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Transcriptional Regulation of the Human μ-Opioid Receptor Gene by Interleukin-6

Christine Börner, Jürgen Kraus, Helmut Schröder, Hermann Ammer[1] and Volker Höllt

Department of Pharmacology and Toxicology, University of Magdeburg, 44 Leipziger Strasse, 39120 Madgeburg, Germany

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Running Title: μ-Opioid Receptor Regulation by Interleukin-6

Corresponding author: Jürgen Kraus

Department of Pharmacology and Toxicology, University of Magdeburg

44 Leipziger Strasse

39120 Magdeburg, Germany

Phone: + 49 391 6715372, Fax: + 49 391 6715869

e mail address: juergen.kraus@medizin.uni-magdeburg.de

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The abbreviations used are: CAT, chloramphenicol acetyl transferase; EMSA, electrophoretic mobility shift assay; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RT-PCR, reverse transcription polymerase chain reaction; S.E.M., standard error of the mean; STAT, signal transducers and activators of transcription.

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Abstract

Inflammatory pain is counteracted by a number of physiological processes. For example, opioid receptors, which are present on peripheral terminals of sensory neurons, are activated by endogenous opioids, which are released from immune cells migrating to the inflamed tissue. Earlier data demonstrated that interleukin-6 contributes to such inflammation-induced analgesia. In this report we demonstrated that interleukin-6 strongly induces μ-opioid receptor mRNA in the human neuroblastoma cell line SH SY5Y, whereas δ-opioid receptor mRNA levels are not influenced. The mRNA increase in these cells is followed by an increase in μ-opioid receptor-specific binding. Using transcription factor decoy oligonucleotides, direct evidence was provided, that the upregulation of μ-opioid receptor mRNA in intact cells is dependent on the transcription factors STAT1 and 3, whereas other transcription factors, such as AP-1, NFκB or NF-interleukin-6 are not involved. STAT1 and 3 bound to a site located at nucleotide –1583 on the promoter of the human μ-opioid receptor gene as shown by transient transfection experiments, electrophoretic mobility shift assays and transcription factor decoy oligonucleotides. A mutation analysis of the 5'-TTCATGGAA-3' STAT1/3 element (palindrome underlined) was performed to determine nucleotide residues that are necessary for binding of STAT1 and 3. It suggested that only the palindromic half sides and the two adjacent central nucleotides are required. Neither mutation of the nucleotides outside the palindrome, nor mutation of the central nucleotide affected STAT1/3 binding.

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Interleukin-6 is a multifunctional cytokine with pro- as well as antiinflammatory properties. It is a major player in haematopoesis, acute-phase response to injury and regulation of fever (for reviews see: Van Snick, 1990; Leon, 2002). In addition, it induces a variety of opposing effects in the nervous system, which are not well understood. On the one hand, interleukin-6 is among the candidates of inflammatory mediators involved in the pathology of Alzheimer's disease. For example, it increases amyloid precursor protein expression in neurons (Del Bo et al., 1995) and synergistically contributes to neuronal damage caused by beta-amyloid peptide and NMDA (Qiu and Gruol, 2003). On the other hand, several reports demonstrated beneficial effects of interleukin-6 in terms of neuronal repair and neuroprotection. Nerve injury in the peripheral and central nervous sytem results in a rapid elevation in interleukin-6 levels (Gadient and Otten, 1997; Flatters et al., 2003) which accelerates post-traumatic neuronal regeneration (Swartz et al., 2001; Hirota et al., 1996). In addition, ischemic brain damage could be reduced by intracerebroventricular injection of interleukin-6 (Loddick et al., 1998). Furthermore, this cytokine is a neuroprotectant in cerebral ischemia in transgenic animal models with CNS-targeted interleukin-6 expression (Penkowa et al., 2003). There is increasing evidence that interleukin-6 also elicits beneficial effects in the regulation of neuropathic and inflammatory pain. Thus, spinal administration to rats with nerve injury results in antinociceptive effects, indicating that this cytokine is a modulator of neuropathic pain (Flatters et al., 2003). In addition, interleukin-6 was shown to induce analgesic effects in an animal model of inflammation contributing to the activation of the endogenous opioid system, which is induced in response to peripheral inflammation (Czlonkowski et al., 1993). This inflammation-induced analgesia involves the release of endogenous opioid peptides from immune cells which activate their cognate receptors, the μ-, δ- and κ-opioid receptors (Pol and Puig, 2004). Furthermore, there is an upregulation of opioid receptors in inflammation as well, which may additionally contribute to the antinociceptive effects of endogenous opioids (Stein et al., 2003; Cahill et al., 2003; Pol et al., 2003; Pol et al., 2001). The μ-opioid receptor

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is an important mediator of the analgesic effects of opioids and is the main subtype for analgesic drugs of the morphine type. A study with interleukin-6-deficient mice supports the idea that this cytokine is important for the regulation of opioid receptors and pain (Bianchi et al., 1999). These mice showed reduced analgesic response to morphine compared to wild type animals. In addition, it was reported that such mice have a lower μ-opioid receptor density in the grey matter of the midbrain, which is known to be critical for the analgesic actions of opioids. The aim of this study was to investigate the influence of interleukin-6 on the expression of the μ- and δ-opioid receptor genes and the molecular mechanisms underlying the interleukin-6-induced upregulation of the μ-opioid receptor gene in the human neuroblastoma cell line SH SY5Y, which serves as a model for neuronal cells.

