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Mechanisms of the Inhibition of Nuclear Factor-κB by Morphine in Neuronal Cells

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ABSTRACT

Opioids potently modulate neuronal functions, for example, by regulating the activity of transcription factors. Here, we investigated the effect of morphine on the activity of the transcription factor nuclear factor κB (NF-κB). Establishing cellular models for our investigations, we demonstrated that NF-κB mediated the tumor necrosis factor (TNF)-induced transcription of the cannabinoid receptor type 1 gene in primary fetal striatal neurons from rats and the human neuroblastoma cell line SH SY5Y. The activity of NF-κB in these models was strongly inhibited by morphine, which was achieved by a marked up-regulation of the inhibitor of nuclear factor-κB (IκB). The opioid-induced up-regulation of IκB was dependent on the transcription factors NF-κB itself and activator protein-1 (AP-1). In fact, stimulation of the cells with morphine resulted in a transient activation of NF-κB and a strong induction of c-Fos, one of the constituents of AP-1. This resulted in IκB levels significantly exceeding the basal, constitutive levels of IκB. These data, together with experiments in which AP-1 and IκB were down-regulated by decoy oligonucleotides and siRNA, suggest that the morphine-induced activation of AP-1 and the subsequent overexpression of IκB are key factors in the inhibition of NF-κB by the drug. In contrast, stimulation of primary neurons from rats and SH SY5Y cells with TNF, which is a classic activator of NF-κB, resulted in a resynthesis of IκB, in which the basal levels of IκB were restored only but did not result in an activation of AP-1 and overexpression of IκB.

Introduction

Many neuronal functions involve the transcription factor nuclear factor κB (NF-κB) (Kaltschmidt and Kaltschmidt, 2009; Park and Bowers, 2010). In this study, we investigated the regulation of NF-κB in neuronal model cells by morphine. As model systems, we used primary fetal neurons from rats and human neuroblastoma SH SY5Y cells, in which NF-κB mediated the induction of the expression of the cannabinoid receptor type 1 (CB1) by tumor necrosis factor (TNF).

Opioids are potent analgesics and are irreplaceable for the treatment of severe pain and in anesthesia. They mediate their effects via three receptors termed μ-, δ-, and κ-opioid receptors. Among these, μ-opioid receptors play an outstanding role, because they mediate effects of morphine and most clinically used opioids (Kieffer and Evans, 2009).

Many effects of cannabinoids on neuronal cells are mediated by CB1. Endocannabinoids and CB1 are key players in neuronal homeostasis, and they regulate, for example, food intake, body weight and emotional responses and control neuroinflammation (Di Marzo, 2008; Börner et al., 2009a; Lutz, 2009). Their precise regulation is of vital importance, and dysregulation may be associated with severe diseases such as Huntington's disease and multiple sclerosis (Centonze et al., 2008; Bisogno and Di Marzo, 2010; Blázquez et al., 2011).

TNF, which communicates key processes in neurons under both physiological and pathological conditions, is a prototypical activator of NF-κB (Baud and Karin, 2001; Park and Bowers, 2010). The activation of NF-κB involves several steps. In brief, the inhibitory protein inhibitor of nuclear factor-κB (IκB) retains NF-κB in the cytoplasm of unstimulated cells, where the transcription factor is inactive. One of the early steps in the activation of NF-κB is the stimulus-triggered phosphorylation of the IκBα-kinase complex. This kinase complex in turn is responsible for the phosphorylation

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ABBREVIATIONS: NF-κB, nuclear factor κB; CB1, cannabinoid receptor type 1; TNF, tumor necrosis factor; PMA, phorbol 12-myristate 13-acetate; cAMPS-RP, adenosine-3′,5′-cyclic monophosphorothioate, *Rp*-isomer; CTAP, D-Phe-Cys-Tyr-D-Trp-Arg-Thr-Pen-Thr-NH_2; H-89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; PD98059, 2′-amino-3′-methoxyflavone; SB203580, 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; ZM336372, 3-(dimethylamino)-*N*-[3-[(4-hydroxybenzoyl)amino]-4-methylphenyl]-benzamide; MAPK, mitogen-activated protein kinase; PKA, protein kinase A; PCR, polymerase chain reaction; RT-PCR, reverse transcription-polymerase chain reaction; IκB, inhibitor of nuclear factor-κB; CAT, chloramphenicol acetyltransferase; ELISA, enzyme-linked immunosorbent assay; PTX, pertussis toxin.

of IκB, which is committed to proteasomal degradation by this signal. After degradation of IκB, NF-κB, which may consist of several proteins among which p65 is most prominent, can enter the nucleus and bind to regulatory DNA sites. In addition, p65 is phosphorylated, which serves as a marker for the transcriptional activity of NF-κB (Baud and Karin, 2001).

Interference with this cascade and thus inhibition of NF-κB may occur at several points. For example, some drugs inhibit the stimulus-triggered degradation of IκB (e.g., Mormina et al., 2006). Alternatively, induction of the expression of IκB, which retains NF-κB in the inactive state, also results in inhibition of NF-κB (e.g., Altman et al., 2008).

Little is known about the precise regulation of NF-κB by opioids. Moreover, these data are not unambiguously clear. Thus, it was reported that NF-κB activity in different cell types is either increased (Liu and Wong, 2005; El-Hage et al., 2008) or decreased (Wang et al., 2008; Börner et al., 2009b) in response to opioids. Therefore, we investigated in detail molecular mechanisms underlying the regulation of NF-κB by morphine in neuronal cells.

Materials and Methods

Cell Culture, Transfection, and Reagents. SH SY5Y cells were cultivated in Dulbecco's modified Eagle's medium supplemented with penicillin/streptomycin (Lonza Verviers SPRL, Verviers, Belgium) and 15% fetal calf serum (Biochrom, Berlin, Germany). The studies in animal cells have been performed in accordance with the *Guide for the Care and Use of Laboratory Animals* (Institute of Laboratory Animal Resources, 1996). Preparation of primary fetal striatal neurons from rats was performed as reported previously (Börner et al., 2007). The neurons were cultivated in Neurobasal medium with glutamine, penicillin/streptomycin, and B-27 supplement (Invitrogen GmbH, Darmstadt, Germany). It has been previously reported that both SH SY5Y cells and primary striatal neurons from rats express μ-opioid receptors, which is the main receptor mediating effects of morphine, and also δ-opioid receptors (Zadina et al., 1994; Mansour et al., 1995; Börner et al., 2007). The transfection of SH SY5Y cells has been described previously (Kraus et al., 2001). The same experimental settings were used for transfection of primary neurons from rats.

TNF (human recombinant TNF-α) was obtained from R&D Systems (Wiesbaden, Germany). Morphine was obtained from Synopharm (Barsbüttel, Germany), and β-endorphin and loperamide were obtained from Sigma-Aldrich (Taufkirchen, Germany). Methadone and fentanyl were obtained from Grünenthal (Aachen, Germany). Cycloheximide, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline (H-89), phorbol 12-myristate 13-acetate (PMA), and forskolin were obtained from Sigma-Aldrich. CTAP, pertussis toxin (PTX), cAMPS-RP, 2′-amino-3′-methoxyflavone (PD98059), 4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)1*H*-imidazole (SB203580), genistein, 3-(dimethylamino)-*N*-[3-[(4-hydroxybenzoyl)amino]-4-methylphenyl]-benzamide (ZM336372), and 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene (U0126) were obtained from Tocris Bioscience (Bristol, UK). To avoid an unspecific effect, the smallest effective doses for all inhibitors were chosen according to our own observations [cAMPS-RP (Börner et al., 2009a,b)] or to published data [H-89 (Roy et al., 2005) and different mitogen-activated protein kinases (MAPK) (Davies et al., 2000)], in which their specificity was demonstrated.

