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**Lehrstuhl für Biotechnologie der Nutztiere**

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# The cannabinoid receptor type 1 in the murine nervous system: physiological roles and cross-talk with other receptor systems


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# 1 INTRODUCTION

## 1.1 Overview of the cannabinoid receptor type 1 (CB1)

### 1.1.1 CB1 ligands (cannabinoids)

The plant *Cannabis sativa* (*C. sativa*), also known as *Marihuana,* is considered as one of the very first plants grown for therapeutic and recreative purposes (reviewed in Peters and Nahas, 1999). First historical reports of the use of *C. sativa* were found in China nearly 5000 years ago, where it was grown rather for fibers than for production of psychoactive extracts. From China, *C. sativa* propagated to all continents over the ages and became more and more important for medical applications besides its usage as pleasure-inducing drug. *C. sativa* contains more than 60 compounds belonging to the chemical family of cannabinoids (Iversen, 2000), although the major psychoactive constituent is $\Delta^9$-tetrahydrocannabinol ($\Delta^9$-THC) (Gaoni and Mechoulam, 1964). Other compounds found in *C. sativa* include $\Delta^8$-tetrahydrocannabinol, cannabidiol and cannabinol. Following the isolation of $\Delta^9$-THC from *C. sativa*, numerous synthetic cannabinoids, based on the structure of $\Delta^9$-THC, were synthesized. These were shown to induce behavioral effects such as hypothermia, catalepsy and hypomobility, similar to the *in vivo* effects of $\Delta^9$-THC, when injected into animals (reviewed in Howlett et al., 2002). Upon the identification and cloning of a specific cannabinoid receptor in the brain (CB1) that mediated the effects of $\Delta^9$-THC (Devane et al., 1988; Matsuda et al., 1990), an endogenous agonist of this receptor, anandamide (AEA; Devane et al., 1992), was identified. This suggested the presence of an endogenous cannabinoid system in the central nervous system (CNS). Later, other endocannabinoids have also been isolated and shown to be present in the CNS. A second cannabinoid receptor (CB2) was cloned from a leukaemic cell line and has a relatively low sequence identity with CB1 (44% overall the whole protein, 68% in the transmembrane regions; Munro et al., 1993). Its expression is limited to cells and organs of the immune system suggesting that the endocannabinoid system may also play a role in modulating the immune system.

Cannabinoid receptor agonists can be classified into four groups: eicosanoid cannabinoids, classical cannabinoids, nonclassical cannabinoids and aminoalkylindoles. Classical cannabinoids include compounds isolated from cannabis, mainly $\Delta^9$-THC (Fig. 1-1), $\Delta^8$-THC, cannabidiol, and cannabinol. The most important compound of the nonclassical cannabinoids is CP55,940 (Fig. 1-1), which has been used extensively to demonstrate the existence of the cannabinoid receptors (Howlett et al., 1986). Aminoalkylindoles are

structurally different from classical and nonclassical cannabinoids and the endocannabinoids themselves. The prototype of this group is WIN55,212-2 (Fig. 1-1). Eicosanoids are derivatives of arachidonic acid and were discovered as endogenous ligands of the cannabinoid receptors (Devane et al., 1992). Prototypes of this group are anandamide (arachidonoylethanolamide, AEA; Fig. 1-1) and 2-arachidonoylglycerol (2-AG), the two major endocannabinoids so far isolated from mammalian tissue.

As soon as cannabinoid receptors were discovered, several newly synthesized compounds were tested as putative specific antagonists of CB1 or CB2. The most potent and well-characterized CB1 antagonist is SR141716A (Fig. 1-1). This compound is a potent antagonist of several of the typical effects of cannabinoids, both *in vitro* and *in vivo*, and is highly specific for CB1, having little or no affinity for CB2 and for a wide range of other membrane receptors (Rinaldi-Carmona et al., 1994; Compton et al., 1996).

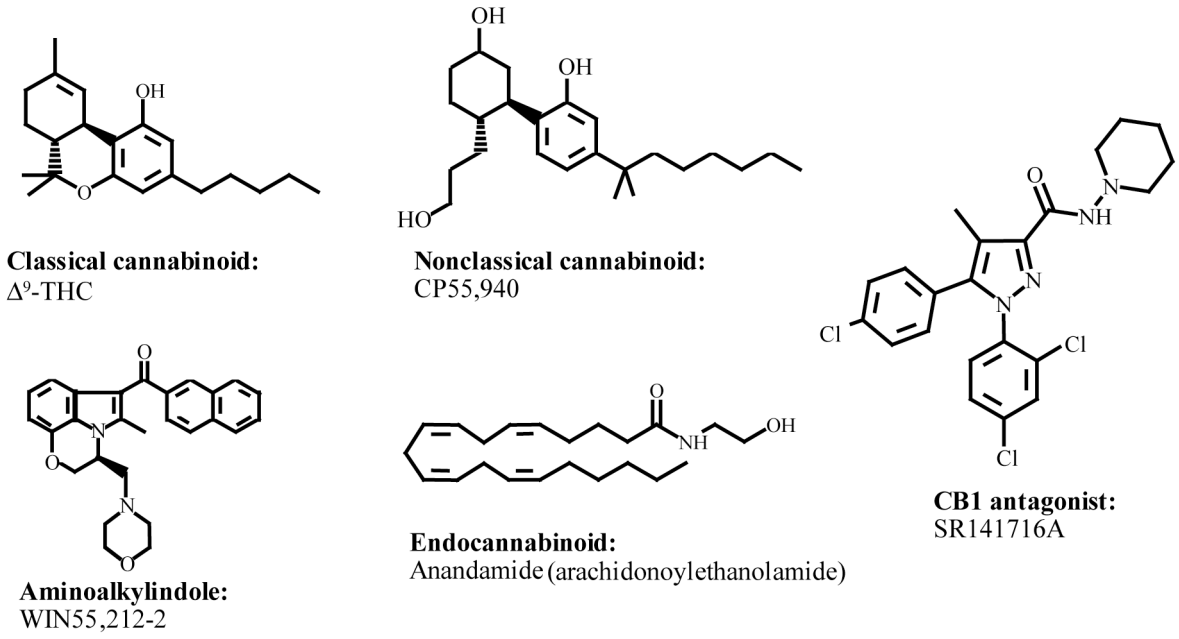


**Fig. 1-1: Chemical structure of CB1 ligands.**

### 1.1.2 CB1-mediated signal transduction pathways

At the moment, two cannabinoid receptors have been cloned: the "brain type" cannabinoid receptor CB1, expressed in the CNS (see in detail in chapter 1.1.4), but also in many peripheral organs although at lower levels, and CB2, whose expression is limited to cells and organs of the immune system. Both CB1 and CB2 are seven transmembrane G protein-coupled receptors, generally coupled to $G_{i/o}$ proteins. CB1-mediated signaling pathways have been extensively characterized. *In vitro* studies, using different neuronal and non-neuronal

culture systems or brain slices have revealed that CB1 exerts its functions presumably through two main intracellular pathways: inhibition of adenylate cyclase (AC) which generates the second messenger cyclic adenosin monophosphate (cAMP) and alterations of ion channel activities. However, in the recent years, also other intracellular pathways have been shown to be triggered by CB1. Here, the most important signaling cascades influenced by CB1 are summarized.

#### *1.1.2.1 Regulation of adenylate cyclase*

The first characterized CB1 signal transduction response was the inhibition of AC in response to cannabinoid agonists as demonstrated in neuroblastoma cells and membranes (N18TG2) (Howlett and Fleming, 1984; Howlett, 1985). Because this response was blocked by pertussis toxin in neuroblastoma cells, in membranes derived from mammalian brain and in primary neuronal cultures (Howlett et al., 1986; Bidaut et al., 1990; Bouaboula et al., 1995), signal transduction was attributed to a member of the $G_i$ family (Fig. 1-2). Pertussis toxin is able to prevent the dissociation of the $\alpha$ and $\beta/\gamma$ subunits of $G_{i/o}$, thereby blocking the G protein-mediated inhibition of AC. Cannabinoid agonists can inhibit AC activity in N18TG2 cells over a range of potencies and efficacies. In purified membranes from N18TG2 cells the percent inhibition of secretin-stimulated AC activity of different cannabinoids was determined in order of potency as followed: CP55,940 > HU210 > $\Delta^9$-THC > $\Delta^8$-THC > cannabinol > cannabidiol (Howlett and Fleming, 1984). In general, the potency of cannabinoids to regulate AC correlates with their affinity for CB1 as determined by heterologous displacement of the CB1 agonist [$^3$H]CP55,940 (Devane et al., 1988; Shim et al., 1998). Activation of G proteins was also shown by receptor-stimulated [$^{35}$S]GTP$\gamma$S binding to brain-derived membranes and to brain sections (Sim et al., 1995; Breivogel et al., 1997). Interestingly, the regional distribution of CB1 as revealed by radioligand binding, and the activation of G proteins by cannabinoid agonists are very similar, indicating that presumably all CB1 receptors are able to activate G proteins. However, quantitative differences between the distribution of receptor and activated G proteins suggest that the receptor can have different coupling efficiencies in various brain regions (Sim et al., 1995; Childers and Breivogel, 1998; Ameri, 1999).

The coupling of CB1 to $G_{i/o}$ proteins is considered as one of the main mechanisms of action of the receptor, but evidence also exists that different G protein subtypes are involved in CB1 signal transduction. Stimulation of AC upon CB1 activation has been reported in pertussis toxin-treated striatal neurons, suggesting that in the absence of functional $G_{i/o}$ coupling, CB1 can activate $G_s$ (Glass and Felder, 1997). The unconventional stimulatory

effect of cannabinoids on AC was also found in other culture systems e.g. N18TG2 cells (Bash et al., 2003), slices of globus pallidus (Maneuf and Brotchie, 1997) and CB1-transfected chinese hamster ovary (CHO) cells (Bonhaus et al., 1998). The complexity of CB1-mediated signaling strengthens the possibility that different behavioral effects of CB1 agonists are not mediated by the activation of the same signaling pathway.

#### *1.1.2.2 Modulation of ion channels*

$G_{i/o}$ is able to couple seven transmembrane receptors not only to AC, but also to ion channels. Agonist activation of CB1 produced inhibition of voltage-activated inward calcium ($Ca^{2+}$) currents in neuroblastoma cells (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie et al., 1993). This effect appears to be mediated by $G_{i/o}$, because it is blocked by pertussis toxin. Application of inhibitors of different $Ca^{2+}$ channel subtypes revealed that N-type and P/Q-type $Ca^{2+}$ channels are the main targets of CB1-induced inhibition of $Ca^{2+}$ influx (Mackie and Hille, 1992; Felder et al., 1995; Hampson et al., 1998a; Fig. 1-2). A recent report showed that cannabinoids inhibited also L-type $Ca^{2+}$ channels in cat brain arterial smooth muscle cells in a pertussis toxin-sensitive manner (Gebremedhin et al., 1999). In contrast Rubovitch et al. (2002) demonstrated a positive modulation of L-type $Ca^{2+}$ channels by a cannabinoid agonist in neuroblastoma cells which was pertussis toxin-insensitive but dependent on protein kinase A (PKA) suggesting a $G_s$-mediated effect (Bash et al., 2003).

CB1 was shown to regulate also the actions of potassium ($K^+$) channels. In AtT-20 pituitary tumor cells, CB1 positively regulates inwardly rectifying potassium channels ($K^+_{ir}$) in a pertussis toxin-sensitive manner, indicating that $G_{i/o}$ proteins serve as transducers of the response (Henry and Chavkin, 1995; Mackie et al., 1995; Fig. 1-2). Moreover, CB1 activates voltage-dependent A-type potassium channels ($K^+_A$) in rat hippocampal cells, which is due to the modulation of intracellular cAMP concentrations, thereby regulating the phosphorylation of ion channel proteins by PKA (Deadwyler et al., 1993; Deadwyler et al., 1995; Fig. 1-2). Interestingly, cannabinoid actions on $K^+_{ir}$ and on P/Q-type $Ca^{2+}$ channels can be strongly attenuated by phosphorylation of CB1 at a single serine residue (S317) in the third cytoplasmic loop of the receptor by the action of protein kinase C (PKC), thus constituting a putative regulatory system of CB1 (Garcia et al., 1998).

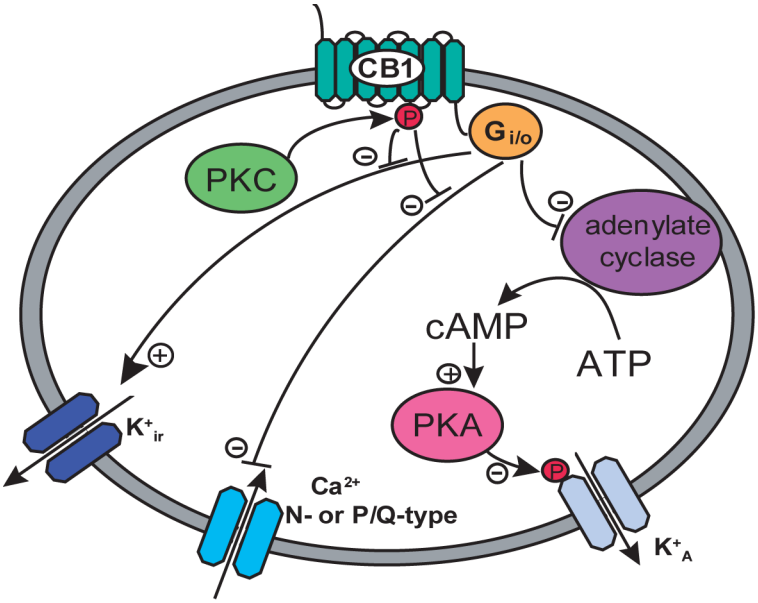


**Fig. 1-2: Schematic representation of the main effects of CB1 on ion channels.**
Activation of CB1 leads to the stimulation of $G_{i/o}$ proteins that, in turn, inhibit the adenylate cyclase-mediated conversion of ATP to cAMP. cAMP molecules can activate protein kinase A (PKA) by binding to its regulatory subunits. Catalytic PKA subunit can phosphorylate $K^+_A$, causing a decrease of the current. Given the negative effect of CB1 on adenylate cyclase, the final result is an activation of voltage-dependent A-type potassium channels ($K^+_A$). $G_{i/o}$ activated by CB1 can also directly inhibit N- or P/Q-type calcium channels ($Ca^{2+}$) and activate inwardly rectifying potassium channels ($K^+_{ir}$). These latter two effects are controlled by protein kinase C (PKC), which, when activated, can phosphorylate CB1 and uncouple the receptor from the effects on these ion channels.

Due to its above-mentioned modulation of $Ca^{2+}$ and $K^+$ channels and its high expression in presynaptic terminals (Herkenham et al., 1990; Tsou et al., 1998a), CB1 plays a major role in the inhibition of neurotransmitter release at synapses. During the past three decades, cannabinoid receptor-induced inhibition of transmitter release has been identified in approximately 40 experimental models demonstrating that cannabinoids acting at CB1 inhibit the release of glutamate, acetylcholine, noradrenaline and γ-aminobutyric acid (GABA) (reviewed in Schlicker and Kathmann, 2001). This important feature of the cannabinoid system can be considered as one of the main cellular mechanisms underlying the diverse physiological actions of cannabinoids (see chapter 1.1.5).

### *1.1.2.3 Regulation of intracellular calcium transients*

Cannabinoids were shown to evoke a rapid, transient increase in intracellular free $Ca^{2+}$ in neuroblastoma cells (N18TG2 and NG108-15) which was blocked by SR141716A, pertussis toxin and a phospholipase C (PLC) inhibitor, suggesting a mechanism whereby a CB1-mediated release of $G_{i/o}$ βγ subunits might activate PLC leading to inositol-1,4,4-trisphosphate

($IP_3$) release and finally results in an increased $Ca^{2+}$ release from intracellular stores (Sugiura et al., 1996; Sugiura et al., 1997). An interaction of CB1 and PLC was also shown in cultured cerebellar neurons, in which cannabinoids augmented the $Ca^{2+}$ signal in response to stimulation of the N-methyl-D-aspartate (NMDA) subtype of glutamate receptors (Netzeband et al., 1999) confering to the same mechanism as suggested by Sugiura et al. (1996).

#### *1.1.2.4 Regulation of several kinases*

CB1 activation was reported to stimulate phosphorylation of focal adhesion kinase (FAK) in hippocampal slices which was blocked by SR141716A and pertussis toxin as evidence for mediation by CB1 and $G_{i/o}$ proteins. As the phosphorylation was reversed by the cAMP analog 8-Br-cAMP and mimicked by PKA inhibitors, $G_{i/o}$-mediated inhibition of AC seems to be involved in this pathway (Derkinderen et al., 1996). FAK is important for integrating cytoskeletal changes with signal transduction events, perhaps playing a role in synaptic plasticity.

Mitogen-activated protein kinase (MAPK, p38) and extracellular signal-regulated kinase (ERK, p42/44) were activated by cannabinoids in several cell lines transfected with CB1 in a SR141716A and pertussis toxin-sensitive manner (Bouaboula et al., 1995; Wartmann et al., 1995; Sanchez et al., 1998; Rueda et al., 2000). Two groups showed that MAPK phosphorylation was blocked by wortmannin, implicating phosphatidylinositol-3-kinase (PI-3-K) as a mediator along this pathway (Bouaboula et al., 1995; Wartmann et al., 1995). From these studies, a pathway is suggested whereby CB1-mediated $G_{i/o}$ release of $\beta\gamma$ subunits leads to activation of PI-3-K, resulting in subsequent MAPK phosphorylation. MAPK/ERK are very important in many aspects of neurophysiology, from differentiation and survival of neurons (reviewed in Fukunaga and Miyamoto, 1998), to the induction of important forms of neuronal plasticity, such as long-term memory (Orban et al., 1999), suggesting that central effects of CB1 on cognitive functions involve modulation of these pathways. The involvement of PI-3-K in the signal transduction cascade of CB1 was also shown by Gomez Del Pulgar et al. (2000). Using CB1-transfected Chinese hamster ovary cells (CHO) and human glioma cells, these authors showed that cannabinoids can stimulate protein kinase B/Akt in a CB1-, $G_{i/o}$- and PI-3-K-dependent manner, thus, indicating a novel potential mechanism of cannabinoid action.

### 1.1.3 The endocannabinoid system

After the identification (Devane et al., 1988) and cloning (Matsuda et al., 1990) of the first cannabinoid receptor, it became clear that natural and synthetic cannabinoids were likely to interfere with an endogenous neuronal system. In 1992, the first endogenous ligand of CB1 was purified from porcine brain, which turned out to be the ethanolamide of arachidonic acid and was denoted as anandamide (AEA; Devane et al., 1992). In 1995, a second type of endocannabinoid, 2-arachidonoyl glycerol (2-AG), was discovered, also a derivative of arachidonic acid (Mechoulam et al., 1995; Sugiura et al., 1995). Also other derivatives of arachidonic acid (docosatetraenoylethanolamide and di-homo-γ-lineoylethanolamide) have been reported to bind to CB1 (Hanus et al., 1993) and, more recently, 2-arachidonoyl glyceryl ether (noladin ether) was reported to act as an endocannabinoid (Hanus et al., 2001).

Studies carried out in rat neurons demonstrated that synthesis and release of AEA could be stimulated by treatment with depolarizing agents such as ionomycin, 4-aminopyridine, kainic acid and high extracellular $K^+$ concentrations (Di Marzo et al., 1994). AEA is inactivated by reuptake via a membranal transport protein and subsequent intracellular enzymatic degradation by fatty acid amide hydrolase (FAAH)-mediated hydrolysis (Cravatt et al., 1996; Day et al., 2001; Deutsch et al., 2001; Giuffrida et al., 2001; Fig. 1-3). In this view, AEA appears to act as a classical neurotransmitter, with depolarization-induced release, action at specific receptors, (re)-uptake by cells and intracellular degradation. However, differently from classical neurotransmitters, AEA and other endocannabinoids are not stored in synaptic vesicles, but are thought to be synthesized "on-demand" from membranal precursors and released from neurons immediately afterwards (Di Marzo et al., 1994; Cadas et al., 1997; Mechoulam et al., 1998; Fig. 1-3). FAAH and the AEA transporter (AT) are distributed in brain areas in a pattern corresponding to that of CB1, i.e. high concentration in hippocampus, cerebellum, cerebral cortex (Egertova et al., 1998; Tsou et al., 1998b; Ueda and Yamamoto, 2000), thus further supporting the notion that endocannabinoids are true neurotransmitters (Self, 1999). 2-AG undergoes similar FAAH-mediated hydrolysis (Ueda and Yamamoto, 2000) and carrier-mediated transmembranal transport (Beltramo and Piomelli, 2000), possibly through the same AEA transporter (Bisogno et al., 2001).

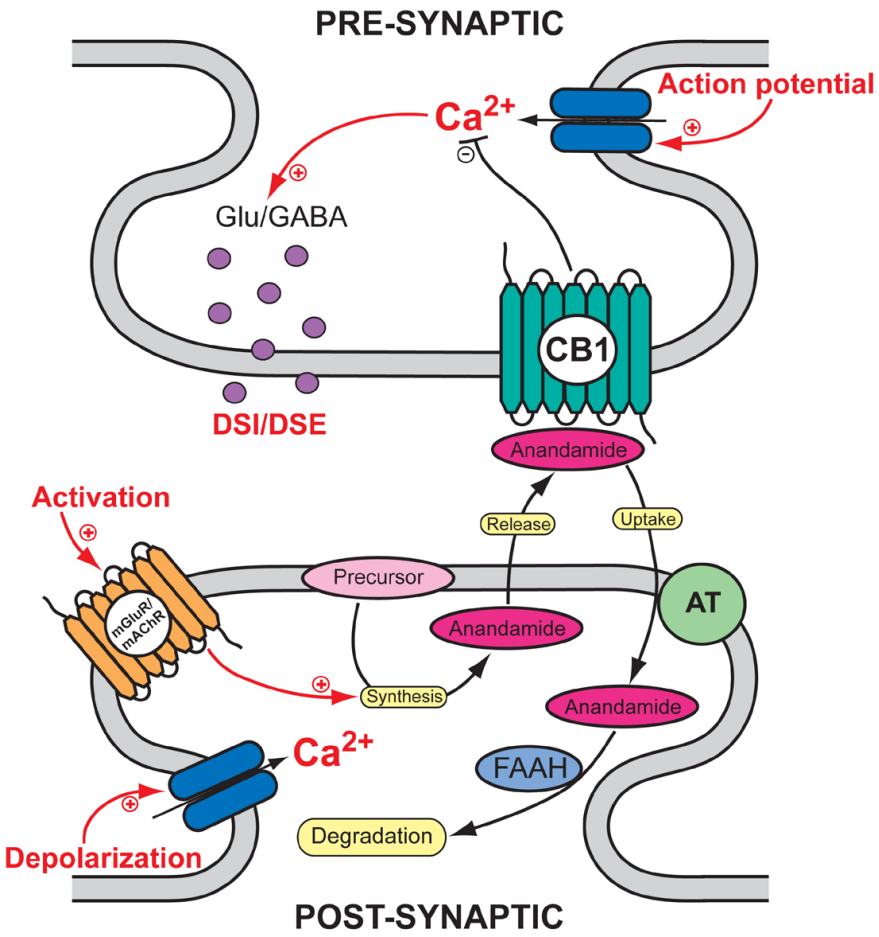


**Fig. 1-3: Schematic representation of the effects of the endocannabinoid system on synaptic transmission.**
Postsynaptic depolarization triggers $Ca^{2+}$ influx by activating voltage-gated $Ca^{2+}$ channels. Elevation of intracellular $Ca^{2+}$ stimulates the biosynthesis of anandamide and induces its release from the postsynaptic neuron. Released anandamide diffuses retrogradely and binds to CB1 on the inhibitory or excitatory presynaptic terminal. This release is enhanced by activation of metabotropic glutamate receptors (mGluR) and muscarinic acetylcholine receptors (mAChR). Binding of anandamide to CB1 suppresses the release of GABA or glutamate (Glu) by inhibiting voltage-gated $Ca^{2+}$ channels and, therefore, mediates DSI (depolarized suppression of inhibtion) or DSE (depolarized suppression of excitation), respectively. Anandamide is reuptaken by the membranal anandamide transporter protein (AT) and is degraded intracellularly by the enzyme fatty acid amide hydrolase (FAAH).

The nature of endocannabinoid-mediated neurotransmission has been greatly clarified in the last years: endocannabinoids are released from a postsynaptic neuron upon stimulation, diffuse back to presynaptic neurons (reviewed in Christie and Vaughan, 2001; Maejima et al., 2001b), where they act on CB1 resulting in a reduced probability of neurotransmitter release such as glutamate, GABA, acetylcholine, and noradrenaline (reviewed in Schlicker and Kathmann, 2001; Fig. 1-3). Because CB1 is widespread in the CNS, these findings suggest that retrograde inhibition by endocannabinoids is an important mechanism in the CNS to control the amount of transmitter release at excitatory and inhibitory synapses. This type of modulation has been found in the hippocampus (Ohno-Shosaku et al., 2001; Wilson and

Nicoll, 2001) and cerebellum (Kreitzer and Regehr, 2001a; Kreitzer and Regehr, 2001b; Diana et al., 2002; Yoshida et al., 2002) and was called depolarization-induced suppression of inhibition (DSI) or excitation (DSE) (Alger and Pitler, 1995). Recent reports show that the activation of group I metabotropic glutamate receptors (mGluRs) enhances the release of endocannabinoids in the cerebellum (Maejima et al., 2001a) and hippocampus (Varma et al., 2001). Independently of the mGluR system, muscarinic acetylcholine receptors (mAChRs) activation also enhances endocannabinoid release in the hippocampus (Kim et al., 2002; Ohno-Shosaku et al., 2003; Fig. 1-3). Both phenomena are thought to result directly from the ability of mGluRs and mAChRs to modulate ionic currents.

### 1.1.4 Distribution of CB1 in the murine brain

After the discovery of the existence of a cannabinoid receptor in the brain, the cannabinoid receptor distribution was first shown by autoradiography of ligand-receptor binding on slide-mounted rat brain sections with different radiolabeled agonists (Herkenham et al., 1990; Herkenham, 1991; Jansen et al., 1992; Glass et al., 1997b). As the quantity of receptors in the brain is very high, CB1 can be considered as one of the most abundant G protein-coupled receptors in the mammalian brain (Herkenham et al., 1990), comparable in quantity and density with glutamate receptors. Moreover, the distribution of CB1 was investigated at the mRNA level, by *in situ* hybridization (ISH) in rodents (Matsuda et al., 1993; Marsicano and Lutz, 1999) and in humans (Mailleux et al., 1992; Westlake et al., 1994). More recently, anti-CB1 antibodies became available allowing to detect the distribution of the protein by immunohistochemistry (IHC; Tsou et al., 1998a; Pettit et al., 1998).

The highest density of cannabinoid receptors has been demonstrated in the basal ganglia (substantia nigra, globus pallidus, enteropeduncular nucleus and dorsolateral caudate putamen) and in the cerebellum. In these areas, discrepancies between mRNA (ISH) and protein (IHC and ligand binding) expression were observed, thus indicating the presence of the receptor on distal neuronal projections. High densities of binding were also described in the CA (Ammon's horn) pyramidal cell layers of hippocampus (Herkenham et al., 1990). This is due to a dense plexus of immunoreactive fibres surrounding the cell bodies of pyramidal cells which appear *per se* devoid of CB1 protein as shown by IHC (Tsou et al., 1998a). However, pyramidal cells of hippocampus have been shown to express low but significant levels of CB1 mRNA (Matsuda et al., 1993; Marsicano and Lutz, 1999), thus again indicating the possibility that CB1 protein is localized on distal projections of pyramidal CA hippocampal neurons. In hippocampus, neocortex (layers II, III, V and VI), entorhinal cortex,

amygdaloid region (basomedial and basolateral amygdala), anterior olfactory nucleus, olfactory tubercle and piriform cortex, CB1 is expressed (at mRNA and protein level) both in scattered cells containing very high levels of the receptor and in more diffuse low-expressing neurons (Matsuda et al., 1993; Tsou et al., 1998a). In these cortical regions, scattered high-expressing cells are likely to be GABAergic interneurons, while diffuse low-expressing cells are probably principal glutamatergic neurons (Katona et al., 1999; Marsicano and Lutz, 1999). Other forebrain areas such as the ventromedial hypothalamic area and some thalamic nuclei contain low levels of CB1. In the hindbrain, besides the molecular and granular layers of cerebellum expressing high levels of the receptor, CB1 is present at low levels in some nuclei of brain stem (Matsuda et al., 1993; Tsou et al., 1998a; Bayatti et al., in preparation).