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Materials and Methods

Cell culture, reagents, and transfection

SH SY5Y cells were cultivated in Dulbecco's modified Eagle's medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 15% fetal calf serum and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin). Twenty hours before stimulations all cells including controls received fresh medium with 1% fetal calf serum. Recombinant human interleukin-6 (R&D Systems, Wiesbaden, Germany) was used in a final concentration of 9 ng/ml, which equals 1000 WHO units/ml. Stock solutions of 10 µg/ml in phosphate buffered saline were kept in frozen aliquots at –20°C to aviod repeated freezing and thawing. Cycloheximide (Sigma, Taufkirchen, Germany) was used at a concentration of 10 µg/ml. The transfection procedure for SH SY5Y cells has been described previously in detail (Kraus et al., 2003a; Börner et al., 2002).

µ-opioid receptor binding studies

^{3}H-DAMGO binding was performed on membranes from SH SY5Y cells essentially as described earlier (Ammer and Schulz, 1997; Koch et al., 1998).

Decoy oligonucleotide approach

The usage of transcription factor decoy oligonucleotides to study interactions with transcription factors in living cells has been described in detail in earlier publications from our laboratory (Kraus et al., 2003a; Kraus et al., 2003b). Decoy oligonucleotides were used in a final concentration of 120 nM.

Oligonucleotides

All oligonucleotides were synthesized by Metabion, Martinsried, Germany. To obtain double stranded decoy oligonucleotides, equimolar amounts of both strands were heated to 90°C in a

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water bath which then was allowed to slowly cool down to room temperature. Then the ends of the oligonucleotides were filled in with Klenow enzyme and the annealed oligonucleotides were stored at –20°C. Sequences of oligonucleotides were: STAT1/3: 5‘-GATCGAGTTTACGAGAACTC-3‘; AP-1: 5‘-CGATTGACTCAGTACTGAGTCAATCG-3‘; NFκB: 5‘-AAAGTTGAGGGGACTTTCCCAGGCCT-3‘; NF-interleukin-6: 5‘-TGCAGATTGCGCAATCTGCA-3‘; AP-2: 5‘-TGCGGGCTCCCCGGGCTTGGGCGAGC-3‘; hSTAT6: 5‘-CAACCTTCTTCTCAGAAGCATATGT-3‘; M1(-1583): 5‘-GATCTTAGTTCATGGAAGAATATGT-3‘; M2(-1546): 5‘-GATCTAGTCTCTAGGAAATCT-3‘; M3(-1520): 5‘-GATCTTTATTGTGTAAATTATATGC-3‘; M4(-1502): 5‘-GATCTGCTTTAATGTAAGAGGATAA-3‘; M5(-1470): 5‘-GATCAACATTGGCAAAATAGCCTAT-3‘; M6(-1422): 5‘-GATCCTGTTTCTAAGATAAATGCCA-3‘; M1 *mu*1: 5‘-TACATTAGTACATGGTAGAATATGT-3‘.

Oligonucleotides M1 *mu*2 to M1 *mu*18 are mutations of M1(-1583), as depicted in figure 5. For all oligonucleotides only the sequences of the sense strands are given.

Western blot analysis

Briefly, 1400 cells/mm^2 were seeded. After one day the medium was changed to 1% fetal calf serum. After another 24h cells were treated for 15 and 30 min with interleukin-6 or vehicle. Then, medium was removed and cells were lysed with 80°C hot sample buffer. Cell lysis, blotting and antibody incubations were performed as suggested in the “western immunoblotting protocol“ (New England Biolabs, Frankfurt, Germany). Aliquots of 20 µl were separated on a 8% polyacrylamide gel. Primary antibodies (Phospho-STAT1(Tyr701) and Phospho-STAT3(Tyr705) from New England Biolabs, and STAT1(E23) and

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STAT3(C20) from Santa Cruz Biotechnology, Heidelberg, Germany) were used in a 1:1000 dilution.

Real-time RT-PCR

RNA isolation, cDNA synthesis and real-time RT-PCR procedure have been described previously (Kraus et al., 2003a). The amounts of μ- and δ-opioid receptor transcripts were calculated relative to those of the housekeeping gene GAPDH. Amplification of GAPDH and μ-opioid receptor transcripts have been described in detail earlier (Kraus et al., 2003a). δ-opioid receptor transcripts were amplified with the primers 5'-ACGTGCTTGTCATGTTCGGCATCGT-3' and 5'-ATGGTGAGCGTGAAGATGCTGGTGA-3' (located on different exons to avoid amplification of genomic DNA) as follows: 1) preincubation for 8 min at 95°C; 2) 50 cycles for 5 s at 95°C, 5s at 63°C and 13 s at 72°C.

Reporter gene plasmids

All reporter plasmids are based on the pBLCAT2/pBLCAT3 vector system (Luckow and Schütz, 1987). The construction of the human μ-opioid receptor promoter reporter constructs -2624, -2229, -1702, -1372, -779, -2229/-1854.tk, -1854/-1227.tk has been described in previous publications (Kraus et al., 2001; Börner et al., 2002; Kraus et al., 2003a). Plasmid -2624/-2291.tk was generated by digestion of the μ-opioid receptor promoter with Sau3A and shotgun cloning of the fragments into pBLCAT2's BamHI site. The plasmid -2229Δ-1580/-1080 was created by opening construct -2229 with BglII (-1372) and cutting with enzyme Bal31. Insertion of oligonucleotides into pBLCAT2 was performed according to a described method (Kang and Inouye, 1993). All plasmids were sequenced from both sides to ensure correct orientations and sequences of the inserts.

