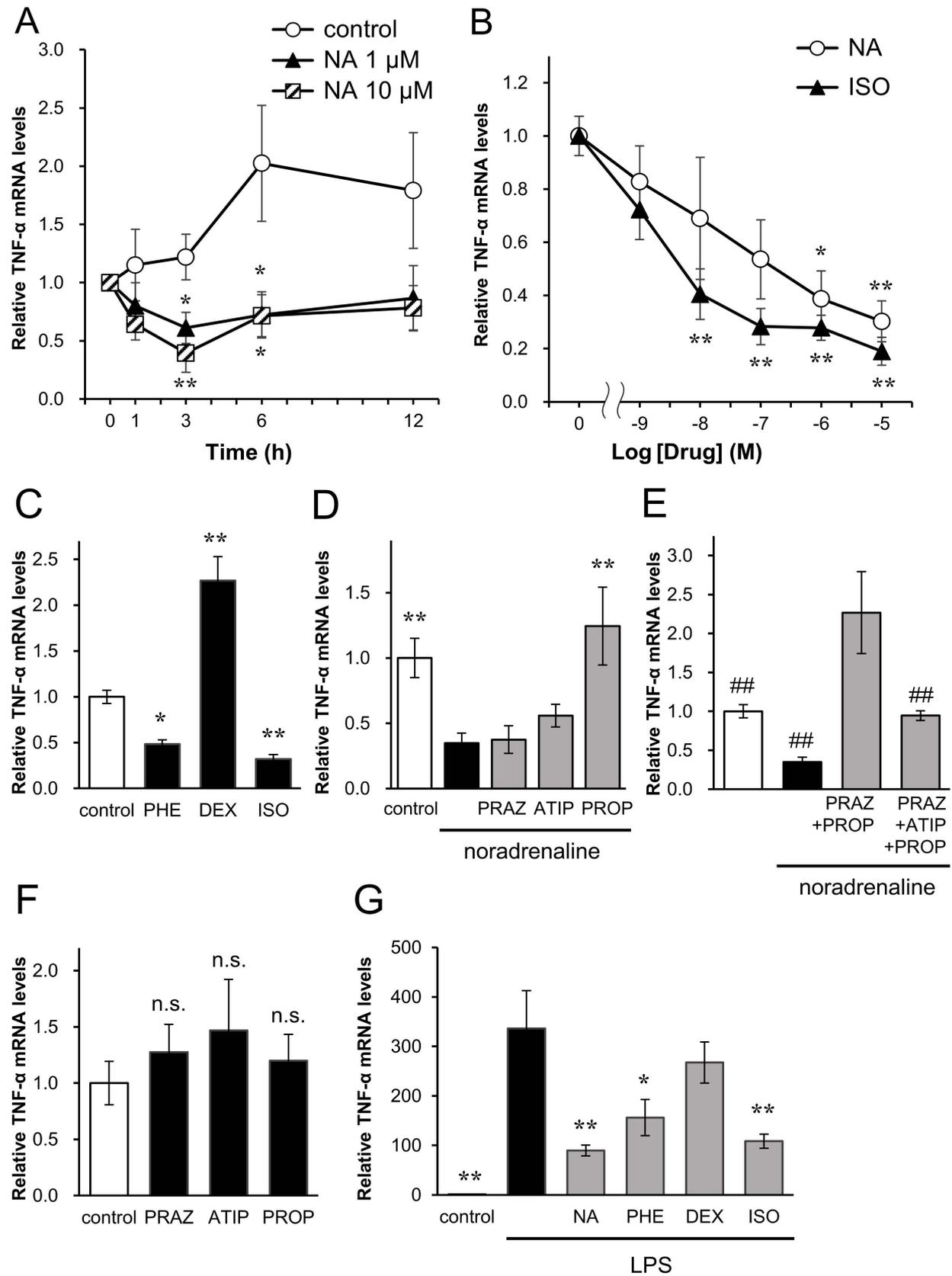
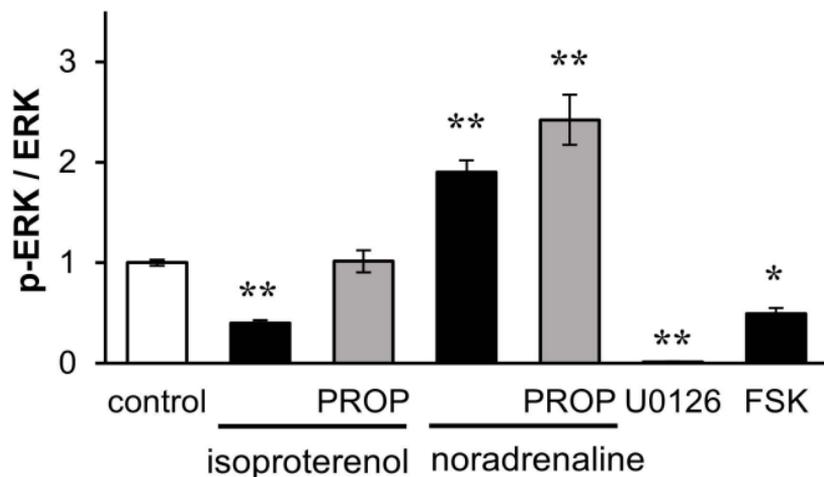
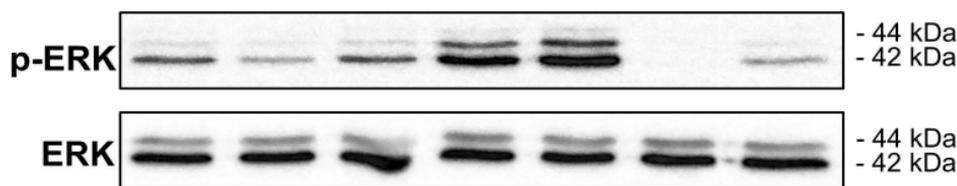
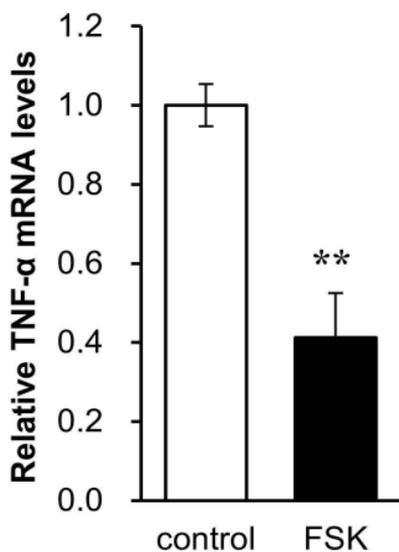
Fig.1