### 1.1.5 Physiological functions and therapeutical implications of the cannabinoid system

The intracellular pathways triggered by CB1, together with the anatomical distribution of the receptor, can account for the numerous effects induced by pharmacological treatment of animals with cannabinoids. An impressive body of studies in the last years has led to many and important clarifications about the physiological and pathophysiological functions which the cannabinoid system might be involved in. Due to the abundance of CB1-mediated effects I restrict to some of the most important roles of the cannabinoid system in the CNS and its therapeutical potential.

#### *1.1.5.1 Neuroprotection*

Neuronal destruction may be caused by the generation of free radicals, reactive oxygen species, pro-inflammatory cytokines or the overstimulation of synaptic excitatory amino acid receptors. Animal models have shown the benefit from early treatment of ischemia and brain trauma by both synthetic cannabinoids and endocannabinoids. Treatment results in long-term functional improvement, survival of neurons and a reduction in infarct volume and brain edema (Leker et al., 1999; Nagayama et al., 1999; Panikashvili et al., 2001; van der Stelt et al., 2001a; van der Stelt et al., 2001b). Several studies have demonstrated the protective effects of cannabinoids on excitotoxicity which is induced through excessive increases of intracellular $Ca^{2+}$ upon stimulation of glutamate receptors (Hampson et al., 1998b; Shen and Thayer, 1998; Abood et al., 2001). These neuroprotective actions of cannabinoids might be explained by the fact that AEA is able to inhibit NMDA-receptor mediated $Ca^{2+}$ influx (Hampson et al., 1998a) and is released on-demand after neurotoxic insults (Schmid et al.,

1996; Cadas et al., 1996; Hansen et al., 1998). In our group it was shown that the endogenous cannabinoid system protects against kainic acid-induced excitotoxicity *in vivo* and *in vitro* by activating a protective signaling cascade indicating an involvement of the cannabinoid system in self-protecting mechanisms of the brain (Marsicano et al., 2003; Khaspekov et al., 2004).

Natural and synthetic cannabinoids have also been shown to possess antinociceptive and anti-inflammatory activities. Today, among the most promising anti-inflammatory compounds from *Cannabis sativa* are the cannabinoid acids (reviewed in Burstein, 1999), compounds that, unlike $\Delta^9$-THC, are devoid of psychotropic activity and are very weak ligands of either CB1 or CB2 receptors. This may suggest that the anti-inflammatory properties of marihuana are not necessarily mediated by cannabinoid receptors. On the other hand, a CB1-mediated effect against thermal hyperalgesia and edema in rats was described later for AEA (Richardson et al., 1998a). A study carried out in our group also suggests a protective role of CB1 during colonic inflammation (Massa et al., 2004). This protection was mediated by an acute activation of the endogenous cannabinoid system since pharmacological blockade of CB1 with the specific antagonist SR141716A led to similar worsening of colitis as observed in CB1-deficient mice. The involvement of the endogenous cannabinoid system in the modulation of the acute phase inflammation is further supported by the increased levels of transcripts coding for CB1 in the wild-type mice after induction of inflammation.

Antioxidant properties of cannabinoids depend on their chemical structure. Classical and nonclassical cannabinoids such as $\Delta^9$-THC and CP55,940 contain a phenolic ring (Fig. 1-1), which has been proposed as an important lead structure for protection against oxidative stress, regardless of any specific receptor-mediated action (Moosmann and Behl, 2002). Therefore, the presence of a phenolic ring in many exogenous cannabinoids could account for their neuroprotective effects. Aminoalkylindole compounds, such as WIN55,212-2, and endocannabinoids do not contain the phenolic ring and, indeed, do not show antioxidant properties (Marsicano et al., 2002a; see also chapter 3.2.2). However, other mechanisms appear to be also involved. WIN55,212-2 was shown to be a potent neuroprotectant in a rat model of global and focal ischemia (Nagayama et al., 1999). Interestingly, in the same report, the neuroprotective action of WIN55,212-2 appeared to be CB1-mediated *in vivo*, but was CB1-independent *in vitro*, thus indicating differences in the mechanisms of action in the whole animal or in isolated neuronal cultures. Taken together, these data suggest complex mechanisms underlying the neuroprotective effects of cannabinoids and the putative neuroprotective role of the endogenous cannabinoid system, ranging from chemical

antioxidant properties of classical and nonclassical cannabinoids to the receptor-mediated effects of aminoalkylindoles and endocannabinoids.

#### *1.1.5.2 Nociception*

In mammals, $\Delta^9$-THC and synthetic CB1 agonists exert strong analgesic effects in several pain paradigms. It is now well established that the antinociceptive effects of cannabinoids are mediated through receptor-dependent mechanisms in the brain that in turn appear to be partly connected to the noradrenergic and kappa opioid systems in the spinal cord to modulate the perception of painful stimuli (reviewed in Martin and Lichtman, 1998; Chaperon and Thiebot, 1999). SR141716A has been demonstrated to block the antinociceptive effects of $\Delta^9$-THC, WIN55,212-2, and CP55,940 while not affecting morphine-induced analgesia (Compton et al., 1996; Lichtman and Martin, 1997). The possibility of cannabinoids acting as analgesic agents resulted in an intensive research of $\Delta^9$-THC in a wide range of antinociceptive assays in rodents. $\Delta^9$-THC has been found to be effective in producing analgesia in both phasic (tail-flick and hot plate test) and tonic (stretching) nociceptive tests (reviewed in Martin and Lichtman, 1998). When exogenously administered, endogenous cannabinoids, such as AEA, are also able to induce analgesia in mice and rats (Stein et al., 1996; Calignano et al., 1998; Walker et al., 2002). However, the opioid system does not appear to be involved in the analgesic effect of exogenously administered AEA (Welch et al., 1995), thus, pointing to pharmacological differences between endocannabinoids and exogenous cannabinoid compounds.

Further human studies are required to determine the efficacy of cannabinoids in analgesia, but promising animal studies suggest that cannabinoids may be useful in pain management if the psychotropic effects can be dissociated from the therapeutic effects.

#### *1.1.5.3 Locomotion*

The finding that the endocannabinoid system might be involved in the regulation of motor behavior is based on several lines of evidence. It has been well demonstrated that synthetic, plant-derived, and endogenous cannabinoids have powerful, mostly inhibitory actions on motor activity (reviewed in Sanudo et al., 1999). It is also well-known that endocannabinoids and CB1 are abundantly distributed in the basal ganglia and the cerebellum, areas that control movement (Herkenham et al., 1991; Mailleux and Vanderhaeghen, 1992; Tsou et al., 1998a). Large doses of natural and synthetic CB1 agonists (including AEA) strongly reduce motor activity and can induce catalepsy, while low doses could stimulate motor activity (Sulcova et

al., 1998; Rodríguez de Fonseca et al., 1998). The CB1 antagonist SR141716A is able to reverse the inhibitory effects of CB1 agonists on locomotor activity (Rinaldi-Carmona et al., 1994; Compton et al., 1996). Dorsolateral caudate putamen and subthalamic nucleus contain CB1 mRNA, thus indicating GABAergic striatonigral and glutamatergic, subthalamonigral neurons as the main CB1-containing cell populations (Mailleux and Vanderhaeghen, 1992; Matsuda et al., 1993). A modulation of dopaminergic, GABAergic and glutamatergic transmission in these regions has been proposed for the cannabinoid-induced motor deficits (Glass et al., 1997a; Sanudo and Walker, 1997).

An increasing number of studies have demonstrated that CB1 receptor binding was altered in the basal ganglia of humans affected by several neurological diseases, and also of rodents with experimentally induced motor disorders (reviewed in Consroe, 1998). Indeed, clinical studies have shown that cannabinoid agonists can be used to reduce the levodopa-induced dyskinesia in a Parkinson´s disease model (Muller et al., 1999; Sevcik and Masek, 2000). Of particular interest is a report concerning the ability of cannabinoids to control spasticity and tremor in a mouse model of multiple sclerosis (Baker et al., 2000; Baker et al., 2001). The authors showed not only that pharmacological treatment with CB1 agonists was able to drastically reduce these signs, but also that treatment of the animals with CB1 and CB2 antagonists was able to exacerbate them. Data from rodent models of Huntington's disease suggest that CB1 might play a role in the pathogenesis of this disease. Several studies have clearly demonstrated that in Huntington's disease, there exists an almost complete disappearance of CB1 receptor binding in the substantia nigra, in the lateral part of the globus pallidus, and, to a lesser extent, in the putamen (Glass et al., 1993; Richfield and Herkenham, 1994; Glass et al., 2000).

#### *1.1.5.4 Learning and memory*

Synthetic and natural cannabinoids are able to inhibit memory formation in animals as assessed by various paradigms (reviewed in Sullivan, 2000). These effects are inhibited by the specific CB1 antagonist SR141716A, thus indicating the involvement of the receptor. Also behavioral studies in humans show that marihuana acutely impairs performance on memory tasks (Belmore and Miller, 1980; Miller and Branconnier, 1983). CB1 is highly expressed in brain areas that are considered as central elements for various forms of memories, that are related to structures such as hippocampus, retrohippocampal areas (entorhinal and perirhinal cortex), amygdaloid nuclei and septal nuclei (Herkenham et al., 1990; Matsuda et al., 1993; Tsou et al., 1998a). In these areas both GABAergic interneurons and glutamatergic pyramidal

cells express CB1 at various levels (Katona et al., 1999; Marsicano and Lutz, 1999). Therefore, the mechanism of action of cannabinoids in these areas can be different depending on the cell types involved, but in most cases it appears to include a presynaptic inhibition of neurotransmitter release. These inhibitory effects on neurotransmitter release are considered to be the basis of the CB1-mediated blockade of long-term potentiation (LTP) and long-term depression (LTD) in the hippocampus. LTP and LTD of synaptic transmission are two *in vitro* models for learning and memory. Activation of CB1 receptors blocks LTP of field potentials in the CA1 region of hippocampal slices (Nowicky et al., 1987; Terranova et al., 1995; Misner and Sullivan, 1999) and has been found to inhibit hippocampal LTD of CA1 field potentials as well (Misner and Sullivan, 1999). The endocannabinoids AEA and 2-AG are present in the hippocampus and are able to inhibit LTP in a CB1-mediated manner (Felder et al., 1996; Stella et al., 1997). The findings of Wilson et al. (2001) provide important clues as to the function of endocannabinoids in the hippocampal network and suggest possible mechanisms for the disruption of memory by cannabinoids. They demonstrated that hippocampal DSI is completely absent in CB1-deficient mice, strongly implying that DSI is mediated by cannabinoid release, and that its presynaptic target is indeed exclusively CB1. Furthermore, the downstream effector of CB1 in this context is likely to be direct inhibition of $Ca^{2+}$ channels by G proteins which accounts for the rapid induction of synaptic depression after postsynaptic depolarization. On the other hand, endocannabinoids have been shown to facilitate LTP induction in single neurons mediated by DSI. Such targeted LTP could underlie behavioral learning associated with LTP induction in limited subsets of cells (Carlson et al., 2002). In addition to this transient disinhibitory action, it was recently shown that endocannabinoids also mediate a persistent depression of inhibitory synaptic transmission. This form of plasticity not only may underlie changes in CA1 pyramidal cell excitability but may also exert long-lasting modulatory actions on the induction of LTP at excitatory synapses (Chevaleyre and Castillo, 2003). However, global activation of CB1 receptors, by disrupting the temporal and spatial selectivity of coding and recall mediated by endocannabinoids, contributes to the learning and memory deficiencies associated with cannabinoid drug abuse.

The endogenous cannabinoid system has been proposed to be tonically stimulated during active forgetting processes in which less important information is deleted from memory storage (Collin et al., 1995; Hampson and Deadwyler, 1998). This notion is supported by several experiments. Treatment with the CB1 antagonist SR141716A was shown to enhance social recognition (Terranova et al., 1996) and, more recently, to increase spatial memory in a delay version of the radial-arm maze task (Lichtman, 2000). Moreover,

CB1-deficient mice showed also enhanced memory in a paradigm of object recognition (Reibaud et al., 1999) and increased hippocampal LTP (Bohme et al., 2000). In our group it was shown that the endogenous cannabinoid system has a central function in extinction of aversive memories (Marsicano et al., 2002b). CB1-deficient mice showed strongly impaired short-term and long-term extinction in auditory fear-conditioning tests, with unaffected memory acquisition and consolidation. Elevated levels of endocannabinoids were measured in the basolateral amygdala complex, a region known to control extinction of aversive memories. In this region cannabinoids were shown to modulate both excitatory and inhibitory synaptic transmission via CB1. The mechanisms underlying this cannabinoid action in the lateral amygdala differ from those described for the hippocampus (Azad et al., 2003).

## 1.2 The cannabinoid system and cross-talk with other receptor systems

Numerous proteins involved in cellular signaling contain protein–protein interaction domains that allow their recruitment and assembly into large complexes. G protein-coupled receptors have been found to interact with a wide variety of proteins containing structural interacting domains (reviewed in Bockaert et al., 2003). These proteins have several important functions. First, they participate in the targeting of G protein-coupled receptors to specific subcellular compartments. Secondly, they are responsible for the clustering of these receptors with various effectors. Thirdly, they can regulate G protein-coupled receptor functions in an allosteric manner. G protein-coupled receptor-associated proteins are known to interact with intracellular loops, transmembrane and C-terminal domains. Besides, interaction of G protein-coupled receptors with various proteins, several pharmacological observations led investigators to propose that G protein-coupled receptors might also couple among each other. Biochemical and biophysical approaches have confirmed the existence of several such complexes in living cells, and there is strong evidence to support the idea that dimerization is important in different aspects of receptor biogenesis and function (reviewed in Bouvier, 2001). While the existence of G protein-coupled receptor homodimers raises fundamental questions about the molecular mechanisms involved in transmitter recognition and signal transduction, the formation of heterodimers raises fascinating combinatorial possibilities that could underlie an unexpected level of pharmacological diversity, and contribute to cross-talk regulation between transmitter systems. Because G protein-coupled receptors regulate such diverse physiological processes as neurotransmission, cellular metabolism, secretion, cellular

differentiation and growth, as well as inflammatory and immune responses, the existence of dimers could have important implications for the development and screening of new drugs. The potential for interaction between cannabinoid signaling pathways and those of other receptor systems has also begun to be investigated in both molecular and integrated system studies and was also the main focus of this work. In the following, some examples of recent research are summarized, aiming to understand interactions of the cannabinoid system with other prominent receptor systems.

### 1.2.1 Interaction with the dopamine system

The neuromodulatory effects of dopamine in the CNS are mediated by two classes of G protein-coupled receptors, each comprising several subtypes. Like CB1, dopamine receptors belong to the family of G protein-coupled receptors and regulate the activity of AC (reviewed in Strange, 1996). Dopamine receptor D1 is the most abundant dopamine receptor in the brain expressed at high levels in the striatum, nucleus accumbens and olfactory tubercle and to a lower extent in the limbic system, thalamus and hypothalamus (Fremeau et al., 1991). A similar expression pattern throughout the murine brain is also shown for dopamine receptor D2 (Meador et al., 1989).

There is evidence for a functional and anatomical link between the dopamine and cannabinoid system, which might be relevant for the understanding of dopamine- and cannabinoid-related disorders. Glass and Felder (1997) found that the activation of either CB1 or D2 receptors in rat primary striatal cells resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an increase of cAMP accumulation. In contrast, another group showed that simultaneous stimulation of CB1 and D2 in striatal membranes produced no greater inhibition than that elicited by either drug alone (Meschler and Howlett, 2001). Pharmacological experiments in mice by Meschler et al. (2000) showed that the application of a D2 agonist was able to attenuate the motor dysfunction caused by the CB1 agonist levonantradol. Similarly, a D1 agonist attenuated the effect of levonantradol, while a D1 antagonist enhanced the effects of levonantradol. A functional interaction of the cannabinoid and the dopamine system was also suggested in memory storage. AEA-mediated impairment of consolidation in mice was antagonized by pretreatment with either D1 or D2 agonists (Castellano et al., 1997) suggesting that D1 and D2 receptors are similarly involved in the effects of AEA on memory consolidation although they are coupled to different G proteins. Similar results were obtained from another group which suggested that impairment of working memory is mediated by the concomitant

activation of D2 and CB1 receptors, as this effect was antagonized not only by SR141716A but also by a D2 antagonist and potentiated by the administration of a D2 agonist (Nava et al., 2000). Giuffrida et al. (1999) showed a functional interaction between AEA and dopamine. AEA release was increased eightfold over baseline after local administration of a D2-like agonist suggesting a functional cross-talk between endocannabinoid and dopaminergic systems which may contribute to striatal signaling.

### 1.2.2 Interaction with the serotonin system

Serotonin (5-HT, 5-hydroxytryptamine), a biogenic amine, acts as a neurotransmitter. Its diversity of pharmacologic actions ranging from modulation of neuronal activity and transmitter release to behavioral changes, is related to a wide variety of receptor and effector mechanisms. Seven serotonin receptor families have been identified so far, comprising a total of 14 structurally and pharmacologically distinct subtypes. The 5-HT receptor family consists of G protein-coupled receptors, except of the 5-HT3 receptor, which is a ligand-gated ion channel (reviewed in Barnes and Sharp, 1999). ISH on murine brain sections revealed characteristic distribution patterns for the serotonin receptors 5-HT1B and 5-HT3 mainly in the basal ganglia, neocortex, hippocampus, hypothalamus, and amygdala (Maroteaux et al., 1992; Tecott et al., 1993).

Several studies carried out so far gave hints to functional cross-talks of the cannabinoid and the serotonin system, which might be relevant in relation to neuropsychological disorders such as schizophrenia, in which both the serotonergic and endogenous cannabinoid systems have been implicated (Hashimoto et al., 1993; Emrich et al., 1997). It was shown that low concentrations of either AEA, WIN55,212-2, and CP55,940 inhibit the function of the serotonin receptor 5-HT3 in rat nodose ganglion neurons (Fan, 1995). Recently, similar results were shown by another group suggesting that several cannabinoids ($\Delta^9$-THC, WIN55,212-2, AEA, and CP55,940) inhibit 5-HT induced currents independently of CB1 by acting allosterically at a modulatory site of 5-HT3. Thus, the functional state of the receptor can be controlled by endogenous AEA (Barann et al., 2002). A cross-talk between CB1 and 5-HT receptors in rat cerebellar membranes was also suggested by Devlin and Christopoulos (2002) who showed that 5-HT can modulate the binding of some cannabinoid agonists in a complex fashion. This involves interaction between components of the CB1 and 5-HT G protein-coupled signaling cascades within the cell membrane environment. Moreover, a correlation between the two neurotransmitter systems was found in memory storage. $\Delta^9$-THC, which impairs spatial memory, decreased 5-HT release in the

ventral hippocampus, whereas it did not in the raphe nucleus, where $\Delta^9$-THC has no effect on spatial memory. This finding suggests that the inhibition of 5-HT transmission in the hippocampus may be involved in the $\Delta^9$-THC-induced impairment of spatial memory (Egashira et al., 2002).

### 1.2.3 Interaction with the vanilloid system

The vanilloid receptor type 1 (VR1) is a six-transmembrane-domain, non-selective cation channel belonging to the family of transient receptor potential (TRP) channels (reviewed in Montell, 2001). So far, VR1 is the only member of a heterogenous family of nociceptors. It is activated by capsaicin, the pungent ingredient of red hot chilli peppers and resiniferatoxin, a toxin of another plant belonging to the genus *Euphorbia* (reviewed in Szallasi and Blumberg, 1999). VR1 was cloned from a rat dorsal root ganglia (DRG) cDNA library (Caterina et al., 1997) and has mainly attracted attention as a transducer of painful stimuli on primary sensory neurons (Tominaga et al., 1998). Interestingly, the endocannabinoid AEA was discovered as its endogenous ligand and acts as a full agonist at VR1 receptors (Zygmunt et al., 1999; Smart et al., 2000). VR1 is mostly expressed in small and medium diameter neurons of DRG and acts as ligand-, proton- and heat-activated molecular integrator of nociceptive stimuli (Caterina et al., 1997). VR1 is also expressed in several brain areas, including hippocampus, striatum, hypothalamus, substantia nigra and locus coeruleus (Mezey et al., 2000), where its function is still a matter for speculation.

Recent evidence points to the existence of functional relationships between CB1 and VR1. The two receptors are colocalized in many small diameter, non-myelinated sensory C-fibers, both at the level of the spinal cord and in DRGs as well as in the peripheral terminals of C-fibers (Ahluwalia et al., 2000). There is now evidence for the coexistence of CB1 and VR1 receptors also in brain nuclei and areas such as substantia nigra, cerebellum, hippocampus, cortex, and amygdala (Mezey et al., 2000; Szabo et al., 2002). It is now established that stimulation of CB1 receptors on sensory neurons with CB1-selective agonists can induce inhibition of VR1-mediated thermal hyperalgesia (Richardson et al., 1998c; Kelly and Chapman, 2001; Millns et al., 2001). Similarly, pretreatment of skin with HU210 significantly reduced the perception of pain following the administration of capsaicin (Rukwied et al., 2003). Several relations have been identified also between the endogenous ligand AEA of CB1 and VR1. Activation of CB1 with AEA reduces the capsaicin-induced peptide release from primary sensory neurons (Richardson et al., 1998c), whereas blocking of CB1 with SR141716A increases capsaicin-induced peptide release from these neurons (Lever

and Malcangio, 2002). These findings suggest that a potential mechanism for the anti-inflammatory actions of cannabinoids might be the inhibition of neurosecretion from the peripheral terminals of nociceptive primary afferent fibers. Recently, it was demonstrated that capsaicin induces AEA production and release in primary sensory neurons, providing a role for AEA as a key endogenous regulator of the excitability of these neurons (Ahluwalia et al., 2003).

### 1.2.4 Interaction with the CRH system

The 41 amino acid neuropeptide corticotropin-releasing hormone (CRH) is the major mediator of the stress response in the CNS (Vale et al., 1981). In addition to modulating the function of the hypothalamic-pituitary-adrenal (HPA) axis, CRH is implicated in affecting other central responses, such as promoting memory and learning (Radulovic et al., 1999), altering synaptic plasticity (Wang et al., 1998), and promoting neuronal viability (Lezoualc'h et al., 2000). These effects are mediated by binding and activation of CRH to two distinct CRH receptors, CRHR1 and CRHR2 (reviewed in De Souza, 1995). Both receptors belong to the family of seven transmembrane, G protein-coupled receptors and are expressed in many regions of the CNS and in the periphery (Van Pett et al., 2000). In the brain, CRHR1 is expressed at particularly high levels in the hippocampus, cortex, and cerebellum. After binding to the receptor, CRH activates a number of intracellular signaling pathways mostly by activation of AC, leading to increased intracellular concentrations of cAMP and activation of PKA (Eckart et al., 2002).

A connection between the cannabinoid and the CRH system exists on the level of the HPA axis. There is evidence that activation of CB1 by exogenous ligands ($\Delta^9$-THC and WIN55,212-2) as well as its endogenous ligand AEA increases the release of adrenocorticotropic hormone (ACTH) in the pituitary gland via the secretion of CRH (Weidenfeld et al., 1994; Pagotto et al., 2001). The enhancement of CRH expression in the hypothalamus and anterior pituitary after chronic exposure to cannabinoids provides evidence of molecular alterations that may be relevant to further understand a variety of behavioral and neuroendocrine effects that occur in cannabinoid drug abuse (Corchero et al., 1999a; Corchero et al., 1999b).

The appetite-stimulating effect of marihuana in humans has been well-known for centuries (Abel, 1975). Several reports have demonstrated that administration of cannabinoids stimulates food intake in animal models (Williams et al., 1998; Koch, 2001). On the basis of the observation that CB1 and endocannabinoids are present in the brain regions controlling

food intake (Howlett et al., 2002), the endogenous cannabinoid system has been proposed as a putative modulator of feeding behavior (Mechoulam and Fride, 2001; Cota et al., 2003a). Therefore, the assumption is obvious that cannabinoids interact with the large number of hypothalamic neuropeptides, e.g. CRH (Raber et al., 1997; Gardner et al., 1998; Huang et al., 1998), which constitute the major neuronal network regulating the neuroendocrine system and energy metabolism. Indeed, the effects of hypothalamic endocannabinoids on energy balance might partially be mediated by CRH (Cota et al., 2003b).

As CB1 and CRHR1 are expressed to a high extent in the same brain regions such as hippocampus, cortex, and cerebellum and both receptors regulate the cAMP signaling cascade, signaling interactions between the two receptor systems and consequential effects on downstream transcriptional target genes would be conceivable.

## 1.3 Aim of the thesis

The main focus of this work was to explore the cannabinoid receptor type 1 for putative cross-talks with other prominent receptor systems to provide insights into the physiological diversity of the cannabinoid system in the murine CNS. Several different approaches were applied to accomplish this aim:

- To obtain further insights into putative interactions of CB1 receptors with dopamine and serotonin receptors, double-*in situ* hybridization experiments on mouse brain sections were performed to determine coexpression at the cellular level.
- For the identification of genes involved in CB1-mediated signaling, brain sections of CB1-deficient mice and wild-type littermates were hybridized with probes encoding several components of various neurotransmitter systems to monitor possible disregulations in CB1-deficient mice.
- Signaling interactions between CB1 and VR1 in HEK-293 cells coexpressing both receptors were investigated by recording the intracellular signaling events after pharmacological activation of both receptors.
- As CB1 and CRHR1 are highly coexpressed in the forebrain and cerebellum, cerebellar primary cultures served as cell culture system to investigate a common signaling pathway by which both receptors control the expression of the target gene BDNF.
- To reveal a mechanism through which CB1 protects against kainic acid-induced excitotoxicity, the expression of different genes (c-fos, zif268, BDNF), known to be

upregulated under these circumstances, was analyzed by *in situ* hybridization in kainic acid-treated mice lacking CB1 in all glutamatergic neurons of cortical forebrain structures.

- To investigate the protective role of BDNF during excitotoxicity in more detail, a time-course of BDNF protein levels after kainic acid treatment and its dependency on CB1 receptors was determined by ELISA, using hippocampal organotypic cultures.
- To determine the involvement of CB1 in the neuroprotective effects of cannabinoids, HT22 cells expressing CB1 were established and assayed for antioxidant properties of different compounds.
- To clarify the role of CB1 during the pathology of inflammation in the colon, *in situ* hybridization experiments were applied to compare the expression levels of CB1 between inflamed and naive animals. Also, the effect of inflammation on the expression of the opioid preproenkephalin was compared between CB1-deficient mice and littermate controls.

# 2 MATERIAL AND METHODS

## 2.1 Drugs

3-Isobutyl-1-methylxanthine (IBMX), forskolin (FRSK) and ionomycin were purchased from Sigma (Deisenhofen, Germany), WIN55,212-2, HU210 and capsaicin from Tocris (Cologne, Germany), wortmannin and LY294002 from Alexis Biochemicals (Lausen, Switzerland), ET-18, U73122 and D609 from Biomol Research Laboratories (Plymouth Meeting, PA, USA) and CRH from Calbiochem (Schwalbach, Germany). AEA was synthesized as previously described (De Petrocellis et al., 2000). SR141716A was from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program.

These compounds were prepared as 10 mM stock solutions in 1x phosphate buffered saline (PBS, consisting of 136.8 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 10.2 mM $Na_2HPO_4$, pH 7.4; in the case of IBMX), in 100% dimethylsulfoxide (DMSO; in the case of WIN55,212-2, HU210, SR141716A, AEA, capsaicin, FRSK and wortmannin), in ethanol (in the case of ET18 and U73122), in methanol (in the case of LY294002), in $H_2O$ (in the case of D609) or in 2% acetic acid (in the case of CRH).

## 2.2 Animals

All animals were housed with a 12 h : 12 h light-dark cycle and allowed to access food and water *ad libitum*. C57BL/6N mice and pregnant Sprague Dawley rats were obtained from Charles River (Sulzfeld, Germany). The generation of the VR1- and CB1-deficient mice lines ($VR1^{-/-}$ and $CB1^{-/-}$) is described in Caterina et al. (2000) and Marsicano et al. (2002b), respectively. The generation of the CB1-deficient mouse line $CB1^{f/f;CaMKII\alpha Cre}$, in which CB1 is deleted in all principal neurons of the forebrain, is described in Marsicano et al. (2003).

## 2.3 *In situ* hybridization

### 2.3.1 Tissue preparation

Experimental adult mice (3-5 months old) were killed by cervical dislocation, brains or colons, respectively, were snap-frozen on dry-ice directly after removal, and stored at -80°C prior to sectioning. The tissue was mounted on Tissue Tek (Polysciences, PA, USA), and 18 µM-thick consecutive sections were cut from the colon or from brain regions of interest,

respectively, on a cryostat (Microtrome HM 560, Microm, Braunschweig, Germany). Sections were mounted onto frozen SuperFrost/plus slides (Fisher Scientific, Hannover, Germany), dried on a 42°C warming plate and stored at –20°C until used.

