Available online at www.sciencedirect.com
ScienceDirect
Neuroscience Letters 412 (2007) 163–167
Neuroscience Letters
www.elsevier.com/locate/neulet

Yohimbine prevents morphine-induced changes of glial fibrillary acidic protein in brainstem and α_2-adrenoceptor gene expression in hippocampus

Elba Alonso [a], Elisa Garrido [a], Carmen Díez-Fernández [a], Carmen Pérez-García [a], Gonzalo Herradón [a,b,*], Laura Ezquerra [b], Thomas F. Deuel [b], Luis F. Alguacil [a]

[a] *Lab. Pharmacology and Toxicology, Universidad San Pablo CEU, 28668 Boadilla del Monte, Madrid, Spain*
[b] *Department of Molecular and Experimental Medicine and Cell Biology, The Scripps Research Institute, La Jolla, CA, USA*

Received 11 September 2006; received in revised form 1 November 2006; accepted 1 November 2006

Abstract

The α_2-adrenoceptor antagonist yohimbine is known to oppose to several pharmacological effects of opioid drugs, but the consequences and the mechanisms involved remain to be clearly established. In the present study we have checked the effects of yohimbine on morphine-induced alterations of the expression of key proteins (glial fibrillary acidic protein, GFAP) and genes (α_2-adrenoceptors) in rat brain areas known to be relevant in opioid dependence, addiction and individual vulnerability to drug abuse. Rats were treated with morphine in the presence or absence of yohimbine. The effects of the treatments on GFAP expression were studied by immunohistochemical staining in Locus Coeruleus (LC) and Nucleus of the Solitary Tract (NST), two important noradrenergic nuclei. In addition, drug effects on α_2-adrenoceptor gene expression were determined by real time RT-PCR in the hippocampus, a brain area that receives noradrenergic input from the brainstem. Morphine administration increased GFAP expression both in LC and NST as it was previously reported in other brain areas. Yohimbine was found to efficiently prevent morphine-induced GFAP upregulation. Chronic (but not acute) morphine downregulated mRNA levels of α_{2A}- and α_{2C}-adrenoceptors in the hippocampus, while simultaneously increased the expression of the α_{2B}-adrenoceptor gene. Again, yohimbine was able to prevent morphine-induced changes in the levels of expression of the three α_2-adrenoceptor genes. These results correlate the well-established reduction of opioid dependence and addiction by yohimbine and suggest that this drug could interfere with the neural plasticity induced by chronic morphine in central noradrenergic pathways.

Keywords: Morphine; Yohimbine; Glial fibrillary acidic protein; α_2-Adrenoceptor; Locus coeruleus; Hippocampus

Opioid drugs produce neuroadaptations in the brain leading to tolerance, dependence and addiction. These changes seem to be related to neural plasticity and are accompanied by the upregulation of several key proteins in the target cells, i.e. glial fibrillary acidic protein (GFAP) [17]. As a consequence, several neurotransmitter systems become functionally affected by chronic opioids. This is the case of the α_2-adrenergic system, which has been shown to be deeply involved in the pharmacological effects of opioid drugs (see review [1]). Yohimbine, a selective α_2-adrenoceptor antagonist, is known to prevent the development of behavioural dependence induced by opioids in rats and mice [11,12,22], to reduce opioid-seeking behaviour in rats [16] and to reduce the symptoms of naloxone-precipitated withdrawal in methadone maintained patients [8], suggesting that adaptations of α_2-adrenoceptor function may play a critical role in opioid dependence and addiction. Furthermore, we recently described that yohimbine prevents the upregulation of the levels of expression of GFAP induced by chronic (but not acute) morphine administration in several brain areas related to drug reinforcement [7]. The present work further examines the link between morphine dependence and α_2-adrenoceptor function by studying possible adaptations of central noradrenergic pathways originating in the brainstem and projecting to the hippocampus, where α_2-adrenoceptors play a role in the regulation of the release of noradrenaline and other neurotransmitters [4]. We have studied the effects of morphine alone or combined with

* Corresponding author at: Lab. Pharmacology and Toxicology, Universidad San Pablo CEU, Urb. Montepríncipe, 28668 Boadilla del Monte, Madrid, Spain. Tel.: +34 91 3724700; fax: +34 91 3510475.
E-mail address: gherradon@yahoo.es (G. Herradón).

