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Title page

Mechanisms of the Inhibition of NF−κb by Morphine in Neuronal Cells

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Running title page

Running title: Inhibition of NF−κB by Morphine

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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 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 upregulation of I−κB, the inhibitor of NF−κB. The opioid-induced upregulation of I−κB was dependent on the transcription factors NF−κB itself and 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 I−κB levels significantly exceeding the basal, constitutive levels of I−κB. These data, together with experiments, in which AP-1 and I−κB were down-regulated by decoy oligonucleotides and siRNA, suggest that the morphine-induced activation of AP-1, and the subsequent overexpression of I−κB 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 I−κB, in which the basal levels of I−κB were restored, only, but not in an activation of AP-1 and overexpression of I−κB.

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Introduction

Many neuronal functions involve the transcription factor 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 irreplaceable for the treatment of severe pain and in anaesthesia. 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 regulate e. g. food intake, body weight and emotional responses and control neuroinflammation (Borner et al., 2009a; Di Marzo, 2008; 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 (Bisogno and Di Marzo, 2010; Blazquez et al., 2011; Centonze et al., 2008).

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 I−κB 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 I−κBα-kinase-complex. This kinase complex in turn is responsible for the phosphorylation of I−κB, which is committed to proteasomal degradation by this signal. After degradation of I−κB, 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,

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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 I−κB (e. g. Mormina et al., 2006). Alternatively, induction of the expression of I−κB, 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 (El-Hage et al., 2008; Liu and Wong, 2005) or decreased (Borner et al., 2009b; Wang et al., 2008) in response to opioids. Therfore, we investigated in detail molecular mechanisms underlying the regulation of NF−κB by morphine in neuronal cells.

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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 AG, Berlin, Germany). The studies in animal cells have been carried out in accordance with the Guide for the Care and Use of Laboratory Animals. Preparation of primary fetal striatal neurons from rats was performed as reported (Borner et al., 2007). The neurons were cultivated in Neurobasal medium with glutamin, penicillin/streptomycin and B-27 supplement (Invitrogen GmbH, Darmstadt, Germany). It has been reported earlier 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 (Borner et al., 2007; Mansour et al., 1995; Zadina et al., 1994). The transfection of SH SY5Y cells has been described earlier (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), Beta-endorphin and loperamide were obtained from Sigma-Aldrich (Taufkirchen, Germany). Methadone and fentanyl were obtained from Grünenthal, Aachen, Germany). Cycloheximide, H-89, PMA and forskolin were obtained from Sigma-Aldrich. CTAP, pertussis toxin, cAMPS-RP, PD 98059, SB 203580, genistein, ZM 336372 and U0126 were obtained from Tocris (Bristol, UK). To avoid unspecific effect, the smallest effective doses for all inhibitors were chosen according to our own observations (cAMPS-RP (Borner et al., 2009a; Borner et al., 2009b)), or to published data (H-89 (Roy et al., 2005)) and different MAPK (Davies et al., 2000)), in which their specificity was demonstrated.

Quantitative real time RT-PCR. 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 diluted to 50 μl. Two microliter of cDNA were used for real-time PCR reactions. Quantitative real time

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RT-PCR was done in a total volume of 20 µl on a LightCycler instrument using the "LightCycler - Fast Start DNA Master SYBR Green I" kit (both from Roche, Mannheim, Germany) according to the manufacturer's suggestions. PCR primers and conditions were as follows: rat beta-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 beta-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 I–κB: 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 was described in detail in previous publications from our group (Kraus et al., 2003a; Kraus et al., 2003b). In brief, short double stranded oligonucleotides with specific binding sequences for transcription factors are introduced into living cells by passive uptake during an over-night-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. Since the decoys act within living cells, they are highly specific. The sequences of the decoy oligonucleotides were (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'; *mu*AP-1 D2, 5'-ACTGAAAGGACTCAGAACTAC-3'. The

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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 (Borner et al., 2002; Kraus et al., 2003a). Putative human I−κB AP-1 decoys had the following sequences: -1607. 5'-TTTCACCTTGCCTCAATCATTGT-3'; -1455, 5'-CTCTCTATCGAGTCAGATTTCTT-3'; -156, 5'-GACCCTAGTGGCTCATCGCAGGG-3'. Decoy oligonucleotides were synthesized by Metabion (Martinsried, Germany) as complementary single strands. The siRNA approach was performed using "I−κB-α 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 (Borner et al., 2008). Construction of the reporter plasmid AP-1-tk-CAT was described earlier (Borner et al., 2002).

Western blot analysis. Western blots were performed as previously described (Borner et al., 2009a; Borner et al., 2009b). Prior to stimulation cells were kept for 16 hours in medium containing 1% fetal calf serum. Then, cells (6 x 10^5 per sample) were incubated at 37°C with stimuli or with vehicle. The incubation was stopped by washing the cells with PBS 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; phospho-NF−κB p65 (Ser536; 93H1) and I−κB-α from Cell Signaling Technology/New England Biolabs (Frankfurt, Germany). Secondary antibodies: anti-rabbit IgG or anti-mouse IgG (both from GE Healthcare, Braunschweig, Germany).

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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, since 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 to 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 five and 24 hours prior to TNF-stimulation, the TNF-mediated induction of CB1 mRNA was significantly inhibited. Similarly, 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 I−κB. The experiments with cycloheximide indicated that protein biosynthesis is needed for

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the inhibitory effect of morphine on the TNF-mediated induction of CB1 mRNA (see fig. 2, lane 7). Searching for a newly synthezised protein that mediates inhibition of NF−κB we found that incubation of primary neurons with morphine resulted in a strong induction of I−κB (fig. 3A). Similarly, I−κB was induced in response to morphine in SH SY5Y cells, where a maximal expression was found three hours after stimulation with the drug (fig. 3B). These protein data are in good accordance with I−κB mRNA data, showing a strong, significant induction in response to morphine in SH SY5Y cells (fig. 3C). Next, the morphine-mediated induction of I−κB was investigated in more detail (fig. 3D). Coincubation of SH SY5Y cells with the μ opioid receptor-specific antagonist CTAP abolished the induction of I−κB mRNA, indicating that μ opioid receptors mediate this effect of morphine. Interestingly, cycloheximide inhibited the induction of I−κB mRNA in response to morphine only partially. This suggests that the induction of I−κB 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, which mediated the induction of I−κB mRNA in response to morphine, we used the decoy oligonucleotide approach. As depicted in figure 3D, different decoy 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 I−κB. 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 I−κB. A similar, NF−κB- and AP-1-dependent induction of I−κB 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 I−κB (Liao et al., 2008; Mormina et al., 2006). However, we did not observe such an effect of morphine in the SH SY5Y cells (fig. 3E). Recent reports suggest that some effects of morphine are different from those of other opioids, e. g. peptide opioids due to 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 I−κB (fig. 3F). However, treatment of SH SY5Y cells with morphine, the endogenous

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opioid peptide beta-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 I–κB.