### 2.3.2 Probe synthesis

Both radioactive ($^{35}$S) and non-radioactive (digoxygenin, DIG) labeled riboprobes were used. The majority of probes was generated by RT-PCR from cDNA derived from total mouse brain RNA. For each of these probes, GenBank accession number (GB No.), sequence and nucleotide positions of the primers and length of the cloned insert are listed in Table 2-1. Nucleotide positions are identical to those used in sequences deposited in GenBank. All remaining probes were purchased from Invitrogen or obtained from other research groups. Their corresponding reference is given in Table 2-2.

| Primer | GB No. | Position [bp] | Sequenz | Length of insert [bp] |
|---|---|---|---|---|
| 5-HT1B-5‘ | Z11597 | 443 | 5‘-GCCAAAGGAGACAAGCCTATA-3‘ | 763 |
| 5-HT1B-3‘ | | 1206 | 5‘-GAGCAGGGTGGGTAAATAGAA-3 | |
| 5-HT3-5‘ | M74425 | 458 | 5‘-GGAAGTCTCCGAACATTCCTT-3‘ | 1121 |
| 5-HT3-3‘ | | 1579 | 5‘-CCCCCATACTTATCCTAACCA-3‘ | |
| CB1-5’ | U22948 | 152 | 5’-GTTGAGCCTGGCCTAATCAAA-3’ | 1530 |
| CB1-3’ | | 1682 | 5’-GTTGACCGAACCTCTGTTTTC-3’ | |
| CCK-5’ | X59520 | 188 | 5’-ACTTAGCTGGACTGCAGCTT-3’ | 411 |
| CCK-3’ | X59522 | 151 | 5’-GGACTACGAATACCCATCGTA-3’ | |
| D2-5’ | X55674.1 | 302 | 5’-CTGTATCACGAGAGAAGGCTT-3’ | 837 |
| D2-3’ | | 1139 | 5’-CTGGGATTGACAATCTTGGCA-3’ | |
| Enk-5’ | M13227 | 57 | 5’-TGCACACTGGAATGTGAAGGA-3’ | 830 |
| Enk-3’ | | 887 | 5’-CACAGACCCTAAAATCACAGC-3’ | |
| FAAH-5’ | NM024132 | 922 | 5’-GCTATGCCTGAAAGCTCTACT-3’ | 820 |
| FAAH-3’ | | 1742 | 5’-TGAACCTCAGACACAGCTCTT-3’ | |
| GAD65-5’ | D42051 | 1055 | 5’-GGCGATGGAATCTTTTCTCCT-3’ | 1041 |
| GAD65-3’ | | 2096 | 5’-CGAGGCGTTCGATTTCTTCAA-3’ | |
| GAD67-5’ | NM017007 | 934 | 5’-GGAGCCATATCCAACATGTAC-3’ | 956 |
| GAD67-3’ | | 1890 | 5’-GATGACCATCCGGAAGAAGTT-3’ | |
| nNOS-5’ | NM008712 | 1985 | 5’-CCTGGTGGAGATTAACATTGC-3’ | 1197 |
| nNOS-3’ | | 3182 | 5’-CTGGTACTGCAACTCCTGATT-3’ | |

**Table 2-1: Riboprobes generated by RT-PCR and corresponding primers used for amplification of probe fragments.**

| Probe | Reference |
|---|---|
| BDNF | IMAGE clone 1397218, Invitrogen, Karlsruhe, Germany |
| c-fos | IMAGE clone 2647069, Invitrogen, Karlsruhe, Germany |
| CRHR1 | gift from W. Wurst, Max-Planck-Institute of Psychiatry, Munich, Germany; see Muller et al. (2001) |
| D1 | gift from T. Lemberger, DKFZ, Heidelberg, Germany |
| zif268 | IMAGE clone 1532857, Invitrogen, Karlsruhe, Germany |

**Table 2-2: Source of external riboprobes.**

PCR products were cloned into pBluescript $KS^-$ (Stratagene, CA, USA) and used as templates for riboprobe synthesis. The identity of all clones used for ISH was checked by sequencing. Linearized template DNA was phenol-extracted, precipitated, resuspended in diethyl pyrocarbonate-treated $H_2O$ at a concentration of 1 µg/µl, and stored at -20°C until use. For $^{35}S$-labeled riboprobes, *in vitro* transcription was carried out for 3 h at 37°C in a total volume of 30 µl containing 1.5 µg of linearized DNA, 1x transcription buffer (supplied with RNA polymerase from Roche Molecular Diagnostics, Mannheim, Germany), 1 mM of rATP/rCTP/rGTP each, 16.7 mM dithiothreitol (DTT), 40 units RNasin (Promega, WI, USA) 10 µl of [$\alpha$-$^{35}S$]-UTP (NEN, MA, USA; 1250 Ci/mmol), and 30 units of T3, T7 or Sp6 RNA polymerase (Roche Molecular Diagnostics). For DIG-labeled riboprobes, *in vitro* transcription was carried out for 3 h at 37°C in a total volume of 50 µl containing 1.5 µg of linearized DNA, 1x transcription buffer, 5 µl of DIG RNA labeling mix (Roche Molecular Diagnostics), 80 units RNasin (Promega), and 100 units of T7 or T3 RNA polymerase. Reactions were treated with 20 units of RNase free DNaseI (Roche Molecular Diagnostics) for 15 min at 37°C, and labeled probes were purified by column exchange (Nucleotide removal kit, Qiagen, Hilden, Germany). Suitable restriction enzymes (New England Biolabs, MA, USA) were used for linearization and the corresponding RNA polymerases T3, T7 or Sp6 (Roche Molecular Diagnostics) were used in order to get labeled antisense and sense fragments, respectively, of the cloned insert (Table 2-3). In ISH experiments, sense controls did not give any detectable signals (data not shown), and antisense probes gave distribution patterns identical to those already published in rat or mouse.

| Probe | Internal number of plasmid | Orientation | Restriction enzyme | RNA polymerase |
|---|---|---|---|---|
| 5-HT1B-5‘ | M328 | AS | EcoRI | T7 |
| 5-HT1B-3‘ | | S | XbaI | T3 |
| 5-HT3-5‘ | M329 | AS | XbaI | T3 |
| 5-HT3-3‘ | | S | EcoRI | T7 |
| BDNF-5’ | M357 | AS | XhoI | T3 |
| BDNF-3’ | | S | EagI | T7 |
| CB1-5’ | M186 | AS | BamHI | T3 |
| CB1-3’ | | S | EcoRI | T7 |
| CCK-5’ | M183 | AS | EcoRI | T7 |
| CCK-3’ | | S | BamHI | T3 |
| c-fos-5’ | M353 | AS | EcoRV | T7 |
| c-fos-3’ | | S | XbaI | Sp6 |
| CRHR1-5’ | M374 | AS | BglII | T7 |
| CRHR1-3’ | | S | BglII | T3 |
| D1-5’ | M331 | AS | SalI | T7 |
| D1-3’ | | S | SacI | T3 |
| D2-5’ | M268 | AS | BamHI | T3 |
| D2-3’ | | S | EcoRI | T7 |
| Enk-5’ | M224 | AS | BamHI | T3 |
| Enk-3’ | | S | EcoRI | T7 |
| FAAH-5’ | M222 | AS | BamHI | T3 |
| FAAH-3’ | | S | EcoRI | T7 |
| GAD65-5’ | G57.1 | AS | BamHI | T3 |
| GAD65-3’ | | S | EcoRI | T7 |
| GAD67-5’ | G62.1 | AS | BamHI | T3 |
| GAD67-3’ | | S | PstI | T7 |
| nNOS-5’ | G58.1 | AS | BamHI | T3 |
| nNOS-3’ | | S | EcoRI | T7 |
| zif268-5’ | M137 | AS | EcoRV | T7 |
| zif268-3’ | | S | XbaI | T3 |

**Table 2-3: Enzymes for generation of antisense and sense riboprobes.**

### 2.3.3 Single-*in situ* hybridization

Slides were warmed up for 30 min at room temperature, fixed in ice-cold 4% paraformaldehyde (PFA) in PBS for 10 min, rinsed three times in PBS, incubated for 10 min in 0.1 M triethanolamine-HCl (pH 8.0) to which 0.63 ml of acetic anhydride was added dropwise, rinsed twice in standard saline citrate (2x SSC, where 1x SSC contains 150 mM NaCl, 15 mM $Na_3$ citrate, pH 7.4), dehydrated in graded series of ethanol, delipidized in chloroform for 5 min, rinsed in 100% and 95% ethanol, and air-dried. Hybridization was carried out overnight at 64°C in 90 µl of hybridization buffer containing $^{35}$S-labeled riboprobe (35,000-70,000 c.p.m./µl). Hybridization buffer consisted of 50% formamide, 20 mM Tris-HCl, pH 8.0, 0.3 M NaCl, 5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, 10% dextran sulphate (D8906, Sigma, Germany), 0.02% Ficoll 400 (F2637, Sigma), 0.02% polyvinylpyrrolidone (MW 40,000, PVP40, Sigma), 0.02% bovine serum albumin (BSA, A6793, Sigma), 0.5 mg/ml tRNA (Roche Molecular Diagnostics), 0.2 mg/ml fragmented herring sperm DNA and 200 mM DTT. After incubation in a humid chamber, slides were rinsed four times for 5 min each in 4x SSC at room temperature, incubated for 30 min at 37°C in 20 µl/ml of RNaseA in 0.5 M NaCl, 10 mM Tris-HCl, pH 8.0, 5 mM EDTA, rinsed at room temperature in decreasing concentrations of SSC (1, 0.5 and 0.1x SSC) containing 1 mM DTT, washed twice for 30 min each at high stringency in 0.1x SSC/1 mM DTT at 64°C and washed twice for 10 min at room temperature in 0.1x SSC. At this point, $^{35}$S-labeled slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Kodak, Germany). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled water). After exposition for 5-20 days at 4°C, slides were developed for 3 min (D-19, Kodak), fixed for 6 min (Kodak fixer), rinsed for 30 min in tap water and air-dried. Slides were mounted in histofluid (Marienfeld, Lauda-Königshofen, Germany).

### 2.3.4 Double-*in situ* hybridization

Slides were warmed up for 30 min at room temperature, fixed in ice-cold 4% PFA in 1x PBS for 20 min, rinsed twice in PBS, quenched for 15 min in 1% $H_2O_2$ in 100% methanol, rinsed twice in PBS, quenched for 8 min in 0.2 M HCl, rinsed twice with PBS, treated with proteinase K 20 µg/ml (Roche Molecular Diagnostics) in 50 mM Tris-HCl (pH 8.0), 5 mM EDTA for 10 min, rinsed once with PBS, fixed in ice-cold 4% PFA in PBS, incubated for 10 min in 0.1 M triethanolamine (pH 8.0) to which 1.2 ml acetic anhydride was added dropwise, rinsed once with PBS, washed with 0.9 % NaCl for 5 min, dehydrated in graded series of

ethanol (30, 50, 70, 80, 95, 100%), and air-dried. Hybridization was carried out in 100 µl of hybridization buffer containing $^{35}$S-labeled riboprobe (70,000-100,000 c.p.m./µl) and DIG-labeled riboprobe (0.2 µg/ml). Hybridization buffer is the same as used for single ISH. Before applying to the tissue, hybridization cocktail was denatured for 2 min at 95°C. Slides were incubated overnight at 54°C in a humidified chamber.

Four high stringency washes were carried out at 62°C with 5x SSC, then with 50% formamide/2x SSC/0.05% Tween-20, with 50% formamide/1x SSC/0.05% Tween-20, and finally with 0.1x SSC/0.05% Tween-20. All of the following posthybridization washes and incubations were carried out at 30°C. Slides were washed with 0.5 M NaCl, 10 mM Tris-HCl (pH 8.0), 5 mM EDTA (NTE)/0.05% Tween-20, incubated with 15 mM iodoacetamide in NTE/0.05% Tween-20 for destruction of intracellular alkaline phosphatase, and washed twice with NTE/0.05% Tween-20. Slides were blocked with 4% heat-inactivated sheep serum in 100 mM Tris-HCl (pH 7.6)/150 mM NaCl/0.05% Tween-20 (TNT), which was filtered through a 0.45 µm filter, washed three times with TNT, incubated for 30 min in blocking buffer (included in TSA Biotin System, NEN Life Science Products, Boston, USA), incubated 1.5 hour with anti-DIG antibody (Roche Molecular Diagnostics) diluted 1:1200 in blocking buffer, washed three times in TNT, incubated for 12 min with biotin-labeled tyramide (TSA Biotin System, NEN Life Science Products), washed with 100 mM maleic acid/150 mM NaCl/0.05% Tween-20 (wash buffer), incubated for 1 hour with streptavidine-alkaline phosphatase (Roche Molecular Diagnostics) diluted 1:1000 with 1% blocking reagent (Roche Molecular Diagnostics) in wash buffer, and washed three times with wash buffer. Chromogenic reaction was carried out with Vector Red kit (Vector Laboratories, CA, USA) at room temperature for 10-30 min. The reaction was stopped with a 10-min incubation in PBS, followed by fixation for 30 min in 2.5% glutaraldehyde in PBS and three washes for 10 min in 0.1x SSC. Slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Scientific Imaging Systems, NY, USA). Dipping and counterstaining was carried out in the same manner as for single ISH.

### 2.3.5 Numerical and densitometric evaluation of expression

*Single ISH*

In single ISH experiments, the mRNA of interest was detected with a $^{35}$S-labeled riboprobe. Densitometric analyses of expression intensities were performed on autoradiographic films using the NIH Image software (http://rsb.info.nih.gov/nih-image/Default.html). In the case of

ISH on colonic tissue, cells positive for CB1 were counted in several sections of treated and untreated animals and values are expressed as percentage of the respective untreated group.

*Double ISH*

In double ISH experiments, CB1 mRNA was detected with a DIG-labeled riboprobe. As CB1 is expressed at various levels, stained cells were classified according to the following criteria: cells expressing CB1 at high levels (termed high CB1-expressing cells) were considered to be those showing a round-shaped and intense red staining surrounding the nucleus or even covering the entire nucleus. Cells expressing CB1 at low levels (termed low CB1-expressing cells) were defined as cells clearly stained above background levels and in a discontinuous shape and/or at uniform and low intensity of staining. Sections were analyzed on a Leica DMRB microscope. Cells positive for CB1 and/or one of the used markers were counted in several brain regions and coexpression values were calculated as percentages.

## 2.4 Immunhistochemistry

### 2.4.1 Tissue preparation

Adult C57BL/6N mice and $VR1^{-/-}$ mice were anesthetized with Isofluran and perfused through the heart with 4% PFA in PBS. The brains were removed and postfixed for 4 h in 4% PFA at 4°C. Dehydration occurred in graded ethanol series at 4°C (70% overnight, 96% 2 h, 100% 2 h) and a final treatment with xylol for 40 min. Brains were kept overnight in liquid paraffin at 63°C and were then embedded in plastic molds. Coronal consecutive sections of 7 µm were cut by using a microtome and sections were mounted onto SuperFrost/plus slides (Fisher Scientific), dried on a 37°C warming plate and stored at 4°C until used.

### 2.4.2 Immunostaining

Paraffin sections were rehydrated in graded alcohol series (xylol 45 min, 2x 100% ethanol 10 min, 2x 95% ethanol 10 min, 2x 70% ethanol 5 min) followed by hydration in water for 10 min. Slides were washed in 0.01 M Na-citrate (pH 6.0) for 3 min, heated for 5 and 3 min, respectively, in the microwave with cooling down for 5 min in between and finally cooling down for 20 min. After rinsing 2x for 2 min in PBS/0.2% Tween, slides were incubated for 5 min 0.1% $H_2O_2$ in PBS, blocked for 1 h (blocking buffer consisting of 1% goat serum, 3% BSA, 10% non-fat milk powder, 0.2% Tween-20) and incubated overnight with anti-VR1

antibodies recognizing the C-terminus (cat.no. ab901, Abcam, Cambridge, UK) or the N-terminus (cat.no. ab5370, Chemicon, Temecula, CA, USA), respectively, of the rat VR1 protein. Either antibody was diluted 1:2000 in blocking buffer. On the next day, slides were rinsed 2x for 2 min in PBS/0.2% Tween-20, incubated for 1 h in secondary biotinylated anti-rabbit antibody (included in Vectastain ABC-kit, Vector Laboratories) diluted 1:200 in blocking buffer, rinsed 2x for 2 min in PBS/0.2% Tween-20, incubated 5 min in ABC-reagent (ABC-kit, Vector Laboratories), rinsed for 5 min in PBS/0.2% Tween-20 and washed for 5 min in Tris-HCl (pH 7.4). Detection of the signal was carried out with the diaminobenzidine (DAB) substrate-kit (Vector Laboratories) according to manufacturer's instruction and reaction was stopped by washing 2x for 5 min in PBS/0.2% Tween-20. Slides were dehydrated in graded alcohol series (2x 70% ethanol 5 min, 2x 95% ethanol 5 min, 2x 100% ethanol 5 min, 2x xylol 10 min) and mounted in Histofluid (Marienfeld).
Negative controls included immunostaining without the primary antibody and immunostaining on paraffin sections derived from $VR1^{-/-}$ animals.

## 2.5 Cell culture

### 2.5.1 Cell lines

Human embryonic kidney (HEK)-293 cells stably expressing human VR1 (hVR1) were obtained from J. Davis (Glaxo Smith Cline). The neuronal cell line HT22 was obtained from D. Schubert (Salk Institue San Diego, CA, USA). Both cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2 mM glutamine and 1% antibiotic-antimycotic (Gibco BRL, Karlsruhe, Germany) at 37°C in a humidified 5% $CO_2$ incubator.

### 2.5.2 Primary cerebellar granule neurons

At postnatal day 3, newborn rats were physically decapitated and cerebellar tissue was removed. After dissection, tissue pieces were incubated for 20 min at room temperature in $Ca^{2+}$-, $Mg^{2+}$-free Dulbecco's PBS (Gibco, Karlsruhe, Germany) containing 0.1% trypsin and 0.02% EDTA. Cells were then transferred to $Ca^{2+}$-, $Mg^{2+}$-free Hank's balanced salt solution (Gibco) supplemented with 10% fetal calf serum (Gibco) and dissociated gently by tituration. Undissociated pieces were filtered through a 50 µm pore-sized Nybolt mesh (Eckardt, Waldkirch, Germany), and cells were centrifuged at 200 xg for 4 min. The pellet was

resuspended in minimal essential medium (MEM; Gibco) supplemented with 10% horse serum (Gibco), and the number of viable cells was counted. Cells were seeded in appropriate multi-well plates (TPP, Trasadingen, Switzerland) that had been previously coated with poly-L-lysine (0.1 mg/ml; molecular weight 100-200 kDa; Sigma, Deisenhofen, Germany) at a density of 150,000 cells/$cm^2$. In all experiments, culture medium was changed after 1 day to serum-free MEM/H12 medium (Gibco) supplemented with N2 (Gibco). After another 24 h, cells were used for experiments.

## 2.6 Establishment of CB1-expressing HT22 cells

### 2.6.1 Electroporation and selection

The mouse CB1 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Karlsruhe, Germany), which was then used for the stable transfection of CB1 into HT22 cells. CB1-expressing HT22 cell lines were generated by electroporation of linearized plasmid as described elsewhere (Adelsberger et al., 2000). In parallel, control cell lines were electroporated with empty pcDNA3. Stable transfectants were selected in medium containing geneticin (G418, 3.5 mg/ml; Calbiochem). Colonies of about 500 cells were picked (about 2 weeks after transfection) and allowed to be expanded, then tested for expression of CB1 mRNA by Northern blot. CB1-HT22 clones containing high levels of CB1 mRNA were tested for functional receptor properties by measurement of FRSK stimulated decrease of cAMP. CB1-HT22 cells were maintained under selection by adding G418 to culture medium every third passage.

### 2.6.2 Northern blot analysis

Standard Northern blotting protocols were used (Sambrook et al., 1989). Briefly, RNA extraction from transfected cell clones was performed using the PeqGold RNApure kit (PeqLab, Erlangen, Germany). Total RNA (20 µg) was loaded onto formaldehyde-containing 1% agarose gels, blotted onto nylon membranes (Hybond NX; Amersham, Freiburg, Germany), and immobilized by UV cross-linking (UV Stratalinker® 2400; Stratagene, Amsterdam, The Netherlands). Blots were prehybridized in rapid-hyb buffer (Amersham) and hybridized in the same solution containing [$^{32}$P]dCTP-labeled probe at 70°C according to manufacturer's instructions. Probe labeling of the full-length cDNA of CB1 was carried out

with a random primer DNA labeling system (Gibco BRL). Blots were exposed at -80°C for 1-2 days to Kodak Biomax films with intensifying screens.

### 2.6.3 cAMP accumulation assay

One day before experiment, cell clones expressing CB1 mRNA were plated onto 48-well plates in 500 µl of complete DMEM at the density of 2 x $10^5$ cells/ml. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Then, 0.5 mM IBMX was added 5 min before initiation of the reaction to prevent the degradation of accumulated cAMP. Cells were preincubated with WIN55,212-2 alone or in combination with SR141716A for 1 h. Then, 5 µM FRSK was added. The reactions were terminated 1 h later by aspiration of the medium and addition of 500 µl ice-cold 6% trichloroacetic acid followed by an overnight incubation at 4°C. DMSO alone served as vehicle control and had no effect on cAMP accumulation. To remove the trichloroacetic acid, the extracts were treated twice with 3 ml diethylether, dried overnight in a lyophilisator and reconstituted in DMEM. Intracellular cAMP levels were measured with a competitive protein binding assay (non-acetylated procedure; Perkin Elmer, Boston, USA). Data obtained in cAMP accumulation assay were expressed as the percentage of FRSK-stimulated cAMP accumulation. Samples were measured in duplicate in two independent experiments and data are given with standard error of mean (SEM).

## 2.7 Establishment of CB1-VR1 expressing HEK-293 cells

### 2.7.1 Electroporation and selection

The pcDNA3 plasmid used for electroporation containing the N-terminal hemaglutinin (HA)-tagged cDNA of rat CB1 (pcDNA3-CB1) was obtained from K. Mackie (University of Washington, Seattle, USA). pcDNA3-CB1 was linearized with XhoI and overhangs were blunted with Klenow. CB1 was released with Acc65I and subcloned into pZeoSV (Invitrogen, Karlsruhe, Germany) linearized with Acc65I and PvuII to obtain pZeoSV-CB1. Plasmid was checked by sequencing. For transfection into HEK-293 cells, pZeoSV-CB1 was linearized with NotI. CB1-VR1-expressing cell lines were generated by transfection of linearized pZeoSV-CB1 into HEK-293 already stably expressing hVR1 by electroporation as described in chapter 2.6.1. Stable transfectants were selected in medium containing Zeocin (0.6 mg/ml; Invitrogen) for CB1 selection and geneticin (G418, 2 mg/ml) for VR1 selection. Colonies of

about 500 cells were picked (about 2 weeks after transfection) and allowed to be expanded, then tested for expression of CB1 mRNA and protein by Northern blot and Western blot, respectively. CB1-VR1-HEK clones containing high levels of CB1 mRNA and protein were tested for functional receptor properties by measurement of FRSK-stimulated decrease of cAMP. CB1-VR1-HEK cells were maintained under selection by adding antibiotics to culture medium every third passage.

### 2.7.2 Northern blot analysis

Northern blotting was carried out as described in chapter 2.6.2.

### 2.7.3 Western blot analysis

For detection of CB1 receptor protein fused to a HA tag, transfected HEK-293 cells were solubilized in a glass homogenizer with 20 mM Tris-HCl (pH 7.4) containing protease inhibitor (Complete™ Mini tablets, Roche). The lysate was centrifuged for 5 min at 1000 xg, the supernatant was collected and assayed for protein content (Bio-Rad, Munich, Germany). Loading buffer (Roti®-load 1; Roth, Karlsruhe, Germany) was added to protein samples, which were denatured for 5 min at 95°C, centrifuged and loaded (20 µg/lane) on a 7.5 % polyacrylamide gel. After electrophoresis, proteins were transferred overnight at 4°C onto a cellulose nitrate membrane (Schleicher & Schüll, Dassel, Germany) with transfer buffer (48 mM Tris-HCl, 390 mM glycine, 0.1% SDS, 20% methanol), using a Bio-Rad blot apparatus. The membrane was blocked for 1 h with blocking buffer (10% non-fat milk powder, 20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, pH 7.6) and then incubated with an anti-HA monoclonal antibody (Santa Cruz, Heidelberg, Germany) diluted 1:200 in blocking buffer overnight at 4°C. After incubation with anti-mouse IgG-horseradish peroxidase as secondary antibody (Dako, Glostrup, Denmark) diluted 1:2000 in blocking buffer, chemiluminescence was performed using Lumi GLO™ reagent (Cell Signaling, Frankfurt am Main, Germany) according to manufacturer's instructions, and the blots were exposed to Biomax films for 1-10 min.

### 2.7.4 cAMP accumulation assay

One day before experiment, CB1-VR1-HEK cells were plated onto 48-well plates in 500 µl of complete DMEM at the density of 2 x $10^5$ cells/ml. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Reaction was initiated by adding

stimulation buffer containing 20 mM HEPES (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 0.1 mg/ml BSA, 5 µM FRSK, 0.5 mM IBMX and different concentrations of the CB1 receptor agonist HU210. FRSK and HU210 were dissolved in DMSO. DMSO alone served as a vehicle control and had no effect on cAMP accumulation. Reactions were terminated 10 min later by aspiration of the medium and the addition of 500 µl ice-cold 6% trichloroacetic acid followed by an incubation overnight at 4°C. Further procedure see chapter 2.6.3. Data obtained in cAMP accumulation assay were expressed as the percentage of FRSK-stimulated cAMP accumulation. Samples were measured in triplicates and data are given with SEM.

## 2.8 Intracellular calcium assay in CB1-VR1-expressing HEK-293 cells

The effect of test substances on intracellular $Ca^{2+}$ $[Ca^{2+}]_i$ in CB1-VR1-HEK and VR1-HEK cells was determined by using Fluo-3 methylester (Molecular Probes, Leiden, The Netherlands), a selective intracellular fluorescent probe for $Ca^{2+}$. One day prior to experiment, cells were transferred into six-well dishes coated with poly-L-lysine (Sigma) and grown in DMEM. On the day of the experiment, cells (50,000–60,000 per well) were loaded for 2 h at 25°C with 4 µM Fluo-3 methylester in DMSO containing 0.04% Pluronic (Molecular Probes). After the loading, cells were washed with Tyrode pH 7.4 (137 mM NaCl, 2.68 mM KCl, 1.8 mM $CaCl_2$ x 2 $H_2O$, 0.32 mM $NaH_2PO_4$ x $H_2O$, 5.56 mM glucose, 1.16 mM $NaHCO_3$), and trypsinized to be suspended in the cuvette of the fluorescence detector (Perkin-Elmer LS50B) under continuous mixing. Experiments were carried out by measuring cell fluorescence at 25°C ($\lambda_{EX}$=488 nm, $\lambda_{EM}$=540 nm) before and after the addition of the test compounds at various concentrations. The efficacy of the effect was determined by comparing it to the effect as observed with 4 µM ionomycin. Data for the compounds tested at varying concentrations were expressed as the concentration exerting a half-maximal effect ($EC_{50}$), calculated by using GraphPad® software. HU210 (50 nM) or FRSK (5 mM) were added, alone or together, 5 min before capsaicin. SR141716A (0.5 µM) was also added 5 min before HU210 or AEA. The PI-3-K and PLC inhibitors were added 5 min before HU210.

## 2.9 Experiments in primary cerebellar granule neurons

### 2.9.1 cAMP accumulation assay

Primary cells were grown in 24-well-plates, and stimulation compounds were added to serum free culture medium. IBMX was added 5 min before the initiation of the reactions to prevent degradation of accumulated cAMP. Reaction were initiated by addition of the CB1 agonist WIN55,212-2 ($10^{-6}$ M) and/or CRH ($10^{-8}$ M). The cells were incubated for 10 min with the drugs and reactions were terminated by aspiration of the medium and the addition of 1 ml ice-cold 6% trichloroacetic acid followed by an incubation overnight at 4°C. DMSO and 2% acetic acid alone served as a vehicle control and had no effect on cAMP accumulation. Further procedure see chapter 2.6.3. Data obtained in cAMP accumulation assay were expressed as the percentage of basal cAMP levels. Extracts from 2 wells were pooled and samples were measured in triplicates and data are given with SEM.