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Extraction of nuclear proteins and EMSA

Both procedures have been described earlier (Kraus et al., 2001). For immunoshift experiments, STAT1(E23)X and STAT3(C20)X antibodies (both Santa Cruz Biotechnology, Heidelberg, Germany) were used.

Statistical Analysis

For statistical evaluation of the experiments Student's *t* tests were performed. Stars indicate significantly different values (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

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Results

Effect of interleukin-6 on μ- and δ-opioid receptor mRNA levels and μ-opioid receptor-specific binding

The human neuroblastoma cell line SH SY5Y serves as a common model for neuronal cells and is known to express μ- and δ-opioid receptors constitutively. In addition, SH SY5Y cells were shown to express interleukin-6 receptors and contain functional signaling pathways for this cytokine, e. g. activated STAT1 and STAT3, and p42/44 MAP kinase (Schumann et al., 1999). Therefore this cell line was used to study the effect of interleukin-6 on the amounts of μ- and δ-opioid receptor mRNA by quantitative real time RT-PCR (fig. 1A, B). It was shown that μ-opioid receptor gene transcription is strongly induced by interleukin-6, initiating after six to eight h. The protein synthesis inhibitor cycloheximide did not influence the effect of interleukin-6 on μ-opioid receptor gene transcription indicating that it is a direct transcriptional effect. Notably, and contrasting with this, δ-opioid receptor gene transcription was not regulated by interleukin-6. Next it was investigated whether the μ-opioid receptor mRNA increase was followed by an increase in μ-opioid receptor-specific binding (fig. 1C). Binding studies on membranes prepared from SH SY5Y cells with ^{3}H-DAMGO, which is highly selective for the μ-opioid receptor subtype, demonstrated a modest increase in the receptor number after stimulation, which was significant at the 48 h time point.

Identification of the transcription factors mediating interleukin-6-induction of μ-opioid receptor mRNA

To address the question which transcription factor(s) mediate(s) the interleukin-6-induced upregulation of μ-opioid receptor transcription, we used an approach that combines use of transcription factor decoy oligonucleotides and quantitative real time RT-PCR. Generally, in the decoy approach double stranded oligonucleotides with specific binding sequences for transcription factors are used, which are brought into living cells to selectively disrupt the function of these factors. In the cells, transcription factors then interact with an excess of

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decoy oligonucleotides instead of binding to the natural regulatory motifs of genes. In our experiments we disrupted the function of STAT1 and STAT3, AP-1, NFκB and NF-interleukin-6 in SH SY5Y cells using decoy oligonucleotides corresponding to sites of the mouse metallothionein-I gene (STAT1/3: Lee et al., 1999), human μ-opioid receptor gene (AP-1 and NFκB: Börner et al., 2002; Kraus et al., 2003a), and the commercial available NF-interleukin-6 binding oligonucleotide (sequence from Santa Cruz Biotechnology, Heidelberg, Germany). These factors are known to directly or indirectly mediate effects of interleukin-6. On the promoter of the human μ-opioid receptor gene two AP-1 (–2388 and –1434) and three NFκB (–2174, –557 and –207) binding sites were already identified (Börner et al., 2002; Kraus et al., 2003a). In addition, there are two putative NF-interleukin-6 binding sites located at -525 and –331 in the promoter region. Furthermore, AP-2 decoy oligonucleotides (corresponding to a site in the mouse δ-opioid receptor gene: Wöltje et al., 2000) were selected as a negative control since AP-2 is unrelated to interleukin-6 signaling. Efficiency and specificity of the decoy oligonucleotides were confirmed earlier in transfection experiments in SH SY5Y cells (data not shown) and identification of NFκB regulatory elements on the μ-opioid receptor gene (Kraus et al., 2003a). Our experiments (fig. 2A, B) revealed, that interleukin-6 induction of μ-opioid receptor transcription was not significantly affected by AP-1, NFκB, NF-interleukin-6 and AP-2 decoy oligonucleotides, suggesting that these transcription factors most likely are not involved in mediating the effect. In contrast, loss of STAT1/3 function attenuated transcriptional induction of the μ-opioid receptor gene in the presence of interleukin-6, demonstrating that this regulation is dependent on STAT1 and/or STAT3. In line with this, increasing amounts of posphorylated STAT1 and STAT3 were observed in western blot experiments after 15 and 30 min of incubation of SH SY5Y cells with interleukin-6 (fig. 2C).

Identification of the interleukin-6-inducible promoter element of the human μ-opioid receptor gene

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To characterize the molecular mechanisms underlying the induction of the µ-opioid receptor gene by interleukin-6 transfection experiments in SH SY5Y cells were performed. Using reporter gene constructs containing various lengths of the 5'-flanking region of the gene, we localized the promoter region required for interleukin-6 inducibility (fig. 3A). Plasmid pBLCAT2, which was used as a vector containing the herpes simplex virus thymidine kinase promoter instead of the µ-opioid receptor promoter in front of the CAT reporter gene, was not responsive to interleukin-6 (lane 10). The basal activity of the –2624 reporter gene construct containing the µ-opioid receptor promoter had approximately 55% of the activity of the herpes simplex virus thymidine kinase promoter in the SH SY5Y cells. Construct -2624, and consecutive 5'-deletions of it up to -1702 were inducible by interleukin-6 (lanes 1 to 3). Two constructs with further 5'- deletions showed no induction by this cytokine (lane 4 and 5). The constructs -2229Δ-1580/-1080 (lane 6), -2624/-2291.tk (lane 7) and -2229/-1854.tk (lane 8) were not interleukin-6-inducible, as well. In contrast, construct -1854/-1227.tk (lane 9) was inducible. These results suggested that the STAT1/3 binding site(s) is located in the region between -1580 and -1372 (marked in grey).