Quantitative Real-Time Reverse Transcription-Polymerase Chain Reaction. Total RNA was extracted using the Nucleospin RNA II kit from Macherey-Nagel (Düren, Germany). One microgram of total RNA was used for cDNA synthesis with Moloney murine leukemia virus reverse transcriptase, RNase H minus (Promega, Mannheim, Germany) and was diluted to 50 μl. Two microliters of cDNA were used for real-time polymerase chain reactions (PCRs). Quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) was performed in a total volume of 20 μl on a LightCycler instrument using the LightCycler and Fast Start DNA Master SYBR Green I kit (both from Roche Diagnostics, Mannheim, Germany) according to the manufacturer's suggestions. PCR primers and conditions were as follows: rat β-actin: 5′-GGTCCACACCCGCCACCAG-3′ and 5′-CAGGTCCAGACGCAGGATGG-3′ primers; preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 60°C, and 22 s at 72°C. Human β-actin: 5′-CGTCCACACCCGCCGCCAGCTC-3′ and 5′-AGCCAGGTCCAGACGCAGGATGG-3′ primers; preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 60°C, and 22 s at 72°C. Human IκB: 5′-CTGCACTTGGCCATCATCCATG-3′ and 5′-CGAAAGTCTCGGAGCTCAGGATC-3′ primers; preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 68°C, and 9 s at 72°C. Human CB1 receptor: 5′-CACCTTCCGCACCATCACCAC-3′ and 5′-GTCTCCCGCAGTCATCTTCTCTTG-3′ primers; preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 68°C, and 10 s at 72°C. Rat CB1 receptor: 5′-CACCTTCCGTACCATCACCAC-3′ and 5′-GTCTCCTGCGGTCATCTTTTCTTG-3′ primers; preincubation for 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 67°C, and 9 s at 72°C.

Decoy Oligonucleotide and siRNA Approaches. The transcription factor decoy oligonucleotide approach, its efficiency, and specificity were described in detail in previous publications from our group (Kraus et al., 2003a,b). In brief, short double-stranded oligonucleotides with specific binding sequences for transcription factors are introduced into living cells by passive uptake during an overnight incubation of the cells in the presence of 160 nM oligonucleotides. In the cells, transcription factors then rather interact with the excess of decoy oligonucleotides than bind to the natural regulatory motifs of genes. Thus, the decoys selectively disrupt the function of a desired transcription factor. Because the decoys act within living cells, they are highly specific. The sequences of the decoy oligonucleotides were as follows (only upper strand is shown): NF-κB D1, 5′-AAAAAAAAGGGACTTTCATTGTACTGGT-3′; NF-κB D2, 5′-GTGGGAGGGGGCTATACGCAGAGG-3′; *mu*NF-κB D1, 5′-TATTATGTGGCTTTTCCTAGAATT-3′; *mu*NF-κB D2, 5′-AGTCTCTAGGAAATCTCTGTAACA-3′; AP-1 D1, 5′-AAACATATGATTCACCAGGCA-3′; AP-1 D2, 5′-TTACCTATGAGTTATCTGTTT-3′; *mu*AP-1 D1, 5′-CCTAAGGAGAGTCAAGAGAAC-3′; and *mu*AP-1 D2, 5′-ACTGAAAGGACTCAGAACTAC-3′. The specificity of the sequences as binding sites for NF-κB and AP-1 or as mutations that do not bind these factors is described elsewhere (Börner et al., 2002; Kraus et al., 2003a). Putative human IκB AP-1 decoys had the following sequences: −1607. 5′-TTTCACCTTGCCTCAATCATTGT-3′; −1455, 5′-CTCTCTATCGAGTCAGATTTCTT-3′; and −156, 5′-GACCCTAGTGGCTCATCGCAGGG-3′. Decoy oligonucleotides were synthesized by Metabion (Martinsried, Germany) as complementary single strands. The siRNA approach was performed using "IκBα siRNA (h)" and "Control siRNA-A," both from Santa Cruz Biotechnology (Heidelberg, Germany).

Reporter Gene Constructs. Cloning of the human hCB1-CAT reporter gene construct (phCB1-CAT-3086) is described in detail elsewhere (Börner et al., 2008). Construction of the reporter plasmid AP-1-tk-CAT was described previously (Börner et al., 2002).

Western Blot Analysis. Western blots were performed as described previously (Börner et al., 2009a,b). Before stimulation, cells were kept for 16 h in medium containing 1% fetal calf serum. Then, cells (6×10^5 per sample) were incubated at 37°C with stimuli or with vehicle. The incubation was stopped by washing the cells with phosphate-buffered saline and subsequent lysis. For protein detection, the following antibodies were used: primary antibodies: actin C-11 and c-Fos K-25 from Santa Cruz Biotechnology; and phospho-NF-κB p65 (Ser536; 93H1) and IκBα from Cell Signaling Technology (Danvers, MA)/New England Biolabs (Frankfurt, Germany); secondary antibodies: anti-rabbit IgG or anti-mouse IgG (both from GE Healthcare, Braunschweig, Germany).

Results

Treatment of Neuronal Cells with TNF Resulted in a NF-κB-Mediated Induction of CB1 mRNA. In primary fetal striatal neurons from rats and in human neuroblastoma SH SY5Y cells TNF (TNF was used at a concentration of 150 pg/ml throughout this study) significantly induced CB1 mRNA (Fig. 1A). This induction was dependent on the transcription factor NF-κB, because inhibiting its activity by decoy oligonucleotides abolished the induction. In contrast, mutated oligonucleotides serving as negative controls, which do not bind NF-κB (Kraus et al., 2003a), did not block this induction (Fig. 1B). In addition, transfection experiments in SH SY5Y cells revealed that the expression of a reporter gene construct containing sequences of the human CB1 gene promoter was significantly induced by TNF (Fig. 1C). Again, this induction was blocked by decoy oligonucleotides directed against NF-κB, but not by mutated oligonucleotides. A construct lacking the CB1 sequences was not responsive to TNF.

Treatment of Neuronal Cells with Morphine Resulted in an Inhibition of the TNF-Mediated Induction of CB1 mRNA. Next, the effect of morphine on the TNF-mediated induction of CB1 mRNA was investigated. Compared with TNF-treated controls (Fig. 2A; lane 2), simultaneous addition of morphine (morphine was used at a concentration of 1 μM throughout this study) together with TNF had no effect on the induction of CB1 mRNA in SH SY5Y cells. However, when the cells were preincubated with morphine for 5 and 24 h before TNF stimulation, the TNF-mediated induction of CB1 mRNA was significantly inhibited. Likewise, the TNF-mediated induction of CB1 mRNA in primary neurons was inhibited by preincubation with morphine (lane 9 versus 10). Furthermore, the inhibitory effect of morphine on the TNF-induced CB1 mRNA was reversible with the μ-opioid receptor-specific antagonist CTAP. In addition, the effect of morphine was abolished in the presence of cycloheximide, an inhibitor of protein biosynthesis. In addition, transient expression of the CB1 reporter construct in SH SY5Y cells demonstrated the inhibitory effect of morphine on the TNF-induced CB1 transcription (Fig. 2B).