### 2.9.2 Semi-quantitative RT-PCR

Total RNA was isolated from primary cells after 48 h of treatment with WIN55,212-2 and/or CRH with peqGOLD RNAPure™ (Peqlab, Erlangen, Germany) according to manufacturer's instructions. 5 µg of total RNA was treated with 1 unit RNase-free DNaseI to digest contaminating genomic DNA, phenol/chloroform extracted, and used for Superscript II (Gibco BRL) reverse transcriptase-mediated synthesis of oligo(dT)12-18-primed (Roche, Mannheim, Germany) cDNA. The reaction was initiated with a 10 min incubation of primer and RNA at 70°C and kept on ice while adding the rest of the reaction mix. The reactions were then incubated for 10 min at 16°C, followed by an amplification cycle at 42°C for 50 min and terminated by a 5 min incubation at 94°C. The resulting cDNA was then subjected to Taq polymerase-catalyzed PCR. After a 5 min denaturation step at 94°C, a total of 28 cycles for hypoxanthineguanine phosphoribosyl transferase (HPRT) and 32 cycles for brain-derived neurotrophic factor (BDNF), respectively, of the following temperature and duration steps were carried out: 94°C for 1 min; 55°C for HPRT and 63°C for BDNF, respectively, for 1 min; 72°C for 1 min, with a 10 min extension at 72°C during the last cycle. The primers used to amplify BDNF corresponded to the following sequences: forward 5'-CTT TTG TCT ATG CCC CTG CAG CCT T-3'; reverse 5'-AGC CTC CTC TGC TCT TTC TGC TGG A-3'. The housekeeping gene HPRT was also amplified as an internal control using the primers: forward 5'-CCT GCT GGA TTA CAT TAA AGC ACT G-3'; reverse 5'-GTC AAG GGC ATA TCC

AAC AAA C-3'. The expected size of the amplicons was 297 bp for BDNF and 351 bp for HPRT. The PCR products were electrophoresed on 2% agarose gels. RNA without reverse transcription did not yield any amplicons, indicating that there was no contamination by genomic DNA (data not shown). For quantitative evaluation of data the optical density of PCR bands was measured with the Kodak-1D software. The results were calculated as ratio of optical density of the BDNF band vs. the HPRT band, and data are given with SEM.

### 2.9.3 Enzyme-linked immunosorbent assay (ELISA)

The Emax$^{TM}$ immunoassay system (Promega, Mannheim, Germany) was used to quantify the expression of BDNF protein in primary cells after 24 h or 48 h of treatment with WIN55,212-2 and/or CRH. Primary cells were grown in 6-well plates, and stimulation compounds were added to serum free culture medium for 24 h or 48 h. DMSO and 2% acetic acid alone served as a vehicle control and had no effect on BDNF expression. After stimulation, cells were lysed in 100 µl lysis buffer (137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1% NP-40; 10% glycerol; 0.5 mM sodium vanadate; protease inhibitor complete Mini tablets, Roche, Basel, Switzerland) per well and 3 wells were pooled per sample. Lysates were briefly sonificated and stored at -80°C. In order to standardize the total amount of protein from each experimental group used for ELISA, protein content was measured using the DC protein assay (Bio-Rad, Munich, Germany). Prior to each assay, lysates were acid-treated. This process increases the detectable amount of free BDNF in solution by dissociating it from its proforms or receptors (Okragly and Haak-Frendscho, 1997). Briefly, samples were acidified with 1 µl 1 M HCl/50 µl sample to have a pH less than 3.0. After 15 min incubation at RT, samples were neutralized with the same amount of 1 M NaOH to a pH of approximately 7.6. Maxisorp 96-well plates (Nunc, Neerijse, Belgium) were used for antibody coating and the ELISA was carried out according to manufacturer's instructions. Samples were measured in duplicates and data are given with SEM.

## 2.10 Statistical analysis

Data were analyzed by one way ANOVA using GraphPad software. Significance between groups was further analyzed using one of the following *post hoc* tests (Tukey, Bonferroni, Newman Keul). $P<0.05$ was considered as statistically significant.

# 3 RESULTS

## 3.1 CB1 and cross-talk with other receptor systems

### 3.1.1 Coexpression of CB1 with dopamine and serotonin receptors in the adult mouse forebrain

*Introduction*

CB1 displays unusual properties, including the dual capacity to inhibit or stimulate AC via $G_{i/o}$ or $G_s$ proteins (Bonhaus et al., 1998; Shire et al., 1999) and a brain density considerably higher than any other known G protein-coupled receptor (Herkenham et al., 1990). Implicit in these properties is the potential of CB1 to modulate the function of other receptor systems such as the dopamine and serotonin system. Indeed, pharmacological studies provide evidence for cross-talks between CB1 and receptors of these neurotransmitter systems (see chapter 1.2.1 and 1.2.2). The first indication to possible interactions between different receptors is given when both receptors are expressed within the same neuron. Indeed, CB1 in rodent forebrain structures (Tsou et al., 1998a; Marsicano and Lutz, 1999; Egertova and Elphick, 2000) displays a significant extent of overlapping expression with various dopamine and serotonin receptors, among which D1 (Mansour et al., 1991), D2 (Meador et al., 1989), 5-HT1B (Maroteaux et al., 1992) and 5-HT3 (Tecott et al., 1993) are the focus of the present study. In trying to obtain further insights into possible functional and/or structural interactions between CB1 and dopamine and serotonin receptors, double-*in situ* hybridization (ISH) are performed at the cellular level on mouse forebrain sections by combining a digoxygenin-labeled riboprobe for CB1 with $^{35}$S-labeled riboprobes for dopamine receptors D1 and D2, and for serotonin receptors 5-HT1B and 5-HT3, respectively.

*Results*

#### *3.1.1.1 CB1 and dopamine receptor D1*

The highest levels of D1 transcripts are observed in the basal ganglia (Mansour et al., 1991; Weiner et al., 1991), including caudate putamen, nucleus accumbens, and olfactory tubercle. High levels of low CB1-expressing cells are detected in the dorsolateral caudate putamen, while the nucleus accumbens contains only few low CB1-expressing cells. The olfactory tubercle shows an intense staining due to a high density of low CB1-expressing cells. Coexpressing cells were counted at a single cell resolution in the olfactory tubercle (Fig. 3-

1A) and caudate putamen (Fig. 3-1B, Table 3-1) but not in the nucleus accumbens, as the signals of CB1-positive cells were too weak. 46% of the medium-sized, CB1-positive neurons in the dorsolateral caudate putamen coexpressed D1. Considering all D1-positive cells containing CB1, the percentage reached 81%. In the olfactory tubercle, D1 mRNA is present in 90% of the CB1-expressing cells. The fraction of D1-positive cells containing CB1 was 76% (Table 3-1). D1 transcripts were observed in two regions of the cortex, but at much lower levels than in the striatum.

A striking finding was that none of the high CB1-expressing cells in these cortical areas contained D1. The highest levels of D1 transcripts were observed in the piriform cortex. This cortical region showed also a high number of low CB1-expressing cells and a sparse number of high CB1-expressing cells. Due to the uniform distribution of cells expressing low levels of D1 in this area, it was not feasible to count coexpressing cells at a single cell resolution. Thus, the numbers reflect an estimate only. 90% of CB1-positive cells in the piriform cortex contain D1 mRNA, whereas 70% of D1-expressing cells showed also signals for CB1 (Table 3-1). Coexpression of CB1 with D1 was also observed in the dorsal endopiriform nucleus where 89% of CB1-positive cells contain D1 mRNA and 69% of D1-positive cells coexpress CB1 (Table 3-1). In other cortical areas such as the neocortex, entorhinal/perirhinal cortex and amygdala as well as in non-cortical areas such as the hypothalamus, no signals for D1 transcripts were detected.

| | **Coexpression in cells expressing low CB1** | | |
|---|---|---|---|
| | % CB1 cells with D1 | % D1 cells with CB1 | (n) |
| Dorsolateral caudate putamen | 46 | 81 | (3798) |
| Olfactory tubercle | 90 | 76 | (102) |
| Piriform cortex | 90* | 70* | (n.c.) |
| Dorsal endopiriform nucleus | 89 | 69 | (94) |

**Table 3-1: Coexpression of D1 in low CB1-expressing neurons of the adult mouse forebrain.**
(n), total number of counted cells
n.c., not counted;
*, estimated percentages

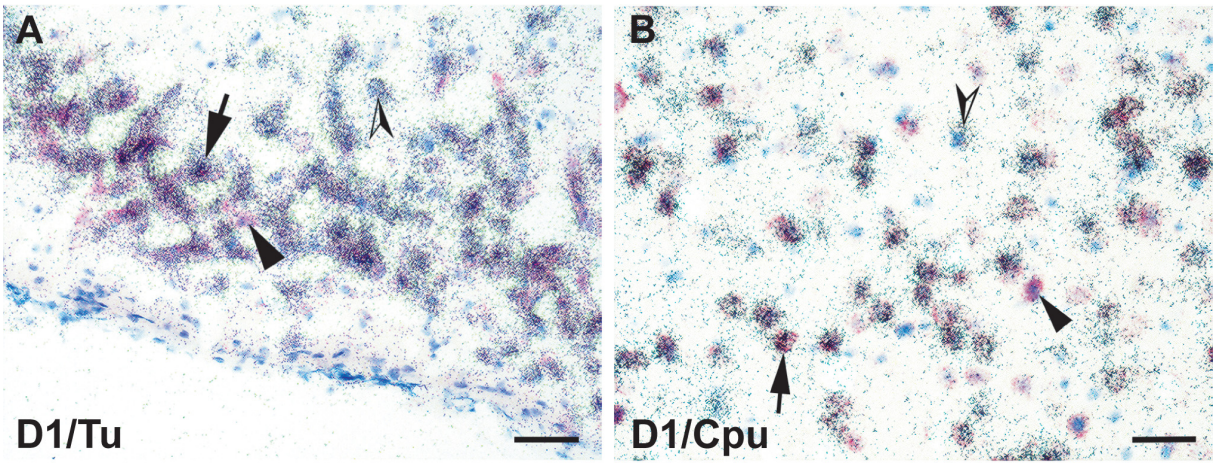


**Fig. 3-1: Bright field micrographs of coronal sections showing examples of coexpression of CB1 (red staining) with D1 (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with toluidine blue.**
(A) Coexpression of CB1 with D1 in the olfactory tubercle (Tu). (B) Coexpression of CB1 with D1 in the caudate putamen (Cpu). Filled arrow, low CB1-expressing cell coexpressing D1; filled arrowhead, low CB1-expressing cell; open arrowhead, D1-expressing cell. Scale bars, 50 µm.

#### *3.1.1.2 CB1 and dopamine receptor D2*

Similarly to D1, the strongest signals of D2 transcripts were detected in the caudate putamen, nucleus accumbens, and olfactory tubercle (Meador-Woodruff et al., 1989; Weiner et al., 1991). In the olfactory tubercle (Fig. 3-2A) and dorsolateral caudate putamen (Fig. 3-2B), 38% of the CB1-positive neurons coexpress D2 (Table 3-2). Higher percentages of coexpression in these two areas were evaluated considering all D2-positive cells containing CB1 with values of 73% in the striatum and 74% in the olfactory tubercle (Table 3-2). Compared to the basal ganglia, the level of D2 transcripts in cortical areas is much lower. D2-CB1 coexpression was detected only in low CB1-expressing cells. Rather high levels of D2 mRNA were observed in the piriform cortex. Due to the uniform distribution of D2-expressing cells in this area, the coexpression with CB1 was estimated to 90% considering all CB1-positive cells, and 70% considering all D2-positive cells, respectively (Table 3-2). In the entorhinal/perirhinal cortex, which contains high numbers of low and high CB1-expressing cells, neurons could be counted at a single cell resolution. 80% of CB1-positive cells coexpress D2, and 77% of D2-positive cells express CB1 (Fig. 3-2C, Table 3-2). In the hippocampus, CB1 signals with intensities ranging from low to very high were observed in all layers. Coexpression with D2 was detected in the polymorph layer of the dentate gyrus (Fig. 3-2D), where D2 hybridization signals were detected in 88% of the low CB1-expressing cells, but only 48% of all D2-positive cells do coexpress CB1 (Table 3-2). In other cortical areas such as the neocortex and amygdala D2 signals were not detected.

| | Coexpression in cells expressing low CB1 | | |
|---|---|---|---|
| | % CB1 cells with D2 | % D2 cells with CB1 | (n) |
| Dorsolateral caudate putamen | 38 | 73 | (4260) |
| Olfactory tubercle | 38 | 74 | (96) |
| Piriform cortex | 90* | 70* | (n.c.) |
| Entorhinal/perirhinal cortex area | 80 | 77 | (104) |
| Dentate gyrus (polymorph layer) | 88 | 48 | (376) |

**Table 3-2: Coexpression of D2 in low CB1-expressing neurons of the adult mouse forebrain.**
(n), total number of counted cells
n.c., not counted;
*, estimated percentages

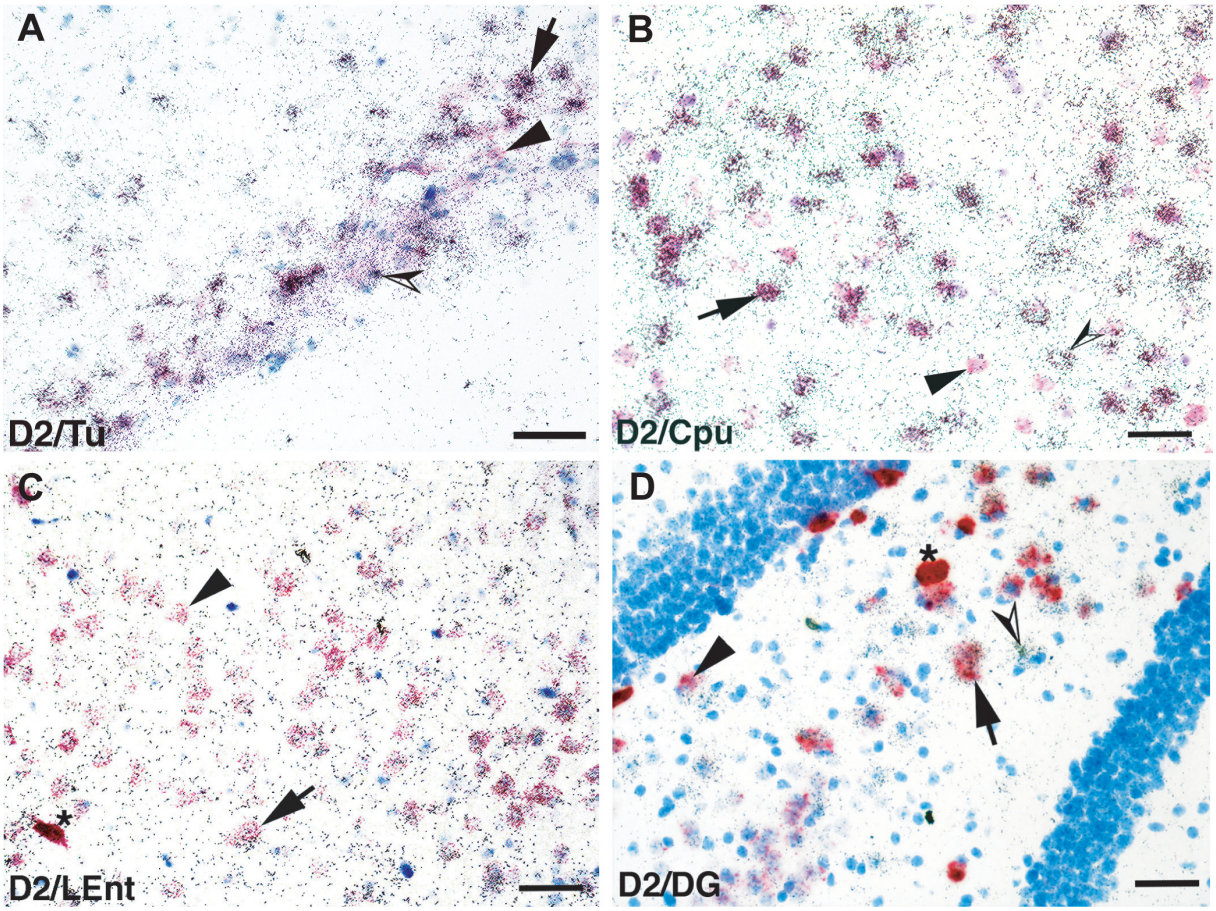


**Fig. 3-2: Bright field micrographs of coronal sections showing examples of coexpression of CB1 (red staining) with D2 (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with toluidine blue.**
(A) Coexpression of CB1 with D2 in the olfactory tubercle (Tu). (B) Coexpression of CB1 with D2 in the caudate putamen (Cpu). (C) Coexpression of CB1 with D2 in the lateral entorhinal cortex (LEnt). (D) Coexpression of CB1 with D2 in the dentate gyrus (DG). Filled arrow, low CB1-expressing cell coexpressing D2; filled arrowhead, low CB1-expressing cell; open arrowhead, D2-expressing cell; asterisk, high CB1-expressing cell. Scale bars, 50 µm.

### *3.1.1.3 CB1 and serotonin receptor 5-HT1B*

High expression levels of 5-HT1B mRNA were detected in striatum and olfactory tubercle, in agreement with the described expression pattern (Maroteaux et al., 1992). Percentages of coexpression in these regions are illustrated in Table 3-3. Evidently, the majority of CB1- and 5-HT1B-expressing cells in the dorsolateral part of the caudate putamen (Fig. 3-3A) and the olfactory tubercle show coexpression. Intense signals for 5-HT1B were also observed in the nucleus accumbens, where most of the cells express 5-HT1B (data not shown). Due to the low expression levels of CB1 in this area, coexpression could not be numerically evaluated by double-ISH, but CB1 is expressed in approximately 20% of the cells (Moldrich and Wenger, 2000). Thus, also in the nucleus accumbens, an estimate of 90% CB1-expressing cells coexpress 5-HT1B. Weaker signals for 5-HT1B were detected in the hippocampus, neocortex and hypothalamus, consistent with the known expression pattern (Maroteaux et al., 1992). In the pyramidal cells of hippocampal CA1 region, which express low levels of CB1 mRNA (Marsicano and Lutz, 1999), 100% coexpression was observed (Fig. 3-3D). 5-HT1B mRNA was observed in a scattered manner throughout layers II-III of the neocortex, whereas both low and high CB1-expressing cells were located primarily in layers II-III and V-VI. In layers II-III, at least 70% of all low CB1- and 5-HT1B-expressing cells show coexpression (Table 3-3, Fig. 3-3B), whereas high CB1-expressing neurons never express 5-HT1B. The ventromedial hypothalamic nuclei showed the presence of low CB1- and 5-HT1B-expressing cells that are uniformly distributed at high cell density. Coexpression was estimated to be more than 90% (Fig. 3-3C).

| | **Coexpression in cells expressing low CB1** | | |
|---|---|---|---|
| | % CB1 cells with 5-HT1B | % 5-HT1B cells with CB1 | (n) |
| Dorsolateral caudate putamen | 72 | 81 | (2983) |
| Olfactory tubercle | 70 | 77 | (226) |
| Hippocampal CA1 area** | 100* | 100* | (n.c.) |
| Layers II-III of neocortex | 70 | 74 | (1084) |
| Ventromedial hypothalamic nuclei | >90* | >90* | (n.c.) |

**Table 3-3: Coexpression of 5-HT1B in low CB1-expressing neurons of the adult mouse forebrain.**
(n), total number of counted cells
n.c., not counted;
*, estimated percentages;
**, principal neurons only

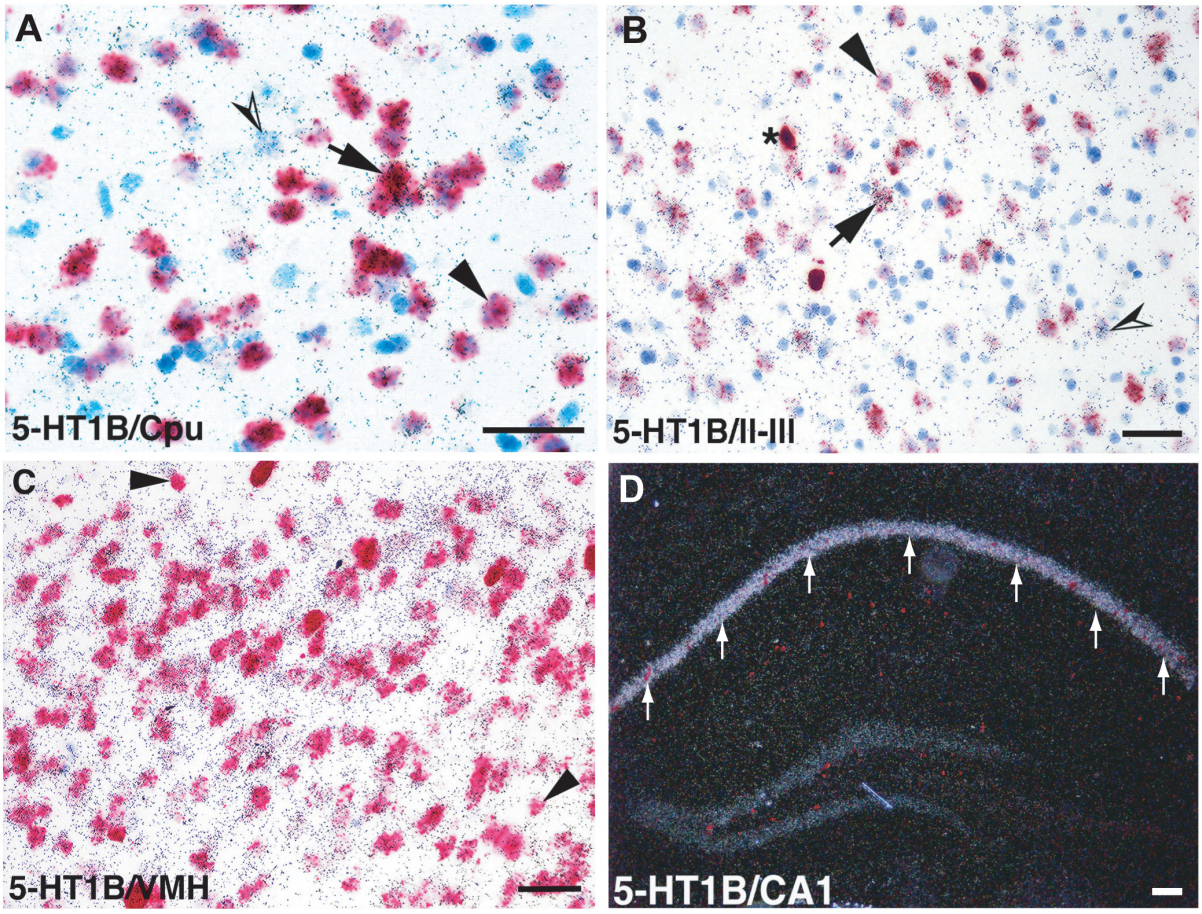


**Fig. 3-3: Bright and dark field micrographs of coronal sections showing examples of coexpression of CB1 (red staining) with 5-HT1B (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with toluidine blue.**
(A) Coexpression of CB1 with 5-HT1B in the caudate putamen (Cpu). (B) Coexpression of CB1 with 5-HT1B in layers II and III of neocortex (II-III). (C) Coexpression of CB1 with 5-HT1B in the ventromedial hypothalamus (VMH). (D) Coexpression of CB1 with 5-HT1B in CA1 region of hippocampus (CA1) indicated by small arrows. Low CB1-expressing cells are not visible in darkfield. Filled arrow, low CB1-expressing cell coexpressing 5-HT1B; filled arrowhead, low CB1-expressing cell; open arrowhead, 5-HT1B-expressing cell; asterisk, high CB1-expressing cell. Scale bars: 50 µm (A, B, C); 200 µm (D).

#### *3.1.1.4 CB1 and serotonin receptor 5-HT3*

Coexpression of CB1 and 5-HT3 was observed in several cortical regions for both low- and high CB1-expressing cells. As compared to CB1 and all other markers described above, the number of 5-HT3-expressing cells in the mouse forebrain is rather low (Tecott et al., 1993). Therefore, the percentages of coexpression considering low CB1-expressing cells that coexpress 5-HT3 are very low, in the range of 0.9% to 3.6%, for all described regions except of the hippocampal formation (Table 3-4). In the hippocampal CA1 and CA3 areas (excluding the pyramidal cells, which do express CB1 but not 5-HT3), the majority of both high and low CB1-expressing cells shows coexpression with 5-HT3, the extent being higher in CA3 (Fig. 3-4A). In the dentate gyrus, coexpression of 5-HT3 with low CB1-expressing cells is only 17%

(Fig. 3-4B). In all parts of hippocampus, the fraction of 5-HT3-expressing cells containing CB1 is between 35% and 39% for high CB1-expressing cells, thus, it is much higher than for low CB1-expressing cells (9% to 14%). This characteristic is also observed in all other forebrain regions analyzed, including neocortex (Fig. 3-4C), anterior olfactory nucleus, piriform cortex and entorhinal/perirhinal cortex. Regarding this feature, the basolateral amygdaloid nucleus is peculiar, as the extent of 5-HT3-expressing cells containing CB1 was approximately the same for high (21%) and low (24%) CB1-expressing cells (Table 3-4, Fig. 3-4D).

| | **Coexpression in cells expressing low CB1** | | | **Coexpression in cells expressing high CB1** | | |
|---|---|---|---|---|---|---|
| | % CB1 cells with 5-HT3 | % 5-HT3 cells with CB1 | (n) | % CB1 cells with 5-HT3 | % 5-HT3 cells with CB1 | (n) |
| Anterior olfactory nucleus | 1.0 | 19 | (1134) | 42 | 36 | (97) |
| Piriform cortex | 2.1 | 26 | (1056) | 41 | 32 | (117) |
| Entorhinal/perirhinal cortex area | 1.0 | 20 | (1715) | 40 | 31 | (165) |
| Neocortex, layers I-II | 2.2 | 22 | (1691) | 46 | 30 | (336) |
| Neocortex, layers III-IV | 0.9 | 20 | (1573) | 40 | 35 | (160) |
| Neocortex, layers V-VI | 3.6 | 18 | (609) | 41 | 35 | (187) |
| Hippocampus, CA1* | 77 | 13 | (313) | 73 | 35 | (480) |
| Hippocampus, CA3* | 96 | 9 | (275) | 82 | 35 | (409) |
| Dentate gyrus | 17 | 14 | (151) | 77 | 39 | (134) |
| Basolateral amygdaloid nuclei (anterior) | 2.8 | 24 | (767) | 31 | 21 | (129) |

**Table 3-4: Coexpression of 5-HT3 in CB1-expressing cells of the adult mouse forebrain.**
*, excluding principal neurons, which do not express 5-HT3; (n), total number of counted cells

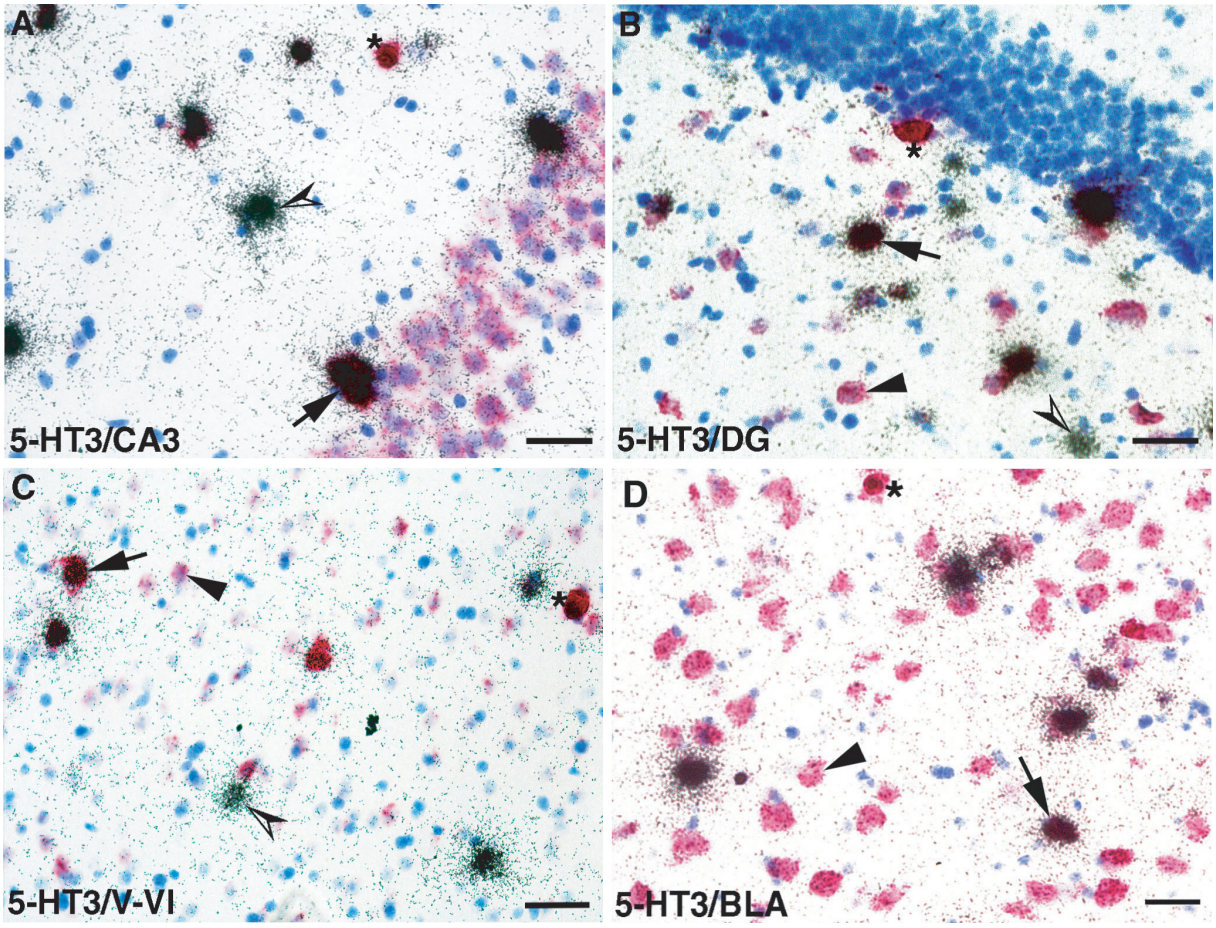


**Fig. 3-4: Bright field micrographs of coronal sections showing examples of coexpression of CB1 (red staining) with 5-HT3 (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with toluidine blue.**
(A) Coexpression of CB1 with 5-HT3 in the CA3 area of hippocampus (CA3). (B) Coexpression of CB1 with 5-HT3 in the dentate gyrus (DG). (C) Coexpression of CB1 with 5-HT3 in layers V and VI of neocortex (V-VI). (D) Coexpression of CB1 with 5-HT3 in the basolateral amygdala (BLA). Filled arrow, high CB1-expressing cell coexpressing 5-HT3; filled arrowhead, low CB1-expressing cell; open arrowhead, 5-HT3-expressing cell; asterisk, high CB1-expressing cell. Scale bars, 20 µm.