Identification of an AP-1-element in the human I–κB gene. Since the sequences of the human I–κB 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 bp palindromic AP-1 binding sequence, 5'-TGA(C/G)TCA-3', revealed three sequence motifs within approximately 2 kb of the human I–κB 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, if they inhibited the induction of I–κB 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 I–κB. 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. Since the morphine-mediated induction of I–κB was dependent on AP-1 and NF–κB itself, we investigated if 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 minutes time-point. At the two hours time-point phosphorylation of p65 was similar to 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

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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 pertussis toxin, 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, 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 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), which both produced similar results. Employing inhibitors of the p42/44 MAPK pathway, which were PD 98059 and U0126 (both are inhibitors of MAPK kinase) and ZM 336372 (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. Similarly, genistein, which is an inhibitor of protein tyrosin 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 SB 203580, an inhibitor of the p38 MAPK pathway.

The AP-1-mediated induction of I–κB 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 I–κB (Baud and Karin, 2001; Renner and Schmitz, 2009). Interestingly, also morphine promotes activation of NF–κB and induction of I–κB. However, in the long run morphine causes an inhibition of NF–κB signaling, as shown above 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 I–κB by TNF and morphine (fig. 7). Incubation of primary

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neurons with TNF resulted in the well established degradation of I–κB within few minutes. Prolonged incubation of the cells with TNF resulted in a resynthesis of I–κB 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 I–κB within few minutes. In sharp contrast to TNF, however, prolonged incubation of the cells with morphine resulted in a marked upregulation of I–κB with levels significantly exceeding basal levels. Although it is well known that TNF activates NF–κB, it is unclear, if 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 I–κB, which is dependent mainly on AP-1 and results in I–κB levels that exceed basal levels. In contrast, TNF, which does not activate AP-1 in neurons, only causes re-synthesis of basal I–κB levels, which is dependent mainly on NF–κB. To further investigate the importance of the AP-1-mediated induction of I–κB 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/AP-1 do/does not interfere with the induction of CB1 by TNF itself. These experiments showed no significant effect of the decoy oligonucleotides (lanes 1 to 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 I–κB were

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performed (fig. 9B, C). Transfection of an I–κB siRNA into SH SY5Y cells and primary neurons from rats inhibited the morphine-mediated increase in I–κB (fig. 9B). In addition, the inhibitory effect of morphine on the TNF-triggered induction of CB1 was significantly inhibited in cells transfected with the I–κB siRNA compared with cells transfected with a scrambled siRNA (fig. 9C). Since the siRNA was more efficient in the SH SY5Y cells compared to 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).

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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, which is related to neuronal cells and expresses μ opioid receptors, and which therefore is relevant for opioid effects in humans, and 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 in future. Probably, it is a physiological mechanism to counteract neuroinflammatory conditions, taking advantage of increased antiinflammatory 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 induction of I−κB. This suggests that most, if not all opioids activating μ opioid receptors induce I−κB 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 to other agonists, is increasingly recognized (Zhang et al., 1998). Also, 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, opioids might have beneficial effects, since 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 (Borner et al., 2009b). It remains to be investigated, if opioids modulate such pathways and diseases in vivo. In this context, it would be important to see in animal models, if I−κB is upregulated in brain regions that express μ opioid receptors in response to single and repeated applications

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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, if both classes of drugs inhibit NF−κB. Inhibition of NF−κB by cannabinoids has indeed been observed repeatedly (e. g. Correa et al., 2010; Nakajima et al., 2006). However, the precise mechanisms and and the question via which receptors such effects are mediated are not completely clear. In CB1 knock-out 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 closer investigate the expression of I−κB. Indeed, morphine induced I−κB in both cell models, which was most pronounced three to five hours 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 five hours resulted in a strong inhibitory effect of morphine on NF−κB. In this context it should be mentioned that elevated I−κB 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 I−κB (see fig. 3D). This observation is in line with the cycloheximide data, which indicated that protein biosynthesis-dependent, and

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-independent mechanisms are involved. While 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). In line with this, 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 I−κB 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 I−κB promoter (Ito et al., 1994). However, regulation of I−κB by AP-1, which was demonstrated in our experiments, is a novel finding. As a basis for this regulation, we identified an AP-1 site on the proximal I−κB 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 neither inflluenced by activation of the cAMP/PKA pathway by forskolin, nor 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 also the opioid receptors belong (Bilecki et al., 2005; Pierce et al., 2001). 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 classically 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 earlier 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 I−κB by AP-1 is the

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key factor: Stimulation of neuronal cells with morphine results in an AP-1- plus NF−κB-mediated synthesis of I−κB, producing levels of I−κB 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 I−κB. 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 electrophoretic mobility shift analysis demonstrating that TNF stimulation of SH SY5Y cells, which was monitored over a period of 24 hours, resulted in an activation of NF−κB only, but not of AP-1 (Borner 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 I−κB 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 I−κB 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 Contributíon

Partcipated in research design: Börner, Höllt, Kraus

Conducted experiments: Börner, Kraus

Contributed new regents or analytic tools: not applicable

Performed data analysis: Börner, Kraus

Wrote or contributed to the writing of the manuscript: Börner, Höllt, Kraus

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Footnotes

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Legends for Figures

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). The cells were either 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 beta-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 over night 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*κD1 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 above. At least two independent experiments performed in duplicate are depicted plus S.E.M. Samples were compared to 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 acetyl transferase (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 hours after transfection and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed (*: $p < 0.05$; **: $p < 0.01$).

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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 beta-actin as measured by quantitative real-time RT-PCR are shown. Below, 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 figure 1), SH SY5Y cells were incubated for five hours and primary neurons for 24 hours 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 hours prior to TNF (as indicated) and then co-incubated with the cytokine. At least three independent experiments were performed in duplicate and are shown plus S.E.M. All samples were compared to 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 one and 16 hours prior to 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, medium was replaced and cells were incubated with TNF (150 pg/ml) and morphine (1 µM) as indicated. Cells were lysed 72 hours 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 (**: $p < 0.01$).

Fig. 3. Treatment of neuronal cells with morphine resulted in a NF–κB- and AP-1-dependent induction of I–κB. A, B: Induction of I–κB 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 I–κB, and, as controls, re-probed for actin. Examples of representative Western blot experiments are depicted, which were performed at least two times in duplicate. C: Detection of I–κB transcripts in response to morphine in SH SY5Y cells by quantitative real-time RT-PCR. Cells were stimulated with morphine (1 µM), lysed and RT-PCR was performed. The amounts of I–κB transcripts are normalized to beta-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 I–κB is mediated by µ opioid

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receptors and dependent on NF–κB and AP-1. I–κB 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 beta-actin. Stimulation with morphine (1 μM) is indicated. CTAP (250 nM) and cycloheximide (CX; 10 μg/ml) were applied one and 16 hours prior to morphine, respectively. Decoy oligonucleotides (160 nM) were added to the cells 16 hours prior to 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 to non-treated 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 I–κB. Cells were stimulated with TNF (150 pg/ml) for 5 min to induce degradation of I–κB (lane 2). Some samples were incubated with morphine for the indicated times (MO; 1 μM) prior to the TNF stimulus (lanes 3-7). Western blots were probed for I–κB and actin as a control. A representative example out of two experiments is depicted. F: Induction of I–κB by various opioids. A representative Western blot (out of two individual experiments) is presented showing the induction of I–κB in response to three hours treatment of SH SY5Y cells with morphine (Mor; 1 μM), beta-endorphin (End; 1 μM), methadone (Met; 1 μM), fentanyl (Fen; 15 nM) and loperamide (Lop; 1.5 μM). The same blot was re-probed for actin.

