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Title: Cannabinoid Receptor Type 2 Agonists Induce Transcription of the  $\mu$ -Opioid Receptor  
Gene in Jurkat T Cells

Authors: Christine Börner, Volker Höllt and Jürgen Kraus

Affiliation: Department of Pharmacology and Toxicology, University of Magdeburg,  
Magdeburg, Germany

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Short title: Cannabinoids Induce  $\mu$ -Opioid Receptors in T Cells

Corresponding author:

Jürgen Kraus, Department of Pharmacology and Toxicology, University of Magdeburg, 44

Leipziger Strasse, 39120 Magdeburg, Germany; Tel. +49 391 6715372; FAX.: +49 391

6715869; e mail: [juergen.kraus@medizin.uni-magdeburg.de](mailto:juergen.kraus@medizin.uni-magdeburg.de)

number of text pages: 25

number of tables: 0

number of figures: 7

number of references: 33

number of words in abstract: 245

number of words in introduction: 406

number of words in discussion: 655

Abbreviations:

$\Delta^9$ -THC, Delta-9-tetrahydrocannabinol; MAPK, mitogen-activated protein kinase; (RT)-PCR,  
(reverse transcriptase)-polymerase chain reaction

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Abstract

Opioids and cannabinoids are both associated with analgetic, psychotropic and immunomodulatory effects. Recently, it has been suggested, that both systems interact on multiple levels. We hypothesized that cannabinoids induce opioid receptors and investigated cannabinoid-dependent expression of the  $\mu$ -opioid receptor subtype in a human T cell model. We report, that activation of the peripheral cannabinoid receptor type 2 leads to a de novo induction of  $\mu$ -opioid receptor transcription in Jurkat E6.1 cells. We show, that interleukin-4 is transcriptionally induced in response to cannabinoids and that an interleukin-4 receptor antagonist blocks cannabinoid-dependent induction of  $\mu$ -opioid receptors, indicating that induced expression of interleukin-4 is required in this process. Induction of interleukin-4 is blocked by decoy oligonucleotides directed against STAT5, indicating the requirement of this transcription factor. In addition, we show cannabinoid-dependent phosphorylation of STAT5. Further experiments demonstrate that interleukin-4 then induces phosphorylation of STAT6, which directly transactivates the  $\mu$ -opioid receptor gene. Additionally, STAT6 induces expression of the transcription factor GATA3, which also contributes to  $\mu$ -opioid receptor gene transcription. The responsive promoter region of the human  $\mu$ -opioid receptor gene with the binding sites for both factors was mapped to nt -1001 to -950. To demonstrate functional  $\mu$ -opioid receptor proteins, morphine-mediated phosphorylation of mitogen-activated protein kinase was investigated. We show that phosphorylation of mitogen-activated protein kinase occurs only in cannabinoid-prestimulated Jurkat E6.1 cells and that it is blocked by the  $\mu$ -opioid receptor antagonist CTOP. In summary, these findings provide a first example for cannabinoid-opioid-interactions in cells of the immune system.

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Effects of cannabinoids are mediated by at least two types of receptors, termed CB1 and CB2, which belong to the family of G<sub>i</sub>/G<sub>o</sub>-protein coupled receptors. CB1 receptors are abundantly expressed in the central and peripheral nervous system. To a lesser degree, they are expressed in peripheral tissues, e. g. in cells of the immune system, where CB2 receptors predominantly are expressed (Howlett et al., 2002). Effects of opioids are mediated by receptors, which also belong to the family of G<sub>i</sub>/G<sub>o</sub>-protein coupled receptors. In particular, three types are distinguished, termed  $\mu$ ,  $\delta$  and  $\kappa$ , which are expressed throughout the nervous system and peripheral tissues (Pol and Puig, 2004). The  $\mu$ -opioid receptor subtype is an important mediator of the analgesic effects of opioids and the main target for drugs of the morphine type. Opioid- and cannabinoid receptors are often co-expressed in cells of the nervous system (Pickel et al., 2004; Rodriguez et al., 2001) and, furthermore, both systems are characterized by various, sometimes synergistic interactions with respect to analgetic, psychotropic and behavioural effects (Cichewicz and McCarthy, 2003; Naef et al., 2003; Navarro et al., 2001). In addition to central effects, agonists of both systems are well known for their modulatory effects on immune cell functions. These effects, which include modulation of T helper cell and natural killer cell functions, are similar for both opioids and cannabinoids (Klein et al., 2004; Roy et al., 2004; Sacerdote et al., 1998; Sacerdote et al., 2000; Yuan et al., 2002). While cannabinoid receptors are constitutively expressed in immune effector cells,  $\mu$ -opioid receptors are normally not expressed in these cells, but known for their de novo inducibility. The most potent inducers of  $\mu$ -opioid receptor gene expression in immune effector cells are cytokines (Borner et al., 2004a; Kraus et al., 2001; Kraus et al., 2003a). In this study we present cannabinoids as a novel group of substances which lead to de novo induction of  $\mu$ -opioid receptor expression in the Jurkat T cell model via a CB2 receptor-mediated mechanism. We show that this effect is indirect and involves induced expression of interleukin-4. It is therefore possible that peripheral cannabinoids indirectly cause upregulation of  $\mu$ -opioid receptors in neuronal cells, since we have demonstrated earlier, that

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expression of this receptor is upregulated by interleukin-4 in neuronal cells, as well (Kraus et al., 2001). Such a mechanism may contribute to analgesic effects of peripheral cannabinoids, that have been reported recently (Hohmann et al., 2004; Ibrahim et al., 2003).