Treatment of Neuronal Cells with Morphine Resulted in a NF-κB- and AP-1-Dependent Induction of IκB. The experiments with cycloheximide indicated that protein biosynthesis is needed for the inhibitory effect of morphine on the TNF-mediated induction of CB1 mRNA (see Fig. 2, lane 7). Searching for a newly synthesized protein that mediates inhibition of NF-κB, we found that incubation of primary neurons with morphine resulted in a strong induction of IκB (Fig. 3A). Likewise, IκB was induced in response to morphine in SH SY5Y cells, where a maximal expression was found 3 h after stimulation with the drug (Fig. 3B). These protein data are in good accordance with IκB mRNA data, showing a strong, significant induction in response to morphine in SH SY5Y cells (Fig. 3C). Next, the morphine-mediated induction of IκB was investigated in more detail (Fig. 3D). Coincubation of SH SY5Y cells with the μ-opioid receptor-specific antagonist CTAP abolished the induction of IκB mRNA, indicating that μ-opioid receptors mediate this effect of morphine. It is noteworthy that cycloheximide inhibited the induction of IκB mRNA in response to morphine only partially. This suggests that the induction of IκB mRNA in response to morphine might be dependent on at least two distinct processes, one of which requires protein biosynthesis and one of which is independent of protein biosynthesis. To identify transcription factors that mediated the induction of IκB mRNA in response to morphine, we used the decoy oligonucleotide approach. As depicted in Fig. 3D, different de-

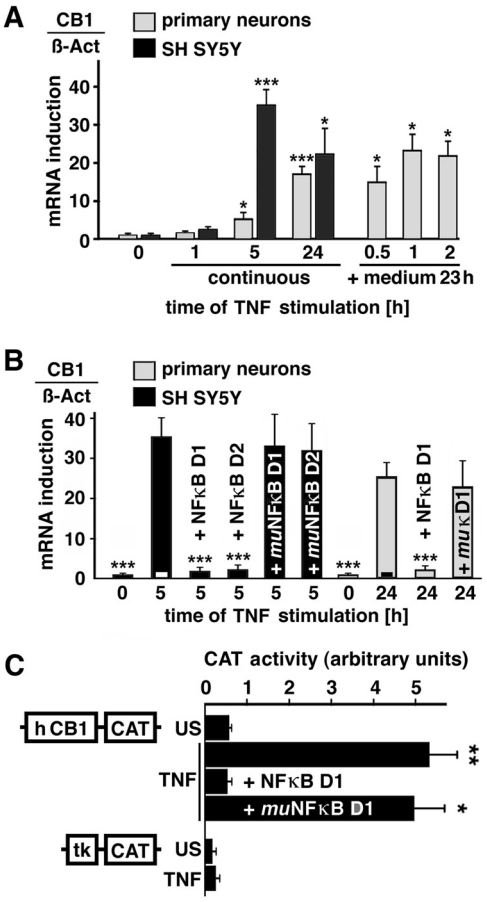


Fig. 1. Treatment of neuronal cells with TNF resulted in a NF-κB-mediated induction of CB1 mRNA. A, induction of CB1 transcription. Primary neurons from rats and SH SY5Y cells were stimulated with TNF (150 pg/ml). Either the cells were incubated continuously with medium containing TNF (left), or the medium was replaced by normal medium without TNF after an initial stimulation of the cells with medium containing TNF (right). After the incubation, cells were lysed and quantitative real-time RT-PCR was performed. The amounts of CB1 transcripts are normalized to those of β-actin. At least two independent experiments performed in duplicate are shown plus S.E.M. (*, $p < 0.05$; ***, $p < 0.001$). B, the TNF-mediated induction of CB1 mRNA is mediated by NF-κB. Primary neurons from rats and SH SY5Y cells were incubated overnight with 160 nM decoy oligonucleotides containing different binding sites for NF-κB (NF-κB D1 and D2) or oligonucleotides containing mutated sites, which do not bind NF-κB (*mu*NF-κB D1/*mu*kD1 and *mu*NF-κB D2). Then, the cells were left untreated or stimulated with TNF (150 pg/ml). Cells were lysed, and RT-PCR was performed as described. At least two independent experiments performed in duplicate are depicted plus S.E.M. Samples were compared with the TNF-stimulated samples of the respective cells. ***, $p < 0.001$. C, inducibility of the human CB1 gene promoter in response to TNF. SH SY5Y cells were transiently transfected with a chloramphenicol acetyltransferase (CAT) reporter gene construct containing the human CB1 gene promoter spanning from nt − 3086 to nt + 142 (hCB1-CAT), or the construction vector pBLCAT2 with the herpes simplex thymidine kinase minimal promoter (tk) instead of CB1 sequences (tk-CAT). The next day, medium was replaced, decoy oligonucleotides (NF-κB D1 or the negative control *mu*NF-κB D1; 160 nM) were added to the medium, and cells were stimulated with TNF (150 pg/ml) as indicated. Cells were lysed 72 h after transfection and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed (*, $p < 0.05$; **, $p < 0.01$).

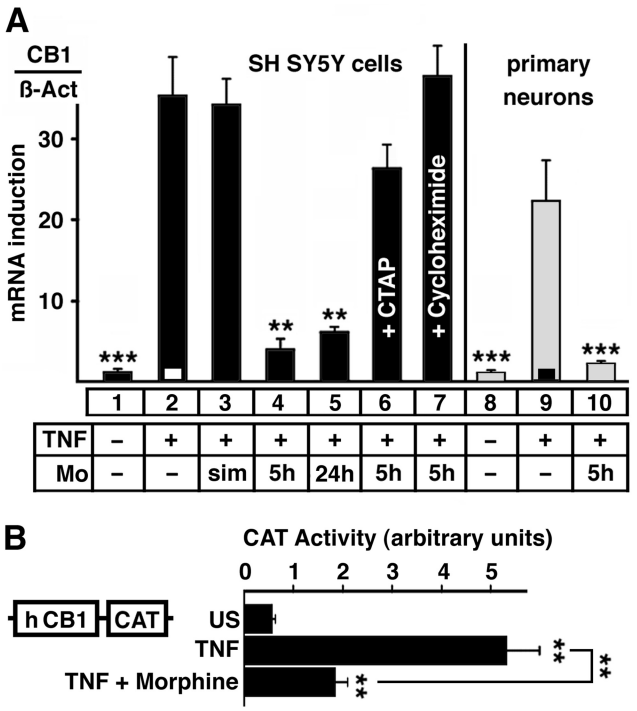


Fig. 2. Treatment of neuronal cells with morphine resulted in an inhibition of the TNF-mediated induction of CB1 mRNA. A, effect of morphine on the TNF-induced CB1 mRNA in primary neurons and SH SY5Y cells. CB1 transcripts normalized to β-actin as measured by quantitative real-time RT-PCR are shown. Bottom, a scheme is plotted showing the incubation of the cells with TNF (150 pg/ml) and morphine (1 μM). For maximal induction of CB1 mRNA (see Fig. 1), SH SY5Y cells were incubated for 5 h, and primary neurons were incubated for 24 h with TNF (lanes 2 and 9, respectively). As shown in the scheme, some samples were additionally treated with morphine, which was added to the cells simultaneously with TNF (sim) or 5 or 24 h before TNF (as indicated), and were then coincubated with the cytokine. At least three independent experiments were performed in duplicate and are shown plus S.E.M. All samples were compared with the TNF-treated samples shown in lanes 2 (SH SY5Y cells) and 9 (primary cells). **, $p < 0.01$; ***, $p < 0.001$. CTAP (250 nM) and cycloheximide (10 μg/ml) were applied 1 and 16 h before morphine, respectively. B, effect of morphine on the TNF-induced promoter activity of CB1. SH SY5Y cells were transiently transfected with the hCB1-CAT construct. The next day, the medium was replaced, and cells were incubated with TNF (150 pg/ml) and morphine (1 μM) as indicated. Cells were lysed 72 h after transfection, and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed. A secondary comparison is indicated by a bracket (**, $p < 0.01$).

coy oligonucleotides directed against NF-κB (NF-κB D1 and D2) and AP-1 (AP-1 D1 and D2) strongly inhibited the morphine-mediated induction of IκB. In contrast, oligonucleotides containing mismatches, which do not bind these transcription factors (mu-), were ineffective. This suggested that NF-κB and AP-1 were involved in mediating the morphine-induced expression of IκB. A similar NF-κB- and AP-1-dependent induction of IκB was also observed in primary neuronal cells (Fig. 3D). An alternative mechanism resulting in the inhibition of NF-κB-dependent signaling is the inhibition of the TNF-induced degradation of IκB (Mormina et al., 2006; Liao et al., 2008). However, we did not observe such an effect of morphine in the SH SY5Y cells (Fig. 3E). Reports suggest that some effects of morphine are different from those of other opioids [e.g., effects of peptide opioids resulting from ligand-specific signaling of μ-opioid receptors (e.g., Zhang et al., 1998)]. To test such a possibility, various μ-opioid receptor ligands were investigated with respect to their ability to induce IκB (Fig. 3F). However, treatment of SH SY5Y cells with morphine, the endogenous opioid peptide β-endorphin, the analgesics methadone and fentanyl, and the peripheral opioid loperamide, in doses that are known to produce similar effects at the receptors, resulted in a similar induction of IκB.