Fig. 4. Identification of an AP-1 site in the I–κB promoter. A: Putative AP-1 sites of the I–κB 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 I–κB gene as decoys, and the classic AP-1 D1 decoy (AP-1), for their ability to inhibit the morphine (Mo)-mediated induction of I–κB. Decoy oligonucleotides (160 nM) were added to the samples 16 hours prior to the morphine-stimulation. Blots were probed for I–κB, and, as controls, re-probed 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 I–κB

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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 chloramphenicol acetyl transferase (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 hours 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, re-probed for actin. Examples of representative experiments are depicted, which were performed at least two times in duplicate.

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 pertussis toxin (PTX). Primary neuronal cells were incubated over night with PTX (10 ng/ml) and then stimulated with morphine (1 µM) for 45 min as indicated. Blots were probed for c-Fos and phospho (P-) p65, and, as controls, re-probed 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, re-probed 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 prior to morphine) and H-89 (20 µM; applied 6 h prior to morphine). Examples of representative experiments are depicted, which were performed at least two times. C: Western blot

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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 kinase inhibitors PD 98059 or U0126, the c-Raf inhibitor ZM 336372, the tyrosin kinase/EGF receptor kinase inhibitor genistein, or the p38 inhibitor SB 203580. All inhibitors were applied 2 h prior to morphine. Examples of representative experiments, performed at least three times are shown. A quantification of these experiments is shown below (asterisks indicate samples significantly different from morphine-treated controls, which were set to 1; *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

Fig. 7. Regulation of I–κB 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 I–κB, and, as controls, re-probed for actin (not shown). Examples of representative experiments are depicted above. Please note: In order to better visualize the initial degradation of I–κB in response to the stimuli within the first hour (left part) and its subsequent regulation (right part), different amounts of the cell lysates were loaded on the gels and also the exposure times were different for the left and the right blots. Therefore, the 0 hour 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 chloramphenicol acetyl transferase (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 (1µM), TNF (150 pg/ml), or PMA (100 nM), or treated with vehicle (US) as indicated. Cells were lysed 72 hours after transfection and a CAT-ELISA was performed. Results of two independent experiments performed in triplicate plus S.E.M. are displayed (*: $p < 0.05$; **: $p < 0.01$).

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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 re-probed for phospho (P-) p65 and actin. A representative example out 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 five minutes time point demonstrates proper activity of TNF.

Fig. 9. The AP-1-dependent induction of I–κB 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 to 11) and primary neurons from rats (lanes 12 to 18) were stimulatd with TNF for five hours (SH SY5Y cells), 24 hours (primary cells), or left untreated. Some cells were prestimulated with morphine for five hours, then TNF was added and cells were further incubated as described above. To some samples decoy oligonucleotides (160 nM) were added 16 hours prior to 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 beta-actin and shown plus S.E.M.. Values are compared to 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 aginst I–κB abolishes the induction of the protein in response to morphine. SH SY5Y cells (SH, left side) and primary neurons from rats (PN, right side; both cell types 7 x 10^5 in 2 ml) were transfected with 60 pmol of an I–κB siRNA, or a non-matching 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 I–κB and actin proteins. C: An siRNA aginst I–κB abolishes the inhibitory effect of morphine on the TNF-triggered induction of CB1. Cells that were simultaneously

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transfected as those that were used for the Western blot described in B were used 16 hours 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 was performed as it is explained in A. Two independent experiments were performed in duplicate and are shown plus S.E.M. Samples were compared to the TNF-treated samples. Secondary comparisons are indicated by brackets (*: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$).

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 I−κB (1) and subsequent activation of NF−κB (2). The activated NF−κB directs resynthesis of I−κB (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 I−κB (4). TNF does not activate AP-1 in neurons. The opioid-triggered overexpression of I−κB results in an inhibition of NF−κB (5).