0304-3940/$ – see front matter © 2006 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.neulet.2006.11.002

yohimbine on GFAP expression in cells of the Locus Coeruleus (LC) and the Nucleus of the Solitary Tract (NST), two brain areas that send ascending and descending noradrenergic projections functionally related to the emergence of the physical manifestations of opioid withdrawal [23]. LC noradrenergic projections to the hippocampus are particularly important since they have been suggested to play a role in determining vulnerability to develop drug seeking behaviours, as shown in comparative studies with different rat strains [9]. Therefore, we have studied if LC changes after repeated opioid exposure are paralleled by yohimbine-sensitive adaptations of hippocampal noradrenergic synapses, through quantification of the levels of α_2-adrenoceptor gene expression in this brain area.

Male Sprague–Dawley rats (San Pablo CEU University breeding) weighing 300–350 g were used. The animals were housed under controlled environmental conditions (22 °C and a 12-h light/12-h dark cycle) with free access to food and water. All experiments were carried out in accordance with the European Communities Council Directive (86/609/EEC) for the care and use of laboratory animals. For the chronic treatment with morphine, the rats were injected i.p. every 12 h during 14 days with 10 mg/kg of morphine sulphate (Alcaliber, Madrid, Spain). Control rats received saline i.p. yohimbine hydrochloride (2 mg/kg i.p.; Sigma, Spain) or saline was injected 30 min before each morphine or saline administration ($n = 3$–5/experimental group).

The animals were sacrificed 12 h after the last injection and perfused transcardially with 0.9% saline containing 10% $NaNO_2$ followed by a fixative solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4). Coronal sections (40 μm) were sampled from brain levels corresponding to LC and NST (−10.04 and −11.60 mm, respectively, in relation to bregma, according to Paxinos and Watson [19] by using a vibratome (VT1000M, Leica)).

After endogenous peroxidase blocking by 0.3% H_2O_2 in methanol, the sections were washed with PBS and placed in a blocking solution containing 5% normal goat serum (NGS) and 0.1% Triton X-100 in PBS (STPBS) for 30 min. Sections were then incubated overnight at 4 °C with rabbit anti-GFAP antibody (1:1000 in STPBS; Chemicon, Temecula, CA, USA) and processed using a Vectastain Elite ABC kit (Vector, Burlingame, CA, USA). Finally, the peroxidase was visualized using a Vector 3,3′-diaminobenzidine (DAB) substrate kit. Immunohistochemical controls were performed as above except for the omission of the primary antibody. No positive immunostaining was found in any controls. The sections were then mounted on gelatin-coated slides, air dried, dehydrated through graded alcohols, cleared in xylene and coverslipped with DPX (Sigma, Spain).

Photomicrographs were captured with a digital camera coupled to an optical microscope (DMLS, Leica, Solms, Germany). The counting of GFAP-positive cells was accomplished with the help of the software Scion Image 4.02 (Scion Corporation, Frederick, MD, USA). The mean was obtained from two sections per animal for each region examined.

Statistical differences were determined using ANOVA followed by Newman–Keuls post hoc test. Comparisons with $p < 0.05$ were considered significant.

Animals and housing conditions for α_2-adrenoceptor gene expression studies were similar to those explained above. The rats were i.p. treated with acute morphine (50 mg/kg), chronic morphine or equivalent injections of saline. Chronic morphine treatment consisted of two daily injections according to the following schedule: days 1 and 2: 2 × 10 mg/kg; days 3 and 4: 2 × 20 mg/kg; days 5 and 6: 2 × 30 mg/kg; days 7 and 8: 2 × 40 mg/kg; days 9 and 10: 2 × 50 mg/kg; day 11, 1 × 50 mg/kg. Thirty minutes prior to each of these injections, the rats were pre-treated either with yohimbine (2 mg/kg) or saline ($n = 3$/experimental group). Animals were sacrificed 2 h after the last injection, the hippocampus dissected and frozen until the analysis of gene expression as follows.

Frozen tissues were homogenized in 1 ml TRIZOL reagent (Invitrogen, Carlsbad, CA) per 50–100 mg tissue and total RNA extracted following the manufacturer's protocol. The concentration of RNA in each sample was measured by A_{260} and RNA integrity confirmed in 1.25% agarose gels after electrophoresis. RNA samples were treated with a preparation of DNAses (Ambion, Austin, TX) following manufacturer's protocol.

