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Cannabinoids attenuate norepinephrine-induced melatonin biosynthesis in the rat pineal gland by reducing arylalkylamine *N*-acetyltransferase activity without involvement of cannabinoid receptors

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Abstract

Cannabinoids modulate neuronal and neuroendocrine circuits by binding to cannabinoid receptors acting upon cAMP/Ca^{2+}-mediated intracellular signaling cascades. The rat pineal represents an established model to investigate intracellular signaling processes because a well defined input, the neurotransmitter norepinephrine, is transformed via cAMP/Ca^{2+}-dependent mechanisms into an easily detectable output signal, the biosynthesis of melatonin. Here we investigated the impact of cannabinoids on norepinephrine-regulated melatonin biosynthesis in the rat pineal. We demonstrated that treatment of cultured rat pineals with 9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC), cannabidiol or cannabinol significantly reduced norepinephrine-induced arylalkylamine *N*-acetyltransferase (AANAT) activity and melatonin biosynthesis. These effects were not mimicked by the cannabinoid receptor agonist WIN55,212–2 and were not blocked by cannabinoid 1 and 2 receptor antagonists. The cannabinoids used did not affect norepinephrine-induced increases in cAMP/Ca^{2+} levels. Notably, cannabinoids were found to directly inhibit AANAT activity in lysates of the pineal gland. This effect was specific in so far as cannabinoids did not influence the activity of hydroxyindole-*O*-methyltransferase (HIOMT), the last enzyme in melatonin biosynthesis. Taken together, our data strongly suggest that cannabinoids inhibit AANAT activity and attenuate melatonin biosynthesis through intracellular actions without involvement of classical cannabinoid receptor-dependent signaling cascades.

Keywords: arylalkylamine *N*-acetyltransferase, cannabidiol, cannabinol, cannabinoid system, delta-9-tetrahydrocannabinol, melatonin biosynthesis.

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The cannabinoid (CB) system consists of selectively produced and inactivated endogenous ligands, the endocannabinoids and their specific CB receptor (R) proteins. Plant-derived exogenous ligands and synthetically designed agonists and antagonists of CBR led to the identification of different CBR subtypes (Iversen 2003; Piomelli 2003). The CB1R and CB2R genes were cloned from different mammalian species (Matsuda *et al*. 1990; Munro *et al*. 1993). CB1R are mainly found in the brain located on neurons and astrocytes. CB2R were generally thought to be absent in healthy brain and mainly associated with the immune system (Stella 2004) but very recently CB2R proteins were found on brainstem neurons (Van Sickle *et al*. 2005). Studies with CB1R and CB2R knockout mice revealed the existence of two other, not yet cloned CBR subtypes: the WINR and the abnormal-cannabidiol

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Abbreviations used: AM251, *N*-1-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide; AM630, 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methasone; AANAT, arylalkylamine *N*-acetyltransferase; AC, adenylyl cyclase; ADR, adrenergic receptor; AEA, anandamide; CB, cannabinoid; CBD, (–)-cannabidiol; CBN, cannabinol; CRE, cyclic AMP responsive element; CREB, cyclic AMP responsive element-binding protein; HIOMT, hydroxyindole-*O*-methyltransferase; NE, norepinephrine; PKA, protein kinase A; R, receptor; THC, 9-carboxy-11-nor-delta-9-tetrahydrocannabinol; WIN, WIN55,212–2.

(abn-CBD)R (Jarai *et al*. 1999; Breivogel *et al*. 2001). WINR activation inhibits glutamatergic neurotransmission in the hippocampus. Abn-CBDRs are located on endothelial cells of blood vessels being involved in the regulation of vascular dilatation (Hajos *et al*. 2001; Offertaler *et al*. 2003).

CBRs are G protein-coupled receptors. CB ligands exert their effects via activation of different G proteins and influence an array of intracellular signal transduction cascades (Howlett *et al*. 2004). Numerous studies describe CBR-mediated activation of $G_{i/o}$ proteins leading to inhibition of adenylyl cyclase (AC) activity and consequently to decrease in intracellular cAMP levels ($[cAMP]_i$) (Childers *et al*. 1993; Pacheco *et al*. 1993; Felder *et al*. 1995). In contrast, some studies report CBR-mediated increases in $[cAMP]_i$ (Glass and Felder 1997; Maneuf and Brotchie 1997). Activated CBRs also modulate the formation of second messengers other than cAMP, such as intracellular Ca^{2+}, nitric oxide or ceramide (Sugiura *et al*. 1996; Prevot *et al*. 1998; Velasco *et al*. 2005). Nevertheless, a couple of studies also report on non-CBR-mediated actions of CBs on intracellular signaling cascades (Felder *et al*. 1992; Jordt *et al*. 2004).

CBR activation is linked to intracellular effector systems thereby modulating short and long-lasting processes such as ion channel activity or gene expression in different cell types of the brain. One important feature of CB-mediated modulation is the regulation of hormone production. For example, the CBR ligand 9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC) affects the neuroendocrine regulation of different hormones, such as gonadotrophins, prolactin or thyroid hormones (Murphy *et al*. 1998).

Very little is known about a possible involvement of the CB system in the regulation of melatonin biosynthesis in the mammalian pineal gland. Melatonin biosynthesis is controlled by one of the best investigated transmembrane signaling cascades: Noradrenergic receptor activation leads to the formation of the second messengers cAMP and Ca^{2+} followed by activation of the penultimate enzyme of melatonin biosynthesis, the arylalkylamine *N*-acetyltransferase (AANAT). Since the impact of receptor activation, second messengers, transcription factors, protein–protein interactions and enzyme activation on melatonin biosynthesis is known in remarkable detail, we have decided to systematically investigate whether and how cannabinoids (CB) interfere with this pathway in order to learn more about the interactions between the CB system and neuroendocrine signaling.

Materials and methods

Materials

Drugs and chemicals were obtained from the following companies or institutions: 3-n-amyl-6,6,9-trimethyl-6-dibenzopyran-1-ol (cannabinol (CBN)), 9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC), ascorbic acid, glucose, HEPES, norepinephrine, paraformaldehyde, poly-L-lysine and sucrose (Sigma-Aldrich, St. Louis, MO, USA); 2-[(1R,6R)-3-methyl-6-(1-methylethenyl)-2-cyclohexen-1-yl]-5-pentyl-1,3-benzenediol ((–)-cannabidiol (CBD), (*R* +)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone mesylate (WIN 55, 212–2 (WIN)), 6-iodo-2-methyl-1-[2-(4-morpholinyl)ethyl]-1*H*-indol-3-yl](4-methoxyphenyl)methasone (AM630) and *N*-(piperidin-1-yl)-5-(4-iodophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1*H*-pyrazole-3-carboxamide (AM251) (Tocris Cookson, Ellisville, MO, USA); Dulbecco's modified Eagle medium (DMEM), Earle's balanced salt solution (EBSS), fetal calf serum, glutamine, penicillin and streptomycin (Life Technologies, Gaithersburg, MD, USA); Fura2/AM (Molecular Probes, Leiden, Netherlands); papain (Boehringer, Mannheim, Germany).