fig. 1

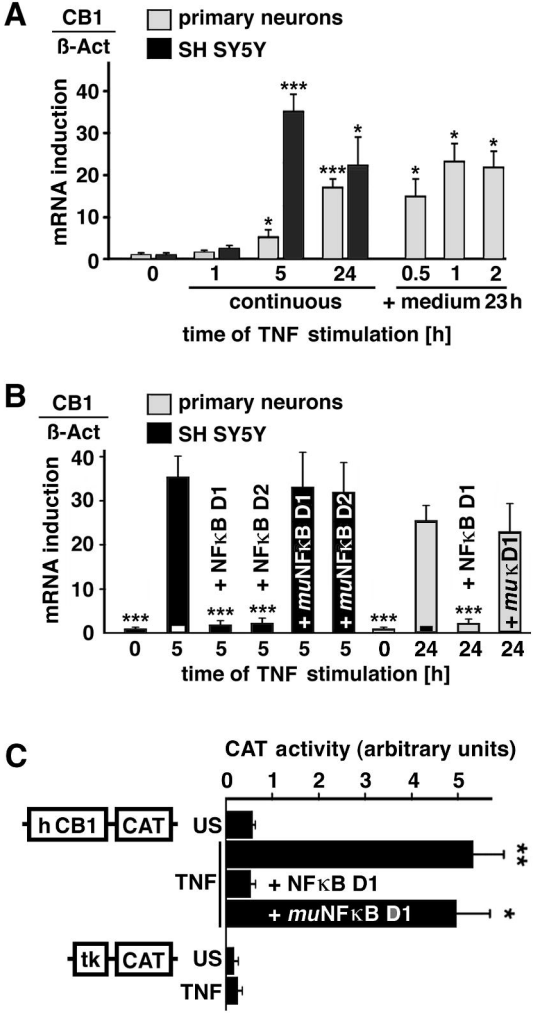
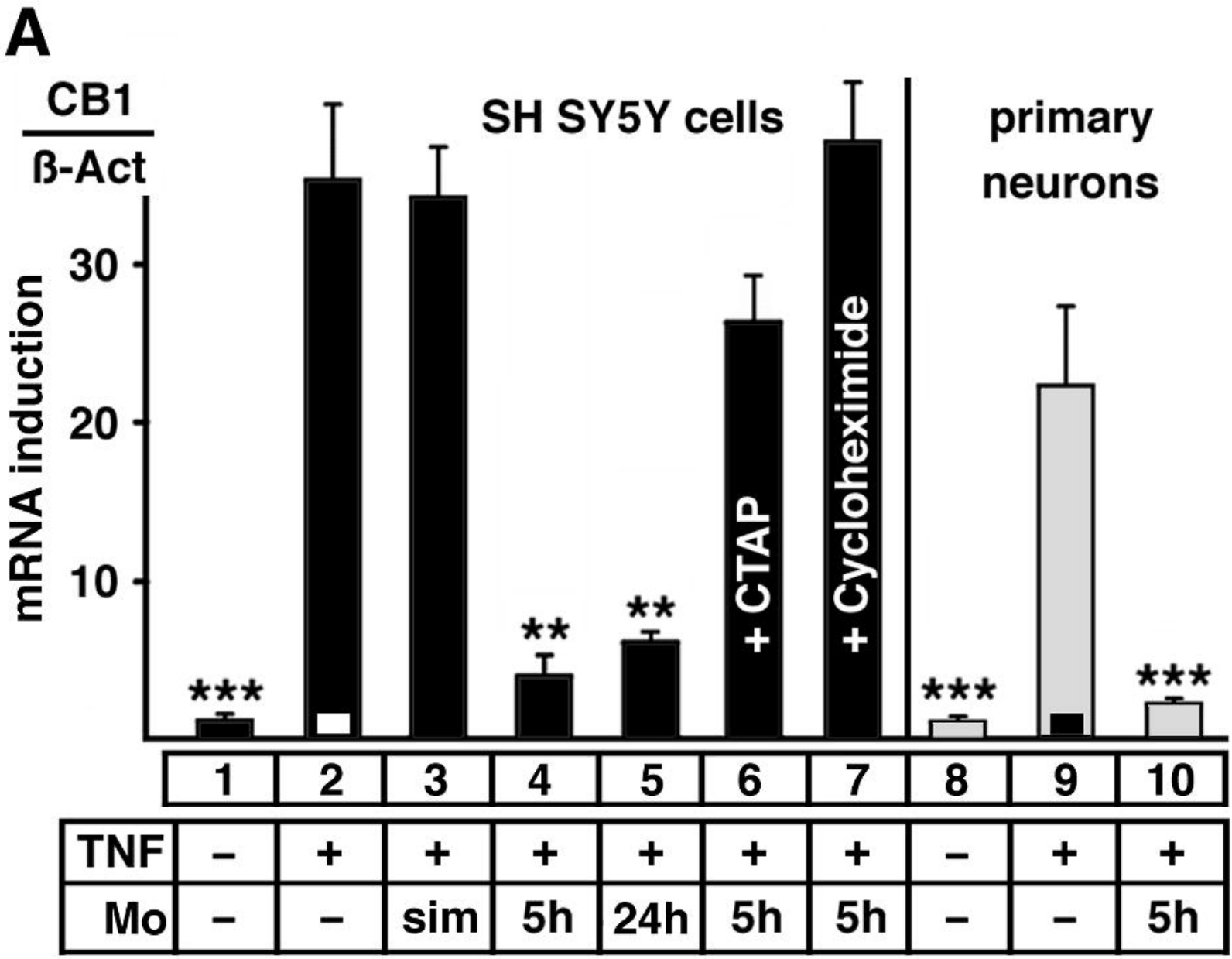

A
CB1
ß-Act
primary neurons
SH SY5Y
mRNA induction
0
10
20
30
40
0
1
5
24
0.5
1
2
continuous
+ medium 23h
time of TNF stimulation [h]
B
CB1
ß-Act
primary neurons
SH SY5Y
mRNA induction
+ NFκB D1
+ NFκB D2
+ muNFκB D1
+ muNFκB D2
+ NFκB D1
+ muκD1
0
5
5
5
5
5
0
24
24
24
time of TNF stimulation [h]
C
CAT activity (arbitrary units)
0
1
2
3
4
5
h CB1
CAT
US
TNF
+ NFκB D1
+ muNFκB D1
tk
CAT
US
TNF

fig. 2


A
CB1
ß-Act
mRNA induction
30
20
10
SH SY5Y cells
primary neurons
+ CTAP
+ Cycloheximide
***
**
**
***
***
1 2 3 4 5 6 7 8 9 10
TNF – + + + + + + – + +
Mo – – sim 5h 24h 5h 5h – – 5h

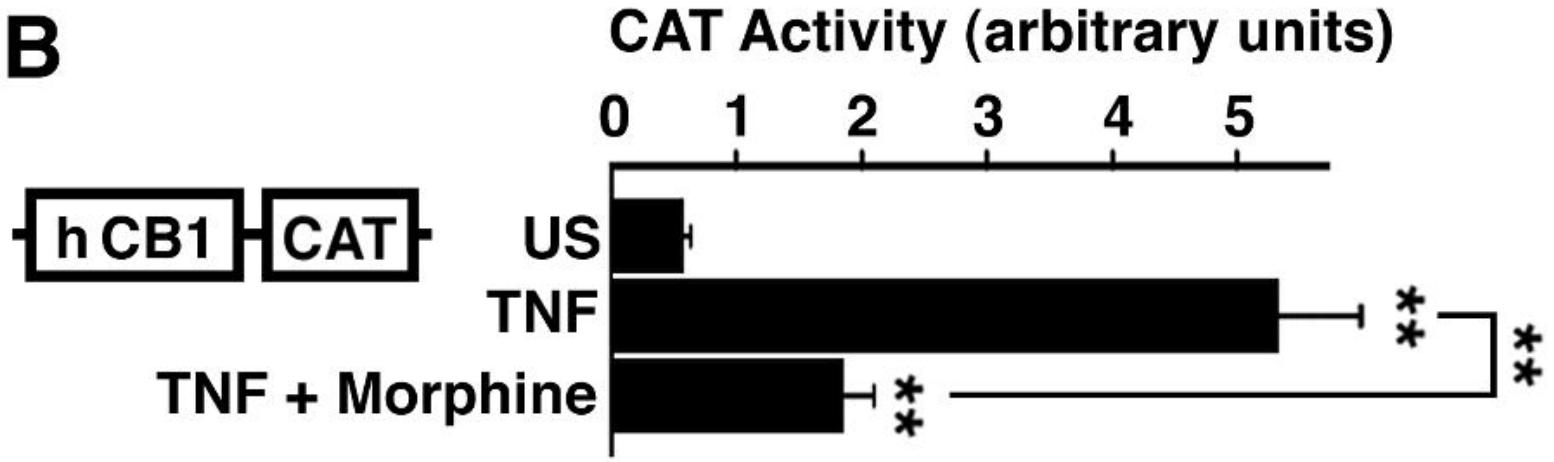

B
CAT Activity (arbitrary units)
0 1 2 3 4 5
h CB1
CAT
US
TNF
TNF + Morphine
**
**
**