Complementary DNAs were synthesized from total RNA using a cDNA synthesis kit (BIO-RAD, Hercules, CA, USA). The SYBR green RT-PCR method (BIO-RAD, Hercules, CA, USA) was used, as previously described [6,10], to determine the relative expression of α_{2A}-adrenoceptor, α_{2B}-adrenoceptor and α_{2C}-adrenoceptor mRNAs in the different tissues. The following primer sets (forward and reverse) were used:

α_{2A}-adrenoceptor:
(5′-CTGTTCACCGTGTTTGGCAAC-3′; 5′-AAAGGGAATGACCAGCGTGG-3′);
α_{2B}-adrenoceptor:
(5′-TGTCATTTCTCTACCGCCCCTC-3′; 5′-ATCCGATGCTGGAAGCCAAG-3′);
α_{2C}-adrenoceptor:
(5′-TGTGTGCCATTACCCTCCAC-3′; 5′-AGATGACAGCCGAGATGAGC-3′).

The relative expression of each gene was normalized against GAPDH (5′-TTCAACGGCACAGTCAAGGC-3′; 5′-CACCAGCATCACCCCATTTG-3′), the reference standard, as described by the manufacturer's user bulletin #2 of ABI prism 7000 Sequence Detection System (Applied Biosystems, Foster City, CA).

As we have previously shown in other brain areas using the same experimental protocol [7], chronic administration of morphine induced a marked increase in GFAP immunostaining compared to saline controls in LC (Fig. 1A) and NST (Fig. 1B). Moreover, yohimbine alone did not influence GFAP expression but markedly reduced morphine-induced GFAP upregulation (Fig. 1). LC noradrenergic projections to the hippocampus are critically important in the development of drug seeking behaviours, therefore we further tested the ability of morphine alone and in combination with yohimbine to regulate the expression levels of the different α_2-adrenoceptor subtypes in the hippocampus. A single injection of a high dose of morphine

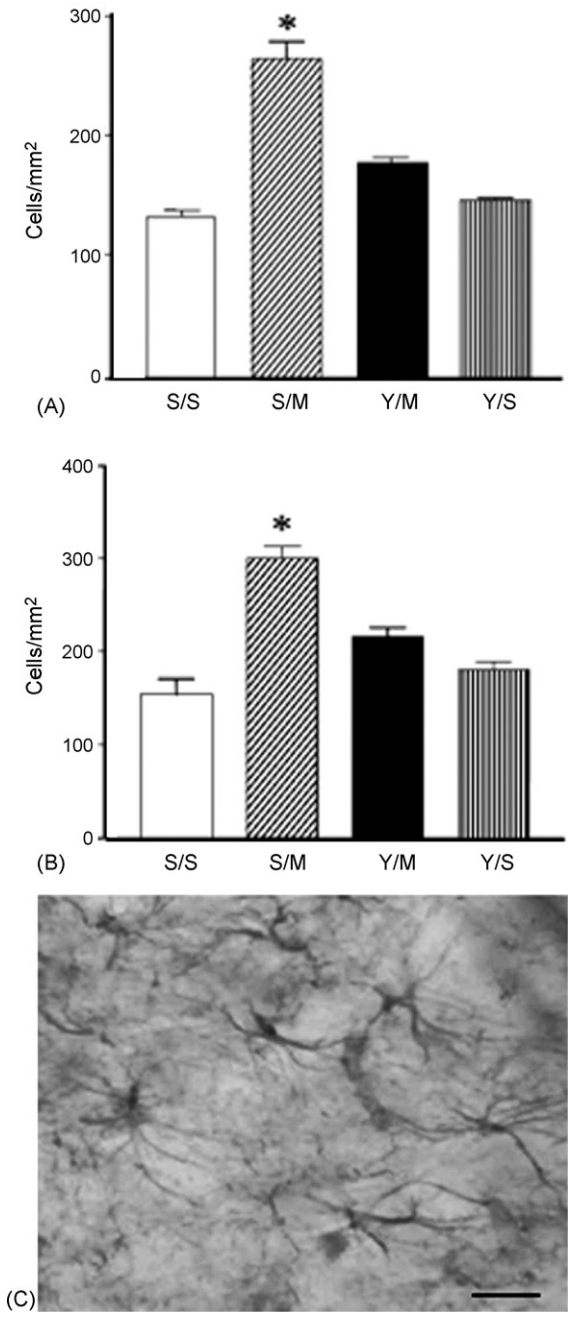

Fig. 1. (A) GFAP immunostaining in the Locus Coeruleus (LC) and (B) the Nucleus of the Solitary Tract (NST) of rats chronically treated with morphine (M) or saline (S). The animals also received before each saline or morphine injection a pre-treatment with yohimbine (groups Y/M and Y/S) or saline (groups S/M, S/S). (C) Representative photomicrograph of GFAP immunostaining in LC. Scale bar = 20 μm. Results are expressed as mean ± S.E.M. of three determinations. $^{*}p < 0.05$ vs. S/S.