The cannabinoid (CB) compounds CBN, THC, AM251, AM630 and WIN were dissolved in DMSO and stored at − 20°C. CBD was dissolved in ethanol (EtOH) and stored at − 20°C. Prior to application of the CB compounds to pineal cell and organ cultures, the CB compounds were diluted in cell culture medium (for composition of cell culture medium, see Pineal cell and organ culture section). Each CB compound was added to immobilized rat pinealocytes or primary cultures of rat pineal glands with a maximal final solvent concentration of 0.1% (v/v) DMSO or 0.1% (v/v) EtOH in cell culture medium. Control experiments with equimolar concentrations of DMSO or EtOH alone did not show any significant effects on the parameters investigated in this study (data not shown).

Pineal cell and organ culture

All animal experimentation was carried out in accordance with the Policy on the Use of Animals in Neuroscience Research and the Policy on Ethics as approved by the Society for Neuroscience and by the European Communities Council Directive (89/609/EEC).

Male Wistar rats (170–200 g; Charles River, Sulzfeld, Germany) were killed by decapitation. Their pineal glands were removed and immediately transferred to ice-cold Earle's balanced salt solution (EBSS) supplemented with 10 mM HEPES and 7 g/L glucose. For organ cultures, pineal glands were cut into halves and incubated at 37°C in Dulbecco's modified Eagle medium (DMEM) containing 5% (v/v) fetal calf serum and supplemented with 10 mM HEPES, 100 μg/mL ascorbic acid, 100 μg/mL streptomycin, 100 U/mL penicillin and 2 mM glutamine. For cell cultures, pineal glands were dissociated by papain digestion and immobilized onto 0.01% (v/v) poly-L-lysine treated glass coverslips for immunostaining or calcium imaging as described earlier (Schomerus *et al*. 1995).

Cell and organ culture treatment

After two days in culture, pinealocytes and pineal glands were stimulated with 1 μM NE for different time periods or left untreated as non-stimulated control preparations (CTL). To achieve maximal effects on AANAT protein amount, AANAT activity and melatonin levels, pineal glands were stimulated with 1 μM NE for 6 h. For investigation of cAMP, intracellular Ca^{2+} and pCREB levels, preparations were stimulated with 1 μM NE for different time periods as indicated in the respective experiments.

The effects of THC, CBD, CBN or WIN on AANAT activity and melatonin levels were investigated by treatment of pineal glands

with different concentrations of THC (10, 5, 1 or 0.1 μM), CBD (10, 1 or 0.1 μM), CBN (10, 1 or 0.1 μM) or WIN (10, 1 or 0.1 μM) for 15 min, followed by concomitant treatment with the respective CB compound and 1 μM NE for 6 h. A possible influence of the CB1R or CB2R on AANAT activity and melatonin levels was investigated by use of the CB1R antagonist AM251 or the CB2R antagonist AM630. Administration of the antagonists to cultured pineal glands started 15 min prior to THC application and was continued during THC and NE application.

To determine the effects of THC on NE-induced cAMP, Ca^{2+}, pCREB levels and AANAT protein, pinealocytes and pineal glands were treated with different concentrations of THC for 15 min, followed by concomitant treatment with THC and 1 μM NE for different time periods as indicated in the respective experiments.

The cannabinoids (THC, CBD, CBN or WIN) used did not influence any of the investigated parameters when applied alone to non-stimulated, cultured pinealocytes or pineal glands (data not shown).

Radiometric assays of AANAT and HIOMT activity

AANAT and HIOMT activity were determined by radiometric assays as described earlier (Champney *et al*. 1984) in homogenates from cultured rat pineal glands. For preparation of pineal gland homogenates, the cultured pineal glands were collected from 24-well culture dishes, pelleted by short centrifugation and immediately frozen on solid CO_2. After thawing, the pineal glands were sonicated in 0.1 M ammonium acetate, pH 6.8 for radiometric assay of AANAT or 0.1 M sodium phosphate, pH 7.9 for determination of HIOMT activity. The samples were centrifuged for 10 min (10 000 *g*) at 4°C and a small amount of the supernatant was used for determination of protein concentration by Bradford protein test (Bradford 1976). To measure AANAT activity, tryptamine (final concentration 0.04 M) and [1–^{14}C]acetyl coenzyme A (1 mM; specific activity 60 μCi/μmol; Perkin Elmer, USA) were added to the remaining supernatant. HIOMT activity was analyzed by application of N-acetylserotonin (final concentration 4 mM) and [^{14}C-methyl]S-adenosylmethionine (0.4 mM; specific activity 51.4 μCi/μmol; Perkin Elmer, USA) to the supernatant. Preparations were then incubated for 20 min at 37°C and enzymatic reactions were stopped in both assays by adding 1 mL chloroform. In AANAT radiometric assay, the organic phase containing the radiolabeled *N*-[^{14}C]acetyltryptamine was washed twice with 0.1 M ammonium acetate, pH 6.8. The radiolabeled [^{14}C]melatonin containing organic phase in the HIOMT radiometric assay was washed twice with 0.45 M sodium borate buffer. Finally, 0.5 mL samples of the organic phase were taken to dryness for determination of radioactivity and activities of AANAT and HIOMT were indicated in nmol/h × mg protein in both assays.

To analyze effects of THC, CBD or CBN on AANAT activity in a cell-free system, homogenates of cultured pineal glands that had been stimulated with 1 μM NE for 6 h were centrifuged for 20 min (20 800 *g*) at 4°C. These lysates were incubated with 10, 1 or 0.1 μM THC, 10 μM CBD or 10 μM CBN for 5 min at 37°C prior to radiometric determination of AANAT activity.

Melatonin measurement

The melatonin concentration in supernatants of cultured rat pineal glands was analyzed by use of a commercially available Melatonin ELISA kit (IHF, Hamburg, Germany) with a minimal detection range of 1.5–2 pg melatonin/mL as described previously (Koch *et al*. 2003).

Immunocytochemical demonstration of pCREB

For immunocytochemical demonstration of pCREB, immobilized pinealocytes were treated with 10, 1, 0.1 or 0.01 μM THC for 15 min, followed by concomitant treatment with THC and 1 μM NE for 30 min. Positive controls were treated with 1 μM NE only for 30 min. The cells were fixed immediately after stimulation with 4% (w/v) paraformaldehyde in phosphate-buffered saline. Subsequently, preparations were incubated over night with a rabbit polyclonal antibody against pCREB (diluted 1 : 250; New England Biolabs, MD, USA). Binding of the primary antibodies was visualized using the ABC method as described above (for further methological methods, see Schomerus *et al*. 1996).

For semiquantitive analysis of pCREB IR, the MeanDENS-percent values were calculated according to von Gall *et al*. (2002).