Identification of an AP-1-Element in the Human IκB Gene. Because the sequences of the human IκB gene are known, we attempted to identify functional *cis*-active AP-1 elements within the promoter region of the gene (Fig. 4). Sequence comparisons with the classic seven-base-pair palindromic AP-1 binding sequence, 5′-TGA(C/G)TCA-3′, revealed three sequence motifs within approximately 2 kb of the human IκB promoter with one mismatch (Fig. 4A). A 100% homologous AP-1 motif is not present within this region. We used the putative sequences as decoys to demonstrate their functionality as AP-1 sites. In a first approach, it was tested whether they inhibited the induction of IκB by morphine (Fig. 4B). Similar to a classic AP-1 decoy, this was indeed the case for the proximal motif, located at nt −156. The other motifs/decoys did not interfere with the morphine-mediated induction of IκB. To demonstrate that the −156 motif binds AP-1, a reporter gene-based approach was chosen (Fig. 4C). Again, similar to a classic AP-1 sequence decoy, the −156 decoy inhibited the PMA-inducible expression of an AP-1-driven reporter gene construct. The other motifs/decoys had no effect.

Treatment of Neuronal Cells with Morphine Resulted in a Transient Activation of NF-κB and an Induction of c-Fos. Because the morphine-mediated induction of I-κB was dependent on AP-1 and NF-κB itself, we investigated whether morphine treatment of neuronal cells resulted in the activation of these factors. Indeed, phosphorylation of p65, which serves as an indicator for the transcriptional activity of NF-κB, as well as induction of c-Fos, was observed in response to morphine in both primary neurons (Fig. 5) and SH SY5Y cells (data not shown) with similar intensities and similar kinetics. The morphine-mediated phosphorylation of p65 peaked at the 30-min time point. At the 2-h time point, phosphorylation of p65 was similar to that of untreated controls (data not shown).

Involvement of μ-Opioid Receptor-Dependent Signaling Pathways in the Phosphorylation of p65 and Induction of c-Fos by Morphine. It is known that μ-opioid receptors are coupled to $G_{i/o}$ proteins. By activation of μ-opioid receptors, adenylyl cyclase/cAMP-dependent pathways and p42/44 MAPK pathways are modulated. We next attempted to characterize mechanisms, which are involved in the morphine-/μ-opioid receptor-mediated phosphorylation of p65 and induction of c-Fos (Fig. 6). Pretreatment of primary neurons (Fig. 6A) and SH SY5Ycells (data not shown) with PTX, which is an inhibitor of G_i protein-mediated processes, inhibited the phosphorylation of p65 as well as the induction of c-Fos. With respect to the cAMP pathway, we found in primary neurons from rats that the activator of adenylyl cyclase forskolin inhibited the morphine-mediated phosphorylation of p65, but not the induction of c-Fos. Forskolin alone had no effect on phosphorylation of p65 or c-Fos levels. The inhibitors of protein kinase A (PKA), cAMPS-RP and H-89, did not interfere with the morphine-mediated phosphorylation of p65 and induction of c-Fos (Fig. 6B). Similar experiments in SH SY5Y cells resulted in comparable effects (data not shown). Next, we tested effects of inhibitors of MAPK

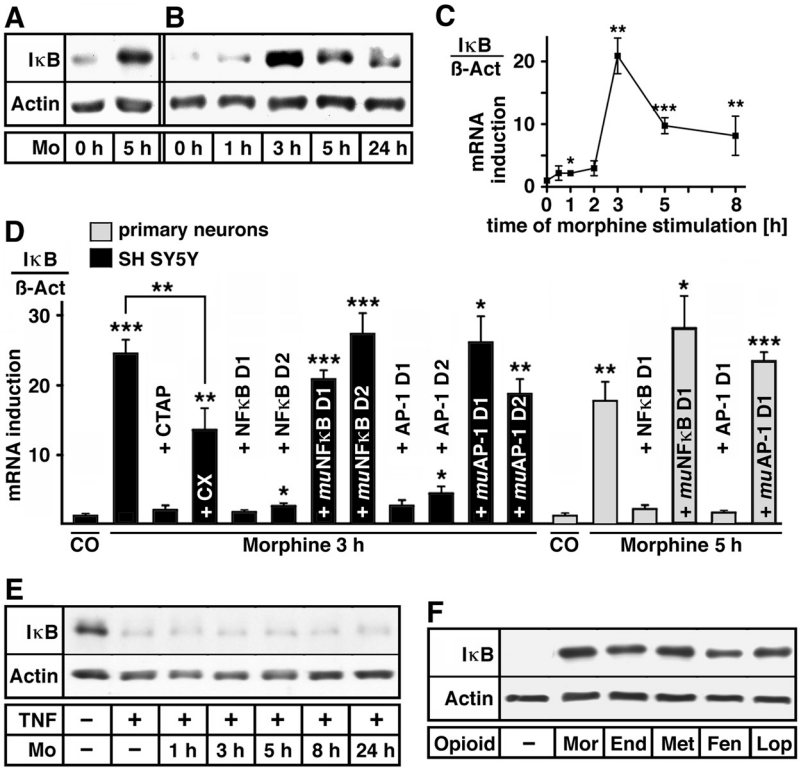


Fig. 3. Treatment of neuronal cells with morphine resulted in a NF-κB- and AP-1-dependent induction of IκB. Induction of IκB in response to morphine in neuronal cells. Primary neurons from rats (A) and SH SY5Y cells (B) were stimulated with morphine (Mo; 1 μM) for the indicated times. Blots were probed for IκB and, as controls, reprobed for actin. Examples of representative Western blot experiments are depicted, which were performed at least two times in duplicate. C, detection of IκB transcripts in response to morphine in SH SY5Y cells by quantitative real-time RT-PCR. Cells were stimulated with morphine (1 μM) and lysed, and RT-PCR was performed. The amounts of IκB transcripts are normalized to β-actin. At least two independent experiments performed in duplicate are shown plus S.E.M. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). D, the morphine-triggered induction of IκB is mediated by μ-opioid receptors and is dependent on NF-κB and AP-1. IκB transcripts in SH SY5Y cells (black columns, left) and primary neurons from rats (gray columns, right) were detected by quantitative real-time RT-PCR and are plotted normalized to β-actin. Stimulation with morphine (1 μM) is indicated. CTAP (250 nM) and cycloheximide (CX; 10 μg/ml) were applied 1 and 16 h before morphine, respectively. Decoy oligonucleotides (160 nM) were added to the cells 16 h before morphine. The decoy oligonucleotides contain different binding sites for NF-κB and AP-1 (NF-κB D1 and D2; AP-1 D1 and D2), or oligonucleotides contain mutated sites, which do not to bind NF-κB and AP-1 (*mu*NF-κB D1 and D2; *mu*AP-1 D1 and D2). Values are compared with nontreated controls (CO). A secondary comparison is indicated by a bracket. At least two independent experiments were performed in duplicate and are shown plus S.E.M. (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). E, morphine treatment of SH SY5Y cells does not inhibit the TNF-triggered degradation of IκB. Cells were stimulated with TNF (150 pg/ml) for 5 min to induce degradation of IκB (lane 2). Some samples were incubated with morphine for the indicated times (MO; 1 μM) before the TNF stimulus (lanes 3–7). Western blots were probed for IκB and actin as a control. A representative example of two experiments is depicted. F, induction of IκB by various opioids. A representative Western blot (of two individual experiments) is presented showing the induction of IκB in response to 3-h treatment of SH SY5Y cells with morphine (Mor; 1 μM), β-endorphin (End; 1 μM), methadone (Met; 1 μM), fentanyl (Fen; 15 nM), and loperamide (Lop; 1.5 μM). The same blot was reprobed for actin.