Fig. 2

A



B



C

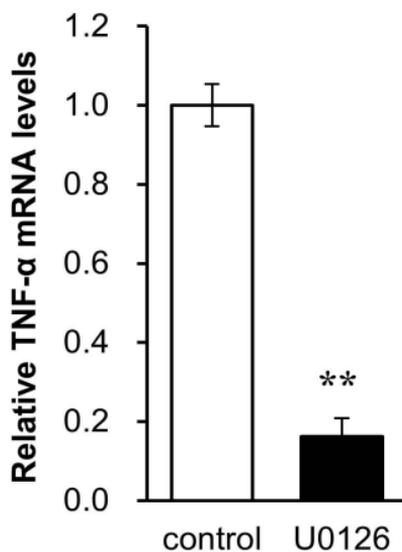


Fig. 3

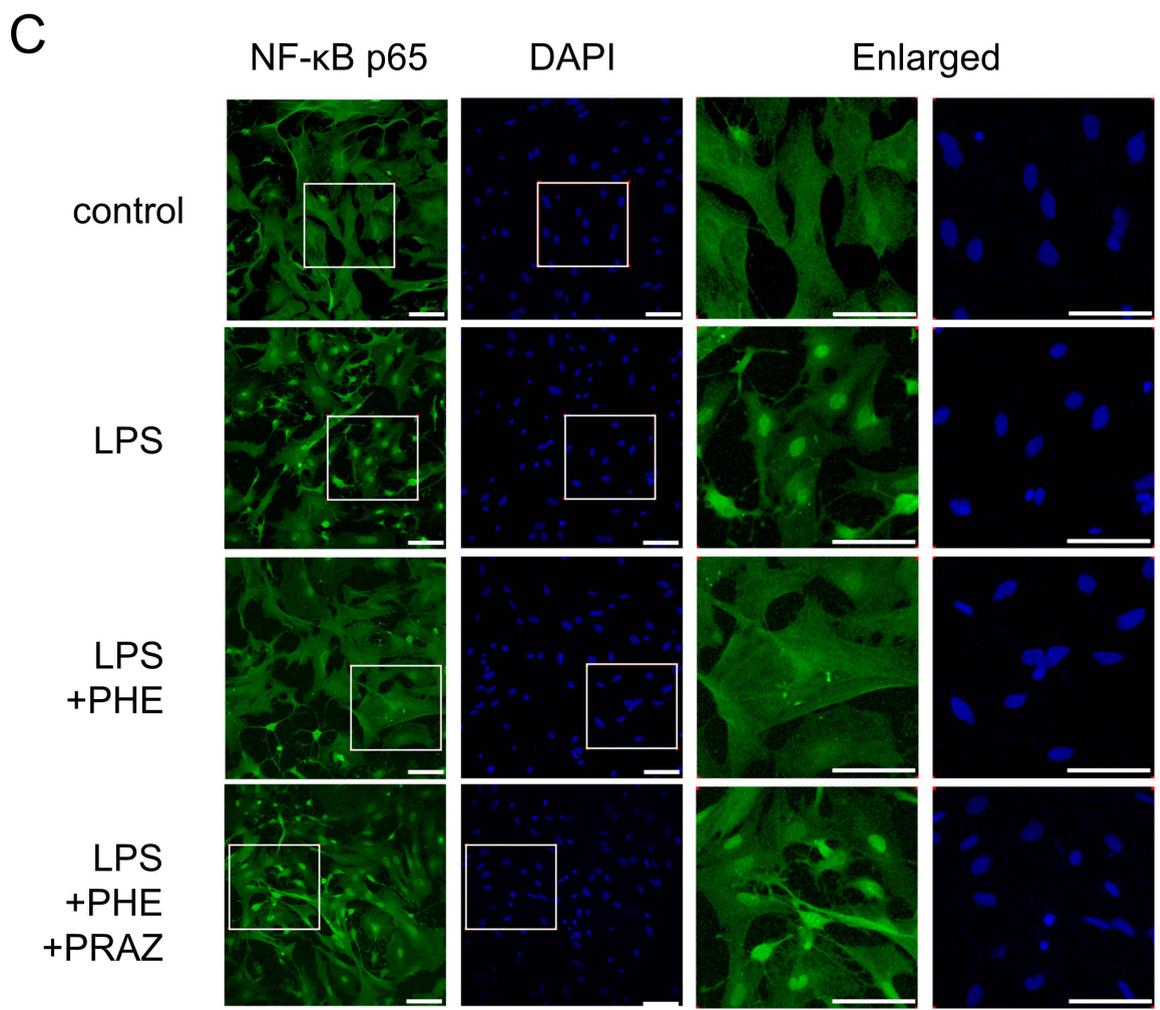
