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

Keywords; astrocyte, noradrenaline, adrenoceptors, TNF-α, transcription factor

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-κB nuclear translocation, and both these effects were blocked by phenylephrine. An NF-κB 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-κB 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.

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

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/cm^2. 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 ΔΔCq 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,

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 ± S.E.M (n = number of independent measurements).

Statistical comparisons between two group 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 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 μM) 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 μM) or ISO (1 μM) but increased by the α_2-agonist dexmedetomidine (DEX, 1 μM) (Fig. 1C). The β-antagonist propranolol (10 μM), but not the α_1-antagonist prazosin (1 μM) and the α_2-antagonist atipamezole (10 μM), 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, lypopolysaccharide (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 ß-ARs also regulate TNF-α transcription
168 under pathological conditions.
169
170 **3.2. Transcriptional regulation of TNF-α via ß-adrenoceptors**
171 In general, ß-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 μM) and the
179 MEK/ERK inhibitor U0126 (10 μM) 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).

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

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

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

Next, we elucidated the mechanisms of the DEX-induced increase in the TNF-α mRNA level. The JNK inhibitor SP600125 (10 μM) inhibited the DEX-induced TNF-α 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 μg/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-κB 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 μM. 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 ß-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 ß-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 ß- 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 ß-ARs under normal conditions.

A $ß_2$-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 ß-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

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

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

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

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

Declaration of competing interest

None.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (No.19K23701).

References

[1] S.E. Loughlin, S.L. Foote, R. Grzanna, Efferent projections of nucleus locus coeruleus: Morphologic subpopulations have different efferent targets, Neuroscience. 18 (1986) 307–319. https://doi.org/10.1016/0306-4522(86)90156-9.

[2] H. Matsuoka, T. Suto, S. Saito, H. Obata, Amitriptyline, but Not Pregabalin, Reverses the Attenuation of Noxious Stimulus-Induced Analgesia after Nerve Injury in Rats, Anesth. Analg. 123 (2016) 504–510. https://doi.org/10.1213/ANE.0000000000001301.

[3] O. Borodovitsyna, M. Flamini, D. Chandler, Noradrenergic Modulation of Cognition in Health and Disease, Neural Plast. 2017 (2017) 57–59. https://doi.org/10.1155/2017/6031478.

[4] M.T. Heneka, V. Gavrilyuk, G.E. Landreth, M.K. O'Banion, G. Weinberg, D.L. Feinstein, Noradrenergic depletion increases inflammatory responses in brain: Effects on IκB and HSP70 expression, J. Neurochem. 85 (2003) 387–398. https://doi.org/10.1046/j.1471-4159.2003.01694.x.

[5] B. Nellgård, G. Burkhard Mackensen, S. Sarraf-Yazdi, Y. Miura, R. Pearlstein, D.S. Warner, Pre-ischemic depletion of brain norepinephrine decreases infarct size in normothermic rats exposed to transient focal cerebral ischemia, Neurosci. Lett. 275 (1999) 167–170. https://doi.org/10.1016/S0304-3940(99)00743-0.

[6] D.M. Jurič, D. Lončar, M. Čarman-Kržan, Noradrenergic stimulation of BDNF synthesis in astrocytes: Mediation via α1- and ß1/ß2-adrenergic receptors, Neurochem. Int. 52 (2008) 297–306. https://doi.org/10.1016/j.neuint.2007.06.035.

[7] J.S. Day, E. O'Neill, C. Cawley, N.K. Aretz, D. Kilroy, S.M. Gibney, A. Harkin, T.J. Connor, Noradrenaline acting on astrocytic ß2-adrenoceptors induces neurite outgrowth in primary cortical neurons, Neuropharmacology. 77 (2014) 234–248. https://doi.org/10.1016/j.neuropharm.2013.09.027.

[8] S. Habbas, M. Santello, D. Becker, H. Stubbe, G. Zappia, N. Liaudet, F.R. Klaus, G. Kollias, A. Fontana, C.R. Pryce, T. Suter, A. Volterra, Neuroinflammatory TNFα Impairs Memory via Astrocyte Signaling, Cell. 163 (2015) 1730–1741. https://doi.org/10.1016/j.cell.2015.11.023.

[9] D. Umbriaco, S. Garcia, C. Beaulieu, L. Descarries, Relational features of acetylcholine, noradrenaline, serotonin and GABA axon terminals in the stratum radiatum of adult rat hippocampus (CA1), Hippocampus. 5 (1995) 605–620. https://doi.org/10.1002/hipo.450050611.

[10] S.H. Christiansen, J. Selige, T. Dunkern, A. Rassov, M. Leist, Combined anti-inflammatory effects of ß2-adrenergic agonists and PDE4 inhibitors on astrocytes by upregulation of intracellular cAMP, Neurochem. Int. 59 (2011) 837–846. https://doi.org/10.1016/j.neuint.2011.08.012.

[11] F. Facchinetti, E. Del Giudice, S. Furegato, M. Passarotto, D. Arcidiacono, A. Leon, Dopamine inhibits responses of astroglia-enriched cultures to lipopolysaccharide via a β-adrenoreceptor-mediated mechanism, J. Neuroimmunol. 150 (2004) 29–36. https://doi.org/10.1016/j.jneuroim.2004.01.014.