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## Materials and methods

### Cell culture, reagents and transfection

Jurkat E6.1 cells were cultivated in RPMI-1640 medium (Cambrex Bio Science, Verviers, Belgium) supplemented with 10% fetal calf serum (Biochrom, Berlin, Germany) and antibiotics (100 units/ml penicillin and 100 mg/ml streptomycin, Cambrex Bio Science). Twenty hours before stimulation experiments cells received fresh medium with 1% fetal calf serum.  $\Delta^9$ -THC, R(+)-methanandamide and cycloheximide were purchased from Sigma (Taufkirchen, Germany), AM 281, AM 630 and JWH 015 were from Tocris (Bristol, UK). The interleukin-4 antagonist IL-4[R121D,Y124D] was a kind gift from Walter Sebald of the Biozentrum, Universität Würzburg, Germany (Tony et al., 1994). Interleukin-4 (R&D Systems, Wiesbaden, Germany) was used at 5 ng/ml. Morphine and CTOP were obtained from Synopharm (Barsbüttel, Germany) and Tocris, respectively. For transfections,  $5 \times 10^6$  cells were pelleted, mixed with 500  $\mu$ l transfection buffer containing 140 mM NaCl, 25 mM HEPES, 0,5 mM  $\text{Na}_2\text{HPO}_4$  and 125 mM  $\text{CaCl}_2$  (pH 7,15) and incubated for 20 min at room temperature. Then, 5 ml medium were added and the cells were incubated for 16 h at 35°C and 3%  $\text{CO}_2$ . After that, cells were pelleted again, resuspended in 7 ml RPMI-1640 medium with 1% fetal calf serum and incubated for 72 h. The reporter gene product chloramphenicol acetyl transferase was assayed by ELISA (Roche, Mannheim, Germany) according to the supplier's manual.

### Oligonucleotides

All oligonucleotides were synthesized by Metabion, Martinsried, Germany. Sequences of oligonucleotides were: STAT5: 5'-GATCGCATTTCGGAGAAGACG-3'; nSTAT5: 5'-GATCGCATTACGGAGTAGACG-3'; STAT6: 5'-CTAGTTCTTCTCAGAAGCATATGT-3'; nSTAT6: 5'-CTAGTTGATCTCAGATCCATATGT-3'; GATA3: 5'-

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CTAGAGGAAGTCTTCAGATAAAAAAGATAACAA-3'; nGATA3: 5'-

CTAGAGGAAGTCTTCACTTAAAAAACTTAACAA-3';

STAT1/3: 5'-GATCGAGTTTACGAGAACTC-3'; AP-1: 5'-

CGATTGACTCAGTACTGAGTCAATCG-3'; AP-2: 5'-

TGCGGGCTCCCCGGGCTTGGGCGAGC-3'; NF $\kappa$ B: 5'-

AAAGTTGAGGGGACTTTCCAGGCCT-3'; NF-AT: 5'-

GATCCGCCCAAAGAGGAAAATTTGTTTCATA-3'; NF-IL-6: 5'-

TGCAGATTGCGCAATCTGCA-3'.

For all oligonucleotides only the sequences of the sense strands are given. The decoy oligonucleotide approach (final concentration of oligonucleotides 160 nM), the efficiency and specificity of the oligonucleotides was described in detail in previous publications from our group (Borner et al., 2004a; Borner et al., 2004b; Kraus et al., 2003a; Kraus et al., 2003b).

#### 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 diluted to 50  $\mu$ l. Two microliter of cDNA was used for RT-PCR reactions. The forward and reverse primers of each primer pair are located on different exons to avoid amplification of genomic DNA. Amplification of  $\mu$ -opioid receptor transcripts by conventional and quantitative RT-PCR was performed as described earlier (Kraus et al., 2001; Kraus et al., 2003a). Quantitative real time RT-PCR was done according to the manufacturer's suggestions as follows:  $\beta$ -actin: 5'-GGTCCACACCCGCCACCAG-3' and 5'-CAGGTCCAGACGCAGGATGG-3' primers; preincubation: 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 60°C and 22 s at 72°C. Interleukin-4: 5'-GTCTCACCTCCCAACTGCTT-3' and 5'-GTTACGGTCAACTCGGTGCA-3' primers (located on exon 1 and exon 2 to avoid