## *Conclusions*

CB1 is differentially coexpressed in the mouse forebrain with dopamine and serotonin receptors either in principal projecting neurons (mainly with D1, D2 and 5-HT1B) or in interneurons (mainly with 5-HT3). Together, these receptor systems might be involved in modulating excitatory circuits as well as inhibitory GABAergic circuits. Particularly in the striatum, high coexpression extent of CB1 with D1, D2 and 5-HT1B, respectively, were observed, suggesting putative cross-talks between the cannabinoid system and other neurotransmitter systems regulating locomotor activity. High levels of coexpressing cells in cortical areas might be an indication for a functional interaction of CB1 with dopamine and serotonin receptors, respectively, having modulatory effects on cannabinoid-induced impairment of working memory and cognitive functions.

### 3.1.2 Expression analysis of different marker genes in CB1-deficient mice

*Introduction*

Numerous signal transduction pathways have been shown to be involved in the action of cannabinoids, which exert most of their known effects through the CB1 receptor (summarized in chapter 1.1.2). However, the role of CB1 and its endogenous ligands in brain function and behavior has not yet been fully understood and remains to be investigated in more details. An area relatively unexplored is how this system affects cellular processes in the brain in terms of changes in expression levels of particular genes. CB1-deficient mice were shown to display significantly increased levels of substance P, dynorphin, enkephalin, and GAD 67 mRNAs in neurons of the striatum (Steiner et al., 1999). These findings demonstrate that elimination of CB1 receptors can result in functional reorganization of brain regions, such as the basal ganglia, which contains high levels of CB1 receptors. The dopamine receptors D1 and D2 as well as the serotonin receptors 5-HT1B and 5-HT3 were shown to be coexpressed with CB1 in several regions of the mouse forebrain (see chapter 3.1.1), which hints to a functional interaction between the cannabinoid system and these neurotransmitter systems. Therefore, dopamine and serotonin receptors might be involved in cannabinoid receptor-mediated signaling and disregulation of expression of these receptor genes in CB1-deficient mice would be conceivable. Following this approach, CB1-deficient mice were generated as described (Marsicano et al., 2002b) and examined for altered expression of the receptors mentioned above. In addition, neurotransmitter-related enzymes, and neuropeptides, which all are associated with the cannabinoid system were investigated for the same purpose. To identify changes in mRNA expression of these candidate genes, ISH was carried out on brain sections of CB1-deficient mice ($CB1^{-/-}$) and expression levels of the different transcripts were compared to wild-type littermates ($CB1^{+/+}$). Expression intensities were evaluated by densitometric quantification from autoradiographic films with the NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/Default.html).

*Results*

Three different G protein-coupled receptors and one cation channel were included into the analysis of putative expression differences in $CB1^{-/-}$ and $CB1^{+/+}$ animals. The dopamine receptors D1, D2 and the serotonin receptors 5-HT1B, 5-HT3 have been shown to colocalize with CB1 in various forebrain regions (see chapter 3.1.1). Together with other studies, these observations suggest a functional cross-talk between CB1 and these neurotransmitter systems (summarized in chapter 1.2.1 and 1.2.2). ISH using $^{35}S$-labeled riboprobes for each of these

receptors mentioned above revealed no expression differences between $CB^{-/-}$ and $CB1^{+/+}$ animals (Fig. 3-5A, A', B, B', C, C', D, D').

γ-aminobutyric acid (GABA) is best known as one of the classical neurotransmitters in the CNS, where it has a predominantly inhibitory function. It is involved in a variety of biological functions such as locomotor activity, learning, reproduction and circadian rhythms. Regulation of GABA-mediated signaling involves several mechanisms, among which modulation of GABA synthesis by the rate-limiting enzyme glutamate decarboxylase (GAD) plays a central role. Molecular cloning studies have shown that in the adult brain, GAD exists as two major isoforms, called GAD65 and GAD67, which are the products of two independently regulated genes (Erlander et al., 1991). Anatomical studies have shown that the two GAD genes are coexpressed in most GABA-containing neurons of the CNS (Feldblum et al., 1993; Esclapez et al., 1994). The effects of cannabinoids on memory pocesses are generally believed to be due to the interaction of CB1 with the GABAergic system (Terranova et al., 1995). High levels of coexpression of GAD65 and CB1 in the mouse forebrain (Marsicano and Lutz, 1999) underline the notion that the endocannabinoid system functionally interacts with the GABAergic system. Moreover, Steiner et al. (1999) showed altered levels of GAD67 mRNA in the striatum of CB1-deficient mice. In contrast, ISH with $^{35}$S-labeled probes for GAD65 and GAD67 carried out in this study did not show different expression levels between $CB1^{-/-}$ and $CB1^{+/+}$ animals (Fig. 3-5E, E', F, F').

The enzyme FAAH plays a central role in regulating the levels and activity of both exogenously administered and endogenously produced AEA (Deutsch and Chin, 1993; Deutsch et al., 2002). The greatly elevated brain levels of AEA observed in FAAH-deficient mice (Cravatt et al., 2001), coupled with the enhanced CB1-dependent analgesia displayed by these animals, promote FAAH as a key regulator of endocannabinoid signaling *in vivo*. Moreover, the endocannabinoid system seems to be disregulated in the hippocampus of CB1-deficient mice, as levels of AEA decrease and FAAH activity increases with age in these animals (Maccarrone et al., 2002). Being one of the key components of the endocannabinoid system, the expression of FAAH might be directly or indirectly regulated by CB1. Using a $^{35}$S-labeled riboprobe for FAAH, ISH demonstrated no differences in signal intensity between $CB^{-/-}$ and $CB1^{+/+}$ mice (Fig. 3-5G, G').

Cholecystokinin (CCK) is a 33 amino acid peptide belonging to the family of gut-brain peptides. Following its identification in the mammalian brain, anatomical and biochemical studies have supported a role for this neuropeptide as a neurotransmitter and/or neuromodulator of classic neurotransmitter action in the CNS. In fact, CCK is synthesized in

neurons and concentrated in synaptic vesicles and is released through sodium and calcium-dependent mechanisms (Emson et al., 1980). In addition, high-affinity binding sites for radiolabeled CCK in the brain of rodents and humans have also been shown (Hays et al., 1981). Many functions which CCK is involved in, are also modulated by cannabinoids, e.g. learning and memory, mediation of painful stimuli and feeding (Crawley and Corwin, 1994). Moreover, both CCK and cannabinoids seem to interact with the dopaminergic and opioid system (Crawley, 1991). A high degree of coexpression for CB1 and CCK was detected in GABAergic interneurons in the forebrain (Marsicano and Lutz, 1999; Tsou et al., 1999). Given these findings, it is tempting to speculate about a possible functional cross-talk between the two systems, e.g. cannabinoids might have an effect on production, processing or release of CCK peptides. Therefore, $CB^{-/-}$ and $CB1^{+/+}$ mice were investigated for possible differences in expression of CCK. ISH with a $^{35}$S-labeled probe for CCK showed no expression differences between genotypes (Fig. 3-5H, H').

Nitric oxide (NO) is produced intracellularly by three isoforms of nitric oxide synthase: the endothelial (eNOS), inducible (iNOS), and neuronal NOS (nNOS; Garthwaite and Boulton, 1995; Huang and Lo, 1998). NO is considered to participate in a variety of physiological and pathological processes such as neuronal plasticity and neurotoxicity (Dawson and Dawson, 1998). Various studies suggest a link between cannabinoid signaling and the NO pathway, e.g. ISH revealed significantly lower levels of CB1 mRNA in the ventromedial hypothalamus and the caudate putamen in nNOS-deficient mice compared to $CB1^{+/+}$ animals (Azad et al., 2001). In cerebellar granule cells it was shown that KCl-induced activation of nNOS was inhibited by various cannabinoids in a pertussis toxin-sensitive manner (Hillard et al., 1999). In light of the widespread role of NO as a modulatory agent in the brain, it is likely that NOS inhibition plays a role in the overall effects of cannabinoids on brain function. Thus, changes of nNOS expression in $CB1^{-/-}$ mice would be conceivable. ISH using $^{35}$S-labeled riboprobes for nNOS revealed no expression differences between $CB^{-/-}$ and $CB1^{+/+}$ animals (Fig. 3-5I, I').

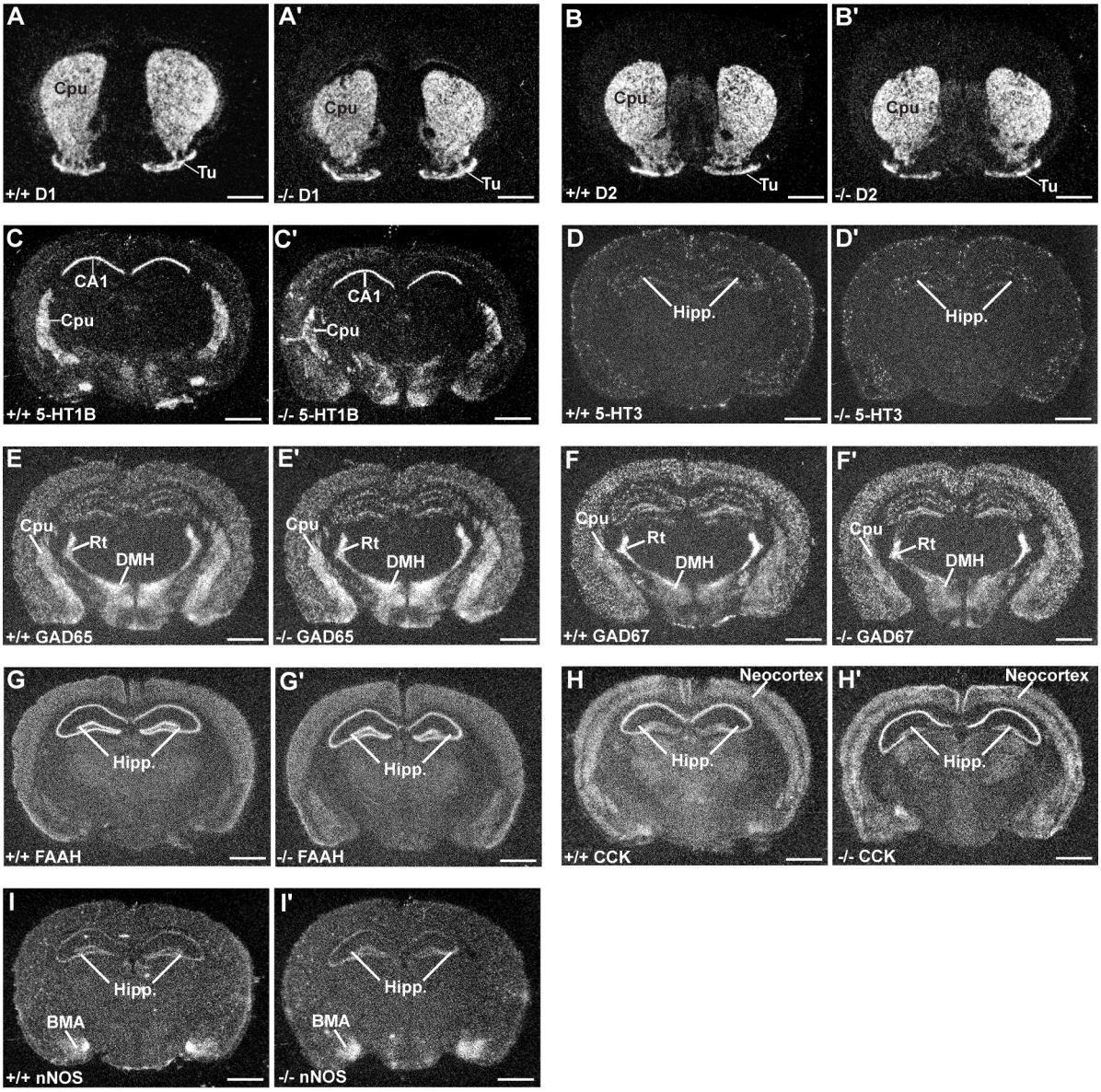


**Fig. 3-5: Expression analysis of different genes in brains of wild-type (+/+) and CB1-deficient (-/-) animals carried out by *in situ* hybridization.**
Darkfield micrographs of representative brain regions are shown for each gene indicated. No apparent differences in expression were found between $CB1^{+/+}$ and $CB1^{-/-}$ animals for all genes examined. D1 expression in $CB1^{+/+}$ (A) and $CB1^{-/-}$ (A') animals; D2 expression in $CB1^{+/+}$ (B) and $CB1^{-/-}$ (B') animals; 5-HT1B expression in $CB1^{+/+}$ (C) and $CB1^{-/-}$ (C') animals; 5-HT3 expression in $CB1^{+/+}$ (D) and $CB1^{-/-}$ (D') animals; note the scattered expression pattern. GAD65 expression in $CB1^{+/+}$ (E) and $CB1^{-/-}$ (E') animals; GAD67 expression in $CB1^{+/+}$ (F) and $CB1^{-/-}$ (F') animals; FAAH expression in $CB1^{+/+}$ (G) and $CB1^{-/-}$ (G') animals; CCK expression in $CB1^{+/+}$ (H) and $CB1^{-/-}$ (H') animals; nNOS expression in $CB1^{+/+}$ (I) and $CB1^{-/-}$ (I') animals. Abbreviations: basomedial amygdala (BMA), CA1 region of hippocampus (CA1); Caudate putamen (Cpu), dorsomedial hypothalamic nucleus (DMH), hippocampal region (Hipp.), olfactory tubercle (Tu), reticular thalamic nucleus (Rt). Scale bars: 1 mm.

## *Conclusions*

In the present study, the effects of CB1 receptor deletion on expression of various receptors, neurotransmitter-related enzymes and neuropeptides, known to be linked to the cannabinoid system, were investigated. Therefore, ISH was used to compare expression levels of these

marker genes between CB1-deficient mice and their wild-type littermates. As none of these genes showed altered levels of mRNA transcripts between genotypes, these results suggest that CB1 receptors have no effect on expression of the genes in basal conditions. However, this does not exclude the possibility that, upon activation of the cannabinoid system, signaling pathways are disregulated under certain circumstances.

### 3.1.3 Expression of VR1 in the adult mouse forebrain

*Introduction*

The vanilloid receptor 1 (VR1) is a non-selective cation channel belonging to a heterogenous family of nociceptors. VR1 is activated by capsaicin, the pungent ingredient of red hot chilli peppers (reviewed in Szallasi and Blumberg, 1999). The endocannabinoid AEA was discovered as its endogenous ligand and acts as a full agonist at VR1 receptors (Zygmunt et al., 1999; Smart et al., 2000), which gives reason to suggest a functional interaction between the vanilloid system and the cannabinoid system. Although the presence of VR1 is firmly established in DRGs, trigeminal and nodose ganglia (Szallasi and Blumberg, 1999; Michael and Priestley, 1999; Ichikawa and Sugimoto, 2001), few studies exists about VR1 expression in the brain where a cross-talk with the CB1 receptor would be of potential interest. There is a moderate level of specific binding of the capsaicin analog resiniferatoxin to membranes obtained from various CNS areas (Acs et al., 1996), and RT-PCR detects VR1 mRNA widely in the brain (Sasamura et al., 1998). Using ISH and immunhistochemistry (IHC), expression of VR1 mRNA and protein was detected in several regions of the rat and human brain (Mezey et al., 2000). However, a detailed expression analysis of VR1 in the mouse brain has not been carried out so far and would provide hints to regions in which VR1 is coexpressed with CB1. Therefore, IHC was performed using two commercially available polyclonal antibodies raised against the N- or C-terminus, respectively, (cat.no. ab901: Abcam, Cambridge, UK; cat.no. ab5370: Chemicon, Temecula, CA, USA) to localize VR1-expressing cells in the forebrain of the mouse.

*Results*

Preliminary ISH-experiments using a $^{35}$S-labeled riboprobe to localize VR1 mRNA did not give any detectable signal on brain sections of wild-type C57BL/6N adult animals which might be due to instability of VR1 transcripts. In the next approach, thus, two antibodies were used to detect VR1 protein. Both antibodies showed identical staining patterns on brain sections from wild-type C57BL/6N adult mice ($VR1^{+/+}$). Corresponding brain sections from

VR1-deficient ($VR^{-/-}$) mice were stained with the same antibodies and served as negative controls. The absence of signals on sections of wild-type mice incubated with the secondary antibody only demonstrated also the specifity of the IHC. In cortical areas, neurons were stained, e.g. in the cingulate cortex, motor cortex (Fig. 3-6A), retrosplenial agranular and granular cortex, somatosensory cortex and piriform cortex. In the limbic system, VR1-positive cells were detected in the hippocampus and the amygdala. Specifically, the pyramidal cell layer of CA1 and the granular layer of dentate gyurs showed a distinct immunolabeling (Fig. 3-6B), whereas only a few cells were visible in the basomedial amygdala. In the hypothalamus, there were numerous immunopositive cells in the lateral hypothalamic area, the ventromedial hypothalamic nucleus and the arcuate hypothalamic nucleus. Also, many immunostained cells were detected in the paraventricular thalamic nucleus and the lateral post thalamic nucleus. In the mesencephalon, the pars compacta of the substantia nigra showed many VR1-positive cells (Fig. 3-6C), whereas only a few cells were detected in the reticular formation. Experiments carried out on brain sections of $VR1^{-/-}$ mice did not show any of these signals (Fig. 3-6A', B', C'), and thus demonstrating the specificity of the two antibodies used in this investigation.

In general, VR1-immunoreactivity was found inside the somata and most proximal dendrites at the light microscopic level. For a detailed subcellular localization further experiments using fluorescent secondary antibodies are necessary. In addition, double-IHC using an antibody for specific neuronal population together with the VR1 antibody would reveal expression of the VR1 receptor in defined neuronal subpopulation of the mouse brain.

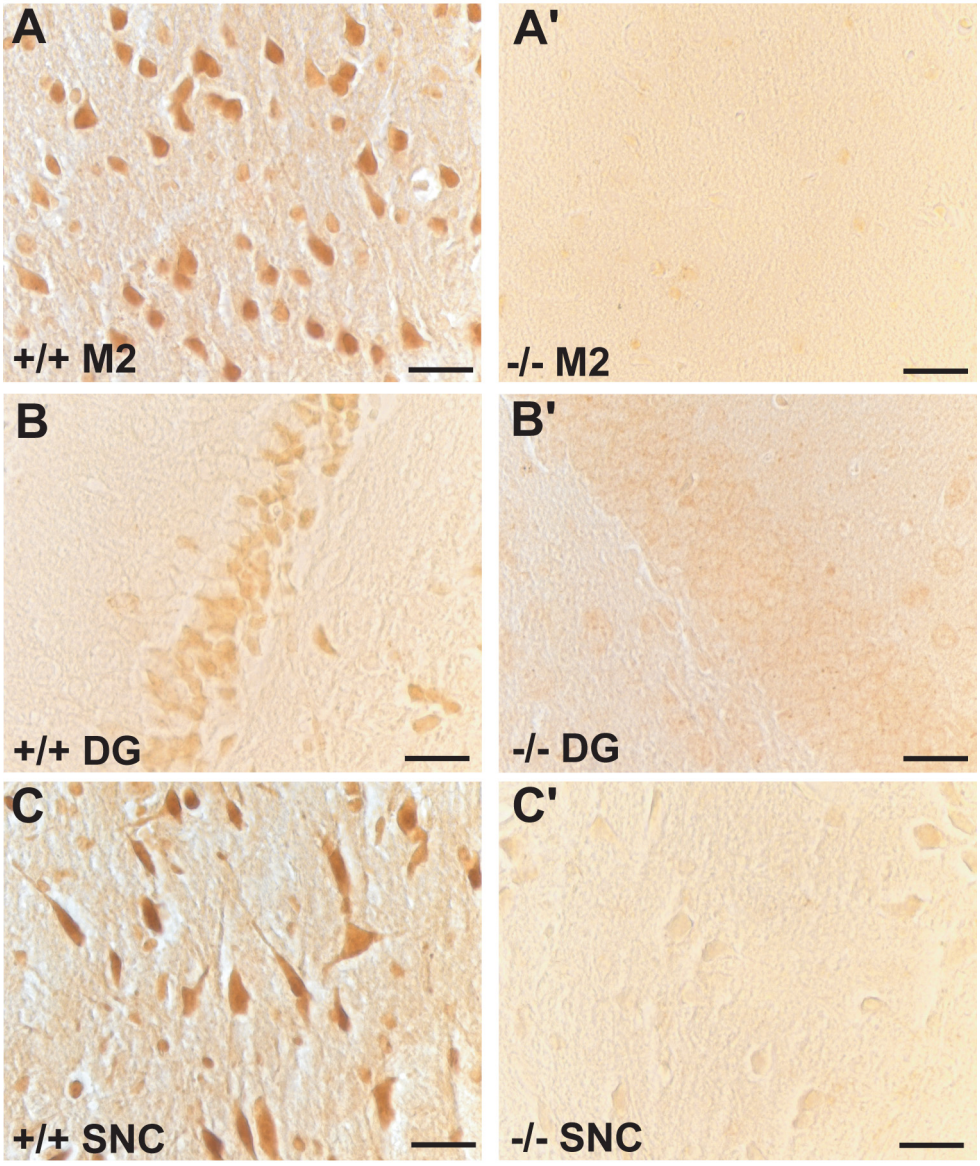


**Fig. 3-6: Bright field micrographs of coronal sections showing expression of VR1 (brown staining) in different regions of the forebrain of C57BL/6N mice (+/+) as detected by immunhistochemistry. VR1$^{-/-}$ mice (-/-) served as negative control.**
Expression of VR1 in the motor cortex 2 (M2; A), dentate gyrus (DG; B) and substantia nigra pars compacta (SNC; C) of C57BL/6N mice. Signals were totally absent in the corresponding brain region of VR1$^{-/-}$ mice (A', B', C'). Scale bars: 50 µm.

## *Conclusions*

VR1-expressing neurons exist in several areas of the mouse brain where also CB1 receptors are known to be present, e.g. neocortex, hippocampus, ventromedial hypothalmus, substantia nigra. These neurons may participate in various, as yet unexplored vanilloid-sensitive pathways and may be regulated by the endogenous compound AEA, which is at the same time a key controller of the endocannabinoid system. Thus, besides the perception of noxious stimuli, VR1-mediated signaling might also control higher brain functions, such as learning and memory, possibly in concert with the cannabnoid system.

### 3.1.4 VR1-induced increase in intracellular calcium is differentially regulated by CB1 activation

*Introduction*

There is evidence for a functional relationship between CB1 and the cation channel VR1. The two receptors are colocalized in many sensory C-fibers, both at the level of the spinal cord, and in DRGs, and there is now evidence for the coexistence of CB1 and VR1 receptors also in the brain. In chapter 3.1.3 it was shown that VR1 protein is present in many areas of the mouse brain which are involved in the control of motor (substantia nigra), cognitive and mnemonic (hippocampus, cortex), and emotional (amygdala) functions. CB1 receptors are known to be expressed in the same regions of the brain where they participate in the regulation of brain functions mentioned above (summarized in chapter 1.1.5). Several studies have been shown that stimulation of CB1 influences VR1-mediated signaling in different manners (see chapter 1.2.3) which might depend on the localization of the receptors either within the same cell or in neighboring neurons. Therefore, in the present study, a cell culture system was established, in which CB1 and VR1 are coexpressed within the same cells to investigate the effect of CB1 receptor stimulation on VR1-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$).

*Results*

#### *3.1.4.1 Double-transfected HEK-293 cells express functional CB1 receptors*

For the investigation of putative signaling interactions between CB1 and VR1, a HEK-293 (human embryonic kidney) cell line, already stably expressing the human VR1 receptor (VR1-HEK cells), was cotransfected with an expression vector containing the whole rat CB1 cDNA fused to the DNA of the hemaglutinin tag. To confirm the presence of CB1 in these double-transfected cell lines (CB1-VR1-HEK cells), Northern and Western blot experiments were performed. Hybridization signals were detected at approximately 6.0 kb for cortex RNA (used as positive control) as previously described (Matsuda, 1997) and at approximately 1.6 kb for Zeocin-resistant CB1-transfected clones, while no band was observed using RNA of cells transfected only with human VR1 (hVR1; Fig. 3-7A). To test whether receptor mRNA is effectively translated into receptor protein, Western blot analysis was carried out and showed a band of the expected size of 80 kD for the hemagglutinin-CB1 fusion protein (Fig. 3-7B), which was not observed by blocking the antigen recognition site of the antibody with the immunizing peptide (data not shown). Two clones of CB1-VR1-HEK cells (#10 and #15)

expressing high levels of mRNA and protein were tested for functional receptor properties. Both clones exhibited functional coupling to $G_i$ proteins, as demonstrated by the inhibition of forskolin (FRSK)-stimulated intracellular cAMP accumulation by WIN55,212-2 (Fig. 3-7C) and HU210 (Fig. 3-7D), whereas VR1-HEK cells did not show any response upon stimulation with WIN55,212-2 (Fig. 3-7C). These two CB1-VR1-HEK clones were subsequently used for the experiments carried out in this study.