[12] M. Paukert, A. Agarwal, J. Cha, V.A. Doze, J.U. Kang, D.E. Bergles, Norepinephrine controls astroglial responsiveness to local circuit activity, Neuron. 82 (2014) 1263–1270. https://doi.org/10.1016/j.neuron.2014.04.038.

[13] S.G. Gaidin, V.P. Zinchenko, A.I. Sergeev, I.Y. Teplov, V.N. Mal'tseva, A.M. Kosenkov, Activation of alpha-2 adrenergic receptors stimulates GABA release by astrocytes, Glia. (2019) 1–17. https://doi.org/10.1002/glia.23763.

[14] P. Ernsberger, L. Iacovitti, D.J. Reis, Astrocytes cultured from specific brain regions differ in their expression of adrenergic binding sites, Brain Res. 517 (1990) 202–208. https://doi.org/10.1016/0006-8993(90)91027-E.

[15] L. Schnell, S. Fearn, H. Klassen, M.E. Schwab, V.H. Perry, Acute inflammatory responses to mechanical lesions in the CNS: differences between brain and spinal cord, Eur. J. Neurosci. 11 (1999) 3648–3658. https://doi.org/10.1046/j.1460-9568.1999.00792.x.

[16] H. Yoon, G. Walters, A.R. Paulsen, I.A. Scarisbrick, Astrocyte heterogeneity across the brain and spinal cord occurs developmentally, in adulthood and in response to

339 demyelination, PLoS One. 12 (2017) e0180697.
340 https://doi.org/10.1371/journal.pone.0180697.
341 [17] Y. Sumiya, K. Torigoe, Z. Gerevich, A. Köfalvi, E.S. Vizi, Excessive release of
342 [3H]noradrenaline by veratridine and ischemia in spinal cord, Neurochem. Int. 39
343 (2001) 59–63. https://doi.org/10.1016/S0197-0186(00)00124-8.
344 [18] R. Eguchi, S. Yamaguchi, K. Otsuguro, Fibroblast growth factor 2 modulates
345 extracellular purine metabolism by upregulating ecto-5′-nucleotidase and
346 adenosine deaminase in cultured rat spinal cord astrocytes, J. Pharmacol. Sci. 139
347 (2019) 98–104. https://doi.org/10.1016/j.jphs.2018.12.002.
348 [19] B.H. Singer, R.P. Dickson, S.J. Denstaedt, M.W. Newstead, K. Kim, N.R.
349 Falkowski, J.R. Erb-Downward, T.M. Schmidt, G.B. Huffnagl, T.J. Standiford,
350 Bacterial dissemination to the brain in sepsis, Am. J. Respir. Crit. Care Med. 197
351 (2018) 747–756. https://doi.org/10.1164/rccm.201708-1559OC.
352 [20] J.B. O'Sullivan, K.M. Ryan, N.M. Curtin, A. Harkin, T.J. Connor, Noradrenaline
353 reuptake inhibitors limit neuroinflammation in rat cortex following a systemic
354 inflammatory challenge: Implications for depression and neurodegeneration, Int.
355 J. Neuropsychopharmacol. 12 (2009) 687–699.
356 https://doi.org/10.1017/S146114570800967X.
357 [21] N. Iida, K. Namikawa, H. Kiyama, H. Ueno, S. Nakamura, S. Hattori,

Requirement of Ras for the activation of mitogen-activated protein kinase by calcium influx, cAMP, and neurotrophin in hippocampal neurons, J. Neurosci. 21 (2001) 6459–6466. https://doi.org/10.1523/jneurosci.21-17-06459.2001.

[22] J.M. Schmitt, P.J.S. Stork, PKA phosphorylation of Src mediates cAMP's inhibition of cell growth via Rap1, Mol. Cell. 9 (2002) 85–94. https://doi.org/10.1016/S1097-2765(01)00432-4.

[23] P.A. Baeuerle, T. Henkel, Function and Activation of NF-kappaB in the Immune System, Annu. Rev. Immunol. 12 (1994) 141–179. https://doi.org/10.1146/annurev.iy.12.040194.001041.

[24] E. Shumay, J. Tao, H.Y. Wang, C.C. Malbon, Lysophosphatidic acid regulates trafficking of β2- adrenergic receptors: The Gα13/p115RhoGEF/JNK pathway stimulates receptor internalization, J. Biol. Chem. 282 (2007) 21529–21541. https://doi.org/10.1074/jbc.M701998200.

[25] M.Y.T. Globus, R. Busto, W.D. Dietrich, E. Martinez, I. Valdes, M.D. Ginsberg, Direct evidence for acute and massive norepinephrine release in the hippocampus during transient ischemia, J. Cereb. Blood Flow Metab. 9 (1989) 892–896. https://doi.org/10.1038/jcbfm.1989.123.