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amplification of the splice variant interleukin-4 delta 2); preincubation: 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 68°C and 10 s at 72°C. Interleukin-13: 5'-GCTCTCACTTGCCTTGGCGGCT-3' and 5'-TCAGCATCCTCTGGGTCTTCTCGATG-3' primers; preincubation: 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 70°C and 11 s at 72°C. GATA3: 5'-AACTGTCAGACCACCACAACCACAC-3' and 5'-GGATGCCTTCCTTCTTCATAGTCAGG-3' primers; preincubation: 8 min at 95°C; 50 cycles: 5 s at 95°C, 5 s at 70°C and 8 s at 72°C.  $\delta$ -opioid receptor: 5'-ACGTGCTTGTCATGTTTCGGCATCGT-3' and 5'-ATGGTGAGCGTGAAGATGCTGGTGA-3' primers; preincubation: 8 min at 95°C; 40 cycles: 5 s at 95°C, 5 s at 63°C and 13 s at 72°C. CB1: 5'-CACCTTCCGCACCATCACCAC-3' and 5'-GTCTCCCGCAGTCATCTTCTCTTG-3' primers; preincubation: 8 min at 95°C; 40 cycles: 5 s at 95°C, 5 s at 68°C and 10 s at 72°C. CB2: 5'-CATGGAGGAATGCTGGGTGAC-3' and 5'-GAGGAAGGCGATGAACAGGAG-3' primers; preincubation: 8 min at 95°C; 40 cycles: 5 s at 95°C, 5 s at 70°C and 24 s at 72°C. Interleukin-5: 5'-GAGGATGCTTCTGCATTTGAGTTTG-3' and 5'-GTCAATGTATTTCTTTATTAAGGACAAG-3' primers; preincubation: 8 min at 95°C; 40 cycles: 5 s at 95°C, 5 s at 65°C and 20 s at 72°C.

#### Reporter gene plasmids

All reporter plasmids are based on the pBLCAT2/pBLCAT3 vector system. The construction of the human  $\mu$ -opioid receptor promoter containing reporter genes -2624, -1854, -1372, -779, -1372 $\Delta$ -1001/-950, -2624/-2291, -2229/-1854 and -1854/-1227 has been described in previous publications (Borner et al., 2002; Borner et al., 2004a; Kraus et al., 2001; Kraus et al., 2003a). Insertion of oligonucleotides into pBLCAT2 was performed according to a described method (Kang and Inouye, 1993). All plasmids were sequenced from both sides to ensure correct orientations and sequences of the inserts.



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### Western blot analysis

Two times  $10^6$  cells were seeded in RPMI-1640 medium without serum. After 18 h, cells were treated with 500 nM  $\Delta^9$ -THC, or 5 ng/ml interleukin-4 (R&D Systems, Wiesbaden, Germany), or vehicle. After stimulation, cells were pelleted and lysed with 80°C hot sample buffer. Cell lysis, blotting and antibody incubations were performed as suggested in the “western immunoblotting protocol“ from Santa Cruz Biotechnology (Heidelberg, Germany). Aliquots of 20  $\mu$ l were separated on a 7% polyacrylamide gel. Primary phospho-STAT-specific (P-STAT5(Tyr694)-R, P-STAT6(Tyr641)-R) and primary antibodies against unphosphorylated STAT proteins were obtained from Santa Cruz Biotechnology (Heidelberg, Germany) and used in a 1:200 dilution. Phospho-p44/42-MAPK antibody (E10, Cell Signaling Technology, Frankfurt, Germany) and ERK 2 antibody (C-14, Santa Cruz Biotechnology) were used in 1:2500 and 1:2000 dilutions, respectively. Secondary antibodies (Anti-rabbit and anti-mouse Ig from Amersham Biosciences, Braunschweig, Germany) were used in a 1:2500 dilution.

### Statistical Analysis

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

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## Results

### Cannabinoids induce $\mu$ -opioid receptor mRNA via CB2 receptors

In Jurkat E6.1 cells  $\mu$ -opioid receptors are normally not expressed. However, stimulation of cells with  $\Delta^9$ -THC markedly induced  $\mu$ -opioid receptor gene transcription (Fig. 1A). To determine the cannabinoid receptor type mediating this induction, CB1 and CB2 receptor agonists and antagonists were used (Fig. 1B). The combination of  $\Delta^9$ -THC with the CB1 specific antagonist AM 281 did not influence the induction of  $\mu$ -opioid receptor mRNA. In contrast, the combination of  $\Delta^9$ -THC with the CB2 specific antagonist AM 630 inhibited  $\mu$ -opioid receptor mRNA induction, indicating a CB2-mediated mechanism. Supporting this, the CB1 specific agonist R-(+)-methanandamide did not cause  $\mu$ -opioid receptor mRNA upregulation, whereas the CB2 specific agonist JWH 015 markedly induced  $\mu$ -opioid receptor mRNA. The protein translation inhibitor cycloheximide prevented the  $\Delta^9$ -THC-mediated  $\mu$ -opioid receptor induction, indicating that protein synthesis is needed for  $\mu$ -opioid receptor induction.