Immunochemical demonstration of pCREB and AANAT

Protein extracts from cultured rat pineal glands were obtained by sonication of cultured pineal glands in lysis buffer containing 9.1 mM NaH_2PO_4, 1.7 mM Na_2HPO_4, 150 mM NaCl, 0.5% (w/v) NaDeoxycholate, 0.5% (v/v) NP40, pH 7.4. Cell debris was removed by centrifugation for 10 min (10 000 *g*). Protein concentrations of the supernatants were determined according to the method of Bradford (Bradford 1976). Sample buffer was added to the preparations and equal amounts of protein were loaded onto a 10% (w/v) SDS-polyacrylamide gel. After gel electrophoresis, the proteins were blotted onto nitrocellulose membranes. The nitrocellulose membranes were incubated with the above-described antibody against pCREB or an antibody against rat AANAT22-37 3 (AS 2500) (Gastel *et al*. 1998). Binding of the primary antibodies was visualized by use of HRP-conjugated secondary antibodies and chemiluminescence (Ultra Signal; Pierce, Rockford, IL, USA). For semiquantitive analysis of pCREB and AANAT immunoreaction (IR), the SUMDENS values of the immunoreactive bands were determined according to Wicht *et al*. (1999).

Calcium imaging

The measurement of $[Ca^{2+}]_i$ in cultured rat pinealocytes was performed as described previously (Schomerus *et al*. 1995). Briefly, pinealocytes immobilized on glass coverslips were loaded with 2.5 μM fura-2/AM for 15 min at 37°C in cell culture medium. Subsequently, coverslips were rinsed with saline (140 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 10 mM HEPES, pH 7.4), placed into a superfusion chamber mounted on the heat-stable stage of an Axiovert 100 microscope (Zeiss, Germany), and superfused with saline at 37°C. All preparations were prestimulated with 1 μM NE to induce $[Ca^{2+}]_i$ responses. After removal of NE, one group of cell preparations was again stimulated with NE to induce $[Ca^{2+}]_i$ responses for a second time. Another group of cell preparations was treated with 5 μM THC followed by concomitant treatment with 5 μM THC and NE. To compare $[Ca^{2+}]_i$ responses of the two different experimental groups, the areas under curve (AUC) of the second response were assessed and used for statistical analysis. Data acquisition and analysis of fluorescence signals

from single cells were performed by use of an Attofluor calcium imaging system (Zeiss, Germany).

cAMP measurement

The cAMP concentration was measured in preparations containing both the homogenates of cultured rat pineal glands and the corresponding cell culture supernatant by use of a commercially available cAMP ELISA kit (IHF, Hamburg, Germany). Following the manufacturers protocol, the cultured pineal glands and the corresponding supernatants were collected together and a final volume of 80% (v/v) EtOH was added. The pineal glands were then sonicated and the preparations were kept on ice for 30 min. Cell debris was removed by centrifugation for 10 min (10 000 *g*). The supernatants were taken to dryness. Prior to determination of the total amount of cAMP by ELISA, the cAMP of the samples was acetylated.

Statistical analysis

The statistical analysis (Graph Pad, San Diego, CA, USA) was performed using an one-way ANOVA with subsequent Bonferroni tests for multiple comparisons or with subsequent Dunnet tests for comparison of multiple groups with one control group with $p < 0.05$ as the criterion of significance.

Results

THC, CBD or CBN-attenuated of NE-induced melatonin biosynthesis and AANAT activity in cultured rat pineal glands

Melatonin levels were determined in the supernatant of primary cultures of rat pineal glands (Fig. 1a). In non-stimulated control preparations (CTL), very low melatonin levels were detected. Stimulation with NE led to a 10-fold increase in melatonin levels, this value was set as 100% in each individual experiment. Treatment with THC (10, 5 or 1 μM), CBD (10 μM) or CBN (10 or 1 μM) for 15 min, followed by concomitant treatment with the respective CB compound and NE, led to a significant and concentration-dependent decline in melatonin levels as compared to preparations that were treated with NE alone. Maximal reduction of NE-induced melatonin levels was observed after coapplication of NE and 10 μM THC (down to 55%), 10 μM CBD (down to 65%) or 10 μM CBN (down to 60%). Application of 25 μM or 50 μM THC, CBD or CBN reduced melatonin levels as effectively as 10 μM THC, CBD or CBN (data not shown). Treatment of preparations with 0.1 μM THC, 1 or 0.1 μM CBD or 0.1 μM CBN did not significantly attenuate NE-induced melatonin levels.

AANAT enzymatic activity was measured in homogenates of cultured rat pineal glands (Fig. 1b). In non-stimulated control preparations (CTL), AANAT activity was barely detectable. Stimulation of cultured rat pineal glands with NE led to a 10-fold increase in AANAT activity, this value was set as 100%. Treatment with THC

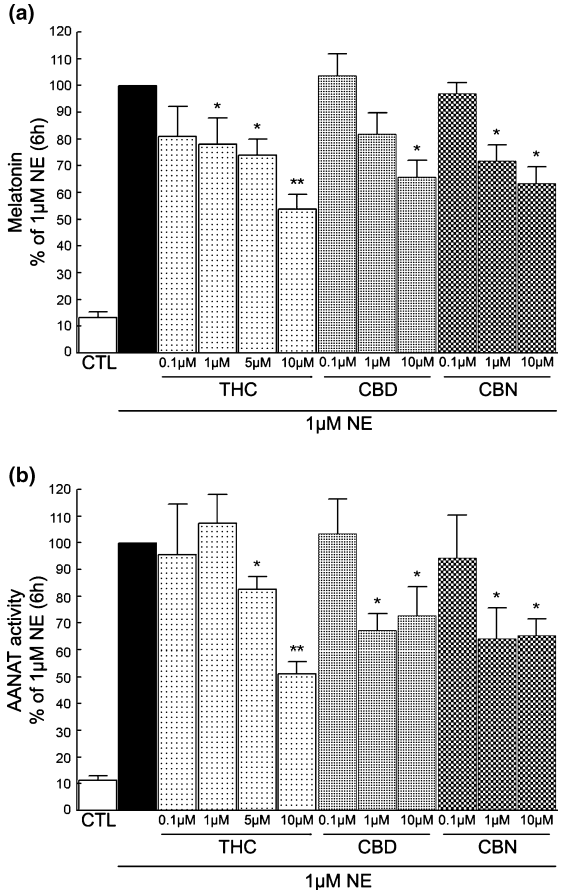

Fig. 1 THC, CBD or CBN-mediated attenuation of norepinephrine (NE)-induced melatonin biosynthesis and AANAT activity in cultured rat pineal glands. (a) Stimulation of preparations with 1 μM NE for 6 h led to a 10-fold increase in melatonin levels as compared to non-stimulated control preparations (CTL). The value obtained after NE stimulation was set as 100%. Application of THC (10, 5 or 1 μM), CBD (10 μM) or CBN (10 or 1 μM) for 15 min, followed by concomitant treatment with the respective CB compound and NE for 6 h, significantly attenuated NE-induced melatonin biosynthesis as compared to preparations stimulated with NE alone (**$p < 0.01$; *$p < 0.05$ vs. 6 h NE). Treatment of preparations with 0.1 μM THC, 1 or 0.1 μM CBD or 0.1 μM CBN did not significantly affect NE-induced melatonin biosynthesis as compared to preparations stimulated with NE alone. (b) Stimulation of preparations with 1 μM NE for 6 h strongly induced AANAT activity about 5–10-fold as compared to non-stimulated control preparations (CTL). The value obtained after NE stimulation was set as 100%. Application of THC (10 or 5 μM), CBD (10 or 1 μM) or CBN (10 or 1 μM) for 15 min, followed by concomitant treatment with the respective CB compound and NE for 6 h, significantly reduced NE-induced AANAT activity as compared to preparations stimulated with NE alone (**$p < 0.01$; *$p < 0.05$ vs. 6 h NE). Treatment of preparations with 1 or 0.1 μM THC, 0.1 μM CBD or 0.1 μM CBN did not significantly affect NE-induced AANAT activity as compared to preparations that were stimulated with NE alone.