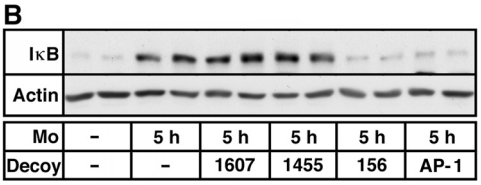
pathways on the morphine-mediated phosphorylation of p65 and induction of c-Fos in primary neurons (Fig. 6C) and in SH SY5Y cells (data not shown), both of which produced similar results. Employing inhibitors of the p42/44 MAPK pathway, which were PD98059 and U0126 (both are inhibitors of MAPK kinase) and ZM336372 (an inhibitor of c-Raf) resulted in a significant inhibition of the morphine-mediated induction of c-Fos. However, the morphine-mediated phosphorylation of p65 was not influenced by these inhibitors. Likewise, genistein, which is an inhibitor of protein tyrosine kinases, inhibited significantly the induction of c-Fos but had no effect on the morphine-mediated phosphorylation of p65. No significant effects on the morphine-mediated induction of c-Fos and phosphorylation of p65 were observed employing SB203580, an inhibitor of the p38 MAPK pathway.

The AP-1-Mediated Induction of IκB Is a Key Event in the Inhibitory Effect of Morphine on NF-κB in Neuronal Cells. It is known that TNF is a strong inducer of the NF-κB pathway and that TNF-induced NF-κB is involved in a negative feedback loop resulting in the termination of the NF-κB-response via induction of IκB (Baud and Karin, 2001; Renner and Schmitz, 2009). It is noteworthy that morphine also promotes activation of NF-κB and induction of IκB. However, in the long term, morphine causes an inhibition of NF-κB signaling, as shown for the inhibition of the TNF-mediated induction of CB1 mRNA. In an attempt to find explanations for the different effects of TNF and morphine on NF-κB, we investigated and compared the regulation of IκB by TNF and morphine (Fig. 7). Incubation of primary neurons with TNF resulted in the well established degradation of IκB within a few minutes. Prolonged incubation of the cells with TNF resulted in a resynthesis of IκB within the first hours, which is in line with the established model (Baud and Karin, 2001; Renner and Schmitz, 2009). Incubation of primary neurons with morphine produced a similar degradation of IκB within few minutes. In sharp contrast to TNF, however, prolonged incubation of the cells with morphine resulted in a marked up-regulation of IκB with levels signifi-

A

putative AP-1 sites of the human IκBα gene	
location	sequence/homology to AP-1 site
-1607	5'-TTTCACCTTGCCTCAATCATTGT-3'
-1455	5'-CTCTCTATCGAGTCAGATTTCTT-3'
-156	5'-GACCCTAGTGGCTCATCGCAGGG-3'

B



C

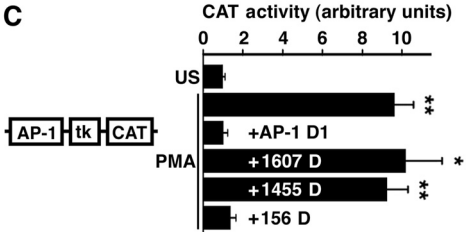


Fig. 4. Identification of an AP-1 site in the IκB promoter. A, putative AP-1 sites of the IκB gene along with their locations with respect to the transcriptional start site. B, Western blot experiment designed to test the putative AP-1 sites of the IκB gene as decoys and the classic AP-1 D1 decoy (AP-1) for their ability to inhibit the morphine (Mo)-mediated induction of IκB. Decoy oligonucleotides (160 nM) were added to the samples 16 h before the morphine stimulation. Blots were probed for IκB, and, as controls, were reprobed for actin. Examples of representative experiments in primary neuronal cells are depicted, which were performed two times. C, transfection experiments designed to test the putative AP-1 sites of the IκB gene and the classic AP-1 D1 decoys for their ability to inhibit the PMA-mediated induction of an AP-1-reporter construct. SH SY5Y cells were transiently transfected with an AP-1-responsive CAT reporter gene construct (AP-1-tk-CAT). The next day, medium was replaced, decoy oligonucleotides (160 nM) were added to the medium, and cells were stimulated with PMA (100 nm). Cells were lysed 72 h after transfection, and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed (*, $p < 0.05$; **, $p < 0.01$).

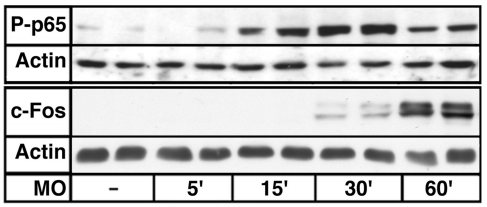


Fig. 5. Treatment of primary neuronal cells from rats with morphine resulted in an activation of NF-κB and AP-1. Western blot experiments showing the phosphorylation of p65 and the induction of c-Fos. Cells were stimulated with morphine (MO; 1 μM) for the indicated times. Blots were probed for phospho (P-) p65 or c-Fos and, as controls, were reprobed for actin. Examples of representative experiments are depicted, which were performed at least two times in duplicate.

cantly exceeding basal levels. Although it is well known that TNF activates NF-κB, it is unclear whether the cytokine also activates AP-1 in neuronal cells. Therefore, the activation of c-Fos and AP-1 by TNF in the neuronal cells was studied next (Fig. 8). Transient transfection studies in SH SY5Y cells demonstrated significant transactivation of an AP-1-dependent reporter gene in response to morphine and, as a control, the phorbol ester PMA, which is known to activate AP-1, but not in response to TNF (Fig. 8A). Furthermore, we demonstrated that incubation of primary neurons with TNF does not result in an induction of c-Fos (Fig. 8B). This suggests that the inhibitory action of morphine on NF-κB in neuronal cells is achieved via a strong induction of IκB, which is dependent mainly on AP-1 and results in IκB levels that exceed basal levels. In contrast, TNF, which does not activate AP-1 in neurons, causes resynthesis only of basal IκB levels, which is dependent mainly on NF-κB. To further investigate the importance of the AP-1-mediated induction of IκB in the inhibitory effect of morphine on NF-κB, we used AP-1 decoy oligonucleotides to block this induction and monitored the effect of morphine on the TNF-triggered induction of CB1 mRNA (Fig. 9A). First, controls with the AP-1 decoy oligonucleotides were performed to guarantee that the oligonucleotides and AP-1 do not interfere with the induction of CB1 by TNF itself. These experiments showed no significant effect of the decoy oligonucleotides (lanes 1–6). The decoy oligonucleotides directed against AP-1, however, strongly and significantly inhibited the effect of morphine, i.e., the inhibition of the TNF-induced CB1 transcription, in the SH SY5Y cells (lanes 8 and 9 versus lane 7) and in the primary neurons from rats (lanes 15 and 16 versus lane 14). Control oligonucleotides that do not bind AP-1 had no significant effect on the morphine-mediated inhibition of the TNF-induced CB1 transcription (lanes 10 and 11 versus lane 7, and lanes 17 and 18 versus lane 14). To obtain further evidence for our hypothesis, experiments with an siRNA directed against IκB were performed (Fig. 9, B and C). Transfection of an IκB siRNA into SH SY5Y cells and primary neurons from rats inhibited the morphine-mediated increase in IκB (Fig. 9B). In addition, the inhibitory effect of morphine on the TNF-triggered induction of CB1 was significantly inhibited in cells transfected with the IκB siRNA compared with cells transfected with a scrambled siRNA (Fig. 9C). Because the siRNA was more efficient in the SH SY5Y cells compared with the primary neurons, which is probably due to a higher transfection efficiency in the SH SY5Y cells (see Fig. 9B), the effect of the siRNA on the inhibition of the TNF-triggered induction of CB1 by morphine was more pronounced in the SH SY5Y cells than in the primary cells (see Fig. 9C).