Identification of the  $\Delta^9$ -THC-inducible promoter region of the human  $\mu$ -opioid receptor gene  
To characterize the promoter region responsible for the induction of the  $\mu$ -opioid receptor gene by  $\Delta^9$ -THC transfection experiments in Jurkat E6.1 cells were performed with reporter gene constructs containing various lengths of the 5'-flanking region of the gene (Fig. 2). The longest  $\mu$ -opioid receptor promoter construct -2624 and consecutive 5'-deletions of it up to nt -1372 were inducible by  $\Delta^9$ -THC, whereas construct -779 showed no induction. Contrasting with construct -1372, a reporter gene with an internal deletion (-1372 $\Delta$ -1001/-950) was not  $\Delta^9$ -THC-inducible. Neither constructs -2624/-2291, -2229/-1854 and -1854/-1227 which contain upstream promoter parts in front of the heterologous thymidine kinase promoter of the

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Herpes simplex virus, nor the reporter gene vector pBLCAT2 alone were responsive, suggesting that the region between nt -1001 and nt -950 is responsible for the induction of the  $\mu$ -opioid receptor gene by  $\Delta^9$ -THC. We earlier demonstrated that this region also confers interleukin-4-responsiveness of the gene and contains a binding site for the transcription factor STAT6 at nt -997 (Kraus et al., 2001). In addition, the experiments with cycloheximide (Fig. 1B) indicate, that novel gene expression is needed for  $\mu$ -opioid receptor regulation. Therefore, a possible role of interleukin-4 expression in the cannabinoid-mediated  $\mu$ -opioid receptor induction was investigated next.

An interleukin-4 antagonist inhibits the induction of  $\mu$ -opioid receptor mRNA by  $\Delta^9$ -THC. Jurkat E6.1 cells were incubated with  $\Delta^9$ -THC and the interleukin-4 antagonist IL-4[R121D,Y124D], and relative amounts of  $\mu$ -opioid receptor mRNA were determined. The interleukin-4 antagonist dose-dependently decreased the  $\Delta^9$ -THC induced upregulation of  $\mu$ -opioid receptor mRNA, suggesting that expression of interleukin-4 and binding to its receptor is necessary (Fig. 3). It is known, that the antagonist binds to the interleukin-4R $\alpha$  receptor subunit (Tony et al., 1994). Thus, interleukin-13 signaling, which is also dependent on this receptor subunit, is blocked, as well. To address the question, which of the two cytokines might mediate the  $\Delta^9$ -THC induced upregulation of  $\mu$ -opioid receptor, the mRNA levels for interleukin-4 and interleukin-13 were determined (Fig. 4). Interleukin-4 mRNA was significantly induced by  $\Delta^9$ -THC. Furthermore, experiments using cycloheximide demonstrated that this induction is independent of protein synthesis. In contrast, interleukin-13 mRNA levels were not significantly influenced by  $\Delta^9$ -THC.

GATA3 is an important transcription factor in the biology of T cells and upregulated by interleukin-4. Its mRNA was therefore assayed, as well. An induction of GATA3 mRNA by

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$\Delta^9$ -THC was indeed observed, and this induction was suppressed by the interleukin-4 antagonist, indicating that this cytokine is a stimulus for GATA3 induction in this scenario.

#### Identification of transcription factors involved

The transcription factor decoy oligonucleotide approach was used to address the question which transcription factors participate in the  $\Delta^9$ -THC regulation in Jurkat E6.1 cells (Fig. 5A). Generally, double stranded decoy oligonucleotides with specific binding sequences for transcription factors are brought into living cells to selectively disrupt the function of these factors. The functional inactivation of the transcription factors is due to their interaction with the excess of specific decoy oligonucleotides instead of binding to the natural regulatory motifs of genes. Induction of  $\mu$ -opioid receptor mRNA by  $\Delta^9$ -THC was strongly inhibited by STAT5 and STAT6 decoy oligonucleotides and significantly reduced by GATA3 decoy oligonucleotides, indicating that these factors are involved in the regulatory events. In addition, we tested decoy oligonucleotides specific for the transcription factors STAT1, STAT3, AP-1, AP-2, NF $\kappa$ B, NF-AT and NF-IL-6, which, however, did not interfere with induction of  $\mu$ -opioid receptor transcription (data not shown).  $\Delta^9$ -THC-induced upregulation of interleukin-4 mRNA was inhibited by STAT5 decoy oligonucleotides only, but not influenced by STAT6 and GATA3 decoy oligonucleotides, indicating that STAT5 is needed for interleukin-4 induction, and that the latter two factors are required downstream of interleukin-4 induction for  $\mu$ -opioid receptor gene trans-activation. GATA3 mRNA upregulation by  $\Delta^9$ -THC was reduced by STAT5 and STAT6 decoy oligonucleotides, indicating that STAT6 not only trans-activates the  $\mu$ -opioid receptor gene directly, as shown earlier (Kraus et al., 2001), but also trans-activates GATA3. As negative controls, nSTAT5, nSTAT6 and nGATA3 decoy oligonucleotides were used, in which the specific transcription factor binding sites were destroyed by mutations. GATA3 belongs to the group of transcription factors that are upregulated during activation, as shown above. In contrast,

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STAT factors are phosphorylated for activation. Thus, phosphorylation of STAT factors was investigated next in our model (Fig. 5B). A robust phosphorylation of both STAT5, and STAT6 was observed after stimulation of Jurkat E6.1 cells with  $\Delta^9$ -THC. In response to interleukin-4, however, only phosphorylation of STAT6 was observed, whereas STAT5 remained unphosphorylated, strengthening our hypothesis that STAT5 acts upstream of the interleukin-4 induction (Fig. 5C).