[26] M. Strittmatter, M. Grauer, E. Isenberg, G. Hamann, C. Fischer, K.H. Hoffmann, F. Blaes, K. Schimrigk, Cerebrospinal fluid neuropeptides and monoaminergic

377 transmitters in patients with trigeminal neuralgia, Headache. 37 (1997) 211–216.
378 https://doi.org/10.1046/j.1526-4610.1997.3704211.x.
379 [27] W.J.G. Hoogendijk, M.G.P. Feenstra, M.H.A. Botterblom, J. Gilhuis, I.E.C.
380 Sommer, W. Kamphorst, P. Eikelenboom, D.F. Swaab, Increased activity of
381 surviving locus ceruleus neurons in Alzheimer's disease, Ann. Neurol. 45 (1999)
382 82–91. https://doi.org/10.1002/1531-8249(199901)45:1<82::AID-ART14>3.0.CO;2-T.
383 [28] D.G. Winder, K.C. Martin, I.A. Muzzio, D. Rohrer, A. Chruscinski, B. Kobilka,
384 E.R. Kandel, ERK plays a regulatory role in induction of LTP by theta frequency
385 stimulation and its modulation by ß-adrenergic receptors, Neuron. 24 (1999) 715–
386 726. https://doi.org/10.1016/S0896-6273(00)81124-1.
387 [29] J.L. Berkeley, A.I. Levey, Cell-specific extracellular signal-regulated kinase
388 activation by multiple G protein-coupled receptor families in hippocampus, Mol.
389 Pharmacol. 63 (2003) 128–135. https://doi.org/10.1124/mol.63.1.128.
390 [30] L. Qian, X. Hu, D. Zhang, A. Snyder, H.M. Wu, Y. Li, B. Wilson, R.B. Lu, J.S.
391 Hong, P.M. Flood, ß2 adrenergic receptor activation induces microglial NADPH
392 oxidase activation and dopaminergic neurotoxicity through an ERK-
393 dependent/protein kinase A-independent pathway, Glia. 57 (2009) 1600–1609.
394 https://doi.org/10.1002/glia.20873.
395 [31] K. Gharami, S. Das, Delayed but sustained induction of mitogen-activated protein

396 kinase activity is associated with ß-adrenergic receptor-mediated morphological
397 differentiation of astrocytes, J. Neurochem. 88 (2003) 12–22.
398 https://doi.org/10.1046/j.1471-4159.2003.02148.x.
399 [32] E.C. Dresselhaus, M.K. Meffert, Cellular specificity of NF-κB function in the
400 nervous system, Front. Immunol. 10 (2019).
401 https://doi.org/10.3389/fimmu.2019.01043.
402 [33] Y. Deng, D. Xie, M. Fang, G. Zhu, C. Chen, H. Zeng, J. Lu, K. Charanjit,
403 Astrocyte-Derived Proinflammatory Cytokines Induce Hypomyelination in the
404 Periventricular White Matter in the Hypoxic Neonatal Brain, PLoS One. 9 (2014)
405 e87420. https://doi.org/10.1371/journal.pone.0087420.
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 μM) 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 μM) or the ß-agonist isoproterenol (ISO, 1 nM-10 μM) 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, 1μM), the α_2-agonist dexmedetomidine (DEX, 1 μM), and ISO (1 μM) 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 μM) in the presence or absence of the α_1-antagonist prazosin (PRAZ, 1 μM), the α_2-antagonist atipamezole (ATIP, 10 μM), and the ß-antagonist propranolol (PROP, 10 μM) 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 ± S.E.M. n = 6.

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

adrenoceptors.

(A) The protein expression levels of phosphorylated or total ERK were quantified and representative blots are shown. Astrocytes were treated with isoproterenol (1 μM), noradrenaline (1 μM), the MEK/ERK inhibitor U0126 (10 μM), or the adenylyl cyclase activator forskolin (FSK, 10 μM) in the presence or absence of the ß-antagonist propranolol (PROP, 10 μM) 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 ± 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-κB inhibitor BAY-11-7082 (BAY, 1 μM) 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 μm. Astrocytes were treated with LPS in the presence or absence of the α_1-agonist phenylephrine (PHE, 1 μM) and the α_1-antagonist prazosin (PRAZ, 1 μM) 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

test). Data are presented as means ± S.E.M. n = 6.

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

(A, B) TNF-α mRNA levels in astrocytes treated with the α_2-agonist dexmedetomidine (1 μM, A) and LPA (10 μg/ml, B) in the presence or absence of the JNK inhibitor SP600125 (SP, 10 μM) for 3 h. **p < 0.01 vs. dexmedetomidine or LPA alone (Dunnett's test). (C) TNF-α mRNA levels in astrocytes treated with SP for 4h. n.s. = not significant (unpaired Student's t-test). (D,E) The protein expression levels of phosphorylated or total JNK (D) and ERK (E) was quantified and representative blots are shown. Astrocytes were treated with dexmedetomidine, noradrenaline (NA, 1 μM), or LPA in the presence or absence of the α_2-antagonist atipamezole (ATIP, 10 μM) and SP for 10 min. **p < 0.01 vs. control (Dunnett's test). Data are presented as means ± 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-κB.
- α_2-Adrenoceptors mediated TNF-α mRNA increase via the JNK/ERK pathway.