fig. 3

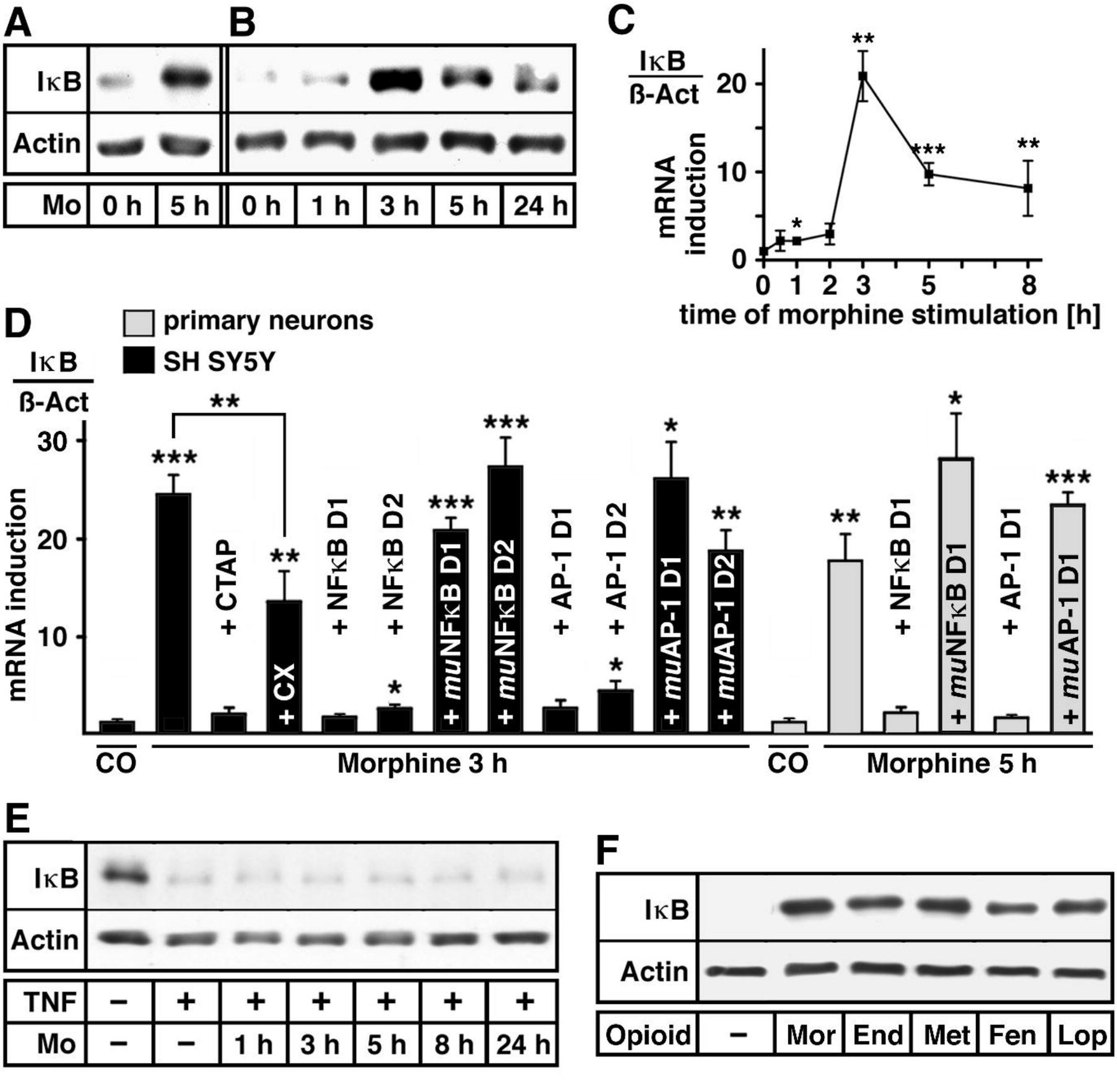
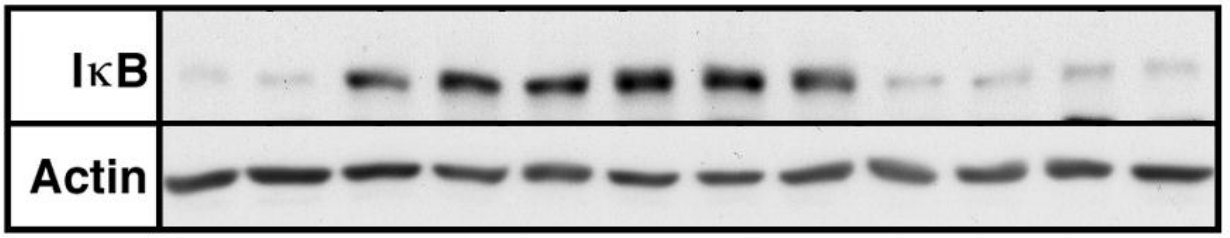

A
B
IκB
Actin
Mo
0 h
5 h
0 h
1 h
3 h
5 h
24 h
C
IκB
ß-Act
mRNA induction
time of morphine stimulation [h]
D
primary neurons
SH SY5Y
IκB
ß-Act
mRNA induction
+ CTAP
+ CX
+ NFκB D1
+ NFκB D2
+ muNFκB D1
+ muNFκB D2
+ AP-1 D1
+ AP-1 D2
+ muAP-1 D1
+ muAP-1 D2
CO
Morphine 3 h
CO
Morphine 5 h
E
IκB
Actin
TNF
Mo
1 h
3 h
5 h
8 h
24 h
F
IκB
Actin
Opioid
Mor
End
Met
Fen
Lop