Discussion

We demonstrated that TNF stimulation of neuronal cells resulted in an induction of CB1 mRNA, which was mediated by NF-κB. Using this transcriptional effect as a model, we show that morphine inhibited the TNF-triggered transcription of CB1 via inhibition of NF-κB. The experiments were performed in SH SY5Y cells, because it is the only human cell line that is related to neuronal cells and expresses μ-opioid receptors, and which is therefore relevant for opioid effects in humans, as well as in primary neuronal cells from rats. The experiments in both cells produced very similar results.

The induction of CB1 by TNF in neuronal cells is a novel finding. Its relevance needs to be investigated further. It is probably a physiological mechanism to counteract neuroinflammatory conditions, taking advantage of increased anti-inflammatory effects of endocannabinoids due to increased numbers of CB1. The inhibition of the TNF-triggered induction of CB1 mRNA by morphine/μ-opioid receptors was used as a model. It should be mentioned that treatment of SH SY5Y cells with various other opioids resulted in a similar

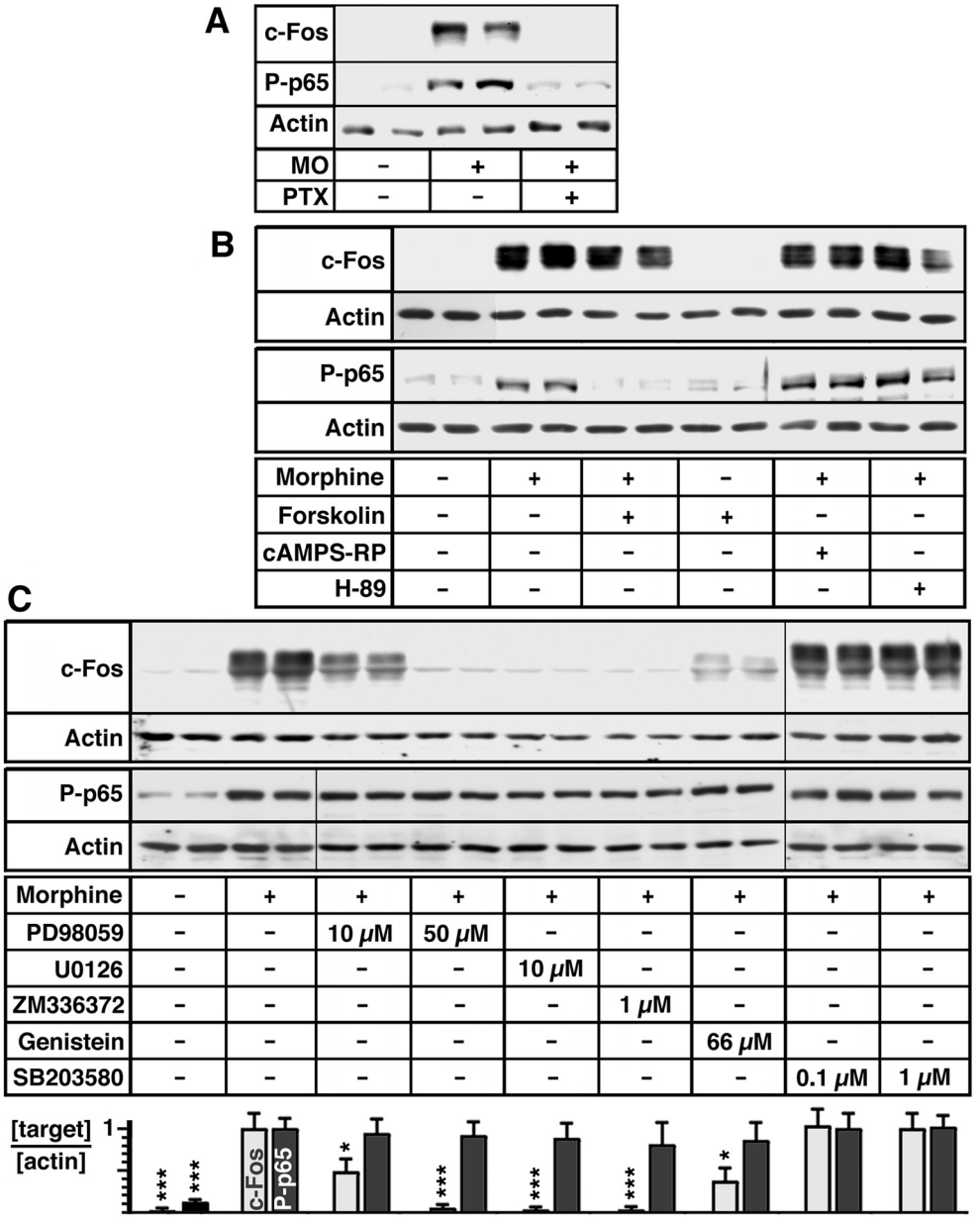


Fig. 6. Involvement of proximal μ-opioid receptor-dependent pathways in the phosphorylation of p65 and induction of c-Fos by morphine. A, the phosphorylation of p65 and the induction of c-Fos by morphine is sensitive to PTX. Primary neuronal cells were incubated overnight with PTX (10 ng/ml) and then were stimulated with morphine (1 μM) for 45 min as indicated. Blots were probed for c-Fos and phospho (P-) p65, and, as controls, were reprobed for actin. Examples of representative Western blot experiments are depicted, which were performed at least two times. B, Western blot experiments showing the involvement of the cAMP/PKA pathway in the induction of c-Fos and the phosphorylation of p65 in response to morphine. Primary neuronal cells were stimulated with morphine (1 μM) for 45 min as indicated. Blots were probed for c-Fos and phospho (P-) p65 and, as controls, were reprobed for actin. As indicated, some samples were additionally treated with the adenylyl cyclase activator forskolin (25 μM; applied simultaneously with morphine, or 30 min alone), or the PKA inhibitors cAMPS-RP (100 μM; applied 1 h before morphine) and H-89 (20 μM; applied 6 h before morphine). Examples of representative experiments are depicted, which were performed at least two times. C, Western blot experiments showing the effect of MAPK pathways on the induction of c-Fos and phosphorylation of p65 in response to morphine in primary rat neurons. Cells were stimulated with morphine (1 μM) for 45 min as indicated. Some samples were additionally treated with the MAPK inhibitors PD98059 or U0126, the c-Raf inhibitor ZM336372, the tyrosine kinase/epidermal growth factor receptor kinase inhibitor genistein, or the p38 inhibitor SB203580. All inhibitors were applied 2 h before morphine. Examples of representative experiments, performed at least three times, are shown. A quantification of these experiments is shown at the bottom (asterisks indicate samples significantly different from morphine-treated controls, which were set to 1; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

induction of IκB. This suggests that most, if not all, opioids activating μ-opioid receptors induce IκB and result in an inhibition of NF-κB. This is worthy to note because ligand-specific signaling of μ-opioid receptors, in which morphine often acts in a different way compared with other agonists, is increasingly recognized (Zhang et al., 1998). In addition, it should be noted that the inhibition of NF-κB by opioids is most likely not restricted to the TNF-triggered induction of CB1 mRNA, but that this is a general effect of opioids on NF-κB. NF-κB is involved in a large number of physiological and pathophysiological neuronal pathways (Kaltschmidt and Kaltschmidt, 2009; Park and Bowers, 2010), all of which could potentially be modulated by opioids. With respect to chronic inflammatory diseases such as multiple sclerosis,