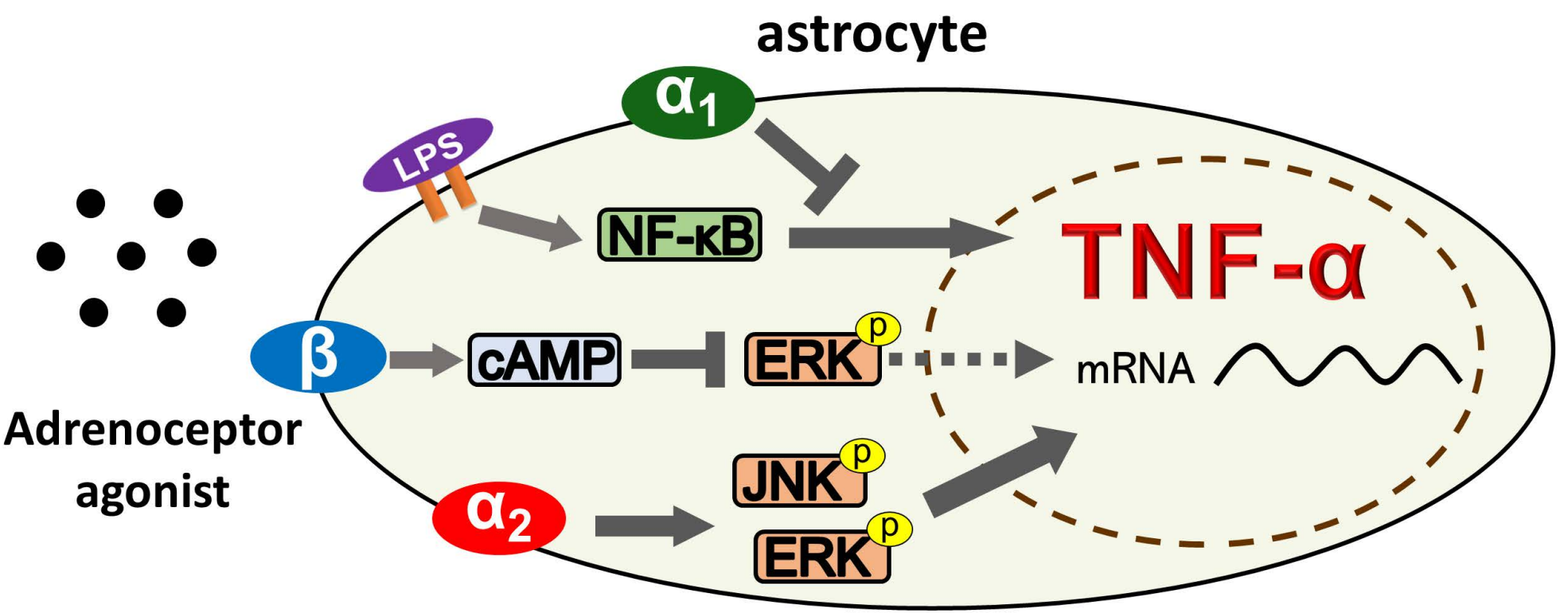

astrocyte
α1
LPS
NF-κB
TNF-α
β
cAMP
ERK
p
mRNA
Adrenoceptor
agonist
JNK
p
α2
ERK
p