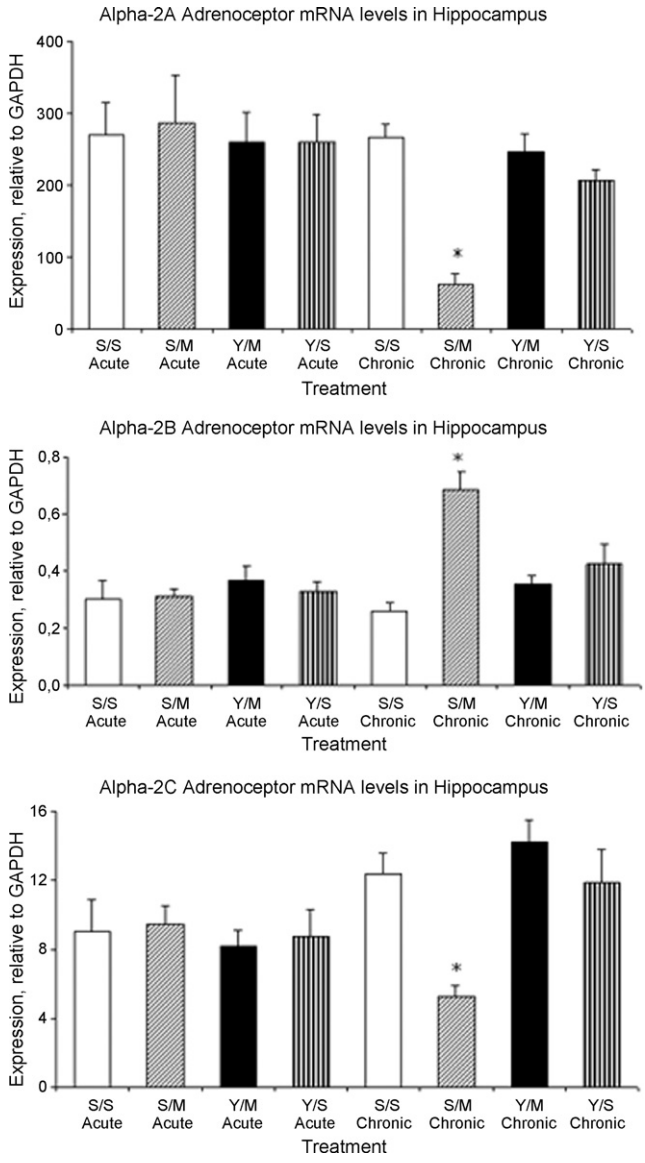

Fig. 2. α_2-Adrenoceptor gene expression in the hippocampus of rats treated with acute or chronic morphine (M) or saline (S). The animals also received before each saline or morphine injection a pre-treatment with yohimbine (groups Y/M and Y/S) or saline (groups S/M, S/S). Results are expressed as mean ± S.E.M. of three determinations. $^{*}p < 0.05$ vs. S/S.

did not change α_2-adrenoceptor gene expression in this brain area (Fig. 2), but chronic morphine provoked a significant downregulation of α_{2A}-adrenoceptor (72% reduction) and α_{2C}-adrenoceptor (62% reduction) gene expression together with a 2.6-fold increase of α_{2B}-adrenoceptor mRNA; all these changes were prevented by yohimbine cotreatment at doses that did not provoke any effect by themselves on the expression levels of α_2-adrenoceptors (Fig. 2).

Our results extend previous evidence that chronic morphine administration produces GFAP overexpression in the rat central nervous system, an effect already described in the spinal cord, ventral tegmental area, nucleus accumbens, frontal cortex and posterior cingulate cortex [3,7,17,20,21]. Although the biological function of GFAP remains poorly understood, the protein is consistently upregulated in gliosis and astrocyte hypertrophy [15], and it has been suggested that these cells could be playing a significant role in opiate tolerance and the induction of neural plasticity [3,17]. GFAP upregulation in LC and NST could be then considered a neurochemical correlate of opioid physical dependence and/or neurotoxicity, since these brain areas are closely related to the manifestations of opioid withdrawal [23]. Importantly, it has been shown that the opiate induction of GFAP expression in the mesolimbic dopamine system is significantly

greater in Lewis rats when compared to F344 rats [3]; taking into account that the former strain is much more prone to develop opioid addiction than the latter, a higher upregulation of GFAP expression induced by opioids was related with greater opioid dependence in that study [3].