(10 or 5 μM), CBD (10 or 1 μM) or CBN (10 or 1 μM) for 15 min, followed by concomitant treatment with the respective CB compound and NE, significantly reduced AANAT activity in a concentration-dependent manner. Maximal reduction of AANAT activity was observed after coapplication of NE and 10 μM THC (down to 50%), 1 μM CBD (down to 65%) or 10 μM CBN (down to 65%). Treatment of preparations with 25 μM or 50 μM THC, CBD or CBN did reduce NE-induced AANAT activity as effectively as 10 μM THC, CBD or CBN (data not shown). Treatment of preparations with 1 or 0.1 μM THC, 0.1 μM CBD or 0.1 μM CBN did not significantly affect NE-induced AANAT activity.

THC did not influence NE-induced pCREB IR in immobilized rat pinealocytes and primary cultures of rat pineal glands

In non-stimulated preparations, almost no pCREB IR was observed (Fig. 2a). Stimulation with NE for 30 min induced a strong nuclear pCREB IR in virtually all rat pinealocytes.

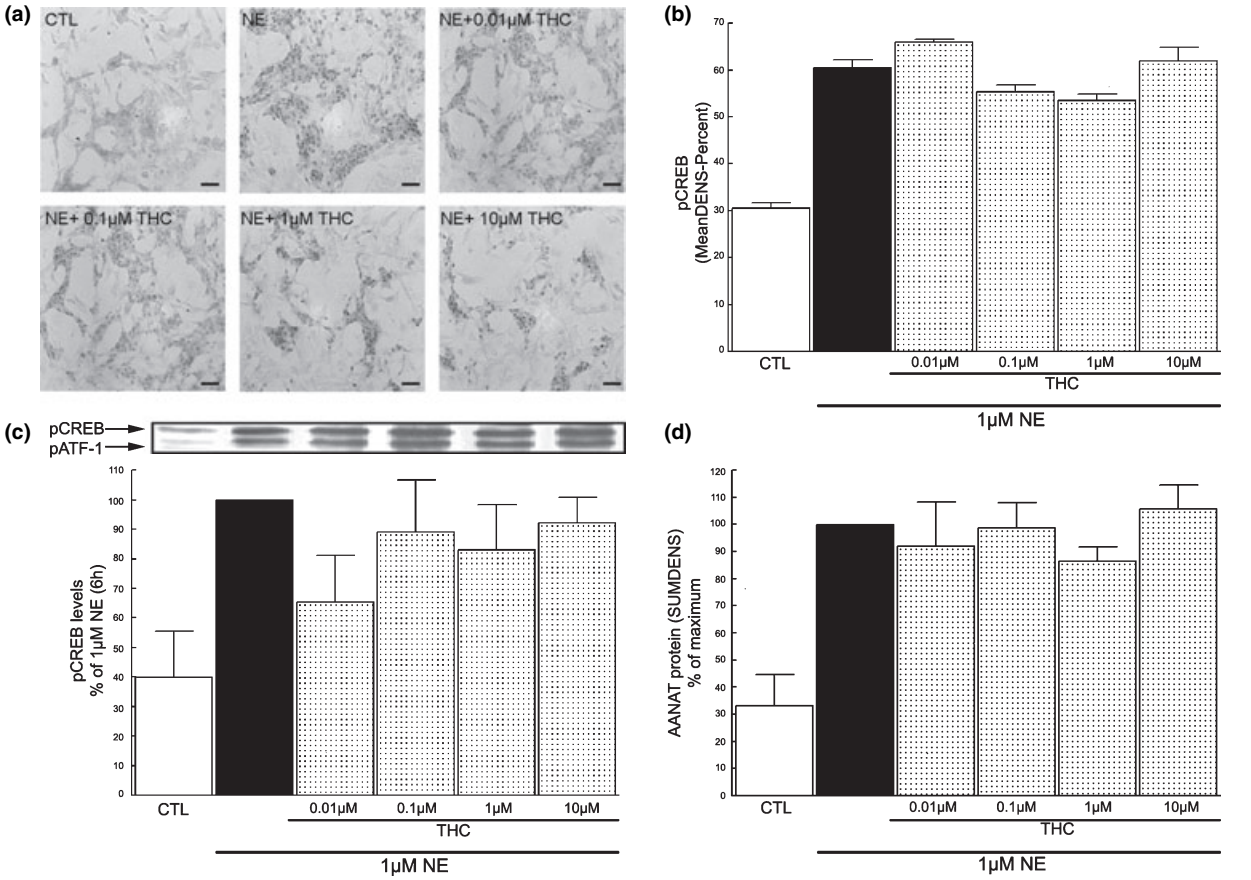

Fig. 2 THC did not influence norepinephrine (NE)-induced pCREB immunoreaction (IR) and AANAT protein levels in immobilized rat pinealocytes and primary cultures of rat pineal glands. (a) Stimulation with 1 μM NE for 30 min led to a strong increase in nuclear pCREB IR in virtually all rat pinealocytes as compared to non-stimulated controls (CTL). Treatment of preparations with 10, 1, 0.1 or 0.01 μM THC for 15 min, followed by coapplication of THC and NE for 30 min, also strongly increased nuclear pCREB IR in virtually all rat pinealocytes. Bars indicate 20 μm (b) Semiquantitative analysis revealed that treatment of immobilized rat pinealocytes with THC (10, 1, 0.1 or 0.01 μM) did not significantly affect NE-induced nuclear pCREB IR 30 min after the onset of NE-stimulation. Data were obtained from 3 independent experiments and are shown in MeanDENS-percent. (c) Stimulation of cultured rat pineal glands with 1 μM NE for 6 h strongly induced both, pCREB and pATF-1 IR as compared to controls (CTL). Treatment of preparations with THC (10, 1, 0.1 or 0.01 μM) for 15 min, followed by concomitant treatment with THC and NE for 6 h, led to a strong induction of both, pCREB and pATF-1 IR. Semiquantitative analysis of pCREB IR did not reveal any significant effects of THC on pCREB IR 6 h after the onset of NE-stimulation. Data were obtained from 5 independent experiments and pCREB SUMDENS values of preparations treated with NE alone were set as 100% in each individual experiment. (d) Stimulation of preparations with 1 μM NE for 6 h strongly induced AANAT protein levels as compared to controls (CTL). For semiquantitative analysis, the SUMDENS values of preparations stimulated with NE alone were set as 100%. Application of 10, 1, 0.1 or 0.01 μM THC for 15 min, followed by concomitant application of THC and NE for 6 h, did not significantly affect NE-induced AANAT protein levels as compared to preparations that were stimulated with NE alone.