Fig.1

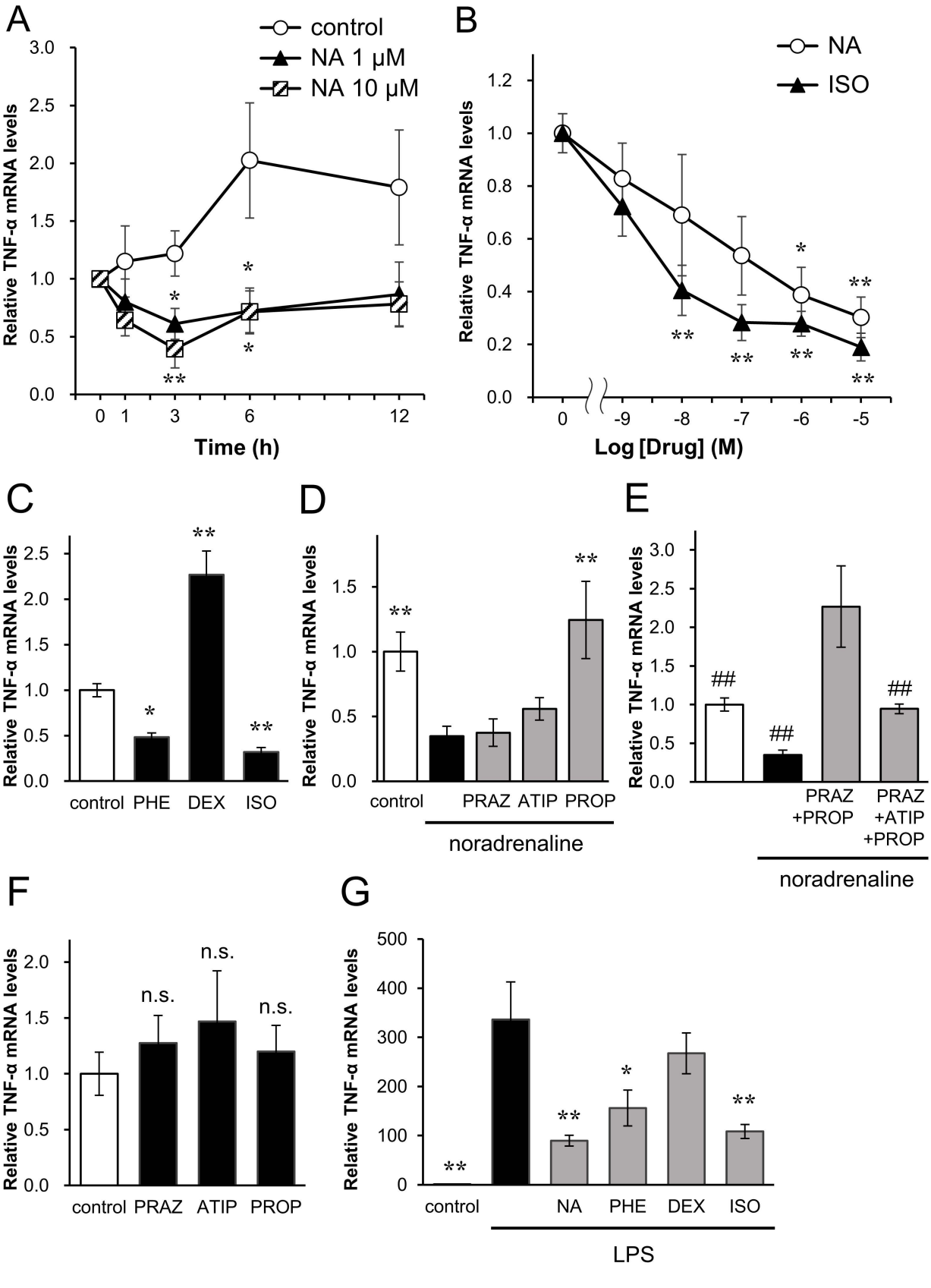

A
control
NA 1 μM
NA 10 μM
Relative TNF-α mRNA levels
Time (h)
B
NA
ISO
Relative TNF-α mRNA levels
Log [Drug] (M)
C
Relative TNF-α mRNA levels
control
PHE
DEX
ISO
D
Relative TNF-α mRNA levels
control
PRAZ
ATIP
PROP
noradrenaline
E
Relative TNF-α mRNA levels
PRAZ +PROP
PRAZ +ATIP +PROP
noradrenaline
F
Relative TNF-α mRNA levels
n.s.
control
PRAZ
ATIP
PROP
G
Relative TNF-α mRNA levels
control
NA
PHE
DEX
ISO
LPS

Fig. 2

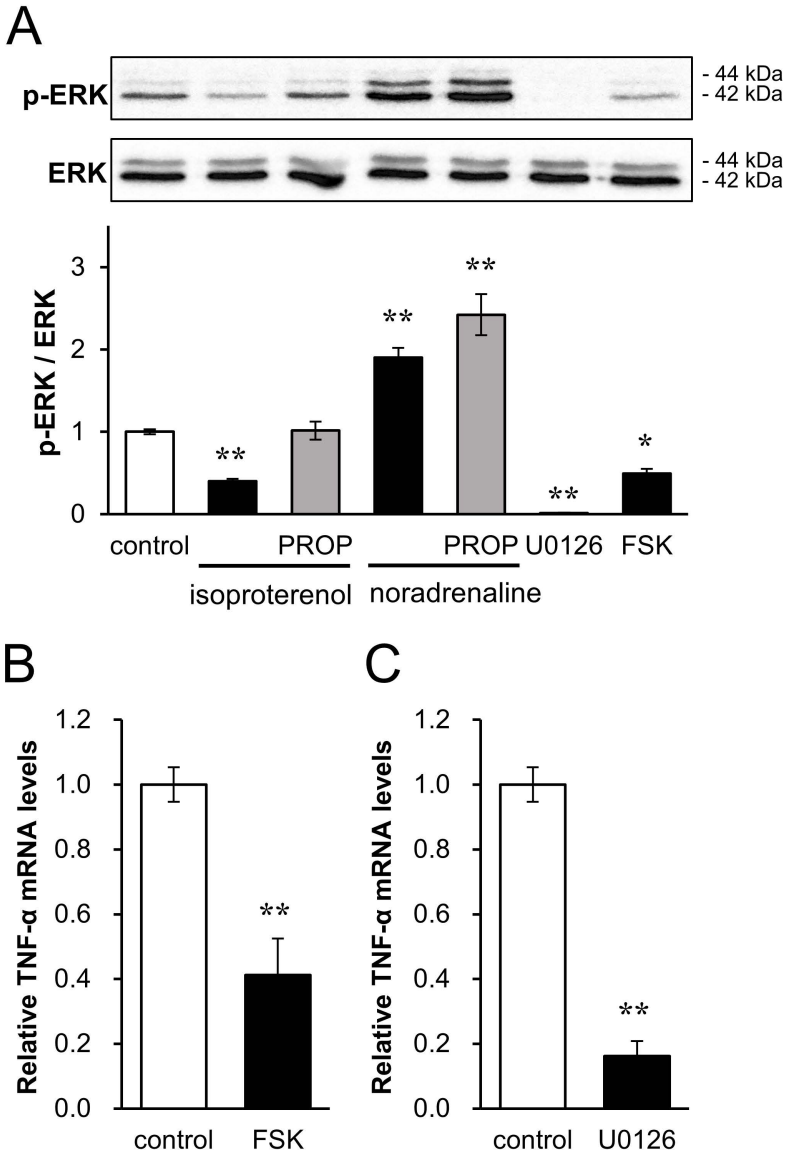

A
p-ERK
ERK
- 44 kDa
- 42 kDa
- 44 kDa
- 42 kDa
p-ERK / ERK
3
2
1
0
**
**
**
**
*
control
PROP
PROP
U0126
FSK
isoproterenol
noradrenaline
B
Relative TNF-α mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
control
FSK
C
Relative TNF-α mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
**
control
U0126