STAT1 and STAT3 transcription factors predominantly bind to sequences with the motifs 5'-TTCNNNGAA-3', and 5'-TTNNNNNAA-3'. Apart from this rule, several STAT1 and STAT3 binding sites with one mismatch in the 5'-TTCNNNGAA-3' motif were identified in studies on other genes (overview: Ehret et al., 2001). These criteria were chosen to designate six sequence motifs within the interleukin-6 responsive region of the µ-opioid receptor promoter as putative STAT1/3 binding sites (M1 to M6, fig. 3B). To identify sequences which really bind STAT1/3 in vivo, we performed experiments in which construct -1702 was cotransfected with decoy oligonucleotides containing the putative binding sites (fig. 3C). Cotransfection of decoy oligonucleotides which do not bind STAT1/3 will retain interleukin-6 inducibility of construct -1702, whereas cotransfection of decoys which bind STAT1/3 will abolish inducibility of the reporter gene construct. The mouse metallothionein-I STAT1/3

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decoy oligonucleotides served as positive controls and decoy oligonucleotides containing the unrelated STAT6 binding site of the human μ-opioid receptor gene promoter (hSTAT6: Kraus et al., 2001) were used as negative controls. The experiments revealed that M1(-1583) is a STAT1/3 binding site, whereas M2(-1546) to M6(-1422) do not bind STAT1/3.

Then, the oligonucleotides used in the decoy experiments shown in figure 3C were cloned in front of the thymidine kinase promoter in pBLCAT2 and tested if they would mediate interleukin-6 responsiveness (fig. 3D). Indeed, the M1(-1583) motif mediated interleukin-6 induction independently of its orientation. In contrast, no induction was observed for the constructs containing motifs M2 to M6, and a mutated M1 motif (M1*mu1*-tk-CAT), confirming the results with the decoy oligonucleotides.

Binding of STAT1/3 to the interleukin-6 response element on the promoter of the human μ-opioid receptor gene in vitro

EMSAs were performed to further characterize binding of STAT1 and STAT3 to the M1(-1583) element (fig. 4). First, M1 (-1583) probe was incubated with nuclear extract of unstimulated and interleukin-6-stimulated SH SY5Y cells (fig. 4A). The intensities of the specific bands were strongly enhanced in extracts of stimulated cells (lanes 1 and 2). Experiments with various competitor DNAs showed that both M1(-1583) and mouse metallothionein-I STAT1/3 oligonucleotides can compete with both M1(-1583) and mouse metallothionein-I STAT1/3 probes for STAT1/3 binding, whereas M1*mu*1 competitor DNA (with an exchange of two nucleotides compared to M1) does not bind the transcription factors (fig. 4A, lanes 3 to 9 and fig. 4B, lanes 1 to 7). In immunoshift experiments a specific antibody can either further retard the electrophoretic mobility of the DNA-protein complex or prevent formation of a specific DNA-protein complex. The latter case was observed in our experiments. Using specific antibodies against STAT1 and STAT3 we showed that both factors can bind to M1(-1583) (fig. 4A, lanes 10 to 12). Efficiency of these antibodies was

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demonstrated in immunoshift experiments with metallothionein-I STAT1/3 probe (fig. 4B, lanes 8 to 10).

Mutational analysis of the interleukin-6 response element M1(-1583)

Next, the question was addressed which nucleotides of the M1(-1583) sequence may be necessary for STAT1/3 binding. The interleukin-6-responsive reporter gene construct –1702 construct was cotransfected with various decoy oligonucleotides containing one or several basepair sequence exchanges compared to M1(-1583) (fig. 5). It was shown that decoy oligonucleotides containing the wild type motif M1(-1583) bind STAT1/3, whereas M1*mu*1 with an exchange of two nucleotides within the core palindrome did not bind to STAT1/3, confirming the EMSA and transfection results. Then, in one series of experiments effects of nucleotide exchanges directly outside of the core palindrome were tested. M1*mu*2 and M1*mu*3 with four altered nucleotides on each side of the core palindrome both bound STAT1/3, indicating that these nucleotides are not required. In another series of experiments, motifs each having one basepair exchange in the 5‘-TTC...GAA-3‘ sequence were tested. These experiments (M1*mu*4 to M1*mu*9) demonstrated that only M1*mu*4 bound STAT1/3, whereas M1*mu*5 to M1*mu*9 showed no STAT1/3 binding. Additionally, effects of mutations of the nucleotides inside the palindromic half sides were investigated. The experiments with M1*mu*10 to M1*mu*12 indicated that the central one of these three nucleotides has no influence on STAT1/3 binding. As nucleotide 5‘ of the central position (M1*mu*16 to M1*mu*18), either an A (wild type) or C (M1*mu*16) residue was required, whereas 3‘ of the central position (M1*mu*13 to M1*mu*15), a G (wild type) residue was needed for STAT1/3 binding.