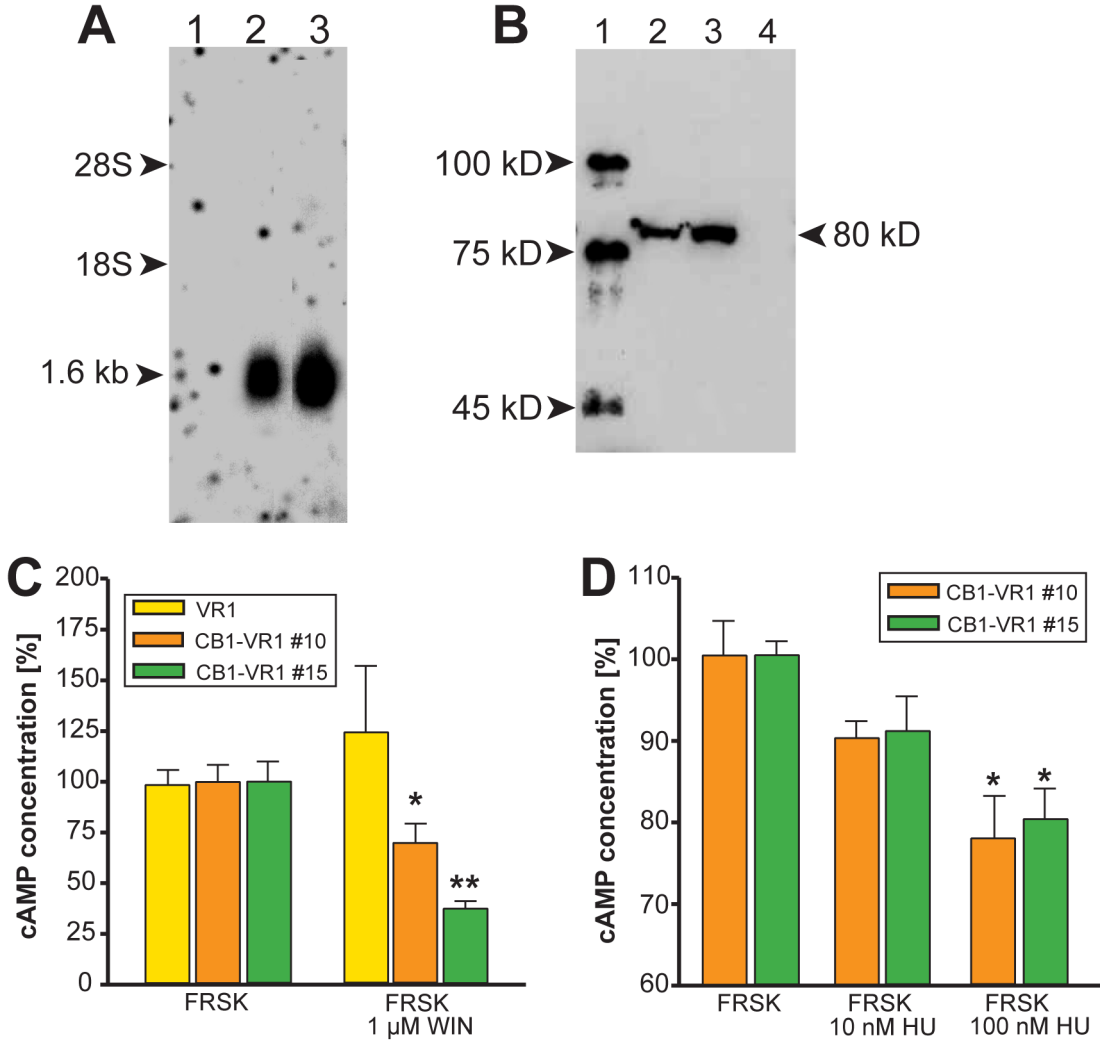


**Fig. 3-7: Analysis of CB1 expression in CB1-VR1-HEK cells.**
(A) Northern blot showing CB1 mRNA in the two different clones of CB1-VR1-HEK cells (lane 2, clone #10; lane 3, clone #15); VR1-HEK cells served as a negative control (lane 1). Ribosomal RNA was used as molecular weight marker (28S, ~4.6 kb; 18S ~1.9 kb). (B) Western blot showing CB1 protein in the same CB1-VR1-HEK clones (lane 2, clone #10; lane 3, clone #15); VR1-HEK cells served as a negative control (lane 4); HA-tagged protein was used as molecular-weight standard (lane 1). (C) Effect of WIN55,212-2 (WIN) on forskolin (FRSK)-induced cAMP accumulation in clone #10 (CB1-VR1 #10) and clone #15 (CB1-VR1 #15) of CB1-VR1-HEK cells, and in cells expressing only VR1 (VR1). (D) Effect of HU210 (HU) on FRSK-induced cAMP accumulation in clone #10 (CB1-VR1 #10) and clone #15 (CB1-VR1 #15) of CB1-VR1-HEK cells. Data are expressed as percentages of the effect of FRSK and are means ± SEM of n=3 independent experiments. *, $P<0.05$ and **, $P<0.01$ vs. FRSK.

### *3.1.4.2 Effect of HU210 on capsaicin response in CB1-VR1-HEK cells*

The increase in $[Ca^{2+}]_i$ is a well-known response of the VR1 agonist capsaicin (Szallasi and Blumberg, 1999) and can be measured by loading VR1-expressing cells with $Ca^{2+}$-sensitive dyes (see chapter 2.8) upon stimulation with capsaicin. The effect of capsaicin on $[Ca^{2+}]_i$ in CB1-VR1-HEK cells (clone #10) is shown in Fig. 3-8A. The compound enhanced $[Ca^{2+}]_i$ in a dose-dependent manner, with an $EC_{50}$=35.0 ± 4.0 nM (mean ± SEM, n=3) that was undistinguishable from that observed in HEK cells overexpressing only VR1 ($EC_{50}$=32.1 ± 5.0 nM, mean ± SEM, n=3). To examine whether the costimulation of CB1 has an effect on VR1-induced increases of $[Ca^{2+}]_i$, the CB1 receptor agonist HU210 was added to the CB1-VR1-HEK cells 5 min before the addition of capsaicin. HU210, at a concentration (100 nM) shown to be fully effective on CB1 receptors (Pertwee, 1997) and shown here to inhibit FRSK-induced cAMP formation in the same cells (Fig. 3-7D), significantly enhanced the effect on $[Ca^{2+}]_i$ of 10-50 nM capsaicin (Fig. 3-8A) when applied 5 min before the stimulation with capsaicin. HU210 alone had no effect on basal $[Ca^{2+}]_i$ (data not shown). The $EC_{50}$ for the effect of capsaicin was decreased from 35.0 ± 4.0 to 17.0 ± 2.1 nM (mean ± SEM, n=6, $P<0.05$). This effect was antagonized by the CB1 receptor antagonist, SR141716A (Fig. 3-8B), at a dose (0.5 μM) selective for CB1 receptors and devoid *per se* of any effect on $[Ca^{2+}]_i$ (data not shown). The effect of HU210 was not observed in VR1-HEK cells (data not shown). HU210 effect was observed also in a second clone (clone #15) of CB1-VR1 HEK cells, which again responded to capsaicin to the same extent as VR1-HEK cells ($EC_{50}$= 27.0 ± 4.3 nM, mean ± SEM, n=3). In these cells, the CB1 agonist decreased the $EC_{50}$ for the effect of capsaicin to 14.5 ± 1.5 nM (mean ± SEM, n=3, $P<0.05$ by ANOVA). Interestingly, simultaneous treatment of CB1-VR1-HEK cells (clone #10) with HU210 and capsaicin did not lead to a potentiation of the effect on $[Ca^{2+}]_i$ of the latter compound (data not shown).

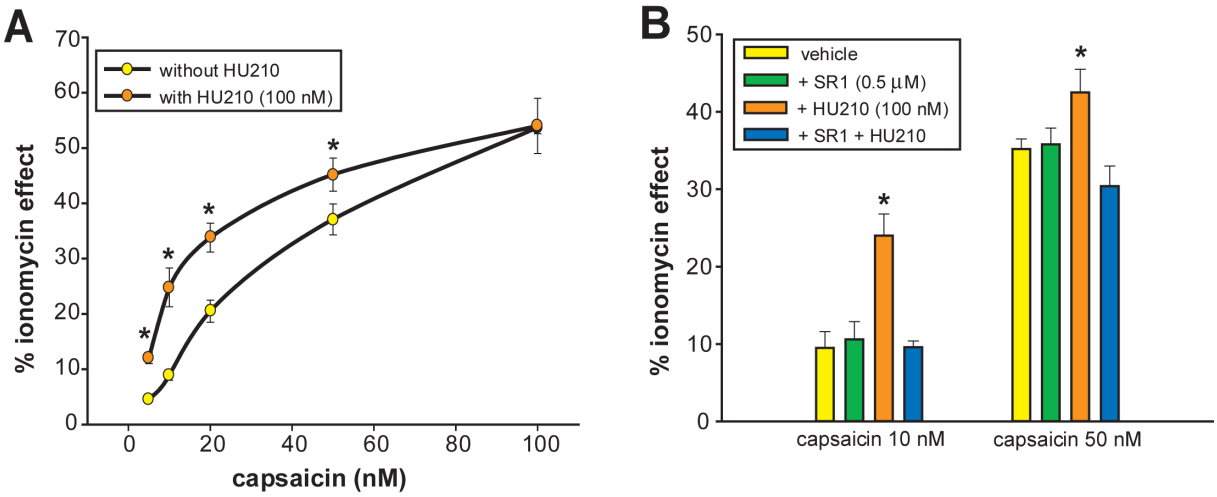


**Fig. 3-8: HU210 enhances VR1-mediated capsaicin effect on intracellular $Ca^{2+}$ in CB1-VR1-HEK cells via a CB1 receptor-mediated mechanism.**
(A) Dose-response for the VR1-mediated effect of capsaicin on intracellular $Ca^{2+}$ with or without HU210 pretreatment (100 nM) of cells (clone #10). (B) Reversal by the CB1 antagonist SR141716A (SR1; 0.5 µM) of HU210-induced potentiation of VR1-mediated capsaicin effect on $[Ca^{2+}]_i$. Data are expressed as percent of the effect of ionomycin (4 µM) and are means ± SEM of at least n=3 independent experiments carried out in duplicate. *, $P<0.05$ vs. vehicle.

### *3.1.4.3 Effect of various inhibitors on HU210 potentiation of capsaicin response*

To reveal a possible mechanism that underlies the reinforcing effect of HU210 on capsain-induced increases in $[Ca^{2+}]_i$ in CB1-VR1-HEK cells, several intracellular signaling pathways, known to be involved in CB1-mediated signaling, were blocked through inhibiting the corresponding enzymes. The two selective inhibitors of phosphatidyl-inositol-3-kinase (PI-3-K), wortmannin (1 µM) and LY294002 (2.5 µM), the two inhibitors of phosphatidyl-inositol-selective phospholipase (PI-PLC), ET18 (1 µM) and U73122 (2 µM), and the inhibitor of phosphatidyl-choline-selective phospholipase (PC-PLC), D609 (10 µM) were inactive *per se* on the response induced by capsaicin alone (Fig. 3-9). When CB1-VR1-HEK cells (clone #10) were preincubated with HU210 (100 nM), all inhibitors completely blocked the HU210 potentiation of capsaicin effect (Fig. 3-9).

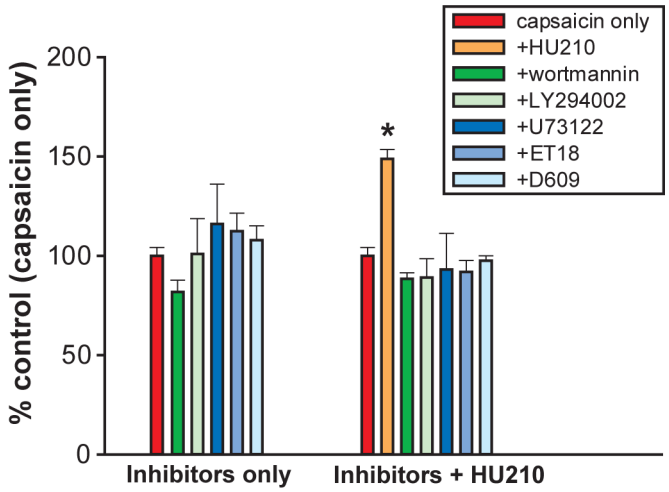


**Fig. 3-9: Effect of various inhibitors of capsaicin action on intracellular $Ca^{2+}$ in CB1-VR1-HEK cells.**
The dose of capsaicin used was 20 nM and led to an increase of intracellular $Ca^{2+}$ concentration to 22.9 ± 1.1% (mean ± SEM, n=12) of the maximal effect of ionomycin (4 µM). The effects of the inhibitors, which were given to cells 5 min before capsaicin, are expressed as percent of the effect of capsaicin alone and are means ± SEM of at least n=3 independent experiments carried out in duplicate. Different inhibitors were tested both on capsaicin alone and on capsaicin + HU210 (100 nM), and the dose was 1 µM for wortmannin (inhibitor of PI-3-K) and ET18 (inhibitor of PI-PLC), 2 µM for U73122 (inhibitor of PI-PLC), 2.5 µM for LY294002 (inhibitor of PI-3-K), and 10 µM for D609 (inhibitor of PC-PLC). The effect of HU210, which was given 5 min before the inhibitors, on capsaicin is also shown as percent of the effect of capsaicin alone. None of the inhibitors *per se* caused any significant change of basal $[Ca^{2+}]_i$. *, $P<0.05$ vs. capsaicin only. Note that the inhibitors used abolished the potentiation of capsaicin by the HU210 pretreatment.

### *3.1.4.4 Effect of anandamide on CB1-VR1-HEK and VR1-HEK cells*

Anandamide (AEA), one of the endogenous ligands of CB1, was discovered to act as full agonist also at VR1 receptors (Zygmunt et al., 1999; Smart et al., 2000). Therefore, the coexpression of CB1 and VR1 in the same cell could lead to a different potency of AEA to increase $[Ca^{2+}]_i$ by activating VR1. To investigate this hypothesis, the effect of AEA on $[Ca^{2+}]_i$ was compared in CB1-VR1-HEK and VR1-HEK cells. Unlike capsaicin, AEA was significantly more efficacious in CB1-VR1-HEK cells (clone #10) than in VR1-HEK cells at the two highest concentrations tested (Fig. 3-10). Importantly, after pretreatment of CB1-VR1-HEK cells with a concentration of SR141716A (0.5 µM) selective for CB1 (De Petrocellis et al., 2001a), the effect of AEA became identical to that observed in VR1-HEK cells (Fig. 3-10). AEA was also more potent and efficacious in clone #15 of CB1-VR1-HEK cells than in VR1-HEK cells (data not shown).

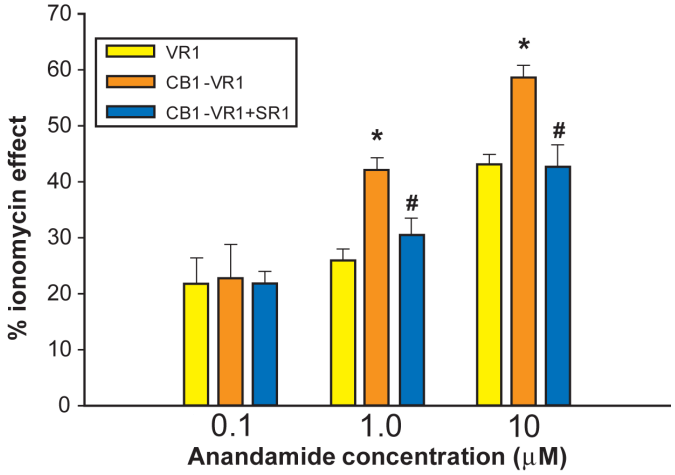


**Fig. 3-10: Dose-dependent effect of anandamide on intracellular $Ca^{2+}$ in CB1-VR1-HEK and VR1-HEK cells.**
The effect on intracellular $Ca^{2+}$ was expressed as percent of the effect of ionomycin (4 μM) and, in CB1-VR1-HEK cells (clone #10), measured after 5 min pretreatment with SR141716A (SR1, 0.5 μM). Data are means ± SEM of at least n=3 independent experiments carried out in duplicate.
*, P<0.05 vs. VR1 cells; #, P<0.05 vs. CB1-VR1 cells without SR1.

### *3.1.4.5 Effect of HU210 on forskolin-induced potentiation of the capsaicin response in CB1-VR1-HEK cells*

Recent studies have shown that the sensitivity of VR1 receptors to ligands can be enhanced by substances that stimulate AC, e.g. FRSK and subsequently activate protein kinase A (PKA) which phosphorylates in turn VR1 (De Petrocellis et al., 2001b; Bhave et al., 2002; Rathee et al., 2002). Since inhibition of AC in response to cannabinoid agonists is a well-known signal transduction response of CB1 (Howlett and Fleming, 1984), HU210 would inhibit, rather than enhance, the stimulatory effect of FRSK on VR1-mediated $Ca^{2+}$-influx. In agreement with this notion, it was found that 5 min pretreatment with FRSK, at a dose (5 μM) inactive *per se* on basal $[Ca^{2+}]_i$, led to a significantly enhanced effect of capsaicin on $[Ca^{2+}]_i$ in CB1-VR1-HEK cells (clone #10; Fig. 3-11). When cells were pretreated with both FRSK and HU210 (100 nM), however, the overall response on $[Ca^{2+}]_i$ was not significantly different from that observed with capsaicin alone (Fig. 3-11).

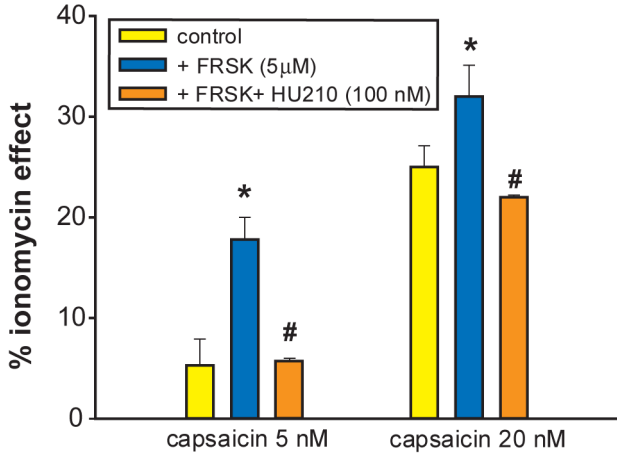


**Fig. 3-11: HU210 inhibits the effect of capsaicin on intracellular $Ca^{2+}$ in CB1-VR1-HEK cells pretreated with FRSK.**
Cells (clone #10) were treated with vehicle, FRSK (5 μM), HU210 (100 nM), or with both HU210 and FRSK 5 min prior to stimulation with capsaicin (5 or 20 nM). HU210 plus FRSK caused no significant change of basal $Ca^{2+}$ (not shown). Data are expressed as percent of the effect of ionomycin (4 μM) and are means ± SEM of at least n=3 independent experiments carried out in duplicate.
*, P<0.05 vs. control. #, P<0.05 vs. FRSK.

*Conclusions*

This study demonstrated that when CB1 receptors and VR1 receptors are coexpressed in the same cells, the sequential stimulation of the two receptors leads to a stronger stimulation of VR1 activity or to its inhibition depending on whether or not the cAMP-signaling pathway is activated. This potentiation of VR1 activity might be mediated by several signaling pathways known to be activated by CB1. Sequential CB1-VR1 stimulation occurs *in vitro* when cells are treated first with HU210 and then with capsaicin, and might occur *in vivo* with extracellular AEA or other endogenous mediators. These findings strengthen the hypothesis that CB1 and VR1 receptors can be regarded as interacting receptor systems for this endogenous compound which regulate different signaling pathways. Whether or not higher brain functions, such as learning and memroy are controlled through this cross-talk remains to be investigated.

### 3.1.5 Cross-talk of CB1 and CRHR1 receptors regulates BDNF expression

*Introduction*

Corticotropine-releasing hormone (CRH), the central mediator of the mammalian stress response, exerts its effects through activation of CRH receptor type 1 (CRHR1; Vale et al., 1981). In the brain, CRHR1 is, like CB1, expressed at particularly high levels in the hippocampus, cortex, and cerebellum (Van Pett et al., 2000) and affects learning and memory (Radulovic et al., 1999), giving the first indication for a putative cross-talk between these two receptor systems. Further studies exist which underline the assumption of a functional interaction between CRHR1 and CB1 (summarized in chapter 1.2.4). CRH binding to CRHR1 typically activates AC, which leads to increased intracellular concentrations of cAMP and activation of PKA (Eckart et al., 2002). The neuroprotective action of CRH is mediated through this CRHR1/cAMP/PKA-dependent signaling (Bayatti et al., 2003), but downstream target genes of CRH have not yet been investigated. One putative target is the brain-derived neurotrophic factor (BDNF), whose expression is controlled by cAMP-elevating agents in neurons (Galter and Unsicker, 2000). As activation of either CB1 and CRHR1 is coupled to the cAMP pathway, these two receptor systems might regulate the expression of BDNF in brain regions where both receptors are coexpressed in the same neurons. Therefore, double-ISH on brain sections was performed to localize coexpression of CB1 and CRHR1 in different regions. Moreover, in primary cerebellar cultures, which are known to contain both CB1 and CRHR1, levels of BDNF expression were measured after stimulation of both receptors.

*Results*

#### *3.1.5.1 Coexpression of CB1 and CRHR1 in the adult mouse brain*

Expression of both CB1 and CRHR1 within the same cells must be first demonstrated to put forward a putative cross-talk between these two receptor systems. Therefore, double-ISH analysis was carried out on coronal sections of the adult mouse brain. Characteristic expression patterns for CB1 and CRHR1 mRNA, respectively, were found as described (Marsicano and Lutz, 1999; Van Pett et al., 2000). The number of coexpressing cells was estimated or counted at a single cell resolution in the basal ganglia, several cortical regions, the hippocampal formation and the cerebellum (Table 3-5). Coexpressing cells comprise only low CB1-expressing cells, as CRHR1 transcripts were not detected in high CB1-expressing cells.

In the olfactory tubercle, CRHR1 mRNA was present in 51% of the CB1-expressing cells. The fraction of CRHR1-positive cells containing CRHR1 was 65% (Table 3-5). In the basal ganglia, coexpressing cells were counted in the lateral globus pallidus, where 51% of CB1-expressing cells also contain CRHR1 and 34% of CRHR1-positive cells coexpress CB1 (Table 3-5). Due to the patchy expression of CRHR1 in the caudate putamen, coexpressing cells could not been counted and were estimated in this area. As the number of CRHR1-positive cells is few as compared to CB1-positive neurons, only 10% of all CB1-expressing cells coexpress CRHR1, whereas 90% of CRHR1-expressing cells contain CB1 mRNA (Table 3-5).

In cortical regions high levels of both CB1 and CRHR1 transcripts were detected. In the piriform cortex, half of all CB1-expressing cells contain CRHR1 mRNA whereas only one third of CRHR1-positive cells coexpress CB1 (Table 3-5). The entorhinal/perirhinal cortex area shows a higher fraction of CRHR1-positive cells also expressing CB1 (89%) but only 60% of CB1-expressing cells contain CRHR1 mRNA (Table 3-5). Transcripts of both receptors were detected in layers II-VI of neocortex but most of the coexpressing cells were counted in layers II and III where 67% of CB1-positive cells express CRHR1 and half of all CRHR1-positive cells coexpress CRHR1 (Fig. 3-12A, Table 3-5). In the basolateral amygdala 38% of CB1-expressing neurons contain CRHR1 mRNA, whereas all CRHR1-positive cells express CB1 (Table 3-5).

Within the hippocampal formation, 100% coexpression of both receptors was observed in the pyramidal cell layer of CA1 and CA3 (Table 3-5). CRHR1-positive neurons are absent outside the pyramidal cell layer except of the polymorph layer of dentate gyrus, whereas CB1 signals with intensities ranging from low to very high were detected throughout

the hippocampus. In this hippocampal subregion, 55% of CB1-expressing cells contain CRHR1 mRNA, and in 72% of CRHR1-positive neurons, CB1 signals were observed (Fig. 3-12B, Table 3-5).

In the cerebellum, 100% coexpression of CB1 and CRHR1 receptors was estimated in all layers of granule cells (Table 3-5). As CB1 positive cells are barely visible in the granular layer due to intense toluidine blue counterstaining, two sections of the cerbellum are shown which are hybridized with radioactive-labeled riboprobes either for CB1 (Fig. 3-12E) or CRHR1 (Fig. 3-12F) and indicate the high expression levels of both receptors in this layer. The molecular layer showed a high density of CB1 signals (Fig. 3-12C, E) and low levels of CRHR1 mRNA (Fig. 3-12C, F). Coexpression could only be detected in few cells (Fig. 3-12C) and was not numerically evaluated. Moreover, coexpression could be detected in the deep nuclei of the brainstem such as the lateral cerebellar nucleus (Lat), where 77% CB1-positive neurons coexpressed CRHR1 and 50% CRHR1-positive neurons coexpressed CB1 (Fig. 3-12D, Table 3-5). A similar expression pattern was found in the anterior interposed nucleus (Alnt), where the fraction of CB1-positive neurons coexpressing CRHR1 was lower (55%). Moderate levels of coexpression could also be detected in the spinal vestibular nucleus, and medial vestibular nucleus (data not shown).

| | **Coexpression in cells expressing low CB1** | | |
|---|---|---|---|
| | % CB1 cells with CRHR1 | % CRHR1 cells with CB1 | (n) |
| Olfactory tubercle | 51 | 65 | (533) |
| Caudate putamen | 10* | 90* | (n.c.) |
| Lateral globus pallidus | 51 | 34 | (426) |
| Piriform cortex | 56 | 33 | (1164) |
| Entorhinal/perirhinal cortex area | 60 | 89 | (607) |
| Basolateral amygdala | 38 | 100 | (726) |
| Dentate gyrus (polymorph layer) | 55 | 72 | (709) |
| Hippocampal CA1 area** | 100* | 100* | (n.c.) |
| Hippocampal CA3 area** | 100* | 100* | (n.c.) |
| Layers II-III of neocortex | 67 | 51 | (2128) |
| Granular cell layer (cerebellum) | 100* | 100* | (n.c.) |
| Lateral cerebellar nucleus | 77 | 50 | (922) |
| Anterior interpose nucleus | 55 | 56 | (785) |

**Table 3-5: Coexpression of CRHR1 in low CB1-expressing neurons of the adult mouse brain.**
n.c., not counted; *, estimated percentages; **, principal neurons only; (n), number of counted cells

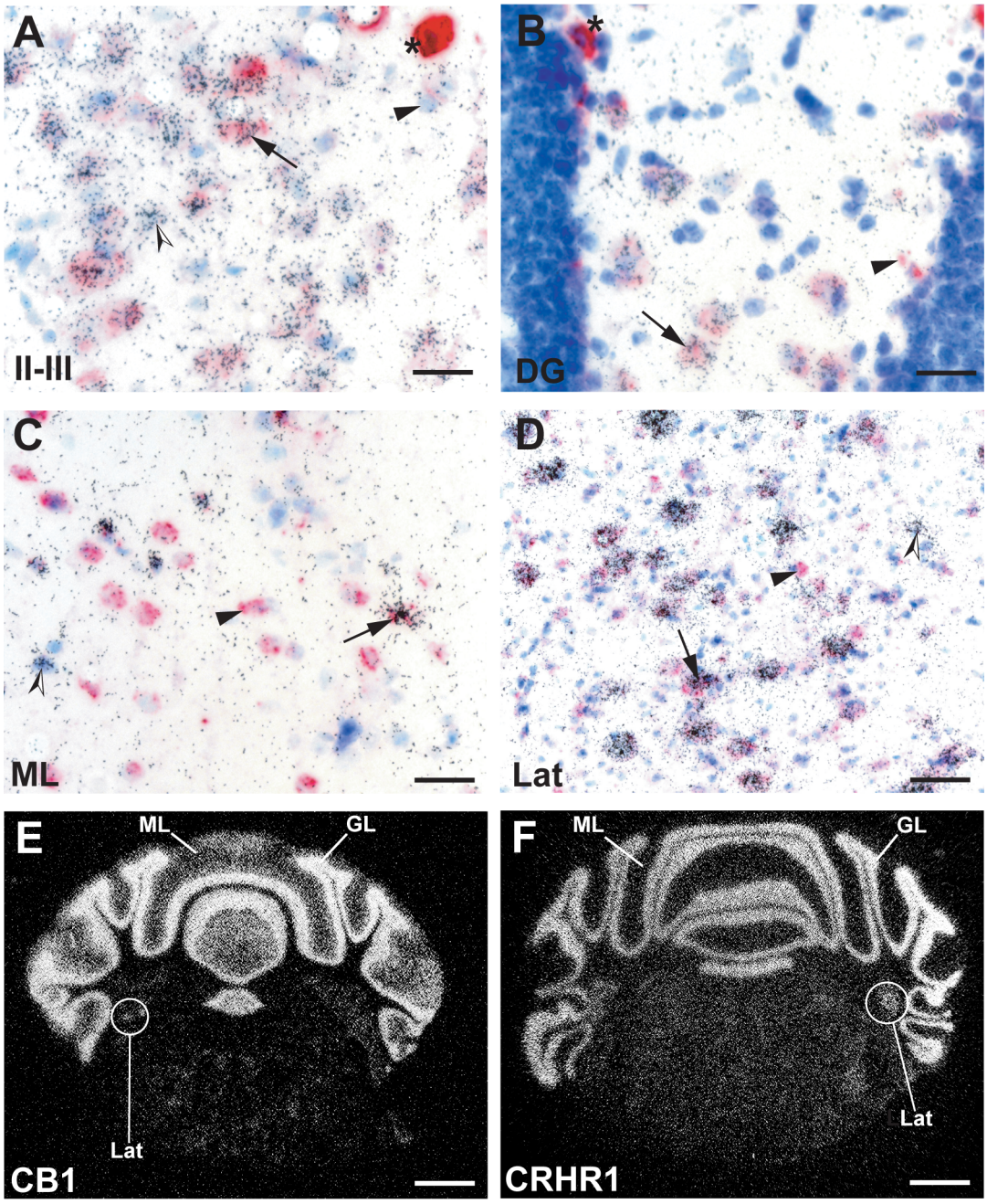


**Fig. 3-12: Bright- and darkfield micrographs of coronal brain sections showing examples of coexpression of CB1 with CRHR1 as detected by single- or double-*in situ* hybridization, respectively. All sections were counterstained with toluidine blue.**
Coexpression of CB1 (red staining) and CRHR1 (silver grains) in (A) layer II and III of neocortex (II-III), (B) molecular layer of dentate gyrus (DG), (C) molecular layer of cerebellum (ML), (D) lateral cerebellar nucleus (Lat). Expression of (E) CB1 and (F) CRHR1 in the cerebellum as detected with $^{35}$S-labeled riboprobes. Note the high expression of both receptors in the granular layer (GL). Filled arrow, CB1-expressing cell that coexpresses CRHR1; filled arrowhead, low CB1-expressing cell; open arrowhead, CRHR1-expressing cell; asterisk, high CB1-expressing cell. Scale bars: 20 µm (A, B); 40 µm (C, D); 1 mm (E, F).