fig. 4

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



Mo	-	5 h	5 h	5 h	5 h	5 h
Decoy	-	-	1607	1455	156	AP-1

C

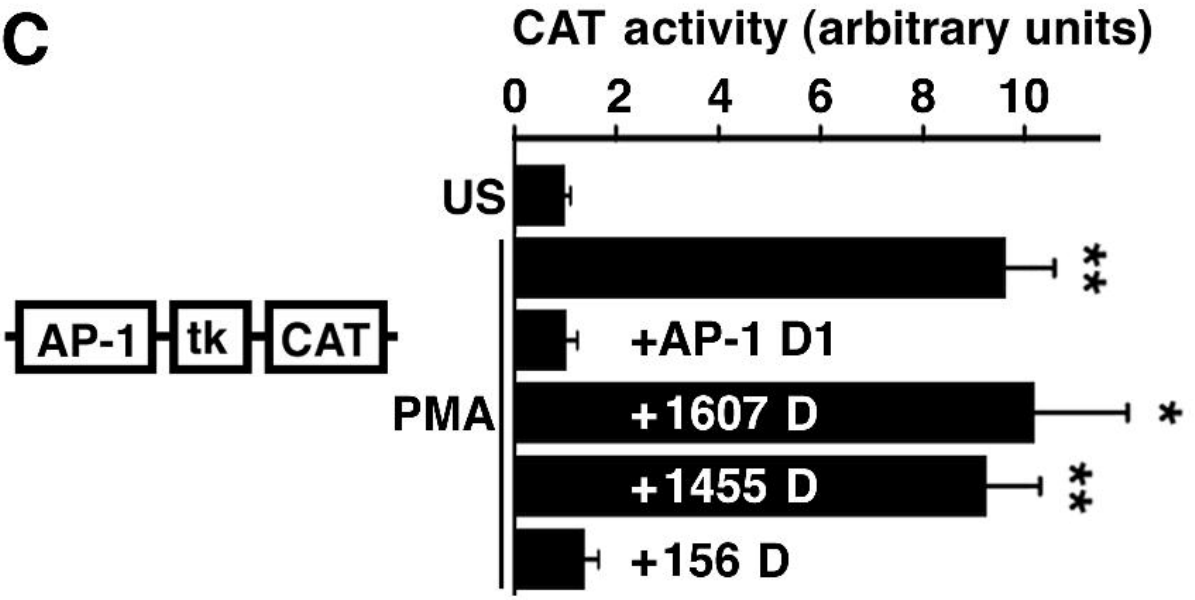


fig. 5

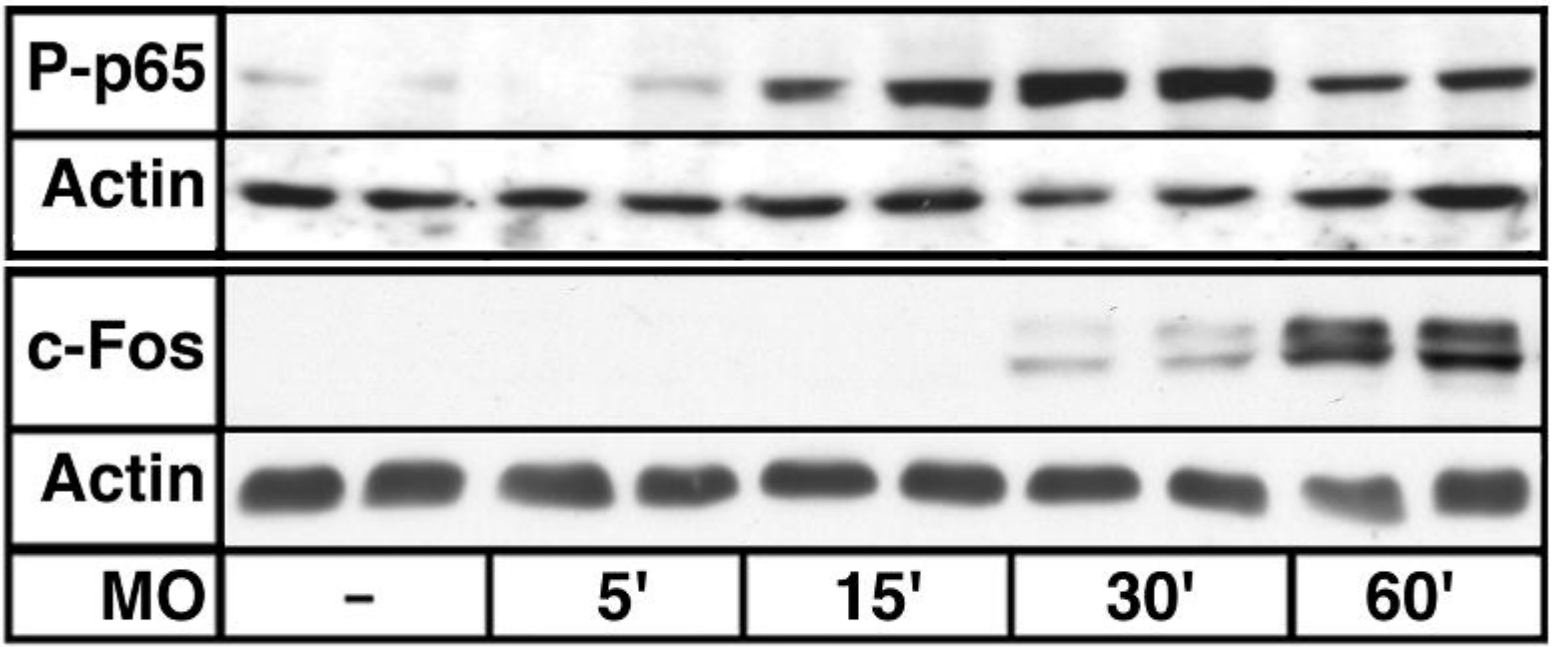


fig. 6

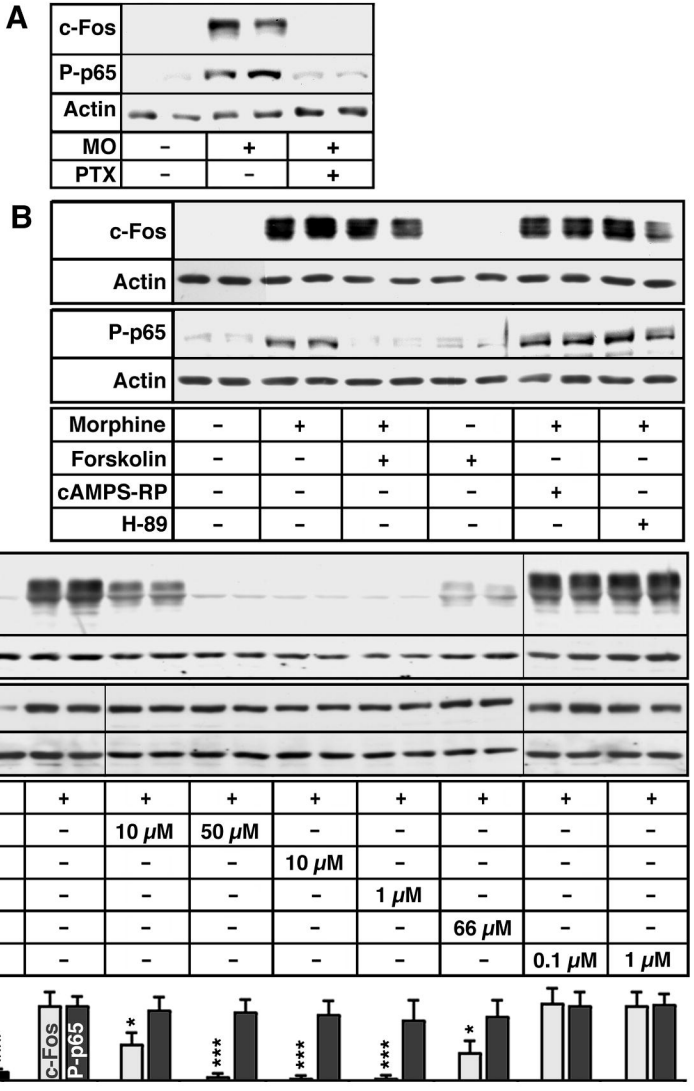

A
c-Fos
P-p65
Actin
MO – + +
PTX – – +
B
c-Fos
Actin
P-p65
Actin
Morphine – + + – + +
Forskolin – – + + – –
cAMPS-RP – – – – + –
H-89 – – – – – +
C
c-Fos
Actin
P-p65
Actin
Morphine – + + + + + + + +
PD98059 – – 10 μM 50 μM – – – – –
U0126 – – – – 10 μM – – – –
ZM336372 – – – – – 1 μM – – –
Genistein – – – – – – 66 μM – –
SB203580 – – – – – – – 0.1 μM 1 μM
[target] / [actin]
1
c-Fos
P-p65
***
***
*
***
***
***
*