Fig. 3

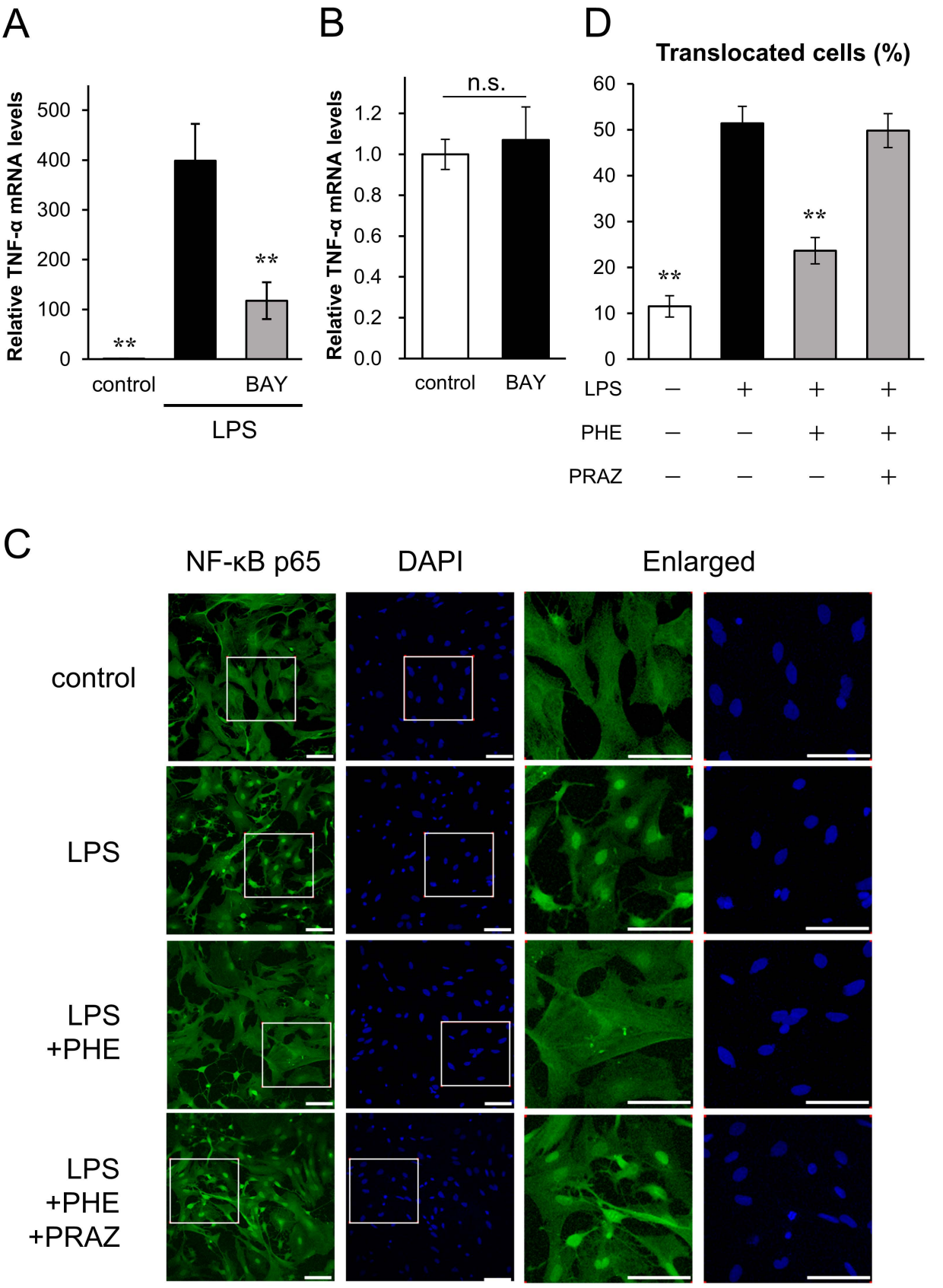
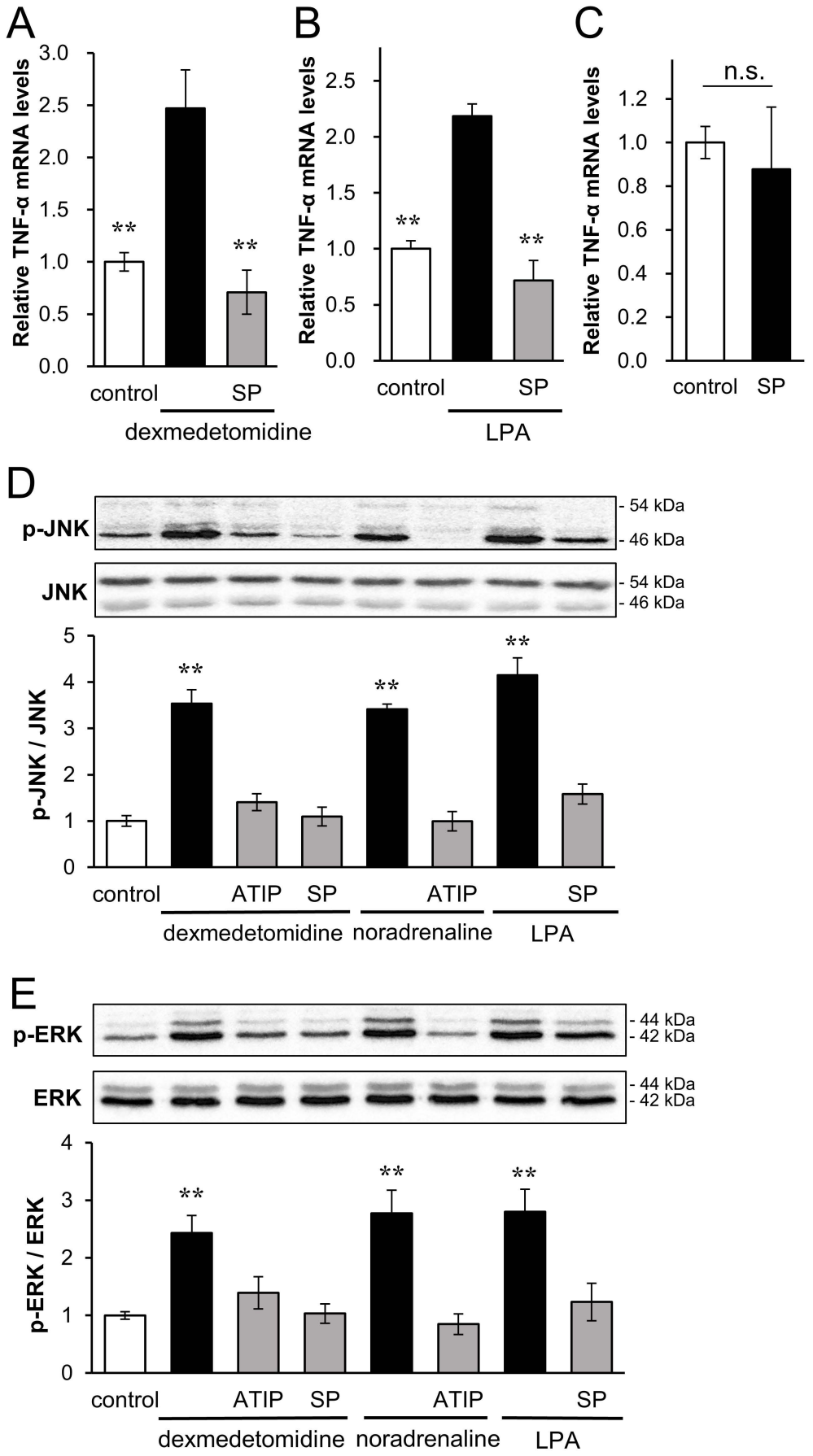