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Discussion

Opioids can mediate their analgesic effects by activating opioid receptors not only within the central nervous system, but also on peripheral sensory neurons (Stein et al., 1991; Stein et al., 1995). The analgesic effects of peripherally applied opioids are augmented under conditions of tissue injury and inflammation (Stein et al., 2003). For example, injection of a low dose of morphine into inflammed submucous tissue of patients undergoing dental surgery reduced supplementally required doses of analgesic agents and induced prolonged postoperative analgesia, whereas adminstration into non-inflamed tissue or perineural was not effective (Likar et al., 2001). One underlying mechanism of such effects is an upregulation of peripheral opioid receptors under inflammatory conditions (Stein et al., 2003; Pol et al., 2001; Pol et al., 2003; Zöllner et al., 2003). Cytokines may play an important role in this mechanism by upregulating μ-opioid receptor expression in neuronal cells, as it was shown for interleukin-4 (Kraus et al., 2001) and interleukin-1β (Ruzicka et al., 1996). Another study indicated a regulation of μ-opioid receptors by interleukin-6, demonstrating that interleukin-6-deficient mice show decreased μ-opioid receptor levels in the grey matter of the midbrain compared to wild type animals, whereas no differences in δ-opioid receptor densities were found (Bianchi et al., 1999). Our study provides evidence that μ-opioid receptor mRNA and protein is induced by interleukin-6 in SH SY5Y cells which serves as a model for neuronal cells. In contrast, δ-opioid receptor mRNA levels are not influenced by this cytokine which is in good accordance with the results described above (Bianchi et al., 1999). An earlier study demonstrated that interleukin-6 has antinociceptive effects in an animal model of peripheral inflammation (Czlonkowski et al., 1993). These effects were abolished by a specific μ-opioid receptor antagonist. In contrast, a specific δ-opioid receptor antagonist did not influence interleukin-6-induced analgesia. This indicates that μ-opioid receptors are involved in these effects, whereas δ-opioid receptors are not. The results obtained from our cell model support the idea that interleukin-6 contributes to antinociceptive effects by enhancing μ-opioid

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receptor expression. However, this model cannot predict whether μ-opioid receptors may be upregulated only at sites of injury or inflammation, or throughout the body. In the whole organism the analgesic effect of interleukin-6 may involve different or additional mechanisms. Interestingly, morphine treatment can increase interleukin-6 levels in plasma (Houghtling et al., 2000), as well as in spinal cord (Raghavendra et al., 2002). In this context, our results indicate that this cytokine is probably involved in mechanisms underlying an upregulation of μ-opioid receptors by morphine.

Interleukin-6 binds to its specific receptors causing an association with two molecules of gp130, thereby activating the intracellular tyrosine kinases of the janus kinase family (Heinrich et al., 2003). Activated janus kinases then activate STAT transcription factors by phosphorylation. Alternatively, a second pathway can be activated involving the G-proteins Ras and Raf, which leads to downstream activation of MAP kinase, resulting in activation of another set of transcription factors. Thus, downstream signaling of interleukin-6 can be mediated by several transcription factors, including STAT1 and 3, NF-interleukin-6, NFκB and AP-1. Using a decoy oligonucleotide approach in living cells we showed that the interleukin-6-induced μ-opioid receptor mRNA upregulation in SH SY5Y cells is mediated via the transcription factors STAT1/3, whereas the other transcription factors mentioned above are most likely not involved in this effect.

STAT1 and STAT3 are members of the STAT transcription factor family (Darnell, 1997). This transcription factor family comprises seven members (STAT1, STAT2, STAT3, STAT4, STAT5A, STAT5B and STAT6) that generally are activated in response to cytokines, growth factors, and hormones. After phosphorylation, STATs form homo- or heterodimers and translocate to the nucleus, where they bind to regulatory motifs in the promoter region and induce gene transcription. STAT1 and STAT3 may form heterodimers and may bind to the same regulatory promoter element. After localizing the STAT1/3 element in the promoter region of the human μ-opioid receptor gene, we performed EMSAs to address the question if

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both transcription factors can bind to the element M1(-1583), which indeed was the case. The question whether STAT1 or STAT3 homodimers or STAT1-STAT3 heterodimers bind to the characterized M1(-1583) element in vivo remains unsolved as these two STAT factors are not distinguable by their size and thus cannot be discriminated by the EMSA technique. Interestingly, the region containing the STAT1/3 element is conserved also in the rat μ-opioid receptor gene promoter with a similar element at nt -1518, but it remains to be shown if this putative STAT binding site is a functional element, as well.

STAT transcription factors generally bind to sequences containing a six basepair 5‘-TTC...GAA-3‘ core palindrome. The different STATs have distinct, non-overlaping functions, as revealed by studying recombinant mice deficient for the different genes coding for STATs (Darnell, 1997; Akira, 1999). Therefore, it can be assumed, that each member of the STAT family binds to different cognate motifs within the regulatory regions of target genes. So far, however, only specific target sequences for regulatory elements for STAT6 were identified (Kraus et al., 2003b). Identification of specific target sequences for the different STAT factors is made even more complex by the fact that STAT1 and 3 may form heterodimers, as already mentioned. Addressing the question which nucleotides in the sequence of this element are required for binding of STAT1/3, we showed that the nucleotides outside of the six basepair 5‘-TTC...GAA-3‘ core palindrome do not influence STAT1/3 binding. This is in contrast to STAT6, which requires an enlarged palindrome of eight basepairs (Kraus et al., 2003b). Nearly all naturally occuring STAT1 and 3 binding sites possess three nucleotides in the middle of the palindromic half sides (Ehret et al., 2001). We demonstrated that the central nucleotide of the three does not influence STAT1/3 binding. Additionally, our results showed that the first nucleotide in the six basepair palindrome of the STAT1/3 site may be A or T residue. Our experiments thus indicate that 5'-WTCMNGGAA-3' (W= A or T; M= A or C) may be a consensus sequence for STAT1/3 binding. This is a first step to establish specific target sequences for STAT1 and 3, but further studies are necessary.