Treatment of immobilized rat pinealocytes with 10, 1, 0.1 or 0.01 μM THC for 15 min, followed by concomitant treatment with THC and NE for 30 min, also caused a strong nuclear pCREB IR in virtually all rat pinealocytes. Semiquantitative analysis of nuclear pCREB IR did not show any significant differences between preparations treated with THC and NE and preparations that were stimulated with NE alone (Fig. 2b).

Immunochemical analysis of pCREB in homogenates of cultured rat pineal glands revealed two faint immunoreactive bands in non-stimulated preparations (Fig. 2c, CTL): the 40 kDa immunoreactive band reflects cross-reactivity of the pCREB antibody with the pCREB-related, phosphorylated activating transcription factor 1 (pATF-1; see New England Biolabs protocol); the 43 kDa immunoreactive band has previously been identified as pCREB (Schomerus *et al.* 1996). Stimulation of cultured rat pineal glands with 1 μM NE for 6 h resulted in a strong induction of pCREB IR. The intensity of this pCREB IR was set as 100% for semiquantitative analysis (Fig. 2c). Treatment of cultured rat pineal glands with THC (10, 1, 0.1 or 0.01 μM) for 15 min, followed by concomitant treatment with THC and NE for 6 h, also caused a strong induction of pCREB IR. Semiquantitative analysis of pCREB IR did not reveal any significant differences between preparations treated with THC and NE and preparations treated with NE alone (Fig. 2c).

NE-induced AANAT protein levels were not affected by THC in primary cultures of rat pineal glands

Semiquantitative analysis showed that stimulation of cultured rat pineal glands with NE for 6 h caused a strong induction of AANAT IR as compared to only weak AANAT IR found in non-stimulated control preparations (Fig. 2d). The intensity of AANAT IR in preparations stimulated with NE was set as 100%. Comparison of NE-stimulated preparations with preparations that were treated with THC (10, 1, 0.1 or 0.01 μM) for 15 min, followed by concomitant treatment with THC and NE, did not show any significant differences in the intensity of the AANAT IR.

NE-induced increases in $[Ca^{2+}]_i$ and cAMP levels were not influenced by THC in immobilized rat pinealocytes and in primary cultures of rat pineal glands

$[Ca^{2+}]_i$ was determined using the fura-2/AM method. Treatment of immobilized rat pinealocytes with 1 μM NE increased $[Ca^{2+}]_i$ in virtually all rat pinealocytes. The NE-induced $[Ca^{2+}]_i$ response was biphasic. Immediately after the onset of NE stimulation, $[Ca^{2+}]_i$ increased to a transient maximum, which decreased to a still elevated plateau that persisted as long as the cells were exposed to the NE-stimulus (Figs 3a,b). When NE was washed out, $[Ca^{2+}]_i$ dropped back to basal levels. To investigate whether THC influences the NE-induced Ca^{2+} response, a second treatment either with NE alone (controls) or with a combination of NE plus THC (THC-group) was performed when $[Ca^{2+}]_i$ levels had returned to basal levels after the first NE-stimulus. In the controls, the second treatment with NE alone evoked a biphasic increase in $[Ca^{2+}]_i$; its amplitude, however, was lower than that of the first response confirming previous data (Schomerus *et al.* 1995). In the THC-group, the second treatment with 5 μM THC, followed by concomitant application of THC and NE (Fig. 3b) also induced a biphasic $[Ca^{2+}]_i$ response closely resembling the second $[Ca^{2+}]_i$ response induced by NE alone. Application of THC alone had no significant effect on $[Ca^{2+}]_i$. To quantify the second $[Ca^{2+}]_i$ response, the area under curve (AUC) was determined in the control and the THC groups and statistically analyzed (Fig. 3c). This confirmed that the second $[Ca^{2+}]_i$ responses did not significantly differ between the two groups.

The cAMP concentrations were determined in homogenates of cultured rat pineal glands plus the corresponding cell culture supernatant (total cAMP) using an ELISA (Fig. 3d). The preparations were left untreated (CTL), stimulated with NE for 10, 15, 20, 30, 45, 120 or 360 min or treated with 5 μM THC for 15 min, followed by concomitant treatment with THC and NE for the same, above indicated time periods.

cAMP was barely detectable in non-stimulated preparations (CTL). Upon stimulation with NE for up to 30 min, the cAMP concentration gradually increased. After application of NE for 45 min cAMP concentrations declined as compared to cAMP concentrations observed after 30 min of NE-stimulation. The highest concentration of cAMP was observed 120 min after the onset of NE-stimulation. As compared to non-stimulated controls (CTL), treatment of pineal glands with 5 μM THC and NE also led to a strong increase in cAMP concentration. The increase in cAMP concentration elicited by concomitant application of THC and NE paralleled the increase in cAMP concentrations observed in preparations that were stimulated with NE alone. Thus, incubation of cultured rat pineal glands with THC did not significantly affect NE-induced cAMP concentrations at any of the indicated time periods.

WIN-, CB1 or CB2 receptors are apparently not involved in CB-mediated attenuation of NE-induced melatonin biosynthesis and AANAT activity

To elucidate the receptor type mediating CB-dependent attenuation of NE-induced melatonin biosynthesis and AANAT activity, primary cultures of rat pineal glands were treated with the CBR agonist WIN, the CB1R antagonist AM251 or the CB2R antagonist AM630. As compared to non-stimulated control preparations (Figs 4a,b; CTL), stimulation with NE for 6 h led to 5–10fold increases in melatonin biosynthesis (Fig. 4a) and AANAT activity (Fig. 4b). The value obtained after NE stimulation was set as 100% (Figs 4a,b). Compared to preparations stimulated with NE