Requirement of STAT6 and GATA3 for the induction of the  $\mu$ -opioid receptor gene

Transfection experiments demonstrated that the promoter region between nt -1001 and nt -950 is responsible for the induction of the  $\mu$ -opioid receptor gene by  $\Delta^9$ -THC (Fig. 2).

Furthermore, the experiments with transcription factor decoy oligonucleotides indicated that STAT6 and GATA3 are involved in this effect (Fig. 5). The involvement of STAT6 in the direct transactivation of the  $\mu$ -opioid receptor gene was previously demonstrated, and a binding site for this factor (nt -997) identified (Kraus et al., 2001; Fig. 5C). Sequence comparisons indicated, that two putative GATA3 binding sites (at nt -962 and nt -953) are located within the  $\Delta^9$ -THC-responsive  $\mu$ -opioid receptor gene promoter region, as well (Fig. 6A). To characterize the involvement of STAT6 and GATA3 in the trans-activation of the  $\mu$ -opioid receptor gene by  $\Delta^9$ -THC, mutational analysis was performed (Fig. 6B). The wild type sequence was fully responsive to  $\Delta^9$ -THC. In contrast, mutation of the STAT6 element completely abolished inducibility of the reporter gene, indicating that STAT6 is essential for the induction of the  $\mu$ -opioid receptor gene by  $\Delta^9$ -THC and that GATA3 alone cannot trans-activate the gene. However, mutations in the putative GATA3 binding sites significantly reduced  $\Delta^9$ -THC-inducibility, indicating that GATA3 additionally contributes to  $\Delta^9$ -THC induction of the gene.

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Functionality of  $\Delta^9$ -THC induced  $\mu$ -opioid receptors in Jurkat E6.1 cells.

It was previously reported that opioids mediate phosphorylation of MAPK in various cell types including immunocytes (Chuang et al., 1997; Schmidt et al., 2000). Assuming that this effect of opioids occurs also in the Jurkat E6.1 model, it was used to demonstrate functional expression of  $\mu$ -opioid receptor proteins after induction by cannabinoids. As shown in Fig. 7, morphine-mediated phosphorylation of MAPK was observed only in cells pretreated with  $\Delta^9$ -THC, but not in unstimulated cells, indicating that the cannabinoid-induced  $\mu$ -opioid receptor mRNA is correctly translated into functional receptor proteins. In addition, the  $\mu$ -opioid receptor-specific antagonist CTOP blocked the effect, indicating that it is indeed mediated by this receptor type.

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## Discussion

We have shown that  $\mu$ -opioid receptor expression is induced by cannabinoids in Jurkat E6.1 cells via a CB2-dependent mechanism. Cannabinoids first induce expression of interleukin-4, which requires STAT5. Interleukin-4 then activates the transcription factors GATA3 and STAT6, which both trans-activate the  $\mu$ -opioid receptor gene (see Fig. 5C). As an early step in the process, the transcription factor STAT5 is phosphorylated in response to cannabinoids. In general, activation of STAT members by G-protein coupled receptors is increasingly recognized (Lo and Wong, 2004; Mazarakou and Georgoussi, 2005; Pelletier et al., 2003). Since cannabinoid and opioid receptors are structurally similar, it is interesting to mention, that a recent report demonstrated STAT5 phosphorylation in response to  $\mu$ -opioid receptor agonists (Mazarakou and Georgoussi, 2005). However, exact molecular mechanisms are largely unknown and need to be elucidated also for our model. In addition, we demonstrated a direct requirement of STAT5, STAT6 and GATA3 by decoy oligonucleotides. These specifically disrupt the function of a chosen transcription factor and, by attenuating downstream effects, directly demonstrate the necessity of a given transcription factor in a specific process. From the phylogenetic point of view it may be interesting, that the  $\mu$ -opioid receptor gene is trans-activated by STAT6 and GATA3, because these transcription factors are typical for type 2 T helper cells. Moreover, immunomodulatory effects of opioids and cannabinoids, which are often similar for both substances, include for example T helper cell regulation. Thus, opioids (Roy et al., 2004; Sacerdote et al., 2000; Wang et al., 2003) as well as cannabinoids direct the balance between T helper cells from the type 1 to the type 2 phenotype. Thus far, inhibition of interferon- $\gamma$  by cannabinoids is well described (Klein et al., 2004; Klein et al., 2000; Yuan et al., 2002). Cannabinoid-induced interleukin-4 and downstream factors like STAT6 and GATA3 may not only be key players in the induction of the  $\mu$ -opioid receptor gene, but may play a central role in T helper cell regulation by

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cannabinoids. In this context it would be interesting to see, whether other typical T helper cell type 2 genes are induced by cannabinoids via an interleukin-4 dependent pathway.