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Footnotes

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Reprint requests to:

Jürgen Kraus

Department of Pharmacology and Toxicology, University of Magdeburg

44 Leipziger Strasse

39120 Magdeburg, Germany

Phone: + 49 391 6715372

Fax: + 49 391 6715869

e mail address: juergen.kraus@medizin.uni-magdeburg.de

[1] Institute of Pharmacology, Toxicology and Pharmacy, University of Munich, Königinstrasse 16, 80539 Munich, Germany

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Legends for figures

Figure 1. Effect of interleukin-6 (IL-6) on μ- and δ-opioid receptor expression in SH SY5Y cells.

A and B, effects on mRNA. SH SY5Y cells were stimulated with interleukin-6 (9 ng/ml) for different periods of time and then subjected to real-time RT-PCR. A, one representative experiment of a real time RT-PCR monitoring amplification of μ-opioid receptor transcripts (1, 2: unstimulated controls; 3, 4: 16 h interleukin-6; 5, 6: 16 h interleukin-6 and cycloheximide). As an insert, amplification of GAPDH mRNA of the same cDNAs is plotted. B, quantification of the results normalized to GAPDH. Results are expressed as fold induction (grey bars, [μ-opioid receptor(MOR)]/[GAPDH]; white bars, [δ-opioid receptor(DOR)]/[GAPDH]). The 16h incubations were tested with and without cycloheximide (CX). The results of three independent experiments performed in triplicate plus S.E.M. are shown. *: $p< 0.05$; **: $p< 0.01$; ***: $p< 0.001$. C, relative ^{3}H-DAMGO binding after incubation of cells for different periods of time with interleukin-6 (9 ng/ml). For the controls, 100% equals to an average of 52 fmol binding sites per mg of protein. The results of two independent experiments performed in tpiplicate plus S.E.M. are shown. *: $p< 0.05$.

Figure 2. Identification of transcription factors mediating μ-opioid receptor mRNA upregulation induced by interleukin-6.

A, one typical real time RT-PCR experiment. SH SY5Ycells were transfected with the indicated decoy oligonucleotides (120nM), stimulated with interleukin-6 (9 ng/ml) for 24h and then subjected to real-time RT-PCR (MOR: μ-opioid receptor). As an insert, amplification of the corresponding GAPDH mRNAs is shown. Below, the sequences of the decoy oligonucleotides are shown with the specific binding sites shaded in grey. B, quantification of the results of three independent experiments performed in triplicate. RNA amounts are normalized to GAPDH and shown as fold induction plus S.E.M. ***: $p< 0.001$

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(interleukin-6-stimulated controls (co) vs interleukin-6-stimulated plus decoy transfected cells). C, western blot analysis showing induction of phosphorylated STAT1 and 3 proteins after 15 and 30 min of incubation with interleukin-6 (9 ng/ml).

Figure 3. Localization of the interleukin-6-responsive element on the human μ-opioid receptor (MOR) promoter.

A, identification of the interleukin-6 responsive region of the promoter. On top, the promoter region with the already identified AP-1 (nt –2388 and nt –1434; black rectangle), NFκB (nt –2174, nt -557 and nt –207; black diamond) and STAT6 (nt –997; black oval) binding sites and the putative NF-interleukin-6 binding sites (nt –525 and nt –331; open triangle) is shown schematically. Below, constructs are depicted relative to the promoter of the gene. CAT activities obtained after transfection of SH SY5Y cells are shown as fold induction (white bars, unstimulated controls; grey bars, interleukin-6 stimulated transfectants (9 ng/ml), tk: thymidine kinase). Results of at least three independent experiments performed in triplicated plus S.E.M. are plotted. **: $p < 0.01$; ***: $p < 0.001$. B, enlargement of the interleukin-6 responsive promoter region with the putative STAT factor binding sites M1 to M6 (open ovals). C, identification of the interleukin-6 responsive element using decoy oligonucleotides. On top, interleukin-6-inducibility of the reporter gene construct –1702 is shown as fold induction. Below, the effect of cotransfected decoy oligonucleotides containing the putative STAT sites on interleukin-6-inducibility of the reporter gene construct is shown. CAT activities are reported as fold induction. Results of at least three independent experiments performed in triplicated plus S.E.M. are shown. *: $p < 0.05$; ***: $p < 0.001$. D, identification of the interleukin-6 responsive element in transfections using reporter gene constructs with the putative elements in front of the herpes simplex virus thymidine kinase promoter. (s) sense, (as) antisense orientation. Cat activities are shown as fold induction as described above.

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Results of at least three independent experiments performed in triplicated plus S.E.M. are plotted. *: p< 0.05; **: p< 0.01.

Figure 4. EMSAs demonstrating binding of STAT1 and STAT3 to the interleukin-6-responsive element on the human μ-opioid receptor gene promoter.