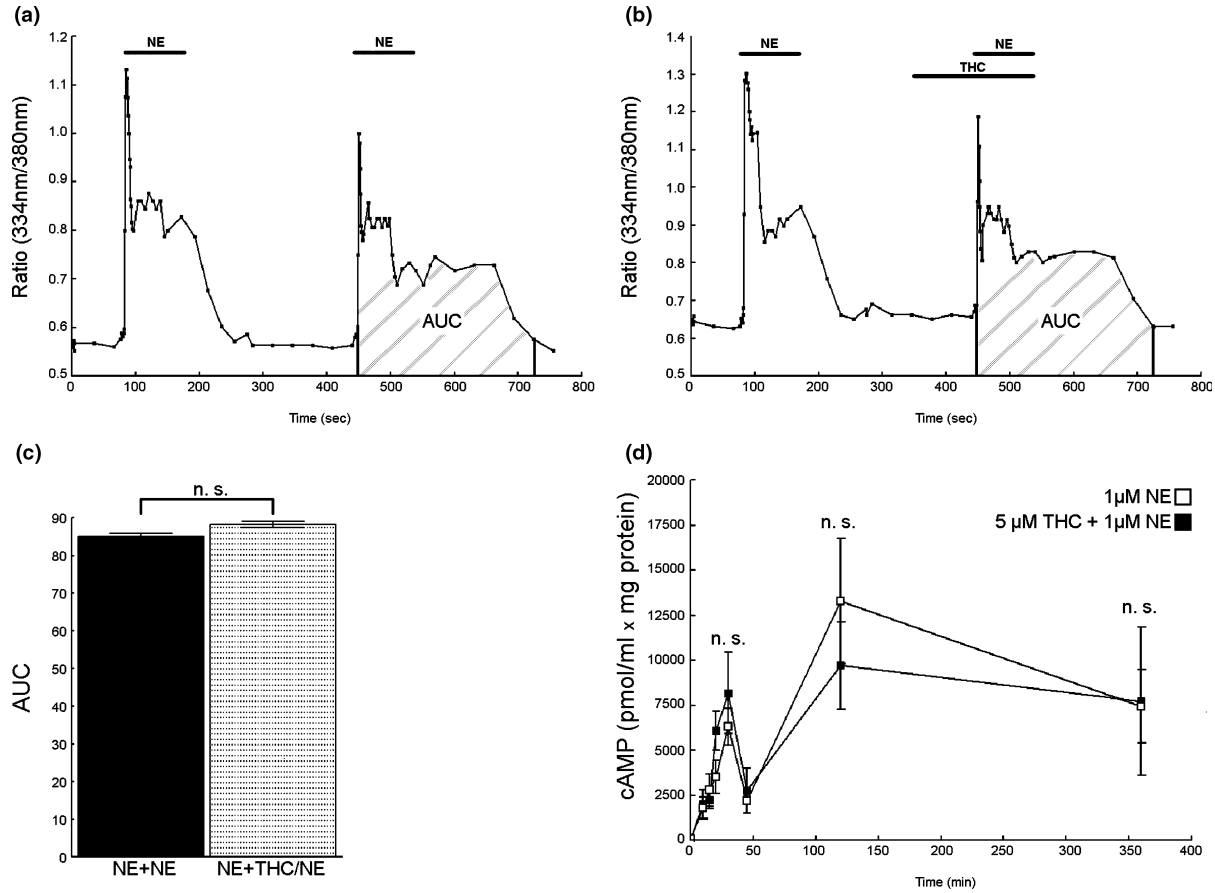

Fig. 3 THC did not affect norepinephrine (NE)-induced $[Ca^{2+}]_i$ in immobilized rat pinealocytes and NE-induced cAMP levels in primary cultures of rat pineal glands. Representative $[Ca^{2+}]_i$ responses of immobilized rat pinealocytes are shown. The NE-induced $[Ca^{2+}]_i$ response was biphasic: immediately after the onset of NE-stimulation, $[Ca^{2+}]_i$ increased to a transient maximum and decreased to a plateau elevated above basal levels. This persisted during the exposure to the NE-stimulus. When $[Ca^{2+}]_i$ levels had reached basal levels after the first NE-treatment, all cells were stimulated for a second time. (a) One group of preparations was stimulated with 1 μM NE only. (b) The other group was treated with 5 μM THC followed by a combination of 1 μM NE plus 5 μM THC. Both treatments elicited a biphasic $[Ca^{2+}]_i$ response, whose amplitude was clearly lower than that of the first response. Application of THC alone had no significant effect on $[Ca^{2+}]_i$. (c) Area under curve (AUC) values of the second responses of the two different experimental groups. Statistical analysis of the AUC values did not show any significant differences between NE-treated preparations and preparations that were concomitantly treated with THC and NE. (d) Stimulation of preparations with 1 μM NE for 10, 15, 20, 30, 45, 120 or 360 min led to a strong induction of cAMP levels as compared to controls (CTL). Induction of cAMP levels peaked at 30 min or 120 min, and maximal cAMP levels were obtained 120 min after the onset of NE-stimulation. Treatment of preparations with 5 μM THC for 15 min, followed by coapplication of THC and NE for 10, 15, 20, 30, 45, 120 or 360 min, also strongly induced cAMP levels as compared to controls (CTL). The cAMP levels peaked again at 30 min or 120 min, and maximal cAMP levels were obtained 120 min after coapplication of THC and NE. Statistical analysis did not show any significant effect of THC on NE-induced cAMP levels at any of the investigated time periods.

alone, treatment with 10, 1 or 0.1 μM WIN for 15 min, followed by concomitant treatment with WIN and NE, did not significantly affect NE-induced melatonin biosynthesis and AANAT activity. Treatment of cultured rat pineal glands with 10 μM AM251 or AM630 for 15 min, followed by concomitant treatment with AM251, THC and NE or AM630, THC and NE, did not block THC-mediated reduction of NE-induced melatonin biosynthesis and AANAT activity as compared to preparations that were treated with THC and NE alone.

THC, CBD and CBN attenuated AANAT activity in a cell-free system

All results described above led us to hypothesize that the THC-, CBD- or CBN-mediated attenuation of AANAT activity may be based on direct interactions between the three, chemically closely related cannabinoids and the AANAT enzyme. In order to test this hypothesis in a cell-free system, lysates were prepared from rat pineal organ cultures that were either stimulated with NE or left untreated. High AANAT activity was detectable in lysates of NE-stimulated

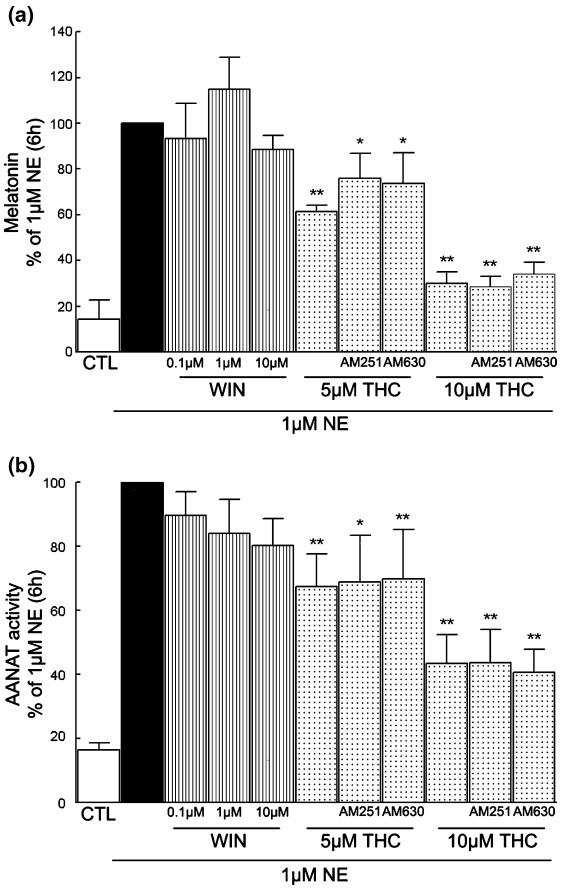

Fig. 4 CB-mediated attenuation of NE-induced melatonin biosynthesis and AANAT activity did not seem to involve WIN-, CB1 or CB2 receptors. Stimulation of cultured rat pineal glands with 1 µM NE for 6 h induced melatonin levels (a) and AANAT activity (b) about 5–10 fold as compared to controls (CTL). The value obtained after NE stimulation was set as 100%. Treatment with WIN (10, 1 or 0.1 µM) for 15 min, followed by concomitant application of WIN and NE for 6 h, did not significantly affect NE-induced melatonin biosynthesis and AANAT activity, as compared to preparations that were stimulated with NE alone. Treatment with 10 or 5 µM THC for 15 min, followed by concomitant stimulation with THC and NE for 6 h, led to a significant, concentration-dependent reduction of NE-induced melatonin biosynthesis and AANAT activity. Treatment with 10 µM AM251 or AM630 for 15 min, followed by concomitant treatment with AM251 or AM630 plus THC and NE for 6 h, did not block THC-mediated reduction of NE-induced melatonin biosynthesis and AANAT activity as compared to preparations that were treated with THC and NE. $^{**}p < 0.01$; $^{*}p < 0.05$ vs. 6 h NE