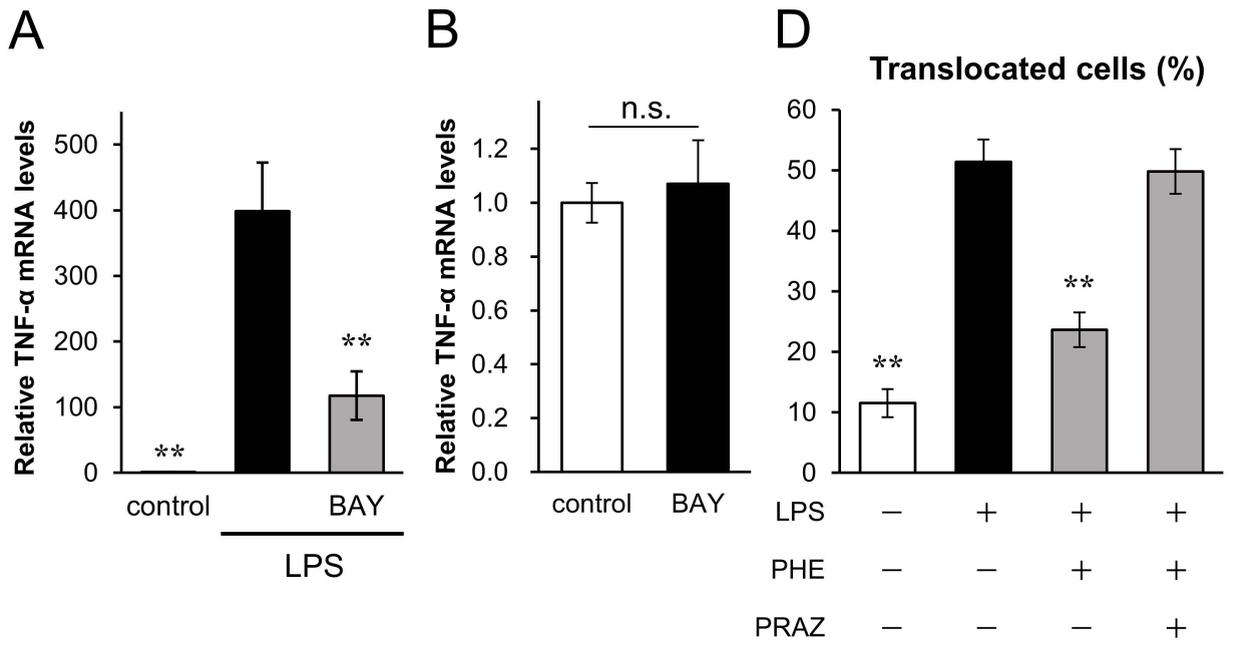
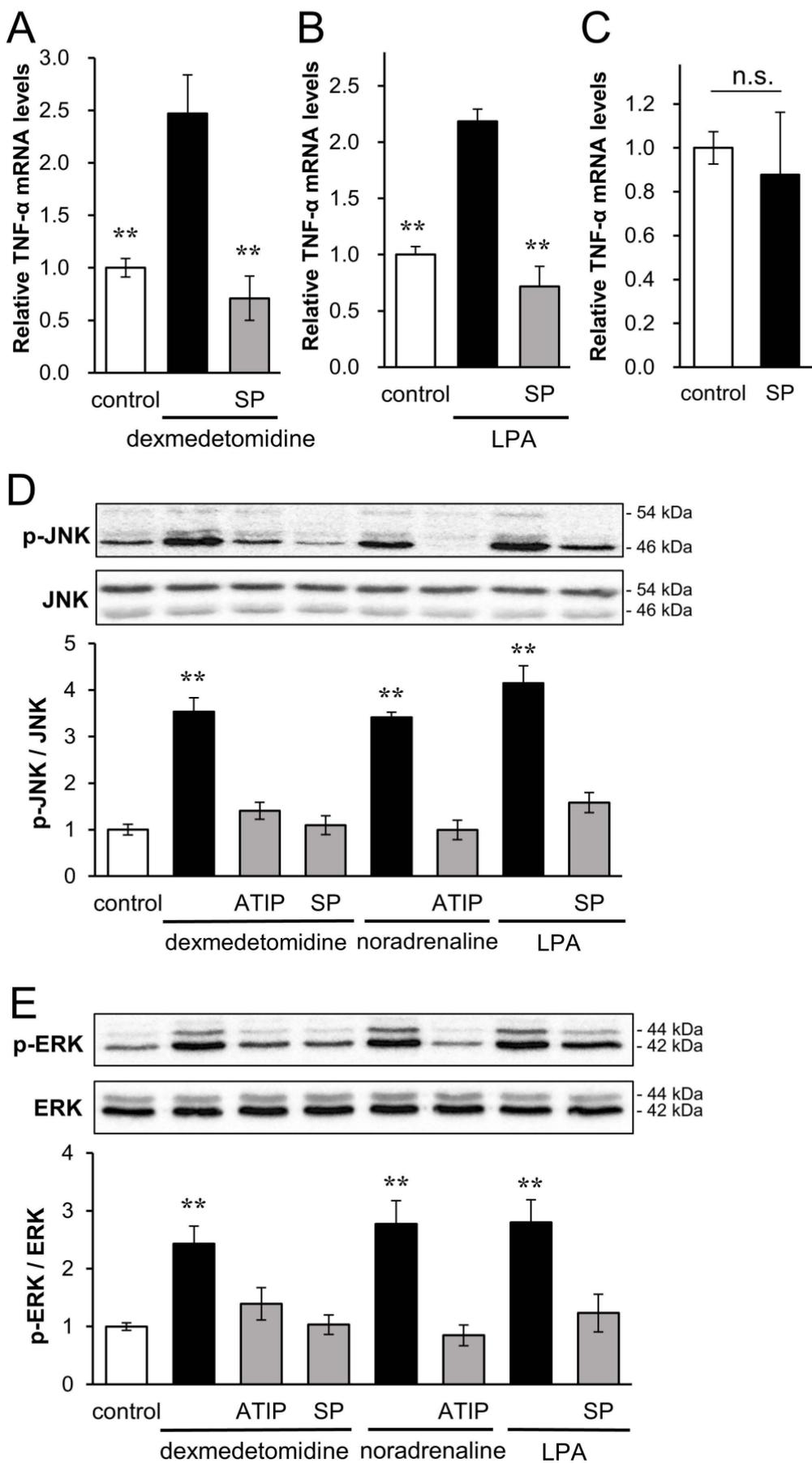