A, labeled M1(-1583) probe was incubated with nuclear extracts (NE) from unstimulated (co, lane 1) and interleukin-6 (IL-6, 9 ng/ml, 1h)- stimulated SH SY5Y cells (lane 2). In lanes 3 to 9, probe was incubated with interleukin-6-stimulated nuclear extract in the absence (-) or presence of competitor DNA as indicated above the gels (1 and 10 pmol competitor DNA, as indicated by the wedges). Immunoshift experiments are shown in lanes 10 to 12 (10: without antibody, 11: with STAT1 antibody, 12: with STAT3 antibody). Samples for immunoshift experiments were preincubated with the antibody for 2h. B, similar experiments with a mouse metallothionein-I STAT1/3 probe. C, sequences of competitor DNAs are shown with the core palindromes shaded in grey.

Figure 5. Mutational analysis of STAT1/3 sites.

On top, interleukin-6-inducibility of the reporter gene construct -1702 is shown as fold induction. This construct contains the STAT1/3 site M1(-1583) and was cotransfected with decoy oligonucleotides containing various mutations compared to the M1(-1583) site (120 nM), as indicated below. On the right, the effect of the decoy oligonucleotides on interleukin-6-inducibility of the reporter gene construct is shown. The results of at least three independent transfection experiments performed in triplicate plus S.E.M. are shown. **: p< 0.01; ***: p< 0.001.

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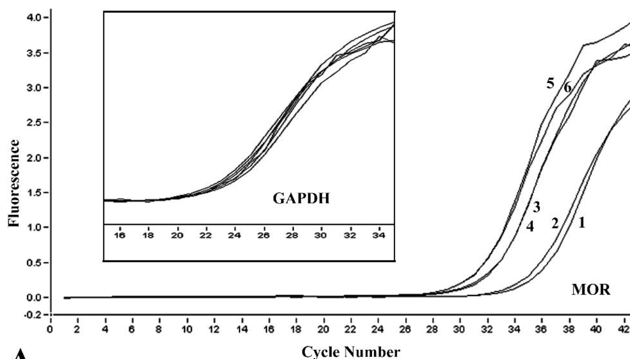
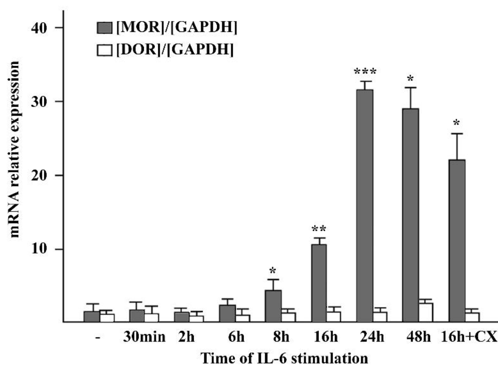


A



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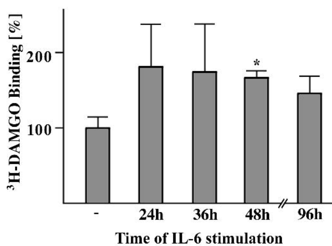
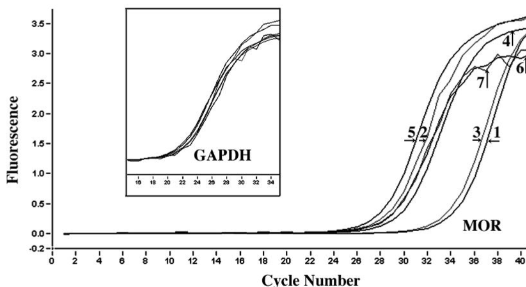


C

Figure 2 MOL 3806



1: unstimulated
2: + IL-6

3: + IL-6 + STAT1/3 decoy 5'-GATCGAGTTTACGAGAACTCGATC-3'
3'-CTAGCTCAAATGCTCTTGAGCTAG-5'

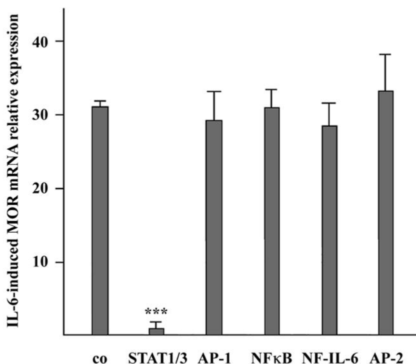
4: + IL-6 + AP-1 decoy 5'-CGATTGACTCAGTACTGAGTCAATCG-3'
3'-GCTAACTGAGTCATGACTCAGTTAGC-5'

5: + IL-6 + NFκB decoy 5'-AAAGTTGAGGGGACTTTCCCAGGCCT-3'
3'-TTTCAACTCCCCTGAAAGGGTCCGGA-5'

6: + IL-6 + NF-IL-6 decoy 5'-TGCAGATTGCGCAATCTGCA-3'
3'-ACGTCTAACGCGTTAGACGT-5'

7: + IL-6 + AP-2 decoy 5'-TGCGGGCTCCCCGGGCTTGGGCGAGC-3'
3'-ACGCCCGAGGGGCCCGAACCCGCTCG-5'

A



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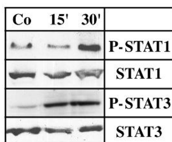


C

Figure 3 MOL 3806

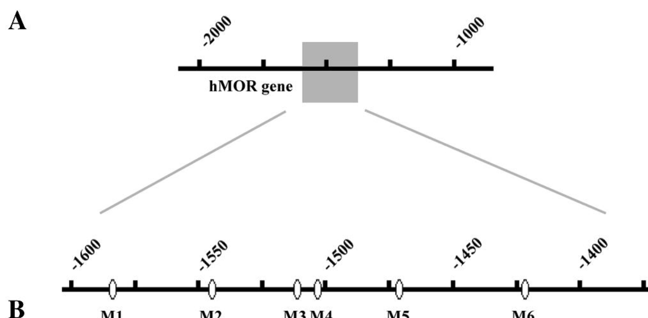
-2000 -1000 +1 (ATG)