fig . 7

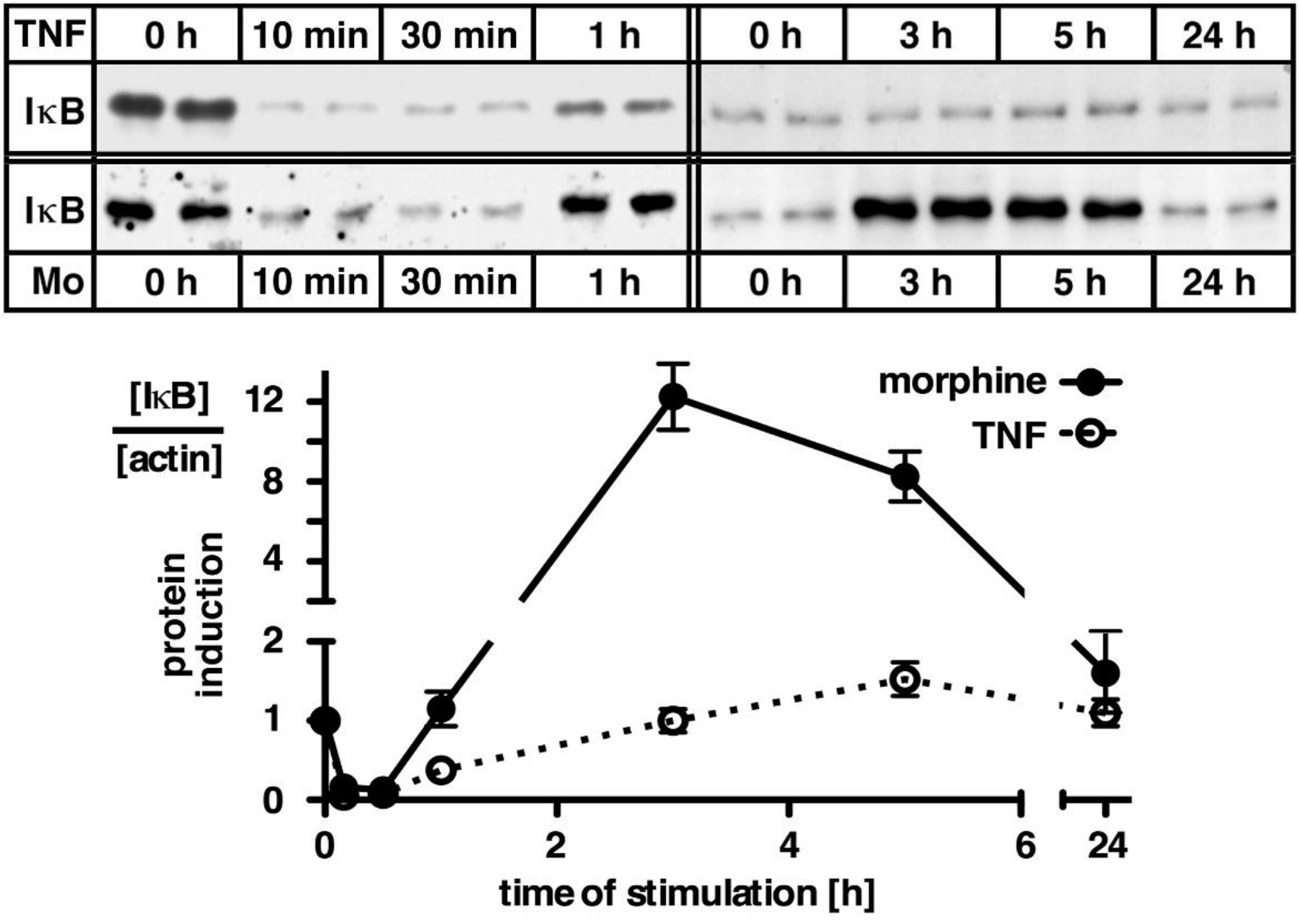

TNF
0 h
10 min
30 min
1 h
0 h
3 h
5 h
24 h
IκB
IκB
Mo
0 h
10 min
30 min
1 h
0 h
3 h
5 h
24 h
[IκB]
[actin]
protein induction
12
8
4
2
1
0
morphine
TNF
0
2
4
6
24
time of stimulation [h]