A
Relative TNF-α mRNA levels
500
400
300
200
100
0
**
control
BAY
LPS
B
Relative TNF-α mRNA levels
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
control
BAY
D
Translocated cells (%)
60
50
40
30
20
10
0
**
**
LPS – + + +
PHE – – + +
PRAZ – – – +
C
NF-κB p65
DAPI
Enlarged
control
LPS
LPS +PHE
LPS +PHE +PRAZ

Fig. 4


A
B
C
Relative TNF-α mRNA levels
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
control
SP
dexmedetomidine
2.5
2.0
1.5
1.0
0.5
0.0
**
**
control
SP
LPA
1.2
1.0
0.8
0.6
0.4
0.2
0.0
n.s.
control
SP
D
p-JNK
JNK
- 54 kDa
- 46 kDa
- 54 kDa
- 46 kDa
p-JNK / JNK
5
4
3
2
1
0
**
**
**
control
ATIP
SP
ATIP
SP
dexmedetomidine
noradrenaline
LPA
E
p-ERK
ERK
- 44 kDa
- 42 kDa
- 44 kDa
- 42 kDa
p-ERK / ERK
4
3
2
1
0
**
**
**
control
ATIP
SP
ATIP
SP
dexmedetomidine
noradrenaline
LPA

Supplementary data

1. Materials and methods

1.1. RT-PCR

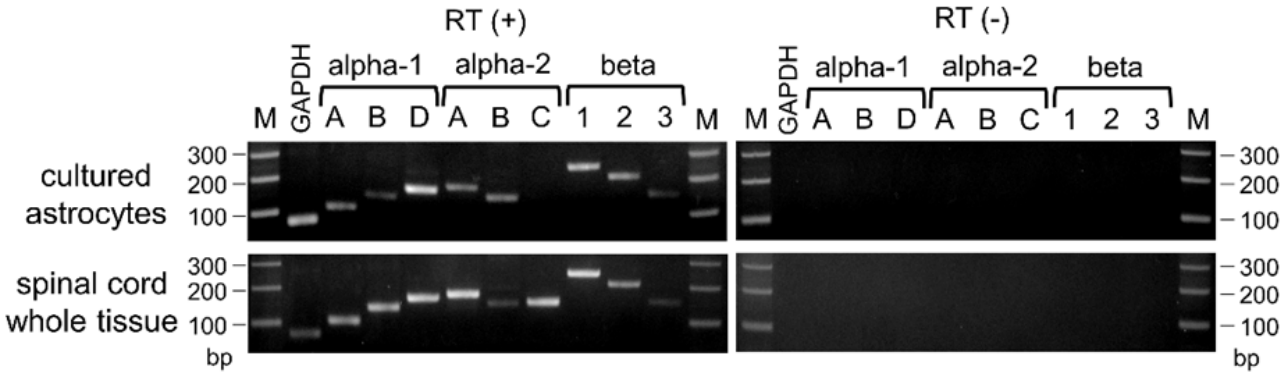
Total RNAs were extracted from cultured astrocytes and spinal cord whole tissue using RNAiso Plus (Takara Bio, Tokyo, Japan). To remove genomic DNA and synthesize cDNA, the RNA sample was then incubated with qPCR RT Master Mix with gDNA Remover (TOYOBO, Osaka Japan). PCR was performed using KOD FX Neo (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)
tumor necrosis factor	125	5'-CATGAGCACGGAAAGCATGA-3' 5'-CCACGAGCAGGAATGAGAAGA-3'
glyceraldehyde -3-phosphate dehydrogenase	74	5'-GCAAGAGAGAGGCCCTCAG-3' 5'-TGTGAGGGAGATGCTCAGTG-3'