Fig. 4

Supplementary data

1. Materials and methods

1.1. RT-PCR

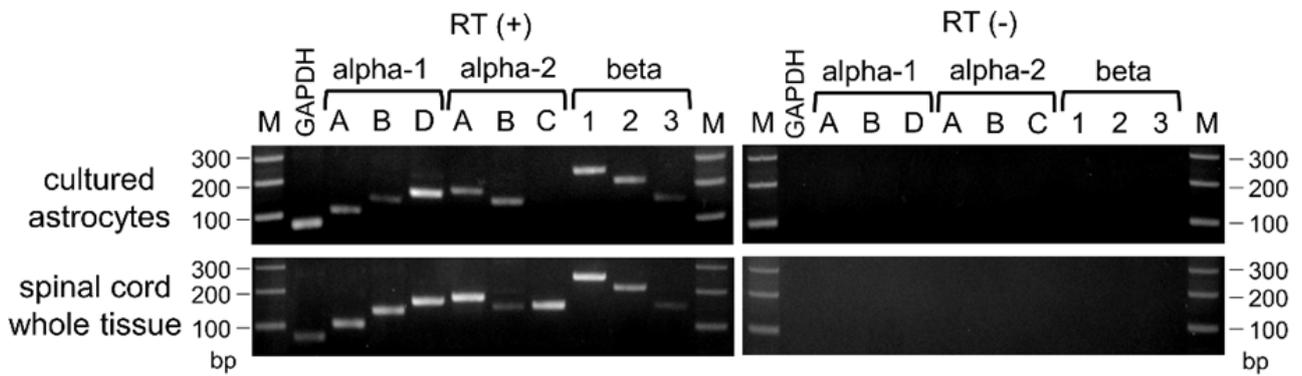
Total RNAs were extracted from cultured astrocytes and spinal cord whole tissue using RNAiso Plus (Takara Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka Japan). PCR was performed using KOD FX Neo (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 (ASTECH, 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'