fig. 8

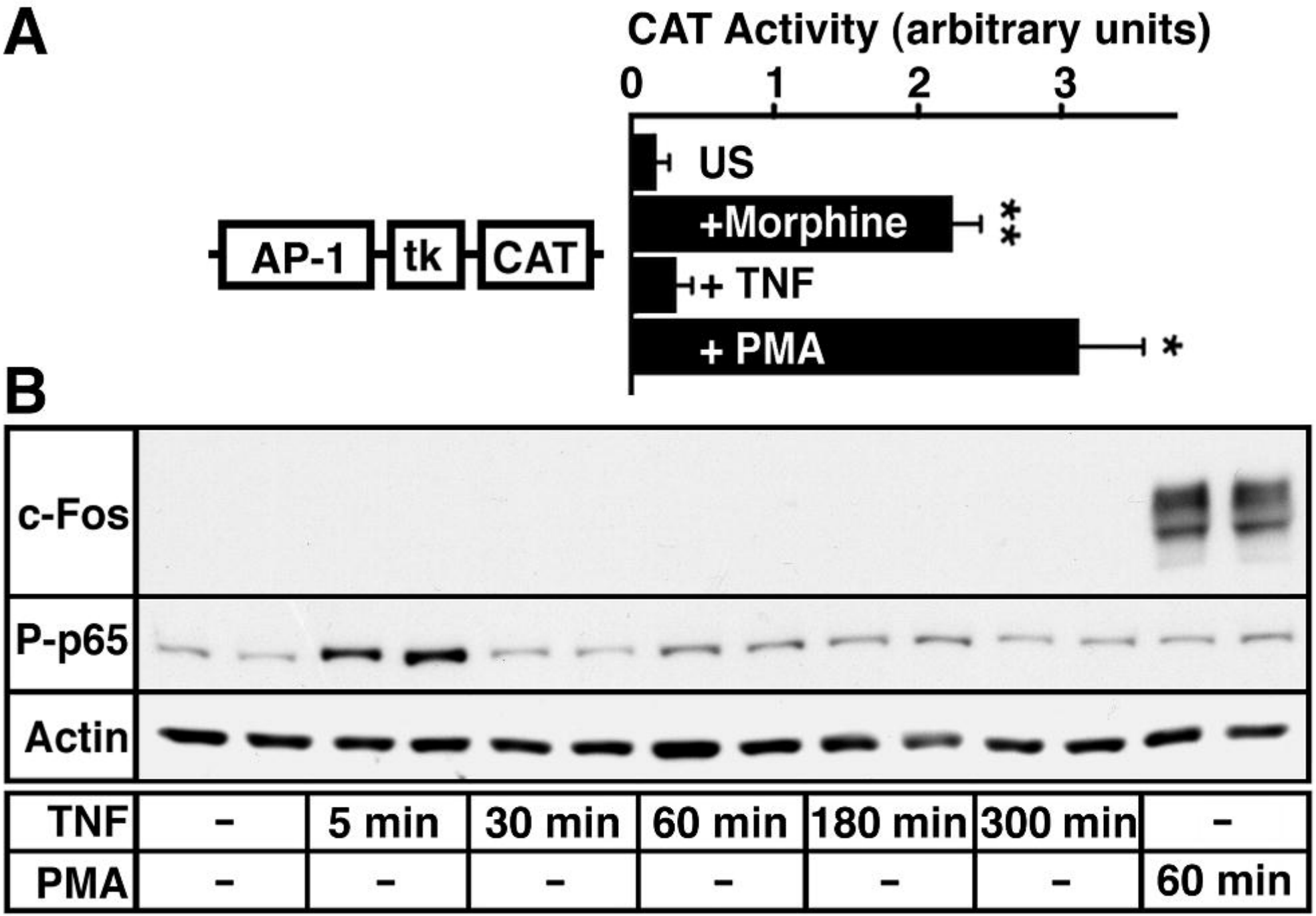

A
CAT Activity (arbitrary units)
0
1
2
3
US
+Morphine
**
+ TNF
+ PMA
*
AP-1
tk
CAT
B
c-Fos
P-p65
Actin
TNF
–
5 min
30 min
60 min
180 min
300 min
–
PMA
–
–
–
–
–
–
60 min

fig. 9

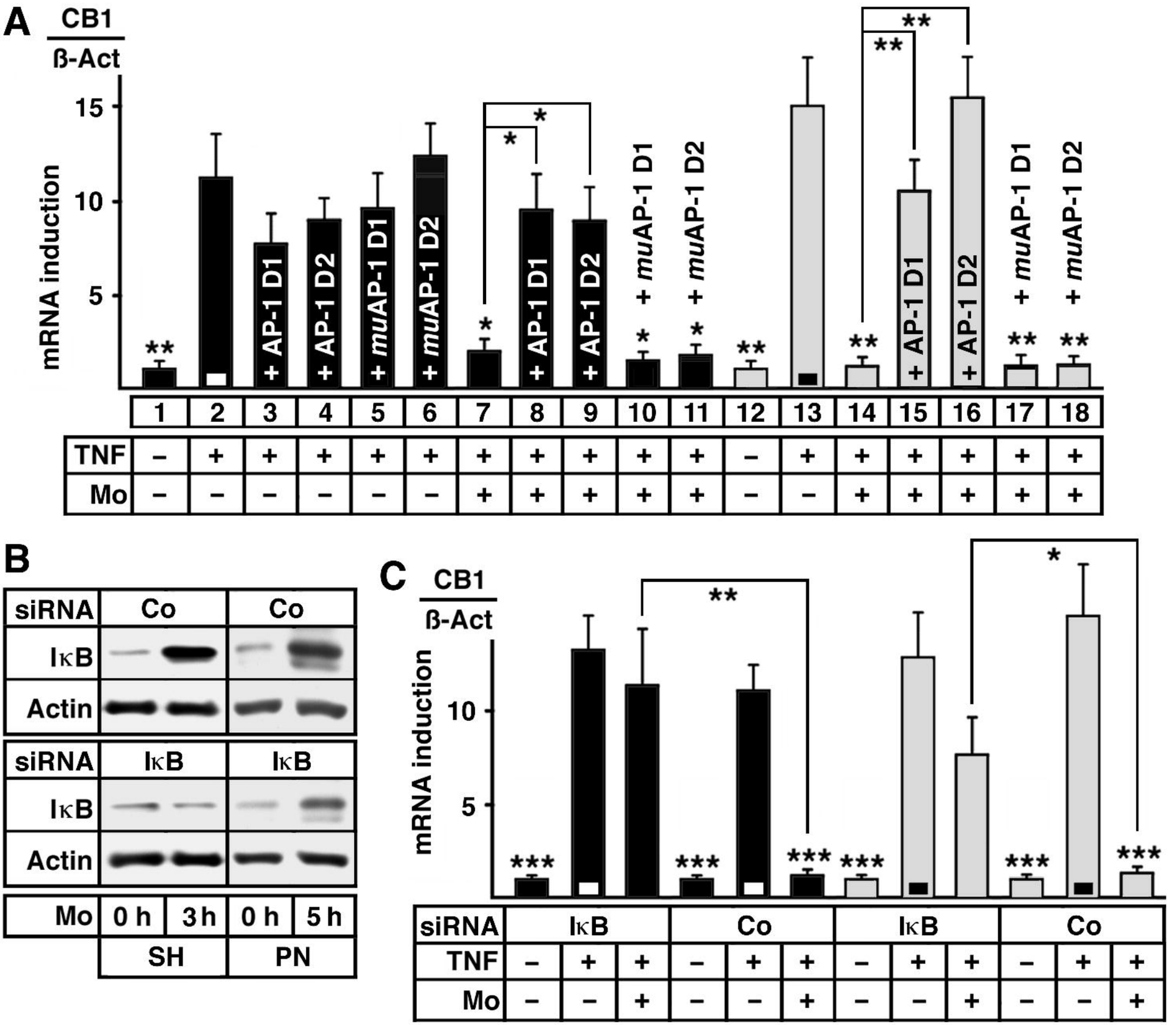

A
CB1
ß-Act
mRNA induction
15
10
5
+ AP-1 D1
+ AP-1 D2
+ muAP-1 D1
+ muAP-1 D2
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18
TNF – + + + + + + + + + + – + + + + + +
Mo – – – – – – + + + + + – – + + + + +
B
siRNA Co Co
IκB
Actin
siRNA IκB IκB
IκB
Actin
Mo 0 h 3 h 0 h 5 h
SH PN
C
CB1
ß-Act
mRNA induction
10
5
siRNA IκB Co IκB Co
TNF – + + – + + – + + – + +
Mo – – + – – + – – + – – +

fig. 10