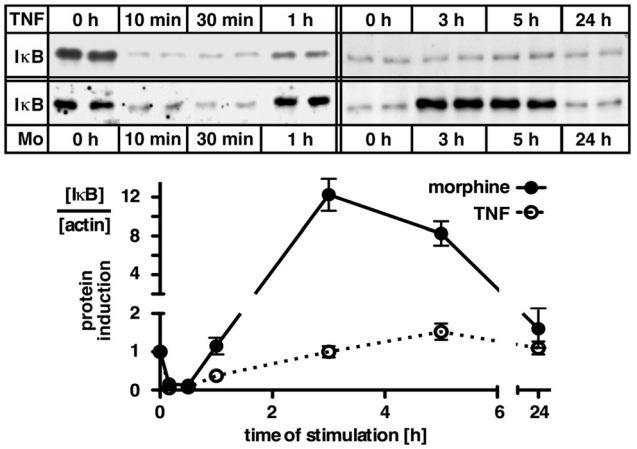


Fig. 7. Regulation of IκB in primary neurons from rats in response to TNF and morphine. Cells were incubated with TNF (150 pg/ml) or morphine (1 μM) for the indicated times. Western blots were probed for IκB and, as controls, were reprobed for actin (data not shown). Examples of representative experiments are depicted at the top. Please note that to better visualize the initial degradation of IκB in response to the stimuli within the first hour (left) and its subsequent regulation (right), different amounts of the cell lysates were loaded on the gels, and the exposure times were different for the left and the right blots. Therefore, the 0-h controls appear different on the two blots. A normalization and quantification of two experiments is shown below.

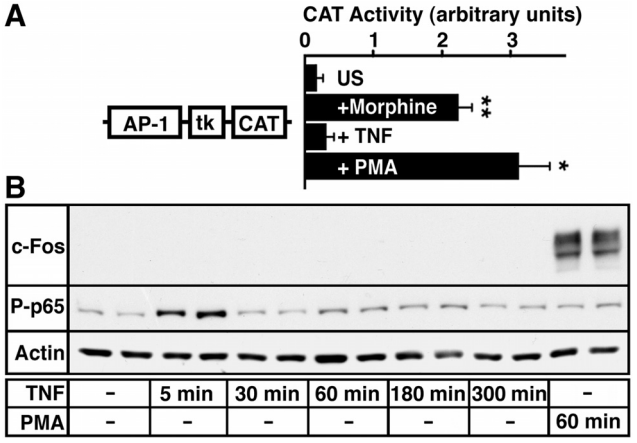


Fig. 8. TNF does not activate AP-1 in neuronal cells. A, effects of morphine, TNF, and the phorbol ester PMA on the activity of an AP-1-responsive reporter gene construct. SH SY5Y cells were transiently transfected with a CAT reporter gene construct containing the herpes simplex thymidine kinase minimal promoter (tk) and one copy of the classic AP-1-binding site 5′-TGACTCA-3′ in front of the promoter (AP-1-tk-CAT). The next day, medium was replaced, and cells were stimulated with morphine (1 μM), TNF (150 pg/ml), or PMA (100 nM) or were treated with vehicle (US, unstimulated) as indicated. Cells were lysed 72 h after transfection, and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed (*, $p < 0.05$; **, $p < 0.01$). PMA, which is a classic activator of AP-1, was used as a control to guarantee proper transfection and responsiveness of the reporter gene construct. B, stimulation of primary neurons from rats with TNF does not result in an induction of c-Fos. Cells were incubated with TNF (150 pg/ml) or PMA (100 nM) for the indicated times (minutes). Western blots were probed for c-Fos and were reprobed for phospho (P-) p65 and actin. A representative example of two experiments is shown. The PMA controls demonstrate that the missing effect of TNF on c-Fos is not due to problems with the antibody or the Western blot procedure. The P-p65 induction at the 5-min time point demonstrates proper activity of TNF.

opioids might have beneficial effects, because they not only inhibit neuronal NF-κB, as shown here, but also the NF-κB-mediated expression of interleukin-2 in lymphocytes, which involves different mechanisms (Börner et al., 2009b). Whether opioids modulate such pathways and diseases in vivo remains to be investigated. In this context, it would be important to see in animal models whether IκB is up-regulated in brain regions that express μ-opioid receptors in response to single and repeated applications of opioids, and to compare these results with those obtained in brain regions that do not express these receptors.

CB1 and μ-opioid receptors are both coupled to $G_{i/o}$ proteins. Therefore, opioids and cannabinoids often show similar effects. Thus, the question is raised whether both classes of drugs inhibit NF-κB. Inhibition of NF-κB by cannabinoids has indeed been observed repeatedly (Nakajima et al., 2006; Correa et al., 2010). However, the precise mechanisms and the question about which receptors mediate such effects are not completely clear. In CB1 knockout mice, an inhibition of neuronal NF-κB by cannabinoids, which is seen in wild-type mice, was not observed, indicating a functional role of CB1 in the inhibition of NF-κB (Panikashvili et al., 2005). This suggests that endocannabinoids might regulate the expression of TNF- and NF-κB-induced CB1 in a feedback loop.

Our experiments showed that CB1 is trans-activated by NF-κB and that sequences between nucleotides −3086 and +142 of the CB1 gene mediate this regulation (see Fig. 1). A comparison of sequences with the consensus binding site for NF-κB, which is 5′-GGG(G/A)NN(C/T)(C/T)CC-3′, revealed one homologous site in the CB1 promoter (located at nucleotide −577; 5′-GGGGGCCTCC-3′), which might serve as a binding site for NF-κB.

Morphine markedly inhibited the TNF-triggered induction of CB1 mRNA by inhibiting NF-κB (see Fig. 2). The cycloheximide experiments indicated that protein biosynthesis is needed for this effect. This prompted us to investigate the expression of IκB more closely. Indeed, morphine induced IκB in both cell models, which was most pronounced 3 to 5 h after the stimulus (see Fig. 3). These kinetics fit well with the kinetics of the morphine-mediated inhibition of the TNF-triggered CB1 induction, which revealed that preincubation of the cells with morphine for 5 h resulted in a strong inhibitory effect of morphine on NF-κB. In this context, it should be mentioned that elevated IκB levels inhibit NF-κB signaling not only by preventing its translocation into the nucleus, but also by shuttling nuclear NF-κB back to the cytoplasm (Arenzana-Seisdedos et al., 1997). The decoy oligonucleotide experiments suggested that NF-κB itself and AP-1 are involved in the morphine-triggered induction of IκB (see Fig. 3D). This observation is in line with the cycloheximide data, which indicated that protein biosynthesis-dependent and biosynthesis-independent mechanisms are involved. Whereas activation of NF-κB is independent of protein biosynthesis, activation of AP-1 often is associated with an induction of c-Fos, which is a subunit of AP-1 (Persico and Uhl, 1996). Likewise, Western blot experiments clearly demonstrated activation of NF-κB by morphine and induction of c-Fos in response to the drug (see Fig. 5) in the neuronal models. The regulation of IκB expression by NF-κB itself leading to an autoregulatory feedback loop that terminates NF-κB activity, e.g., after a TNF stimulus, is established (Baud and Karin, 2001; Kearns et al., 2006). As a structural basis, three NF-κB sites were identified on the proximal IκB promoter (Ito et al., 1994). However, regulation of IκB by AP-1, which was demonstrated in our experiments, is a novel finding. As a basis