Preliminary data indicate that cannabinoids regulate not only  $\mu$ -opioid receptors in Jurkat cells, but also other genes. Whereas mRNA for interleukin-13 (Fig. 4) and interleukin-5 (data not shown), which are typical for T helper cells type 2, and mRNA for CB2 (data not shown) were not changed at the 48 h time-point, increased mRNA levels were found not only for interleukin-4 and GATA3, but also for  $\delta$ -opioid receptors and CB1 receptors (data not shown). Thus, cannabinoids do not specifically induce  $\mu$ -opioid receptors. Mechanisms for regulatory events on other genes will be topics of future reports. When regarding mRNA data it is a general question, whether changes in mRNA really reflect changes in functional protein levels. Using phosphorylation of MAPK, which is a typical effect of opioids in various cell types including immunocytes (Chuang et al., 1997; Schmidt et al., 2000), we demonstrated that cannabinoids indeed induce functional  $\mu$ -opioid receptor proteins. In general, regulation of  $\mu$ -opioid receptor mRNA in immunocytes may thus cause changes in the efficacy of opioids modulating immune functions.

We previously reported that interleukin-4 induces  $\mu$ -opioid receptor transcription not only in various immune effector cells, but also in primary neuronal cells (Kraus et al., 2001). It would be worth to investigate if peripheral cannabinoids, via an interleukin-4-dependent mechanism, induced  $\mu$ -opioid receptor expression in neuronal cells, as well. Although not unequivocally established, an upregulation of antinociceptive receptors in neurons may enhance analgetic effects, as indicated by reports investigating the role of  $\mu$ -opioid receptors in inflammation (Brack et al., 2004), and after viral transduction into dorsal root ganglia (Xu et al., 2003).

Hypothetically, an upregulation of  $\mu$ -opioid receptors in neuronal cells by cannabinoids might thus contribute to synergistic effects of cannabinoids and opioids (Cichewicz and McCarthy, 2003; Tham et al., 2005; Yesilyurt et al., 2003) and to antinociceptive effects of CB2 agonists (Hohmann et al., 2004; Ibrahim et al., 2003).



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## Acknowledgements

The authors wish to thank Walter Sebald (Biozentrum, Universität Würzburg, Germany) for generously providing the interleukin-4 antagonist IL-4[R121D,Y124D].

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Footnotes

Financial support: Supported by grants from the Doktor-Robert-Pfleger-Stiftung (J.K.)  
and the Deutsche Forschungsgemeinschaft (KR 1740) (J.K.).

Reprint address: Jürgen Kraus, Department of Pharmacology and Toxicology,  
University of Magdeburg, 44 Leipziger Strasse, 39120 Magdeburg, Germany; e mail:  
[juergen.kraus@medizin.uni-magdeburg.de](mailto:juergen.kraus@medizin.uni-magdeburg.de)

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Legends for Figures

**Figure 1. CB2 receptor agonists induce  $\mu$ -opioid receptor mRNA in Jurkat E6.1 cells.**

**A:**  $\mu$ -opioid receptor (MOR) mRNA induction by  $\Delta^9$ -THC. Jurkat E6.1 cells were stimulated with  $\Delta^9$ -THC (500 nM, 48 h, +; unstimulated controls, -) and  $\mu$ -opioid receptor specific transcripts were detected by conventional RT-PCR. A representative example is shown (arrow:  $\mu$ -opioid receptor-specific amplification product; left:  $\phi$ X174 DNA-HaeIII digest size marker). **B:**  $\mu$ -opioid receptor mRNA induction is mediated by CB2. Jurkat E6.1 cells were stimulated with the unspecific cannabinoid receptor agonist  $\Delta^9$ -THC,  $\Delta^9$ -THC together with CB1- (AM 281) and CB2- (AM 630) antagonists, or CB1- (R(+)-methanandamide, MAEA) and CB2- (JWH 015) agonists, and cycloheximide (CX, 5  $\mu$ g/ml) and then subjected to quantitative real time RT-PCR. Results of three independent experiments performed in triplicate relative to  $\beta$ -actin plus S. E. M. are shown. Values are compared to relative  $\mu$ -opioid receptor induction by  $\Delta^9$ -THC, which was set to 100%.  $\Delta^9$ -THC: 500 nM [ $K_i$  (CB1): 53.3 nM;  $K_i$  (CB2): 75.3 nM], AM 281: 500 nM [ $K_i$  (CB1): 12 nM;  $K_i$  (CB2): 4200 nM], AM 630: 500 nM [ $K_i$  (CB1): 5152 nM;  $K_i$  (CB2): 31.2 nM], MAEA: 500 nM [ $K_i$  (CB1): 17.9 nM;  $K_i$  (CB2): 868 nM], JWH 015: 250 nM [ $K_i$  (CB1): 383 nM,  $K_i$  (CB2): 13.8 nM].  $K_i$  values were taken from (Howlett et al., 2002). \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ .

**Figure 2. Identification of the  $\Delta^9$ -THC-responsive  $\mu$ -opioid receptor gene promoter region.**

On top, the human  $\mu$ -opioid receptor gene promoter with an earlier identified STAT6 binding site (nt -997; dashed vertical line) is shown. Below, chloramphenicol acetyl transferase (CAT) reporter constructs are depicted relative to the promoter. CAT activities obtained after transfection of Jurkat E6.1 cells are shown as fold induction on the right (white bars:

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unstimulated controls; grey bars:  $\Delta^9$ -THC (500 nM) stimulated transfectants). The bottom lane shows the vector pBLCAT2. Results of at least three independent experiments performed in triplicate plus S. E. M. are plotted (tk: thymidine kinase promoter; \*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ).