### 3.1.5.2 Inhibition of CRH-mediated signaling by the CB1 agonist WIN55,212-2

As CB1 and CRHR1 are highly coexpressed in the granular layer of the cerebellum (Fig. 2-12E, F), a signaling interaction between both receptor systems was investigated using primary cerebellar cultures, which are known to contain mainly granule cells. CRH-induced activation of CRHR1 leads to the production of cAMP and CB1 has conversely been reported to inhibit cAMP production. Therefore, the effects of the CB1 agonist WIN55,212-2 on CRH-induced cAMP accumulation in cultures from postnatal cerebellar granule neurons were analyzed. While application of CRH ($10^{-8}$ M) for 10 min induced a significant increase in intracellular cAMP levels to 259.6 ± 17.04% ($P<0.05$ vs. vehicle; n=6) as compared to basal levels (100%), simultaneous incubation for 10 min with the CB1 agonist WIN55,212-2 ($10^{-6}$ M) reduced CRH-mediated cAMP production, but did not reach significant difference as compared to CRH alone (CRH and WIN55,212-2, 193.6 ± 17.92%; CRH, 259.6 ± 17.04%; $P<0.05$ vs. CRH, n=6). When given alone, WIN55,212-2 had no effect on intracellular cAMP levels (Fig. 3-13A).

Increases in intracellular cAMP concentrations lead to the activation of PKA, which in turn promotes the phosphorylation of cAMP response element binding protein (CREB). In order to monitor interactions between CB1 activation and CRH-mediated signaling, Western blot analysis was carried out using phospho-specific antibodies directed against the activated form of CREB (pCREB). Treatment of cerebellar neurons with CRH ($10^{-8}$ M) for 30 min led to a 469% ± 153% ($P<0.05$ vs. vehicle control; n=3) increase in pCREB levels as compared to untreated controls (100%). However, cotreatment of cell cultures with WIN55,212-2 ($10^{-6}$ M) for 30 min inhibited CRH-mediated CREB phosphorylation (128% ± 27% of control, n=3; $P<0.05$ vs. CRH; n=3; Fig. 3-13B).

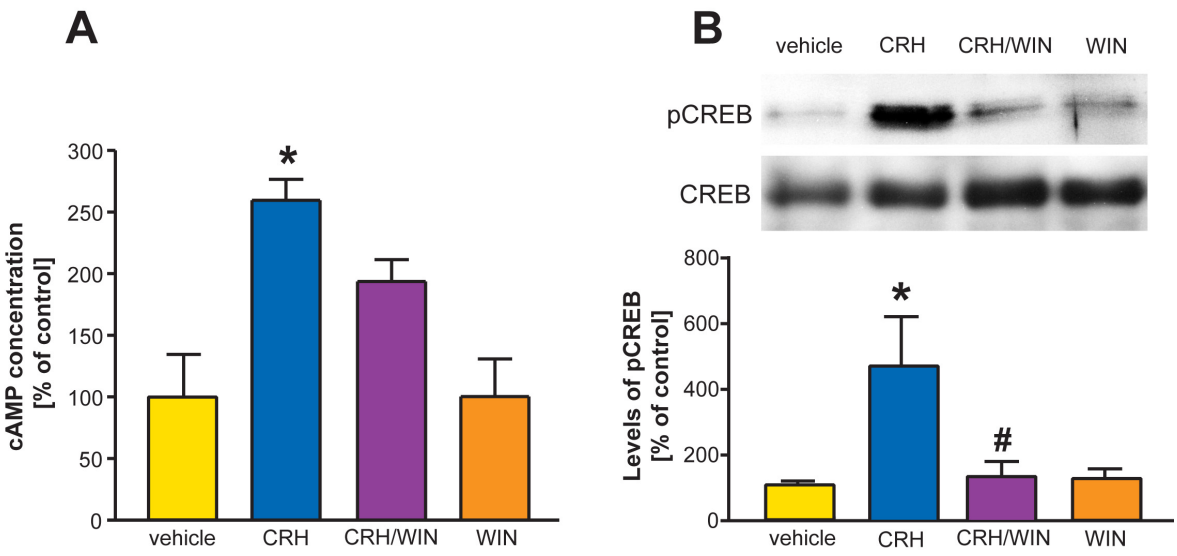


**Fig. 3-13: Modulation of CRH-induced signaling by WIN55,212-2 in cerebellar granule neurons.** (A) cAMP accumulation assays with neurons treated for 10 min with CRH ($10^{-8}$ M) and/or WIN55,212-2 ($10^{-6}$ M) as indicated. Samples were measured in triplicates, and data are expressed as the mean ± SEM of the percentage of basal cAMP levels (considered as 100%). *, $P<0.05$ vs. vehicle (n=6). (B) Western blot analyzing the phosphorylation status of CREB (one representative blot is shown). Neurons were treated for 30 min with CRH ($10^{-8}$ M) and/or WIN55,212-2 ($10^{-6}$ M) as indicated. Phosphorylated CREB levels were normalized to total unphosphorylated levels and depicted as percentage increase ± SEM of vehicle controls (considered as 100%). *, $P<0.05$ vs. control; #, $P<0.05$ vs. CRH; (n=3).

### *3.1.5.3 Inhibition of CRH-mediated increases in BDNF expression by the CB1 agonist WIN55,212-2*

Following phosphorylation, CREB recruits the transcriptional cofactor CREB-binding protein (CBP) and binds to the cis-regulatory cAMP response element (CRE). CREs are located in the promoter regions of target genes and mediate CREB-induced transcription of these genes (Andrisani, 1999). One well documented CREB target gene is BDNF. To see whether CRHR1 receptor activation has an effect on BDNF expression and might be modulated by the concomitant stimulation of CB1, semi-quantitative RT-PCR analysis using BDNF-specific primers was carried out with mRNA extracted from cultures of postnatal rat cerebellar neurons treated with CRH ($10^{-8}$ M), WIN55,212-2 ($10^{-6}$ M), or a combination of the two. Its expression was determined as normalized to the levels of hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA. Treatment of neurons with CRH alone had no effect on BDNF mRNA expression within 24 h (data not shown), but resulted in an increase in BDNF mRNA expression after 48 h to 200 ± 40.76% ($P<0.05$ vs. vehicle; n=4) as compared to untreated controls (100%; Fig. 3-14A). Simultaneous treatment with WIN55,212-2 significantly reduced the stimulatory actions of CRH alone (CRH and WIN55,212-2, 128.3 ± 10.61%; $P<0.05$ vs. CRH; n=4). WIN55,212-2 alone had no

observable effects on BDNF expression as compared to controls (Fig. 3-14A). To determine whether these drug treatments do not only change mRNA levels of BDNF but also protein levels, BDNF protein content in the cultures was determined using ELISA. CRH alone had no effect on BDNF protein levels after 24 h (data not shown), but significantly increased BDNF protein levels 48 h after application of CRH to 149.5 ± 13.08% ($P<0.05$ vs. vehicle; n=3) as compared to untreated control (100%; Fig. 3-14B). This increase was inhibited by addition of WIN55,212-2 (CRH and WIN55,212-2 86.86 ± 16.17%; $P<0.05$ vs. CRH; n=3). Application of WIN55,212-2 alone did not show any significant changes of BDNF protein levels as compared to controls (Fig. 3-14B).

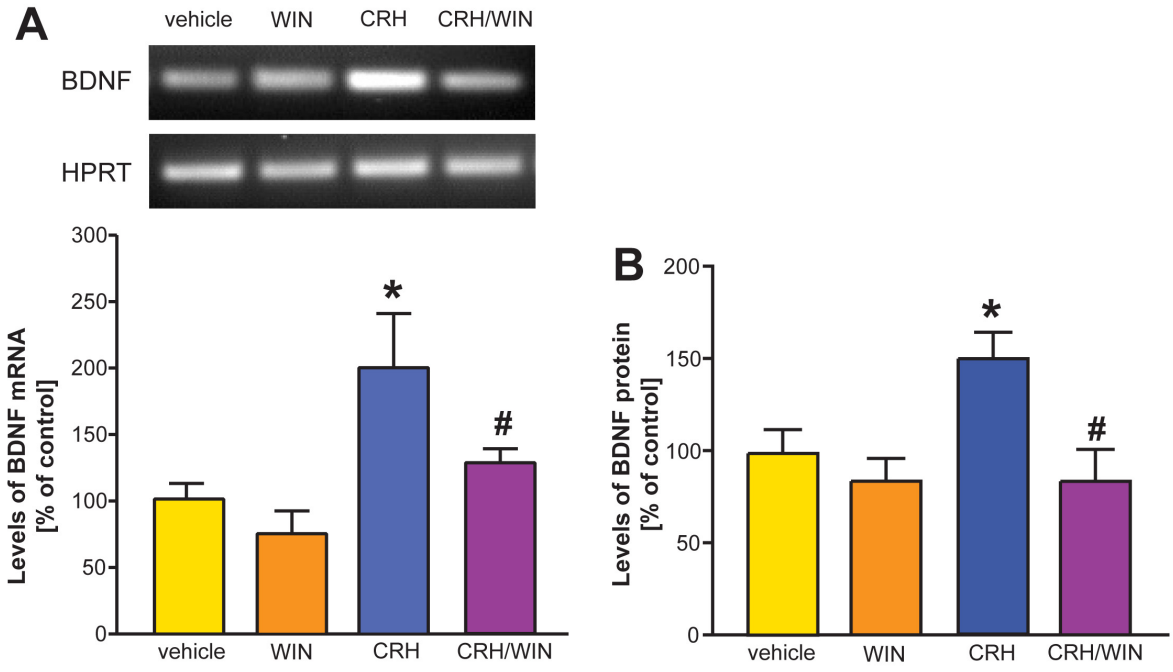


**Fig. 3-14: Effects of WIN55,212-2 on CRH-induced BDNF expression in cerebellar granule neurons.**
(A) Semi-quantitative RT-PCR for BDNF, using HPRT as an internal standard. Neurons were treated with CRH ($10^{-8}$ M) and/or WIN55,212-2 ($10^{-6}$ M) as indicated for 48 h. Results were calculated as ratios of optical density of the BDNF band vs. the HPRT band and expressed as the mean ± SEM of the percentage of control (considered as 100%). *, $P<0.05$ vs. vehicle; #, $P<0.05$ vs. CRH (n=4). (B) ELISA for BDNF; Neurons were treated with CRH ($10^{-8}$ M) and/or WIN55,212-2 ($10^{-6}$ M) as indicated for 48 h. Samples were measured in duplicates and data were expressed as the mean ± SEM of the percentage of basal BDNF levels (considered as 100%). *, $P<0.05$ vs. vehicle; #, $P<0.05$ vs. CRH (n=3).

## *Conclusions*

This study highlights an important role of the stress response peptide CRH in activating BDNF expression and a counter-regulatory role of CB1 activation which takes place in granule cerebellar neurons, where ISH revealed a high degree of coexpression of CRHR1 and

CB1 receptors. BDNF is a CREB target gene and phosphorylation of CREB is regulated through the cAMP signaling pathway. As CB1 and CRHR1 have opposite effects on the cAMP pathway, this provides a possible mechanism how activation of these receptors regulates BDNF expression. However, BDNF transcription is also known to be induced by other signaling pathways, all of them driven through $Ca^{2+}$ influx. Further studies should therefore concentrate on a detailed pharmacological analysis revealing the exact mechanism by which the CRH and the cannabinoid system influence BDNF expression and eventually synaptic plasticity in the brain.

## 3.2 Neuroprotective and anti-inflammatory properties of the cannabinoid system

### 3.2.1 Cross-talk of CB1 with the glutamatergic system protects from kainic acid-induced excitotoxicity *in vitro* and *in vivo*

*Introduction*

Glutamate, the major excitatory neurotransmitter in the mammalian brain, is a key mediator of intercellular communication, plasticity, growth and differentiation. Through a family of membrane receptors, glutamate transduces signals that govern postsynaptic depolarization but also mediate excitotoxicity, the process responsible for triggering neurodegeneration through glutamate receptor overactivation. In cortical areas, the CB1 receptor is highly expressed in GABAergic interneurons (Katona et al., 1999; Marsicano and Lutz, 1999), but evidence exists for its presence also in principal, glutamatergic neurons of, e.g. hippocampus (Matsuda et al., 1993; Marsicano and Lutz, 1999). As exogenous natural and synthetic cannabinoids have been shown to exert neuroprotective functions in several models of neurotoxicity (Mechoulam et al., 2002; van der Stelt et al., 2002; Veldhuis et al., 2003; Croxford, 2003), and neuronal depolarization increases the production of endocannabinoids (Cadas et al., 1996; Di Marzo et al., 1998; Howlett et al., 2002; van der Stelt et al., 2002), the endocannabinoid system might protect against excitotoxicity through modulating glutamate signaling in principal neurons.

Excitotoxicity is a pathological process, in which excessive neuronal excitation induces neuronal damage and death. Several acute and chronic diseases of the CNS are believed to converge onto excitotoxic processes to produce neuronal loss and malfunctioning (Segovia et al., 2003; Lo et al., 2003). Therefore, it is conceivable that protective mechanisms exist that are able to provide on-demand defense in case of abnormally high spiking activity. A balance between these mechanisms determines the final outcome of the excitotoxic insult. Kainic acid (KA), an excitatory amino acid isolated from seaweed, is widely used in studies of excitotoxicity. Local or systemic administration of KA in rodents leads to a pattern of repetitive limbic seizures and status epilepticus, which can last for several hours (Cavalheiro et al., 1982; Hellier et al., 1998). In this model, the hippocampus appears as the brain region most susceptible to KA-induced effects (Ben Ari and Cossart, 2000). Also *in vitro*, application of KA in hippocampal slice cultures induces typical excitotoxic profiles (Zimmer et al., 2000; Kristensen et al., 2001).

The involvement of the endogenous cannabinoid system in a physiological protection against the consequences of excessive neuronal activity is still a matter of debate (van der Stelt et al., 2002), and even CB1 receptor-mediated neurotoxic effects were reported (Chan et al., 1998; Hansen et al., 2002; Clement et al., 2003). To test the role of the endogenous cannabinoid system in the control of excessive neuronal activity in the brain, two model systems were treated with KA and examined for the activation of signaling cascades known to be implicated in the development of KA-induced excitotoxicity: I) CB1-conditional mutant mice, lacking CB1 in forebrain principal, glutamatergic neurons, and II) organotypic cultures of hippocampal tissue in which CB1 was pharmacologically blocked.

*Results*

#### *3.2.1.1 CB1 expression is restricted to GABAergic interneurons in CB1 conditional knock-out mice*

There is evidence that the cannabinoid system protects against excitotoxicity through modulating glutamate signaling in principal neurons. To investigate the role of CB1 in principal, glutamatergic neurons, genetically modified mice were generated, in which CB1 is specifically absent in all principal neurons of the forebrain. In detail, a mouse line was generated in which the CB1 coding region is flanked by two loxP sites (CB1-floxed mice, $CB1^{f/f}$). By crossing this mouse line with mice expressing Cre recombinase under the control of the regulatory sequences of $Ca^{2+}$/calmodulin-dependent kinase IIα (here called $CB1^{CaMKII\alpha Cre}$ mouse line; Casanova et al., 2001), mice ($CB1^{f/f;CaMKII\alpha Cre}$) were obtained, in which the CB1 receptor is deleted in all principal neurons of the forebrain, maintaining its expression in cortical GABAergic interneurons (including the hippocampus; Fig. 3-15E, H) and in cerebellar neurons (Fig. 3-15F). To visualize this different expression pattern in transgenic animals, ISH with a radioactive-labeled CB1 probe was performed using coronal brain sections of $CB1^{f/f;CaMKII\alpha Cre}$ and $CB1^{f/f}$ littermates. In the cerebellum, CB1 is highly expressed in the granular and molecular layers and some deep nuclei of the brainstem showing no differences between genotypes (Fig 3-15C, F). Forebrain sections of $CB1^{f/f}$ mice show the typical distribution of CB1 mRNA with particularly high expression levels in the neocortex (Fig. 3-15A, B), dorsolateral caudate putamen (Fig. 3-15A) and hippocampus including the pyramidal cells (Fig. 3-15B, G; Marsicano and Lutz, 1999). In forebrain sections from $CB1^{f/f;CaMKII\alpha Cre}$ animals, CB1 expression is restricted to a neuronal population within cortical regions which contain high levels of transcripts (Fig. 3-15D, E, H). To show that this subpopulation belongs to the cortical GABAergic type of interneurons, double-ISH

was carried out using a radioactive-labeled probe for the GABAergic-specific marker GAD65 (glutamate decarboxylase, 65 kD) together with a DIG-labeled probe for CB1, revealing that all remaining high CB1-expressing neurons in CB1$^{f/f;CaMKII\alpha Cre}$ coexpress GAD65. Here, the hippocampal region of both genotypes is depicted showing low levels of CB1 in the pyramidal cell layer and coexpressing cells containing high levels of CB1 in the molecular layer of CA1 and CA3 of CB1$^{f/f}$ mice (Fig. 3-15I). In contrast, the hippocampus of CB1$^{f/f;CaMKII\alpha Cre}$ animals lacks CB1 expression in the pyramidal cell layer and CB1 transcripts were only detected in GAD65-positive neurons (Fig. 3-15J).

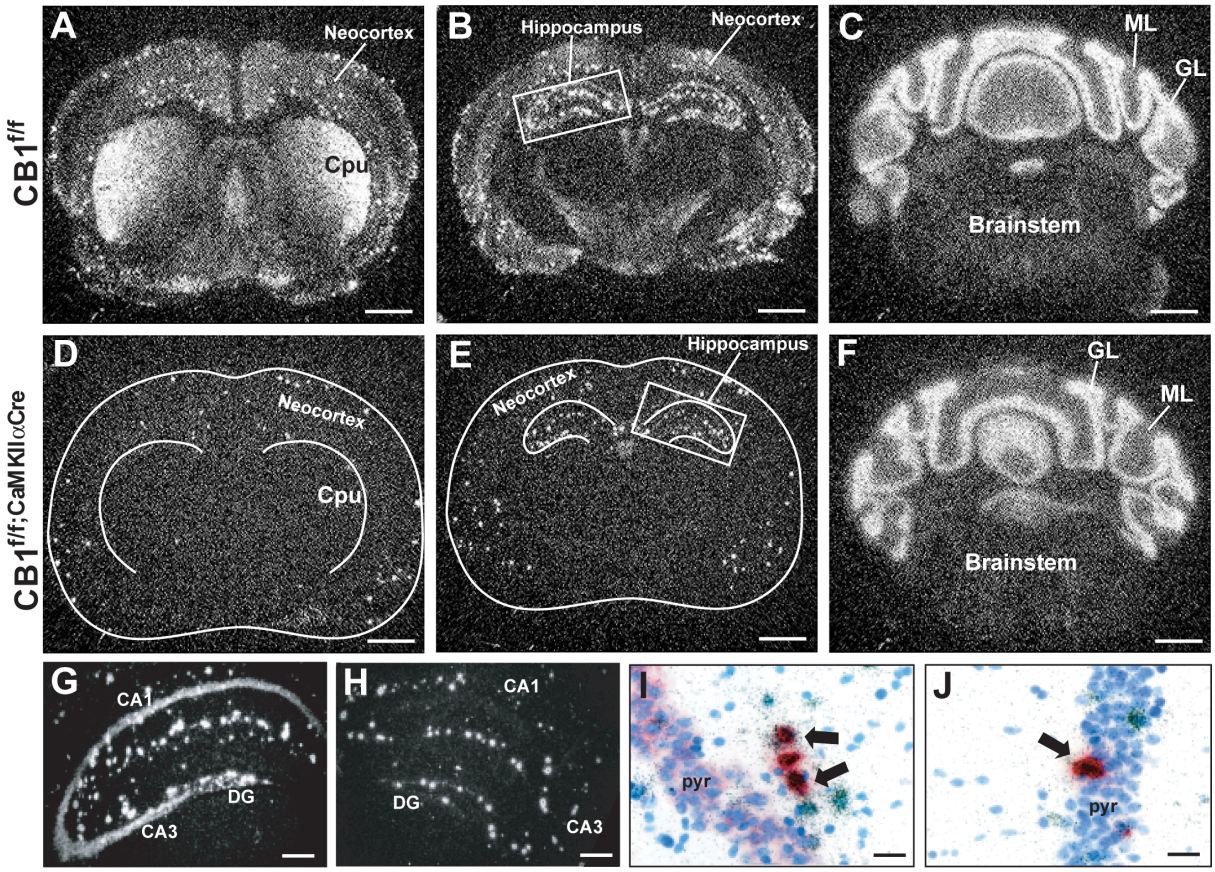


**Fig. 3-15: In CB1$^{f/f;CaMKII\alpha Cre}$ mice, CB1 is expressed only in cortical GABAergic interneurons and in the cerebellum.**
Expression of CB1 mRNA (dark-field) in the forebrain from CB1$^{f/f}$ (A, B) and CB1$^{f/f;CaMKII\alpha Cre}$ (D, E) mice and in the cerebellum from CB1$^{f/f}$ (C) and CB1$^{f/f;CaMKII\alpha Cre}$ (F) mice. Note the high levels of CB1 expression in the neocortex (A, B), dorsolateral caudate putamen (A) and hippocampus (B) in CB1$^{f/f}$ animals which are totally absent in CB1$^{f/f;CaMKII\alpha Cre}$ animals except high CB1-expressing neurons in cortical areas (D, E). CB1 expression levels in the cerebellum remain unchanged in both genotypes (C, F). (G, H) Higher magnification of CB1 expression (dark-field) in hippocampi from CB1$^{f/f}$ (G) and CB1$^{f/f;CaMKII\alpha Cre}$ (H) mice. (I, J) Expression of CB1 mRNA (red staining), in combination with the GABAergic-specific marker GAD65 (silver grains) in CA3 region of hippocampus in CB1$^{f/f}$ (I) and in CB1$^{f/f;CaMKII\alpha Cre}$ (J) mice. Note the presence of CB1 mRNA in pyramidal neurons in CB1$^{f/f}$, but not in CB1$^{f/f;CaMKII\alpha Cre}$ mice. Arrows, interneurons coexpressing CB1 and GAD65; blue stain, toluidine-blue counterstaining. Abbreviations: Caudate putamen (Cpu), molecular layer of cerebellum (ML), granule layer of cerebellum (GL), dentate gyrus (DG), CA3 pyramidal layer (pyr). Scale bars: 1 mm (A-F); 40 µm (G, H); 20 µm (I, J).

### 3.2.1.2 CB1 in glutamatergic neurons activates a protective signaling cascade against kainic acid-induced excitotoxicity

To test the role of the endogenous cannabinoid system in the control of excessive neuronal activity in the brain, CB1-null mice ($CB1^{-/-}$; Marsicano et al., 2002b) and their $CB1^{+/+}$ control littermates were compared in the KA model of excitotoxic epileptiform seizures (Ben Ari and Cossart, 2000). Injection of KA (30 mg/kg) into $CB1^{-/-}$ mice induced clearly more severe seizures and increased the death rate as compared to $CB1^{+/+}$ littermates indicating that genetic ablation of the CB1 receptor lowers the threshold for KA-induced seizures (Fig. 3-16A). To see whether CB1 specifically in glutamatergic neurons protects against seizures, the same experiments were carried out with the mice strain in which CB1 is deleted in all glutamatergic neurons of the forebrain. Similar results were obtained in $CB1^{f/f;CaMKII\alpha Cre}$ in which KA injection induced more severe seizures and lowerd the survival rate (Fig. 3-16B). A comparison of behavioral scores of $CB1^{-/-}$ and $CB1^{f/f;CaMKII\alpha Cre}$, and of respective littermate controls, revealed that the development of seizures did not differ between the CB1-null mutants and the conditional CB1 knock-out thus, indicating that the effects of the drug are specifically mediated by CB1 receptors on glutamatergic neurons (Fig. 3-16C; Marsicano et al., 2003).

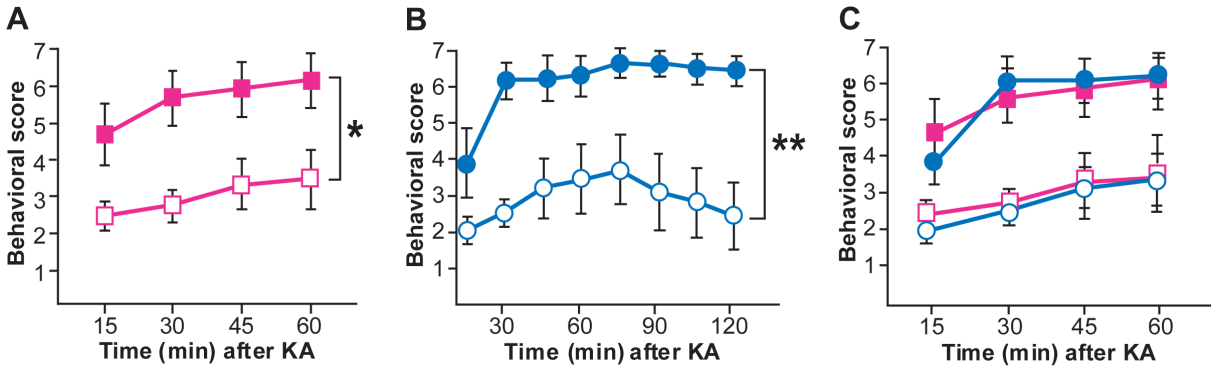


**Fig. 3-16: CB1 receptors in glutamatergic forebrain neurons protect against kainate-induced seizures.**
KA was administered intraperitoneally at 30 mg/kg body weight. Mice were monitored for 1 h to 2 h and behavioral scores were recorded every 15 min. Scores were quantified by trained observers blind to genotype and drug treatment according to Croxford (2003), with stage 7 indicating death. (A) Seizure scoring of $CB^{+/+}$ mice (open squares, n=7) and $CB^{-/-}$ mice (filled squares, n=8). (B) Seizure scoring of $CB^{f/f}$ mice (open circles, n=8) and $CB^{f/f;CaMKII\alpha Cre}$ mice (filled circles, n=10). (C) Comparison between seizure scoring of $CB^{-/-}$ mice (filled squares) and $CB^{f/f;CaMKII\alpha Cre}$ mice (filled circles) and respective control littermates ($CB^{+/+}$ mice, open squares, and $CB^{f/f}$ mice, open circles). Data are means ± SEM. *, $P<0.05$; **, $P<0.01$. These data were kindly provided by Marsicano et al. (2003).

Injection of KA activates the endogenous cannabinoid system, which, in turn, protects neurons from the excitotoxic effects of this drug via the activation of CB1 receptors. How

does CB1 receptor activation reduce excitotoxicity? Several intracellular pathways have been implicated in the development of KA-induced excitotoxicity (Ferrer et al., 2002). KA administration rapidly induces expression of immediate early genes (IEGs) such as c-fos or zif268 (O'Donovan et al., 1999). In particular, the activation of the c-fos gene plays a central role in protection against KA-induced excitotoxicity (Zhang et al., 2002). Because the pharmacological stimulation of CB1 receptors induces the expression of these IEGs (Derkinderen et al., 2003), the levels of c-fos and zif268 transcripts were analyzed in hippocampi from $CB1^{f/f;CaMKII\alpha Cre}$ and $CB1^{f/f}$ littermates 75 min after KA or saline injection by ISH. In saline-injected mice, the hippocampal levels of c-fos (Fig. 3-17A, C, M) and zif268 transcripts (Fig. 3-17E, G, N) were similar between genotypes. However, all subregions of the hippocampi derived from KA-treated $CB1^{f/f}$ mice showed strongly increased levels of both c-fos (Fig. 3-17B, M) and zif268 transcripts (Fig. 3-17F, N). In the hippocampi derived from KA-treated $CB1^{f/f;CaMKII\alpha Cre}$ mice, the induction of c-fos (Fig. 3-17D, M) and zif268 expression (Fig. 3-17H, N) was abolished.

The brain-derived neurotrophic factor (BDNF) plays pivotal roles in development, survival and maintenance of neurons (Huang and Reichardt, 2001). It has been described that experimentally induced seizures resulted in a rapid but transient increase in BDNF transcript and protein levels in hippocampal and cortical neurons (Isackson et al., 1991; Dugich et al., 1992). As BDNF participates in the c-fos-dependent neuronal protection against KA-induced excitotoxicity (Zhang et al., 2002) which was shown here to be CB1-dependent, the cannabinoid system could also be involved in regulation of BDNF levels under these circumstances. Therefore, BDNF mRNA levels were analyzed by ISH in the hippocampi of the same mice used for the analysis of c-fos and zif268 expression. In saline-treated mice, BDNF mRNA is expressed at moderate levels in all subregions of the hippocampus (Fig. 3-17I, K, O). Slightly, but significantly lower levels of BDNF were observed in the CA3 region of $CB1^{f/f;CaMKII\alpha Cre}$, possibly indicating a role of CB1 receptors in the basal control of BDNF expression (Fig. 3-17O). In KA-treated $CB1^{f/f}$ mice, BDNF expression was strongly enhanced as compared to saline-treated littermates in all hippocampal subregions (Fig. 3-17J, O). However, similarly to c-fos and zif268, no increase of BDNF expression was observed in KA-treated $CB1^{f/f;CaMKII\alpha Cre}$ mice as compared to saline-treated controls (Fig. 3-17L, O).