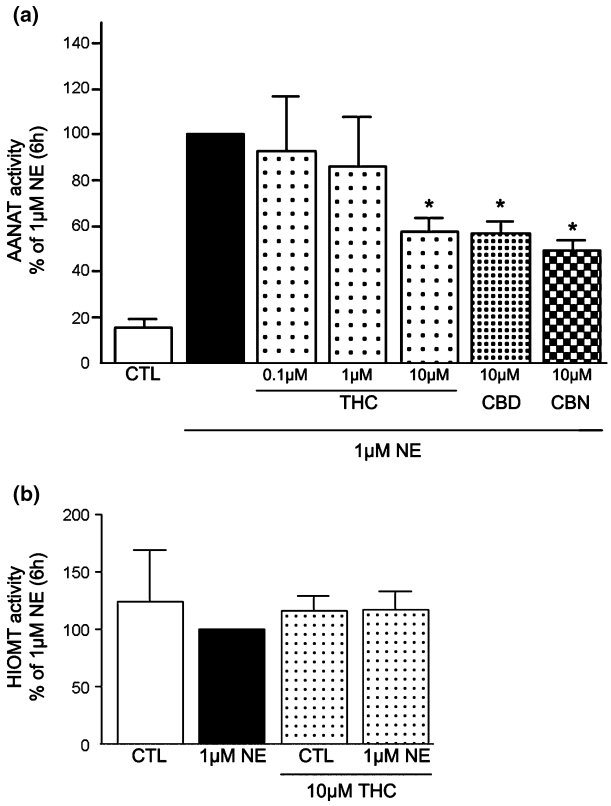

Fig. 5 CBs directly attenuate AANAT activity in cell-free lysates and did not affect activity of HIOMT, the final enzyme of melatonin biosynthesis cascade.(a) In lysates prepared from NE-stimulated rat pineal organ cultures AANAT activity was strongly detectable and set as 100% in contrast to only weak activity of AANAT enzyme observed in lysates from non-stimulated control cultures (CTL). Application of 10 µM THC, CBD or CBN to lysates of NE-stimulated cultures significantly reduced AANAT activity down to 50–60% as compared to lysates from organ cultures treated with NE only. Application of 1 or 0.1 µM THC did not significantly attenuate AANAT activity in this cell-free system. (b) HIOMT activity was evenly detectable in non-stimulated control (CTL) and NE-stimulated preparations. HIOMT activity in pineal organ cultures stimulated with NE for 6 h was set as 100%. Both, treatment of controls with 10 µM THC for 6 h and pre-treatment of pineal cultures with 10 µM THC for 15 min, followed by concomitant application of NE and THC for 6 h, did not affect HIOMT activity as compared to preparations left untreated or stimulated with NE only. $^{*}p < 0.05$ vs. 6 h NE

cultures, this value was set as 100% (Fig. 5a). In contrast, very low AANAT activity was found in lysates prepared from non-stimulated control cultures (Fig. 5a; CTL). Application of 10 µM THC, CBD or CBN to lysates of NE-stimulated cultures led to a significant reduction of AANAT activity (down to 50–60%) as compared to lysates of organ cultures treated with NE alone (Fig. 5a). Application of 1 or 0.1 µM THC did not significantly reduce AANAT activity in this cell-free system.

THC did not affect HIOMT activity in cultured rat pineal glands

To test the influence of CBs on other enzymes involved in melatonin biosynthesis, the activity of hydroxyindole-*O*-methyltransferase (HIOMT), the final enzyme of melatonin biosynthesis, was determined in cultured rat pineal glands. HIOMT activity was very similar in untreated and

NE-stimulated preparations (Fig. 5b). The values from preparations stimulated with NE for 6 h were set as 100%. Both, treatment of non-stimulated preparations with 10 μM THC for 6 h or pretreatment of pineal cultures with 10 μM THC for 15 min, followed by concomitant application of NE and THC for 6 h, did not affect HIOMT activity as compared to preparations left untreated or stimulated with NE only.

Discussion

The cannabinoid (CB) system is involved in the modulation of many neuronal and neuroendocrine circuits in the central nervous system. In the present study we demonstrated for the first time that CBs influence the formation of melatonin in the rat pineal gland which is induced by the release of norepinephrine (NE) from sympathetic intrapineal nerve fibers during night (Klein 1985). The plant-derived CBs 9-carboxy-11-nor-delta-9-tetrahydrocannabinol (THC), (–)-cannabidiol (CBD) or cannabinol (CBN) attenuated the NE-induced melatonin biosynthesis by approximately 50% in a dose-dependent manner.

NE-induced stimulation of melatonin biosynthesis requires activation of the penultimate enzyme of the melatonin biosynthesis, the arylalkylamine *N*-acetyltransferase (AANAT) and we have therefore investigated in a first step whether THC, CBD or CBN affect AANAT activity. Indeed, the three CBs reduced the NE-induced AANAT activity in the same magnitude as the NE-stimulated melatonin secretion. Thus, the THC, CBD or CBN-mediated attenuation of melatonin biosynthesis appears to be based on THC-, CBD- or CBN-mediated reduction of NE-induced AANAT activity.

In the rat pineal gland, AANAT activity is controlled through different mechanisms including protein kinase A (PKA)-dependent phosphorylation of AANAT protein and protein/protein interactions between AANAT and 14-3-3 proteins (Ganguly *et al*. 2001; Obsil *et al*. 2001; Ganguly *et al*. 2005). Indispensable for AANAT activation is the formation of AANAT protein. AANAT protein level is regulated via proteasomal proteolysis (Gastel *et al*. 1998), but, in rodents, the initial increase in AANAT protein requires the NE-induced transcriptional activation of the *Aanat* gene (Borjigin *et al*. 1995; Roseboom *et al*. 1996; Maronde *et al*. 1999) by PKA-dependent CREB phosphorylation (Maronde *et al*. 1999; Koch *et al*. 2003). The NE-induced > 150-fold rise in *Aanat* mRNA levels is correlated with the large increase in AANAT protein levels and activity. cAMP is the essential second messenger mediating the NE-induced activation of AANAT and melatonin formation. Intracellular cAMP levels are increased upon β_1-adrenergic stimulation and activation of the AC mediated by G_s proteins (Babila *et al*. 1992). Calcium has no effect on its own on AANAT activation, but potentiates the formation of cAMP. The concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) is controlled through α_1-adrenergic receptors (ADR) (Vanecek *et al*. 1985; Schomerus *et al*. 1995). Stimulation of α_1-ADR is sufficient to elicit maximal increases in $[Ca^{2+}]_i$, but does not increase AANAT activity and melatonin biosynthesis on its own. α_1-ADR stimulation and increases in $[Ca^{2+}]_i$ affect AANAT activity and melatonin biosynthesis in rat pineal only if β_1-ADR are stimulated concomitantly (Sugden *et al*. 1985). Under these conditions, increases in $[Ca^{2+}]_i$ raise the amplitude of β_1-adrenergic induced increase in cAMP levels ($[cAMP]_i$) by modulating AC activity. This amplification involves phospholipase C (PLC)γ-driven Ca^{2+}-release from IP3-sensitive intracellular Ca^{2+}-stores and opening of Ca^{2+}-channels located in the pinealocyte membrane (Ho *et al*. 1988a,b; Schomerus *et al*. 1995).