**Figure 3. Effect of an interleukin-4 antagonist on  $\mu$ -opioid receptor mRNA induction by  $\Delta^9$ -THC.**

Jurkat E6.1 cells were incubated with  $\Delta^9$ -THC (500 nM, 48 h) and the indicated amounts of the interleukin-4 antagonist IL-4[R121D,Y124D] and then subjected to real time RT-PCR. Induction of  $\mu$ -opioid receptor mRNA [MOR]/[ $\beta$ -actin] by  $\Delta^9$ -THC without antagonist was set to 100%. Results of three independent experiments performed in triplicate plus S. E. M. are shown (\*:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

**Figure 4. Induction of interleukin-4, interleukin-13 and GATA3 mRNA after  $\Delta^9$ -THC stimulation.**

Jurkat E6.1 cells were stimulated with  $\Delta^9$ -THC (500 nM, 48 h, +; unstimulated controls, -) and then subjected to real time RT-PCR for the indicated genes. Interleukin-4 and GATA3 mRNA was additionally assayed in the presence (+) and absence (-) of cycloheximide (5  $\mu$ g/ml) or the antagonist IL-4[R121D,Y124D] (300 nM). Quantifications of the results of three to four independent experiments performed in triplicate normalized to  $\beta$ -actin are shown (†:  $p < 0.05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

**Figure 5. Identification of transcription factors involved.**

**A:** Effect of decoy oligonucleotides. The effects of transcription factor decoy oligonucleotides (160 nM), or mutated oligonucleotides as controls (e. g.  $\underline{n}$ STAT6; 160 nM) on  $\Delta^9$ -THC (500



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nM; 48 h)-induced mRNA for  $\mu$ -opioid receptor (MOR), interleukin-4 and GATA3 in Jurkat E6.1 cells are shown. Induction of the mRNAs by  $\Delta^9$ -THC normalized to  $\beta$ -actin was set to 100%. An inhibition of the  $\Delta^9$ -THC induction demonstrates involvement of the indicated transcription factor. Quantification of the results of at least three independent experiments performed in triplicate plus S. E. M. are shown (\*:  $p < 0,05$ ; \*\*:  $p < 0,01$ ; \*\*\*:  $p < 0.001$ ). **B:** Phosphorylation of STAT5 and STAT6 by Western blot analysis. Jurkat E6.1 cells were stimulated with  $\Delta^9$ -THC (500 nM, 48 h) or interleukin-4 (5 ng/ml, 20 min). The membrane was then hybridized with antibodies against phosphorylated STATs. Afterwards, the same membrane was reprobbed for unphosphorylated STAT proteins. **C:** Model for the induction of the  $\mu$ -opioid receptor gene (MOR) by  $\Delta^9$ -THC.

**Figure 6. STAT6 and GATA3 transactivate the  $\mu$ -opioid receptor gene.**

**A:**  $\Delta^9$ -THC responsive promoter region with the STAT6 binding site and putative GATA3 binding sites. **B:** Transfection experiments in Jurkat E6.1 cells.  $\Delta^9$ -THC-inducibility of reporter gene constructs with wild type and mutated (underlined) sequences are shown as fold induction (white bars: unstimulated controls; grey bars:  $\Delta^9$ -THC (500 nM) stimulated transfectants). Results of at least three independent experiments performed in triplicated plus S. E. M. are shown (CAT: chloramphenicol acetyl transferase; tk: thymidine kinase promoter; †:  $p < 0,05$ ; \*\*:  $p < 0.01$ ; \*\*\*:  $p < 0.001$ ).

**Figure 7.  $\Delta^9$ -THC induces functional  $\mu$ -opioid receptor proteins.**

Jurkat E6.1 cells were prestimulated with  $\Delta^9$ -THC (500 nM, 5 d) to allow sufficient protein expression or left unstimulated (-). Then, morphine-induced phosphorylation of MAPK was assayed by Western blot analysis (morphine (mo): 5  $\mu$ M, 5 min, 37°C). The antagonist CTOP (1  $\mu$ M) was added 45 min prior to morphine stimulation. After gel electrophoresis, proteins

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were blotted and membranes containing the same probes were hybridized with either antibodies against phosphorylated MAPK (P-MAPK) or unphosphorylated ERK 2 (MAPK).

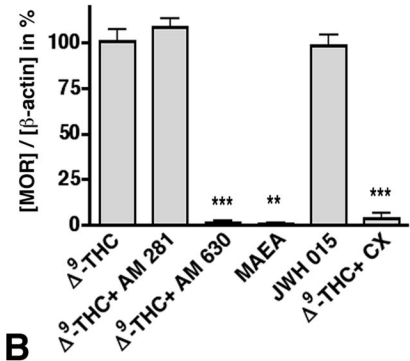
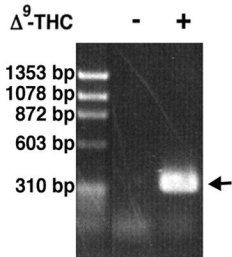