It is interesting to note that a link between increased GFAP expression and changes in α_2-adrenoceptor binding after brain injury has been suggested [5]. Thus, morphine-induced GFAP upregulation could affect noradrenergic projections to the hippocampus, where α_2-adrenoceptor gene expression was profoundly altered after chronic morphine injection. Interestingly, the relative predominance of α_{2B} gene expression under these conditions is similar to that found in the hippocampus of Lewis rats compared to F344 [9] suggesting that upregulation of α_{2B}-adrenoceptor gene expression in the hippocampus could be related to the development of opioid dependence and addiction.

This suggestion may be important, however functional confirmation is required. The task is imposing since the knowledge of α_2-adrenoceptor physiology has improved through the use of knockout mice, but selective antagonists of every α_2-adrenoceptor subtype are needed to fully understand the pharmacology of these receptors. Recent studies with transfected PC12 cells revealed interesting differences among receptor subtypes concerning induction of the transcriptional activity of AP-1, NF-κB and $p21^{waf\text{-}1}$, thus suggesting that the different pharmacological effects mediated by α_2-adrenoceptors could not be explained exclusively on the basis of ligand recognition or tissue distribution, but also on signal transduction [14]. A wider knowledge of α_2-adrenoceptor physiology and pharmacology is obviously needed to progress in this field.

Cotreatment with yohimbine completely prevented GFAP and α_2-adrenoceptor expression changes induced by morphine in the LC/NST and hippocampus, respectively, a finding that correlates with yohimbine prevention of opioid-induced dependence and addiction in behavioural studies [12,16,22]. This interaction could be extended to opioid neurotoxicity, which could be partially related to central noradrenaline hypoactivity; in fact, opioid drugs are known to reduce LC noradrenergic activity (see review [18]) and noradrenaline depletion has negative effects on cell survival or neurogenesis in several brain areas including the hippocampus, where proliferation of dentate gyrus progenitor cells is markedly reduced in these conditions [13]. Yohimbine could oppose these effects by the same mechanism than other α_2-adrenoceptor blockers provide neuroprotection in different experimental models, i.e. activation of the LC-noradrenergic system [2].

In summary, we have found that chronic morphine produces marked changes in LC and NST, as revealed by overexpression of GFAP, and profound modifications of α_2-adrenoceptor gene expression in target areas such as the hippocampus, where α_{2B} overexpression develops as it happens in rat strains that are prone to exhibit opioid seeking behaviours. Gene expression changes produced by chronic morphine are prevented when the animals are cotreated with the α_2-adrenoceptor antagonist yohimbine, as it happens with other pharmacological effects of opioid drugs, thus suggesting that noradrenergic activity in the hippocampus is critical to explain opioid addiction and/or neurotoxicity.

Acknowledgements

This study was supported by grants from Laboratories ESTEVE and USP (11/03).