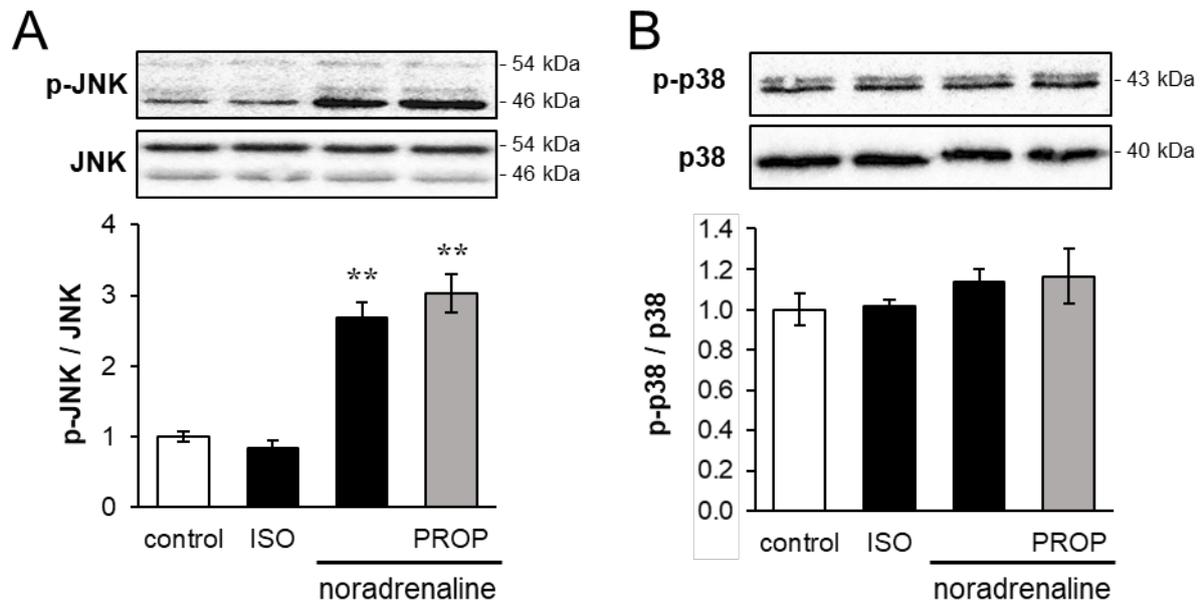
Table S2. Primers used to determine adrenoceptor subtype by RT-PCR.

Target gene	Product size (bp)	Sequence (upper; sense, lower; antisense)
<i>adrenoceptor alpha 1A</i>	104	5'-AGAAGAAAGCTGCCAAGACG-3' 5'-GAAATCCGGGAAGAAAGACC-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'



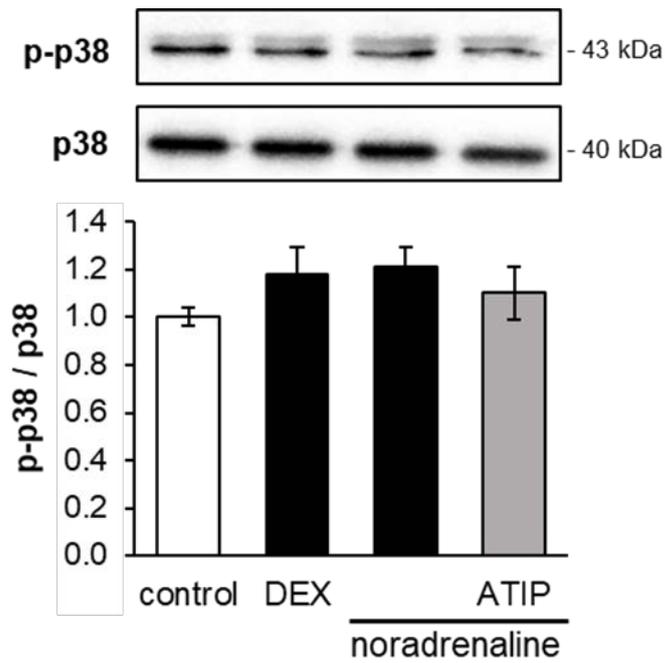
Supplementary Figure S1. Adrenoceptor subtype mRNA expression in the cultured astrocytes

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.