Figure 1

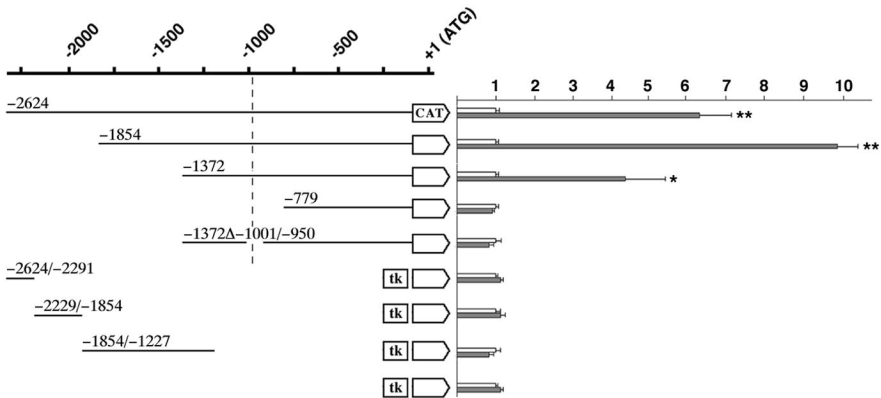


Figure 2

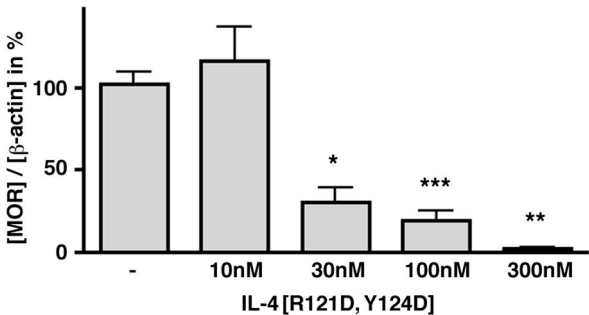
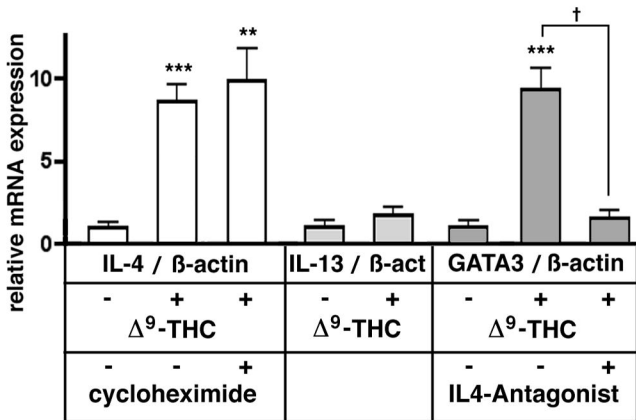


Figure 3



	MOR mRNA	IL-4 mRNA	GATA3 mRNA
no decoy	100.0±6.6	100.0±8.1	100.0±4.3
STAT6	7.8±3.9 <sup>***</sup>	144.6±31.2	19.3±3.2 <sup>***</sup>
nSTAT6	81.1±14.4	125.9±41.1	145.2±21.8
GATA3	43.8±4.9 <sup>**</sup>	137.2±32.1	n.d.
nGATA3	98.3±11.2	143.2±19.3	n.d.
STAT5	1.4±1.1 <sup>***</sup>	10.1±4.4 <sup>***</sup>	5.2±1.2 <sup>**</sup>
nSTAT5	86.4±7.5	73.4±20.8	130.7±43.7

**A**

	$\Delta^9$ -THC		IL-4	
	-	+	-	+
P-STAT5				
STAT5				
P-STAT6				
STAT6				

**B**

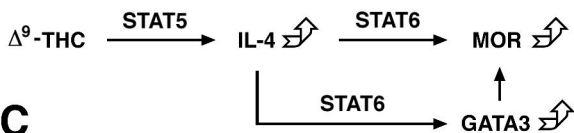
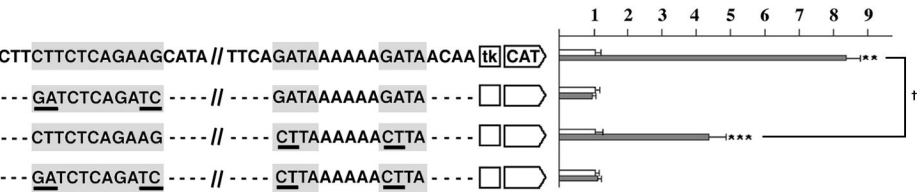


Figure 5

nt -1000  
 5'-CTTCTTCTCAGAAGCATATGTCTATCGAGGAAGTCTTCAGATAAAAAAGATAACAA-3'  
 nt -950

**A** STAT6 element putative GATA3 elements



**B**

Figure 6



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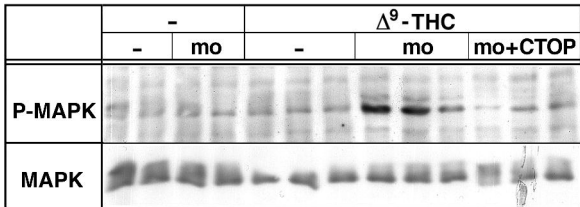


Figure 7