To identify the mechanisms through which CBs attenuate AANAT activity and melatonin formation in the rat pineal we have systematically investigated their impact on key components of the above-described NE-dependent signal transduction cascade. Notably, the CBs used attenuated neither the NE-induced phosphorylation of CREB nor the increase in AANAT protein levels. At variance to the rat pineal gland, CBs were shown to interfere in other neuronal circuits with protein kinase-driven activation of immediate early gene expression and the subsequent regulation of protein synthesis, including the mitogen activated protein kinase (MAPK) pathway, the transcription factor Krox-24 or the cAMP/PKA/pCREB pathway (Howlett *et al*. 2002). Acute intracerebral THC injection activated the MAPK pathway and formation of ERK, pCREB and c-fos in caudate/putamen and cerebellum whereas chronic THC treatment increased ERK, pCREB and FOS B in prefrontal cortex and hippocampus (Rubino *et al*. 2004). In the cerebellar granule cell layer acute THC-treatment induced CB1 receptor(R)-mediated CREB phosphorylation, whereas chronic THC exposure attenuated it (Casu *et al*. 2005).

As a next step we investigated whether CBs affect the NE-induced increase in second messenger concentrations in the rat pineal which are involved in the control of AANAT activity and melatonin biosynthesis (Korf *et al*. 2003). In other systems, CBs were shown either to decrease $[cAMP]_i$ via $G_{i/o}$ protein dependent inhibition of AC activity (Childers *et al*. 1993; Pacheco *et al*. 1993; Felder *et al*. 1995) or to increase $[cAMP]_i$ (Glass and Felder 1997; Maneuf and Brotchie 1997). CBs can also affect the PLCγ-mediated modulation of Ca^{2+} formation (Sugiura *et al*. 1996) and inhibit different types of Ca^{2+} channels, finally causing a decrease in Ca^{2+} conductance (Mackie and Hille 1992). The effects on calcium channels are directly mediated via G proteins and independent of cAMP (Caulfield and Brown 1992; Shen *et al*. 1996). None of these mechanisms appear to be involved in the CB-mediated attenuation of melatonin formation, since THC did not affect the NE-induced formation of cAMP or the elevation of $[Ca^{2+}]_i$ in the rat pineal organ.

To clarify whether the CB-dependent attenuation of melatonin biosynthesis and AANAT activity depends on activation of CB receptors, we tested the effects of several CBR antagonists. THC and CBN act as partial agonists on CB1R and CB2R (Gerard *et al*. 1991; Condie *et al*. 1996), but the THC-mediated attenuation of NE-induced melatonin biosynthesis was not blocked by the respective CB1R and CB2R antagonists AM251 and AM630. Moreover, CBD which does not act upon CB1R and CB2R (Showalter *et al*. 1996) elicited the same attenuating effect on melatonin biosynthesis as THC and CBN. Thus, CB-mediated attenuation of NE-induced melatonin biosynthesis does not appear to involve activation of CB1R and CB2R.

In addition to CB1R and CB2R, two other receptors, WINR and abnormal (abn)-CBDR, have been pharmacologically identified (Jarai *et al*. 1999; Breivogel *et al*. 2001). WINR are coupled to $G_{i/o}$-protein and activated by anandamide (AEA) and WIN, but not by other ligands such as THC (Di Marzo *et al*. 2000). In hippocampus, they were found to modulate glutamatergic neurotransmission (Breivogel *et al*. 2001; Hajos *et al*. 2001). In the rat pineal, WIN did not influence NE-induced melatonin biosynthesis suggesting that the CB effects on melatonin biosynthesis are not mediated by WINR. The abn-CBDR, pharmacologically identified in mesenteric arteries of rodents, is activated by AEA and abn-CBD (Jarai *et al*. 1999; Wagner *et al*. 1999). Its activation leads to vasodilatation (Kunos *et al*. 2000) and this effect is antagonized by CBD. Treatment of cultured rat pineal glands with CBD attenuated NE-induced melatonin biosynthesis and AANAT activity. However, it is unclear whether this effect is mediated by the abn-CBDR since THC that does not act upon abn-CBDR in blood vessels (Jarai *et al*. 1999) has a significant effect on melatonin biosynthesis.

All these data prompted us to hypothesize that the THC-, CBD- and CBN-mediated attenuation of melatonin biosynthesis and AANAT activity may reflect an intracellular interaction between CBs and the activated AANAT enzyme. To test this hypothesis we investigated the effects of THC, CBD and CBN on AANAT activity in cell-free lysates. Indeed, we found that AANAT activity was reduced by the used CBs in a cell-free system nearly to the same magnitude as found in whole pineal organ cultures. The fact that THC, CBD and CBN, which all possess a similar chemical structure, reduced AANAT activity in pineal lysates nearly to the same amount, provides further evidence for the proposed intracellular interaction between CBs and the AANAT enzyme. The direct effect of CBs on AANAT activity appears specific as the activity of hydroxyindole-*O*-methyltransferase (HIOMT), the final enzyme in biosynthesis cascade of melatonin was not affected.

Our observation that CBs directly interact with the activated AANAT enzyme and thereby reduce AANAT activity and melatonin biosynthesis in the rat pineal gland expands the list of studies on non-CBR-mediated effects. The endocannabinoid AEA was shown to modulate the activity of rat brain protein kinase C *in vitro* by binding to the enzyme (De Petrocellis *et al*. 1995). The plant-derived THC was reported to cause slowly developing vasorelaxation in rat isolated arteries by binding to and activating the transcription factor peroxisome proliferator-activated receptor gamma (PPAR-γ, O'Sullivan *et al*. 2005). Furthermore, AEA induced COX-2 mRNA and protein levels leading to prostaglandin synthesis by activation of the MAPK pathway in human neuroglioma cells (Ramer *et al*. 2001) or activated the MAPK pathway leading to cell activation and proliferation in hematopoietic cell lines in a CBR-independent mechanism (Derocq *et al*. 1998).

In conclusion, we demonstrate for the first time that CBs attenuate NE-induced activation of melatonin biosynthesis and AANAT activation in the rat pineal gland. We provided experimental evidence that these effects are not mediated via CB-dependent signal transduction mechanisms identified so far in other neuronal and neuroendocrine systems and identified an intracellular interaction between CBs and the AANAT enzyme as mechanism reponsible for the CB-dependent attenuation of melatonin biosynthesis. It will now be of considerable interest to examine whether direct interactions between CBs and activated enzymes also operate in other neuronal and neuroendocrine systems.

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