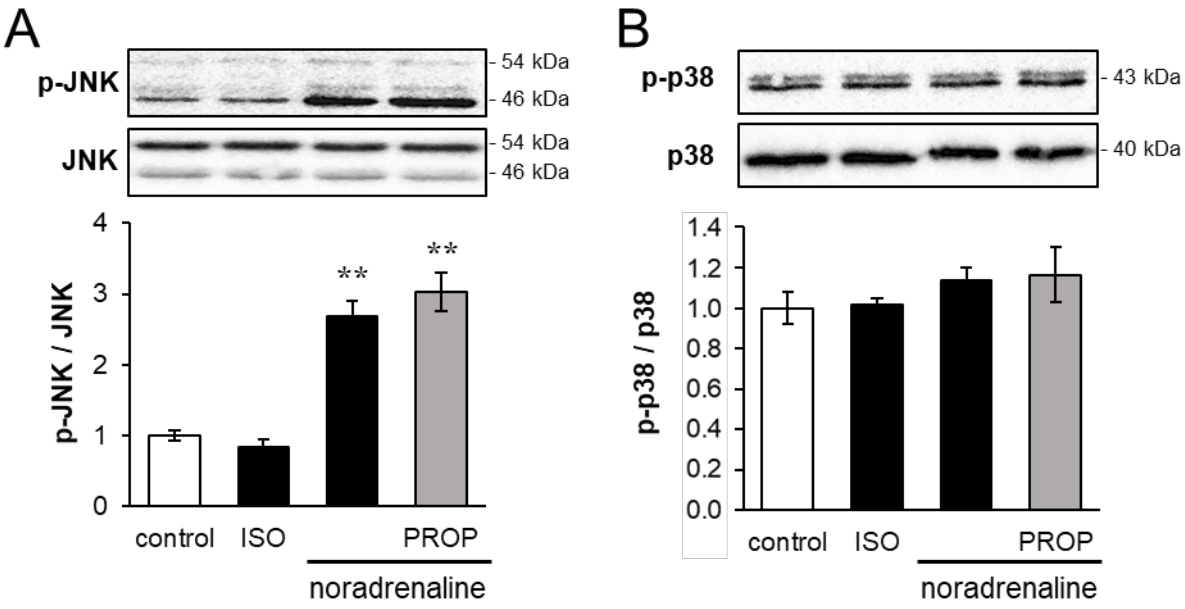
Table S2. Primers used to determine adrenoceptor subtype by RT-PCR.

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



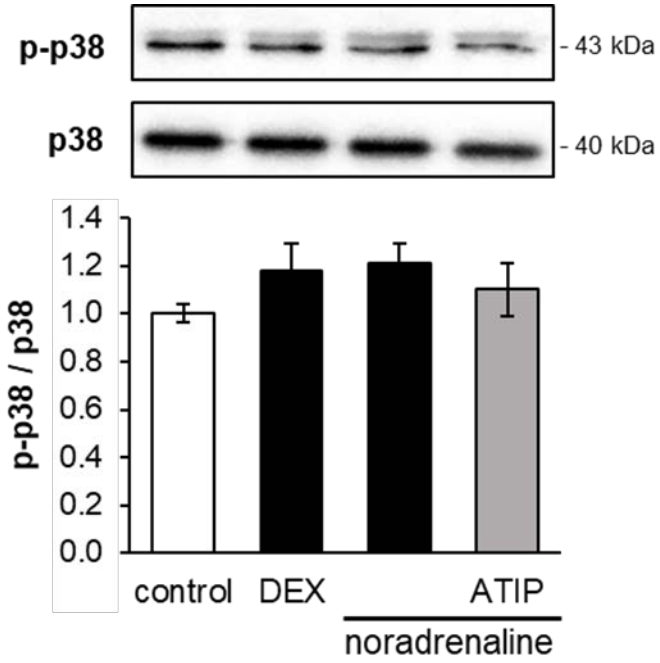
Supplementary Figure S1. Adrenoceptor subtype mRNA expression in the cultured astrocytes

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 ß-agonist isoproterenol (ISO, 1 μM), or noradrenaline (1 μM) in the presence or absence of propranolol (PROP, 10 μM) for 10 min. **$p < 0.01$ vs. control (Dunnett's test). Data are presented as means ± 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 μM), or noradrenaline (1 μM) in the presence or absence of atipamezole (ATIP, 10 μM) for 10 min. n = 6. Data are presented as means ± S.E.M.