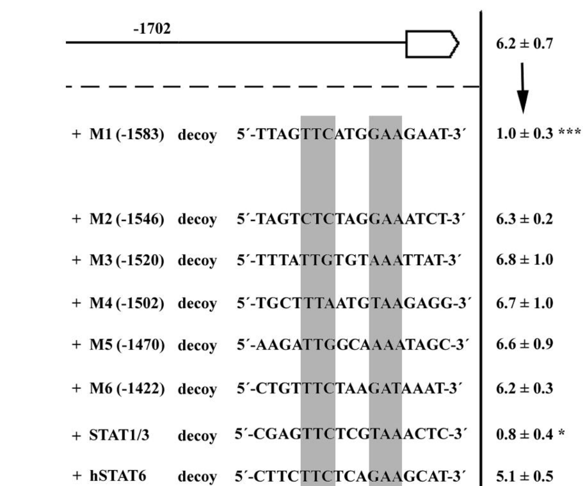
hMOR gene

1 2 3 4 5 6 7 8 9

1 -2624 CAT ***
2 -2229 **
3 -1702 ***
4 -1372
5 -779
6 -2229 Δ -1580/-1080
7 -2624/-2291.tk tk
8 -2229/-1854.tk
9 -1854/-1227.tk ***
10 pBLCAT2

A





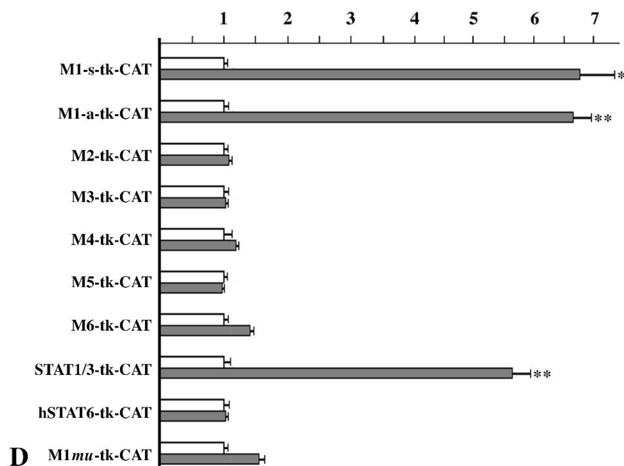
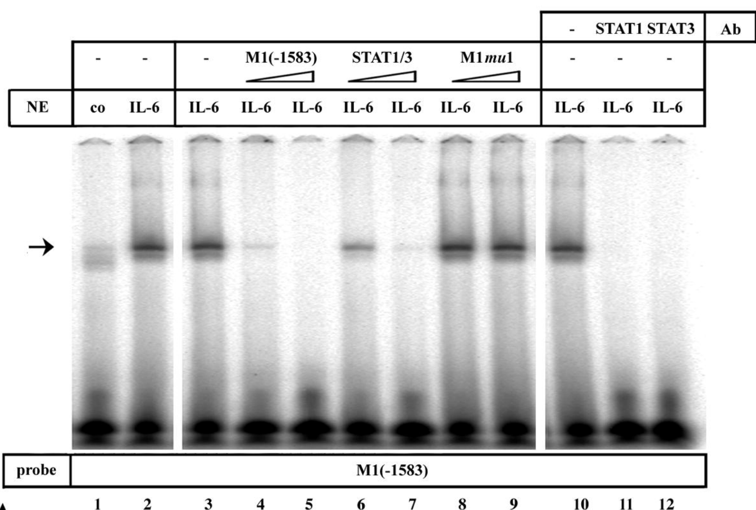
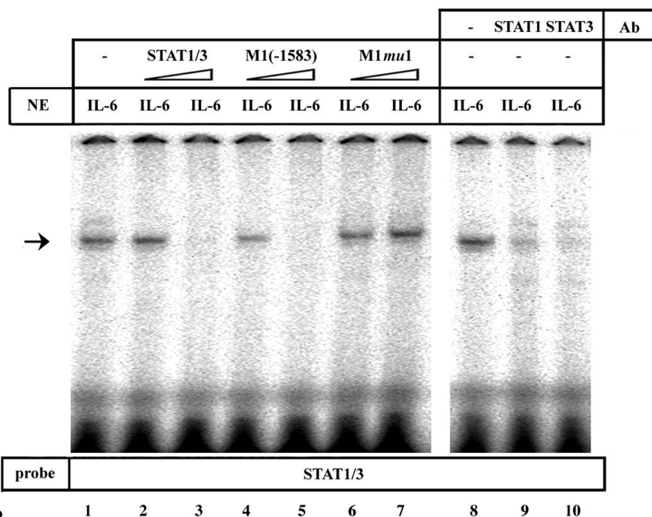


Figure 4 MOL 3806





M1(-1583): 5'-GATCTTAGTTCATGGAAGAATATGTGATC-3'
3'-CTAGAATCAAGTACCTTCTTATACACTAG-5'

STAT1/3: 5'-GATCGAGTTTACGAGAACTCGATC-3'
3'-CTAGCTCAAATGCTCTTGAGCTAG-5'

M1*mu*1: 5'-TACATTAGTACATGGTAGAATATGT-3'
3'-ATGTAATCATGTACCATCTTATACA-5'

C

Figure 5 MOL 3806