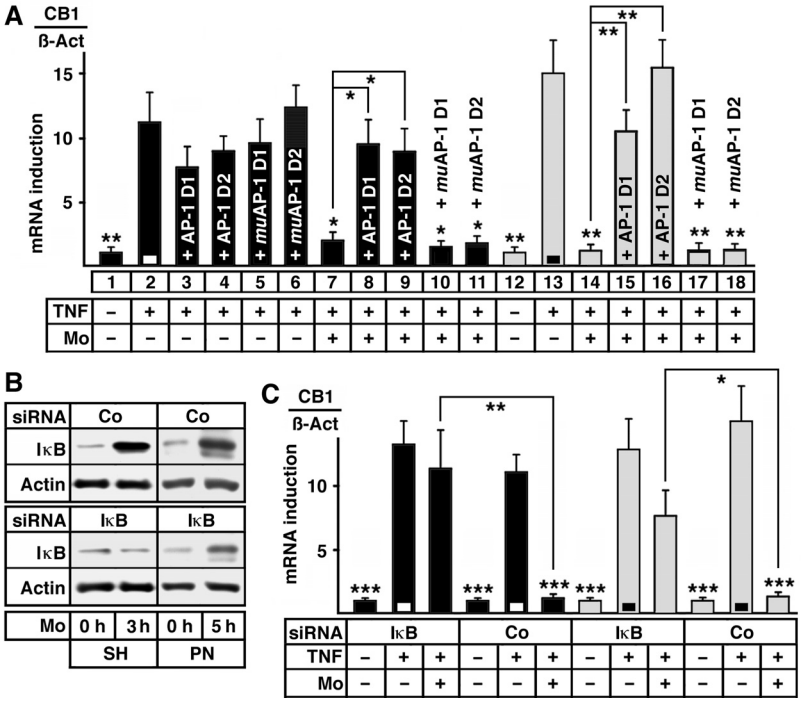


Fig. 9. The AP-1-dependent induction of IκB is a key factor in the inhibition of the TNF-triggered induction of CB1 transcription by morphine. A, decoy oligonucleotides directed against AP-1 abrogate the inhibitory effect of morphine on the TNF-triggered induction of CB1. SH SY5Y cells (lanes 1–11) and primary neurons from rats (lanes 12–18) were stimulated with TNF for 5 (SH SY5Y cells) or 24 h (primary cells) or were left untreated. Some cells were prestimulated with morphine for 5 h, then TNF was added, and cells were further incubated as described. To some samples, decoy oligonucleotides (160 nM) were added 16 h before morphine. The oligonucleotides contain different binding sites for AP-1 (AP-1 D1 and D2) or mutated sites, which do not to bind AP-1 (*mu*AP-1 D1 and D2). After stimulation, cells were subjected to quantitative real-time RT-PCR. The amounts of CB1 transcripts are normalized to those of β-actin and are shown plus S.E.M. Values are compared with TNF-treated samples shown in lanes 2 and 13. Secondary comparisons are indicated by brackets. Two independent experiments were performed in duplicate and are shown plus S.E.M. (*, $p < 0.05$; **, $p < 0.01$). B, an siRNA against IκB abolishes the induction of the protein in response to morphine. SH SY5Y cells (SH, left) and primary neurons from rats (PN, right; both cell types 7×10^5 in 2 ml) were transfected with 60 pmol an IκB siRNA or a nonmatching control siRNA. Sixteen hours after the transfection, cells were stimulated with morphine for the indicated time or left untreated. Western blot experiments were performed to quantify IκB and actin proteins. C, an siRNA against IκB abolishes the inhibitory effect of morphine on the TNF-triggered induction of CB1. Cells that were simultaneously transfected with those that were used for the Western blot described in B were used 16 h after the transfection for stimulation experiments. Stimulation of the SH SY5Y cells (black columns, left) and primary neuronal cells from rats (gray columns, right) and quantitative real-time RT-PCR were performed as explained in A. Two independent experiments were performed in duplicate and are shown plus S.E.M. Samples were compared with the TNF-treated samples. Secondary comparisons are indicated by brackets (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$).

for this regulation, we identified an AP-1 site on the proximal IκB promoter at nucleotide −156.

Using the specific antagonist CTAP, we demonstrated that the effects of morphine are mediated by μ-opioid receptors. Therefore, we attempted to identify μ-opioid receptor-mediated mechanisms involved in the activation of NF-κB and induction of c-Fos (see Fig. 6). The induction of c-Fos was not influenced by activation of the cAMP/PKA pathway by forskolin or by inhibition of this pathway, suggesting that it may not be involved. In contrast, we found that activation of the p42/44 MAPK pathway is essential for the induction of c-Fos by morphine. It is known that this pathway plays a pivotal role in the signal transduction of G-protein-coupled receptors, to which the opioid receptors also belong (Pierce et al., 2001; Bilecki et al., 2005). The activation of NF-κB seemed to be independent of MAPK pathways. However, the experiments manipulating the cAMP/PKA pathway suggested a regulatory role of this pathway in the activation of NF-κB. In particular, activation of this pathway inhibited the phosphorylation of p65, which was also observed by others (Kamthong et al., 2000; Takahashi et al., 2002). Therefore, it might be speculated that decreased cAMP levels, which are traditionally associated with the G_i-protein-coupled μ-opioid receptors, are important for the activation of NF-κB by opioids.

Activation of NF-κB and induction of c-Fos by morphine in SH SY5Y cells has been reported previously by different groups (Chang et al., 1993; Gutstein et al., 1998; Liu and Wong, 2005). However, a physiological relevance of these events was not demonstrated. Our data suggest that both events contribute to the inhibition of NF-κB signaling by morphine. In this scenario, the induction of IκB by AP-1 is the key factor: Stimulation of neuronal cells with morphine results in an AP-1-mediated plus NF-κB-mediated synthesis of IκB, producing levels of IκB that markedly exceed the basal levels. This results in inhibitory effects on the activity of NF-κB. Stimulation of neuronal cells with TNF, which is a strong inducer of NF-κB, also results in a NF-κB-mediated resynthesis, but not overexpression of IκB. The difference between TNF and morphine is the lack of activation of AP-1 by TNF in a neuronal context. This is a novel and astonishing finding, because it seemed well established that TNF induces both, NF-κB and AP-1 in various cell types, especially immune effector cells (e.g., Brenner et al., 1989; Redhu et al., 2011). However, to the best of our knowledge, we found no indication in literature that this applies also to neuronal cells. In contrast, our data indicate that TNF does not activate AP-1 in neuronal cells (see Fig. 8). This is further supported by earlier data obtained in transfection studies and in

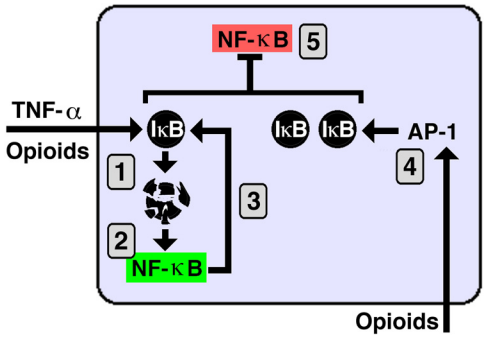


Fig. 10. Scheme summarizing the regulation of NF-κB by opioids and TNF in neuronal cells. On the one hand, stimulation of neurons with opioids results in a degradation of IκB (1) and subsequent activation of NF-κB (2). The activated NF-κB directs resynthesis of IκB (3). The same loop (1–3) is characteristic for stimulation of neurons with TNF. On the other hand, stimulation of neurons with opioids also results in an AP-1-dependent overexpression of IκB (4). TNF does not activate AP-1 in neurons. The opioid-triggered overexpression of IκB results in an inhibition of NF-κB (5).

electrophoretic mobility shift analysis demonstrating that TNF stimulation of SH SY5Y cells, which was monitored over a period of 24 h, resulted in an activation of NF-κB only, but not of AP-1 (Börner et al., 2002). In this context, it is interesting to mention that AP-1 suppresses NF-κB-activity in a colitis model (Takada et al., 2010). Our experiments, in which the induction of IκB by AP-1 was inhibited using decoy oligonucleotides and siRNA, and in which the inhibitory effect of morphine on NF-κB was abrogated (see Fig. 9), further strengthen the importance of the induction of IκB via AP-1 in response to morphine for the inhibitory effect of the drug on NF-κB. Parts of the results of this report are summarized in a scheme (Fig. 10).

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Authorship Contributions

Participated in research design: Börner, Höllt, and Kraus.
Conducted experiments: Börner and Kraus.
Performed data analysis: Börner and Kraus.
Wrote or contributed to the writing of the manuscript: Börner, Höllt, and Kraus.

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