References

[1] L.F. Alguacil, L. Morales, Alpha-2 adrenoceptor ligands and opioid drugs: pharmacological interactions of therapeutic interest, Curr. Neuropharmacol. 2 (2004) 343–352.
[2] S. Bauer, E. Moyse, F. Jourdan, F. Colpaert, J.C. Martel, M. Marien, Effects of the alpha-2 adrenoceptor antagonist dexefaroxan on neurogenesis in the olfactory bulb of the adult rat in vivo: selective protection against neuronal death, Neuroscience 117 (2003) 281–291.
[3] D. Beitner-Johnson, X. Guitart, E.J. Nestler, Glial fibrillary acidic protein and the mesolimbic dopamine system: regulation by chronic morphine and Lewis-Fischer strain differences in the rat ventral tegmental area, J. Neurochem. 61 (5) (1993) 1766–1773.
[4] S. Bohem, Presynaptic alpha2-adrenoceptors control excitatory, but not inhibitory, transmission in rat hippocampal slices, J. Physiol. 519 (1999) 439–444.
[5] E.L. Conway, A.L. Gundlach, J.A. Craven, Temporal changes in glial fibrillary acidic protein messenger RNA and [3H]PK11195 binding in relation to imidazoline-I2-receptor and alpha 2-adrenoceptor binding in the hippocampus following transient global forebrain ischaemia in the rat, Neuroscience 82 (3) (1998) 805–817.
[6] L. Ezquerra, G. Herradon, T. Nguyen, T.F. Vogt, R. Bronson, I. Silos-Santiago, T.F. Deuel, Pleiotrophin is a major regulator of the catecholamine biosynthesis pathway in mouse aorta, Biochem. Biophys. Res. Commun. 323 (2004) 512–517.
[7] E. Garrido, C. Pérez-García, L.F. Alguacil, C. Díez-Fernández, The alpha-2 adrenoceptor antagonist yohimbine reduces glial fibrillary acidic protein upregulation induced by chronic morphine administration, Neurosci. Lett. 383 (2005) 141–144.
[8] F.A. Hameedi, S.W. Woods, M.I. Rosen, H.R. Pearsall, T.R. Kosten, Dose dependent effects of yohimbine on methadone maintained patients, Am. J. Drug Alcohol Abuse 23 (1997) 327–333.
[9] G. Herradon, L. Ezquerra, L. Morales, B. Franklin, I. Silos-Santiago, L.F. Alguacil, Lewis and Fischer 344 strain differences in alpha-2 adrenoceptors and tyrosine hydroxylase expression, Life Sci. 78 (2006) 862–868.
[10] G. Herradon, L. Ezquerra, T. Nguyen, I. Silos-Santiago, T.F. Deuel, Midkine regulates pleiotrophin organ-specific gene expression: evidence for transcriptional regulation and functional redundancy within the pleiotrophin/midkine developmental gene family, Biochem. Biophys. Res. Commun. 333 (2005) 714–721.
[11] V. Iglesias, C. Alamo, E. Cuenca, L. Morales, C. Pérez-García, L.F. Alguacil, Effect of oral yohimbine on withdrawal jumping behaviour of morphine-dependent mice, Addict. Biol. 3 (1998) 459–463.
[12] V. Iglesias, L.F. Alguacil, C. Alamo, E. Cuenca, Effects of yohimbine on morphine analgesia and physical dependence in the rat, Eur. J. Pharmacol. 211 (1992) 35–38.
[13] V.A. Kulkarni, S. Jha, V.A. Vaidya, Depletion of norepinephrine decreases the proliferation, but does not influence the survival and differentiation of granule cells progenitors in the adult rat hippocampus, Eur. J. Neurosci. 16 (2002) 2008–2012.
[14] A. Lymperopoulos, G. Karkoulias, W.J. Koch, C.S. Flordellis, Alpha-2 receptor subtype-specific activation of NF-κB in PC12 cells, Neurosci. Lett. 102 (2006) 210–215.
[15] A. Messing, M. Brenner, GFAP: functional implications gleaned from studies of genetically engineered mice, Glia 43 (2003) 87–90.
[16] L. Morales, C. Perez-Garcia, L.F. Alguacil, Effects of yohimbine on the antinociceptive and place conditioning effects of opioid agonists in rodents, Br. J. Pharmacol. 133 (2001) 172–178.
[17] M. Narita, M. Suzuki, M. Narita, Y. Yajima, R. Suzuki, S. Shioda, T. Suzuki, Neuronal protein kinase Cγ-dependent proliferation and hypertrophy of spinal cord astrocytes following repeated in vivo administration of morphine, Eur. J. Neurosci. 19 (2004) 479–484.

[18] E.J. Nestler, M. Alreja, G.K. Aghajanian, Molecular and cellular mechanisms of opiate action: studies in the rat locus coeruleus, Brain Res. Bull. 35 (1994) 521–528.
[19] G. Paxinos, C. Watson, The Rat Brain in Stereotaxic Coordinates, Academic Press, New York, 1986.
[20] V. Raghavendra, M.D. Rutkowski, J.A. DeLeo, The role of spinal neuroimmune activation in morphine tolerance/hyperalgesia in neuropathic and sham-operated rats, J. Neurosci. 22 (2002) 9980–9989.
[21] P. Song, Z.Q. Zhao, The involvement of glial cells in the development of morphine tolerance, Neurosci. Res. 39 (2001) 281–286.
[22] J.R. Taylor, V.O. Lewis, J.D. Elsworth, P. Pivirotto, R.H. Roth, D.E.J. Redmond, Yohimbine co-treatment during chronic morphine administration attenuates naloxone precipitated withdrawal without diminishing tail-flick analgesia in rats, Psychopharmacology (Berlin) 103 (1991) 407–414.
[23] J.T. Williams, M.J. Christie, O. Manzoni, Cellular and synaptic adaptations mediating opioid dependence, Physiol. Rev. 81 (2001) 299–343.