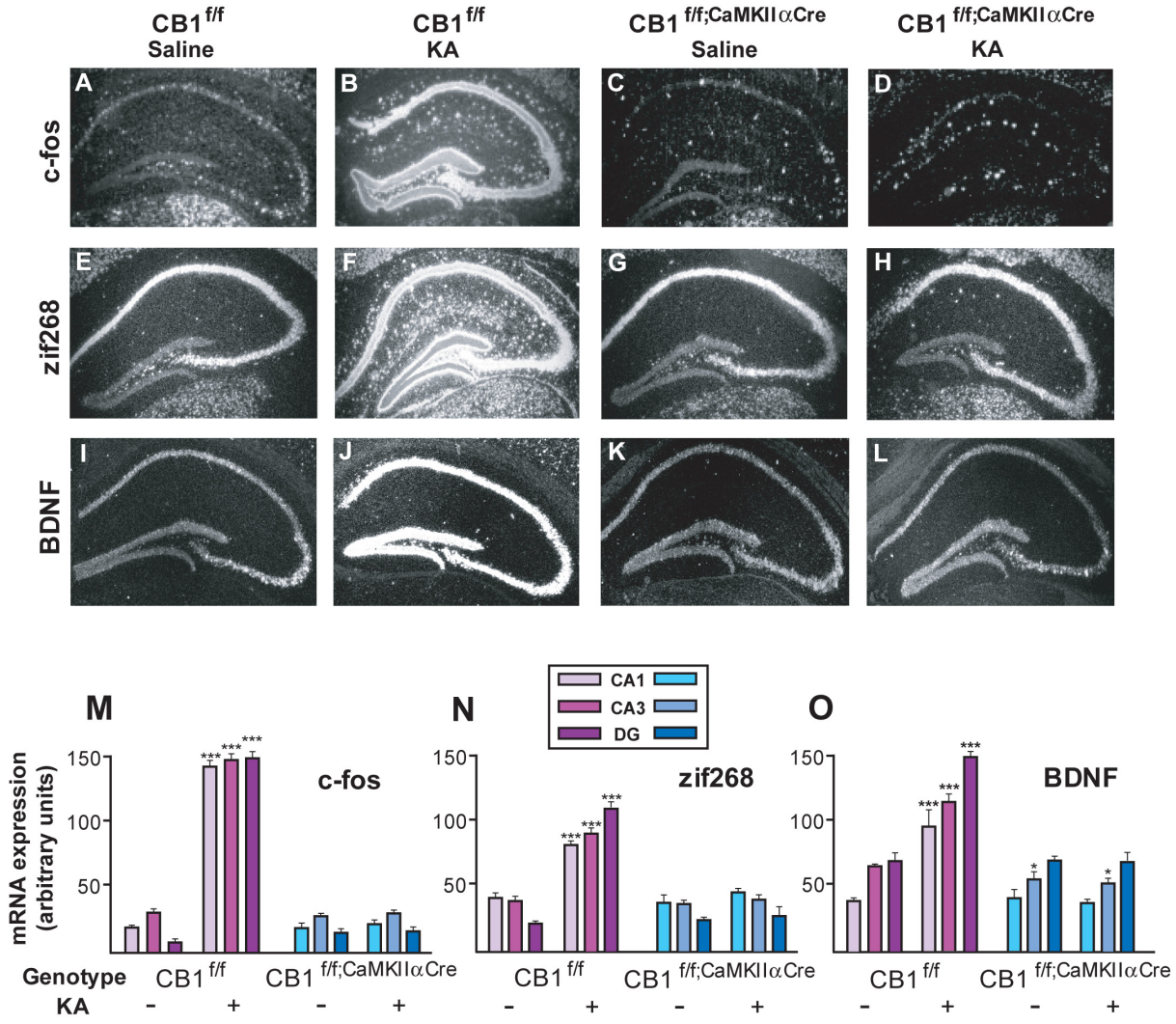


**Fig. 3-17: On-demand activation of the endogenous cannabinoid system in principal hippocampal neurons is required to induce protective molecular cascades.**
(A-L) Representative dark-field micrographs showing expression of c-fos (A-D), zif268 (E-H) and BDNF (I-L) mRNA in $CB1^{f/f}$ and $CB1^{f/f;CaMKII\alpha Cre}$, 75 min after injection of KA (15 mg/kg) or saline; dark halos in (B) and (F) are artefacts, due to excessive presence of silver grains. (M-O) Densitometric quantification from autoradiographic films for mRNA expression of c-fos (M), zif268 (N) and BDNF (O) in CA1, CA3 and DG of hippocampus (n=5-6 mice/group). Means ± S.E.M.; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$ vs. saline-treated $CB1^{f/f}$. Expression intensities were evaluated by densitometric quantification from autoradiographic films with the NIH Image software (National Institutes of Health; http://rsb.info.nih.gov/nih-image/Default.html).

#### 3.2.1.3 CB1-dependent expression of BDNF protects against kainic acid-induced excitotoxicity in organotypic hippocampal slice cultures

In the kainic acid (KA) model of epileptiform seizures (Ben Ari and Cossart, 2000), it was shown that KA treatment increased levels of transcripts encoding BDNF in a CB1 receptor-dependent manner (see chapter 3.2.1.2). However, the function of BDNF in the CB1-dependent protection against excitotoxicity has not yet been fully understood. To more closely analyze the relationship between CB1 and BDNF during excitotoxicity, a series of experiments were performed using hippocampal organotypic cultures. This technique represents an adequate model to study mechanisms of excitotoxicity because it reproduces *in vitro* the basic morphological and functional properties of the hippocampal neuronal network and allows to perform experiments using distinct pharmacological treatments and to circumvent some of the technical limitations that may occur in *in vivo* approaches (Stoppini et al., 1991; Kristensen et al., 2001).

For the determination of BDNF protein levels in hippocampal explants after KA treatment, an ELISA method was used. To assess a time-course, the amount of intracellular BDNF protein was measured at different time points after KA treatment (6 μM). The levels of BDNF protein significantly increased 9 h after KA treatment and decreased after 21 h (untreated, 100 ± 17.1%; 1 h, 82.5 ± 20.9%; 9 h, 246.7 ±3 8.3%; 21 h, 153.2 ± 75.3%), indicating that KA treatment induces a transient increase of BDNF protein in organotypic hippocampal cultures (Fig. 3-18A). To test whether this increase in BDNF levels was dependent on the acute activation of CB1 receptors, explants were exposed to the same dose of KA in the presence of vehicle or SR141716A (5 μM) and BDNF protein levels were evaluated 9 h later. As expected, KA treatment induced an increase in BDNF levels as compared to vehicle-treated slices (Fig. 3-18B). Interestingly, treatment of the slices with 5 μM SR141716A almost completely abolished the induction of BDNF by KA (BDNF levels, percent of vehicle; KA, 240.4 ± 42.0%; KA+SR1, 131.2 ± 22.8%, $P<0.05$; Fig. 3-18B). These results show that, in conditions in which blockade of CB1 receptors increases the sensitivity of hippocampal cultures to KA (Fig. 3-18A), the KA-induced overexpression of BDNF protein is also dependent on CB1 activation.

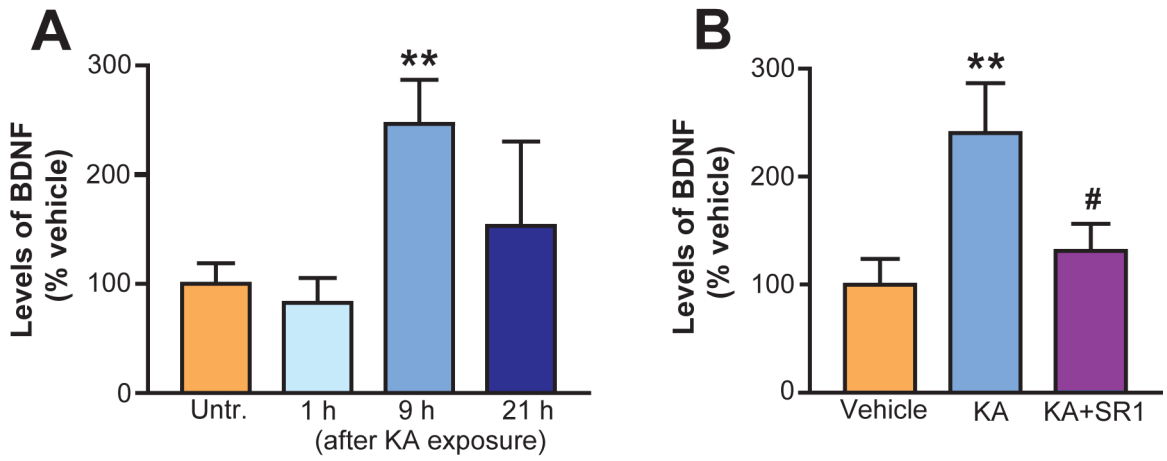


**Fig. 3-18: CB1 mediates increases in BDNF protein levels after KA treatment of hippocampal slices.**
(A) Effects of KA (6 μM) on BDNF protein levels as measured by ELISA after 1, 9 and 21 h after 3 h KA exposure; Untr., untreated cultures. (B) Pharmacological blockade of CB1 receptors prevents the increase of BDNF protein levels induced by KA (6 μM). Cultures were treated with vehicle or SR141716A (SR1; 5 μM), and BDNF protein levels were evaluated 9 h after KA exposure. Samples were measured in triplicates and data were expressed as the mean ± SEM of the percentage of basal BDNF levels (considered as 100%). **, $P<0.01$ vs. vehicle; #, $P<0.05$ vs. KA (n=3).

To test the causal relationship between CB1 receptor-mediated neuroprotection and enhancement of BDNF levels, hippocampal cultures were treated with SR141716A or vehicle in presence or absence of exogenous BDNF protein and were challenged with a low dose of KA (6 μM). In these experiments, 6 μM KA did not induce significant neuronal damage, whereas 5 μM SR141716A strongly increased the effect of the excitotoxin ($P<0.001$, Fig. 3-19). Intriguingly, this effect of the CB1 receptor antagonist was almost completely abolished by the presence of BDNF (100 mg/ml; $P<0.001$, Fig. 3-19). BDNF had no effect on the viability of the slices when SR141716A treatment was absent (Fig. 3-19). These results show that exogenous BDNF is sufficient to prevent the increased sensitivity of organotypic hippocampal cultures to KA induced by blockade of CB1 receptors, indicating that this neurotrophin mediates, at least in part, the on-demand protection of the endogenous cannabinoid system against excitotoxicity (Khaspekov et al., 2004).

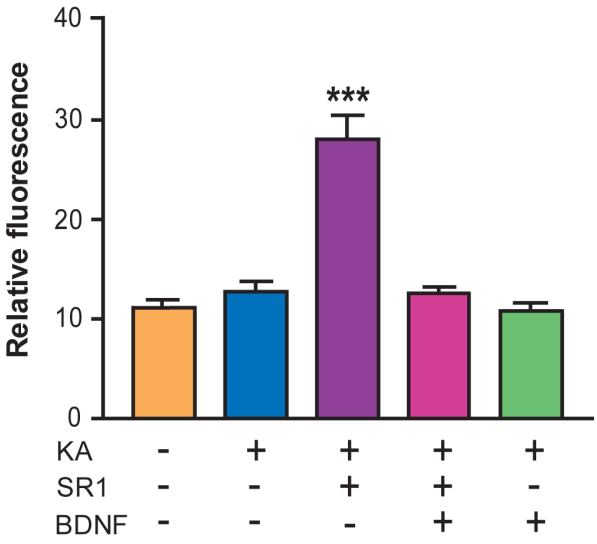


**Fig. 3-19: BDNF mediates CB1 receptor-dependent neuroprotection.**
BDNF treatment (100 ng/ml) completely prevents the increased vulnerability to KA neurotoxicity (6 µM) induced by SR141716A (SR1; 5 µM). Cultures were treated as depicted and relative propidium iodide (PI) staining was evaluated 21 h after KA treatment. n=15-24 explants per group; ***, P<0.001 vs. all other groups. PI was used as an indicator of neuronal membrane integrity and cell death. The intensity of PI fluorescence in pyramidal layer of the hippocampal explants was used as an index of cell death and was measured by quantitative densitometric analysis. Values before KA treatment were subtracted from the values after KA treatment for each slice, and data were expressed as relative fluorescence of each slice. These data were kindly provided by Khaspekov et al. (2004).

*Conclusions*

These results clearly demonstrate the physiological importance of the endogenous cannabinoid system in glutamatergic neurons of the mouse forebrain to protect against KA-induced excitotoxicity. Under these circumstances, CB1 receptor activation is a necessary, early step to induce a protective signaling cascade in the hippocampus. Especially BDNF seems to be an important downstream mediator to rescue neurons from excitotoxic insults. The functional cross-talk between CB1 receptors in glutamatergic neurons and BDNF signaling might represent a promising target for treatment of neurodegenerative disorders characterized by the occurrence of excitotoxic events.

### 3.2.2 CB1 receptors in transfected HT22 cells are not involved in the neuroprotective action of cannabinoids against oxidative stress

*Introduction*

Neuroprotective effects have been described for natural, synthetic and endogenous cannabinoids in several neurotoxicity models (Hampson et al., 1998b; Nagayama et al., 1999; Sinor et al., 2000). Most of the actions of cannabinoids in the CNS appear to be exerted by CB1 (Matsuda et al., 1990; Zimmer et al., 1999). A clearly CB1-dependent protection against excitotoxicity was shown in the kainic acid model of excitotoxicity where CB1 activation induced protective signaling cascades (Marsicano et al., 2003; Khaspekov et al., 2004, see also chapter 3.2.1). However, some non-CB1-binding cannabinoids such as cannabinol and cannabidiol, were shown to protect cells from neurotoxic insults (Hampson et al., 1998b;

Chen and Buck, 2000). These observations would indicate a completely CB1-independent mechanism of neuroprotection of cannabinoids. To elucidate the role of CB1 in another model of neurotoxicity than excitotoxicity, one part of this study was to examine the protective potential of CB1 in a mouse hippocampal cell line (HT22) in oxidative stress assays. Therefore, stably transfected HT22 cells containing CB1 were established and the protective potential of cannabinoids was compared with that observed in the control HT22 cell line lacking CB1.

### *Results*

To examine the participation of CB1 in the cannabinoid-mediated neuroprotection against oxidative stress, it is necessary to test the protection potential of the drugs in identical cellular model systems that differ only in the expression of CB1 and to compare the pharmacological effects in its presence or absence. Therefore, the neuronal cell line HT22 was stably transfected with an expression vector coding for the mouse CB1 receptor, and G418-resistant clones were analyzed by Northern blot. Hybridization signals were detected at approximately 6.0 kb for cortex RNA (used as positive control) as previously described (Matsuda, 1997) and at approximately 1.6 kb for many G418-resistant CB1-transfected clones (Fig. 3-20A). cAMP accumulation assays revealed that some of the clones expressing CB1 mRNA also expressed a functional receptor, as the presence of WIN55,212-2 was able to decrease the FRSK-induced cAMP accumulation (considered as 100%) in CB1-expressing cells (HT22 CB1), but not in cells transfected with an empty vector (HT22 WT) or in parental cells (Fig. 3-20B and data not shown). The extent of reduction of FRSK-induced cAMP accumulation was approximately 30% (FRSK and WIN, of HT22 CB1 69.46 ± 4.19; $P<0.05$ vs. FRSK of CB1-HT22), consistent with reported values in other heterologous CB1 expression systems (Song and Bonner, 1996). Additional application of SR141716A abolished the WIN55,212-2-induced decrease of cAMP showing the involvement of the receptor (FRSK, WIN and SR1 of HT22 CB1 103.4 ± 10.84; $P<0.01$ vs. FRSK and WIN of HT22 CB1; Fig. 3-20B).

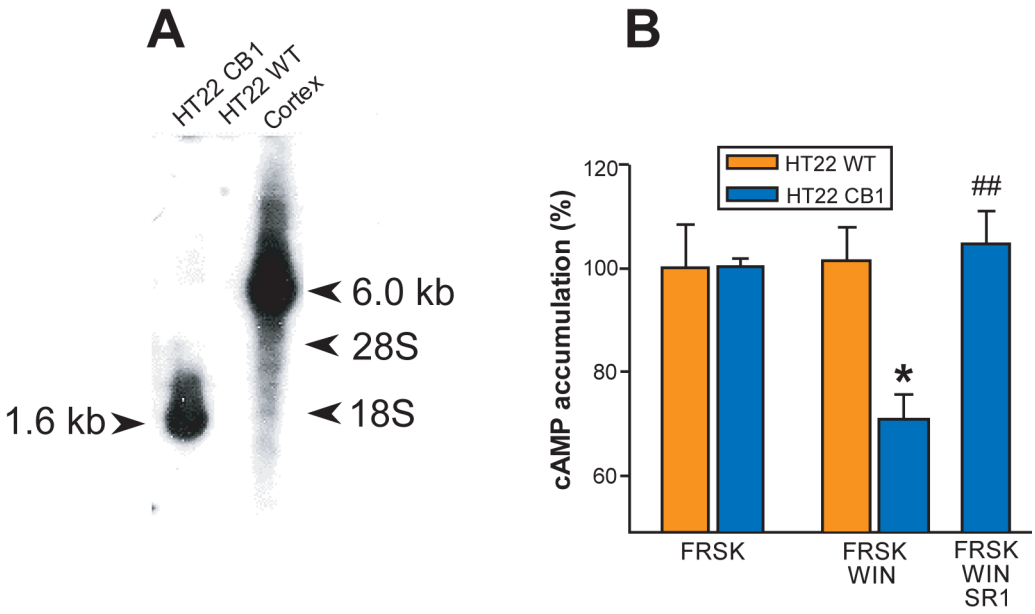


**Fig. 3-20: Analysis of CB1 expression in HT22 cells.**
(A) Northern blot analysis of HT22 cells showing one transfected clone (HT22 CB1) and an empty vector control (HT22 WT). Ribosomal RNA was used as molecular weight marker (28S, ~4.6 kb; 18S ~1.9 kb). Mouse cortex RNA, containing high levels of CB1 mRNA (6.0 kb), was used as positive control (Cortex). (B) Effect of WIN55,212-2 (WIN, 1 µM) and WIN+SR141716A (SR1, 1 µM) on forskolin (FRSK)-induced cAMP accumulation in the same clones as in (A). Samples were measured in duplicates and data are expressed as mean ± SEM of the percentage of FRSK-stimulated cAMP levels (considered as 100%); n=3. *, $P<0.05$ vs. FRSK of HT22 CB1; ##, $P<0.01$ vs. FRSK+WIN of HT22 CB1.

After confirming that the clonal cells expressed a functional CB1, HT22 CB1 and HT22 WT were used for oxidative stress assays in the presence of cannabinoids. As there are four different groups of CB1 agonists (see chapter 1.1.1), one compound from each group was assayed: $\Delta^9$-THC (classical cannabinoids) and CP55,940 (non-classical cannabinoids) both containing a phenolic antioxidant group; methanandamide (eicosanoids) and WIN55,212-2 (aminoalkylindoles) representing non-phenolic and non-antioxidant compounds. As shown in Fig. 3-21A and B, the two phenolic compounds $\Delta^9$-THC and CP55,940 were able to protect HT22 WT cells up to values of approximately 70% and 60%, respectively. However, no differences were observed between the HT22 WT and the CB1-expressing cells HT22 CB1. The dose-response curves were basically identical, thus indicating that the presence of CB1 was altering neither the efficacy nor the potency of the drugs. Methanandamide (Fig. 3-21C) showed no ability to protect cells from oxidative stress, neither in the absence nor in the presence of CB1. Also WIN55,212-2 (Fig. 3-21) did not show any significant protective effect even at concentrations as high as 10 µM. These observations indicate that CB1 is not required for the protective activity of cannabinoids in *in vitro* oxidative stress toxicity paradigms in neuronal cell lines (Marsicano et al., 2002a).

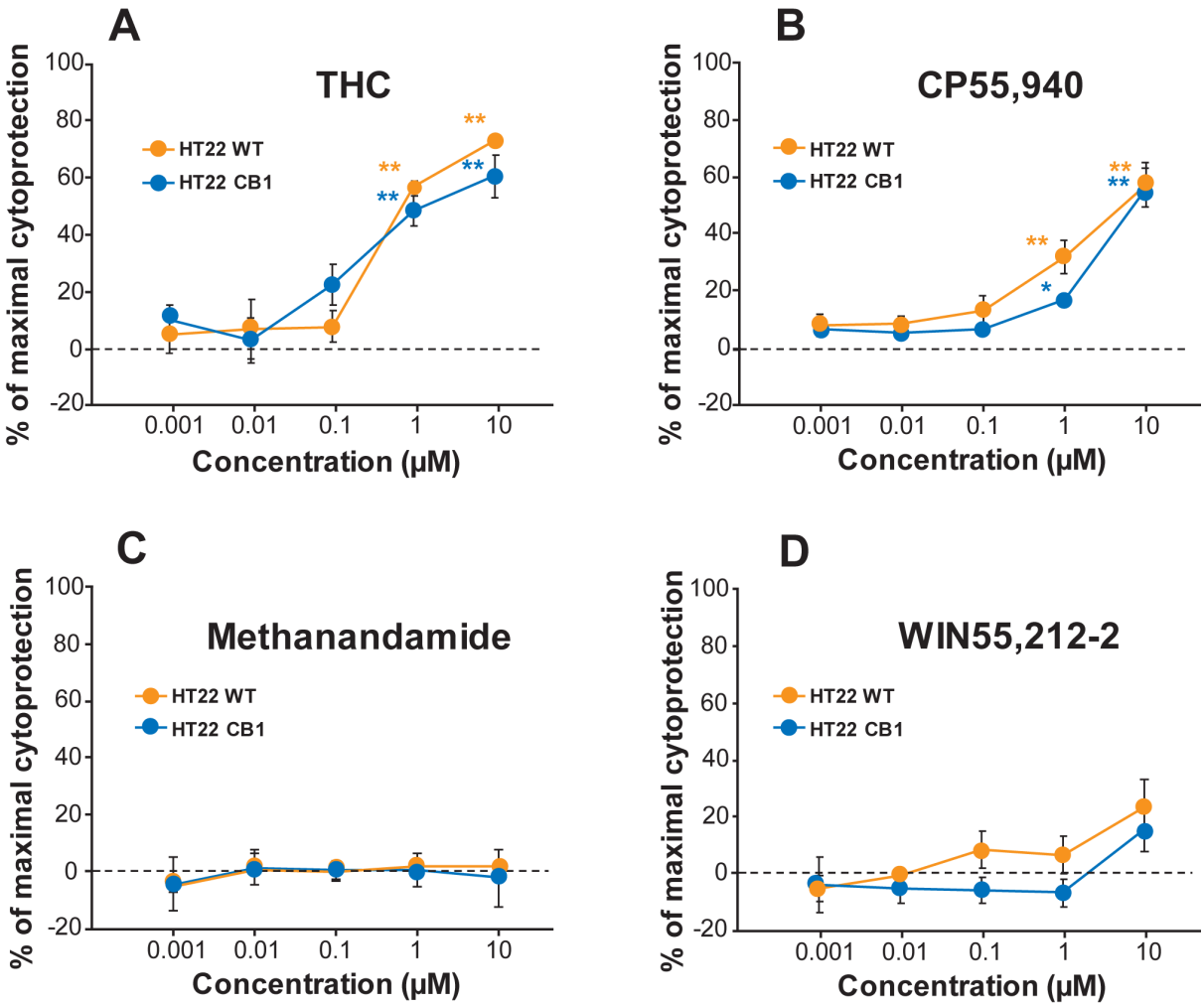


**Fig. 3-21: CB1 is not involved in cannabinoid-mediated protection against oxidative stress in HT22 cells.**
Antioxidant properties of cannabinoids as measured after the application of different concentrations of (A) $\Delta^9$-THC (THC), (B) CP55,940, (C) Methanandamide, (D) WIN55,212-2 to HT22 cells expressing CB1 and not expressing CB1 as indicated. 0% (dotted line) and 100% indicate the value in the absence of cannabinoids (only $H_2O_2$) and the value for untreated cells (without cannabinoids and $H_2O_2$), respectively. Data are expressed as mean ± SEM of n=4; *, P<0.05 and **; P<0.01 vs. control (0%). No significant differences were observed between the two genotypes. These data were kindly provided by Marsicano et al. (2002a).

## *Conclusions*

The results strongly suggest that CB1 is not involved in the cellular antioxidant neuroprotective properties of cannabinoids. These findings are in contrast to the observations gained from the KA model of excitotoxic epileptiform seizures in CB1 mutant mice, where a CB1-dependent protection against neurotoxicity was clearly demonstrated (see chapter 3.2.1). However, the use of cannabinoids could find therapeutical application for targeting different aspects of neurodegenerative diseases.

### 3.2.3 The endogenous cannabinoid system protects against colonic inflammation

*Introduction*

Inflammation-related colon pathologies span a wide range of different conditions, from frankly inflammatory bowel diseases (ulcerative colitis and Crohn's disease), to so-called "functional" bowel diseases (irritable bowel syndrome or colon irritable) and represent an important and widespread health problem in modern society (Drosmann et al., 2000; Mayer and Collins, 2002). The occurrence of an enteric infection, traumata or inflammation has been suggested to be related to the initiation of these diseases (Barbara et al., 2002; Tornblom et al., 2002). Among several experimental animal models of inflammatory bowel diseases, the intrarectal administration of dinitrobenzenesulphonic acid (DNBS) or trinitrobenzenesulphonic acid has been extensively used in the past to study the mechanisms of colonic inflammation and to test anti-inflammatory drugs (Selve, 1992). Infections, traumata or chemical insults are believed to induce several cellular reactions, which eventually lead to an inflammatory status of the colon. However, simultaneous protective mechanisms intrinsic to the organism are also induced during inflammation. These pathways try to counteract the pathological outcome of the inflammatory insults. A balance between pro- and anti-inflammatory processes is likely to determine the progress and the severity of colitis. Therefore, a better understanding of the mechanisms underlying these intrinsic protective activities against inflammation would provide progress aiming to develop novel therapeutic treatments against colitis.

A variety of natural and synthetic cannabinoids have been shown in the past to possess anti-nociceptive and anti-inflammatory activities (Richardson et al., 1998b; Piomelli et al., 2000; Zurier, 2003). Functional CB1 receptors are present on myenteric neurons, although the cellular localization of CB1 receptors is less well characterized (Lynn and Herkenham, 1994; Jaggar et al., 1998; Kulkarni-Narla and Brown, 2000) and the gastrointestinal tract produces endocannabinoids (Pertwee, 2001; Izzo et al., 2001b; Izzo et al., 2001c; Pertwee, 2001;). In fact, the endogenous cannabinoid system plays a role in the control of various functions in these organs (Di Carlo and Izzo, 2003).

To understand the role of the endocannabinoid system in intestinal inflammation, one part of the study was to examine differences in expression levels of CB1 transcripts in colons of DNBS-treated and naive mice by ISH and to investigate putative interactions with other neuronal receptor systems which participate in the protection against inflammation. The endogenous opioidergic system was recently suggested to play a role in the protection against

inflammation in the DNBS model of colitis (Philippe et al., 2003) and is known to functionally interact with the cannabinoid system in the brain (Ledent et al., 1999; Manzanares et al., 1999; Valverde et al., 2000; Valverde et al., 2001). To test whether the protective functions of the endogenous cannabinoid system involve the activation of endogenous opioid signaling, the expression of the opioid preproenkephalin (Enk) mRNA in untreated and DNBS-treated colons derived from CB1-deficient mice ($CB^{-/-}$) and wild-type littermates ($CB1^{+/+}$), respectively, was analyzed by ISH.

*Results*

#### *3.2.3.1 CB1 mRNA is upregulated in the colon after DNBS-induced inflammation*

In order to study the involvement of CB1 and the endogenous cannabinoid system in colon inflammation, C57BL/6N mice were used in the DNBS model of colitis. Intrarectal administration of vehicle (100 µl of 50% ethanol) did not induce detectable inflammation, as macroscopically evaluated (data not shown) and as compared to untreated animals (Fig. 3-22A). Conversely, after intrarectal administration of DNBS the macroscopic evaluation of colons revealed strong inflammation indicated by localized hyperaemia and ulceration (Fig. 3-22B).

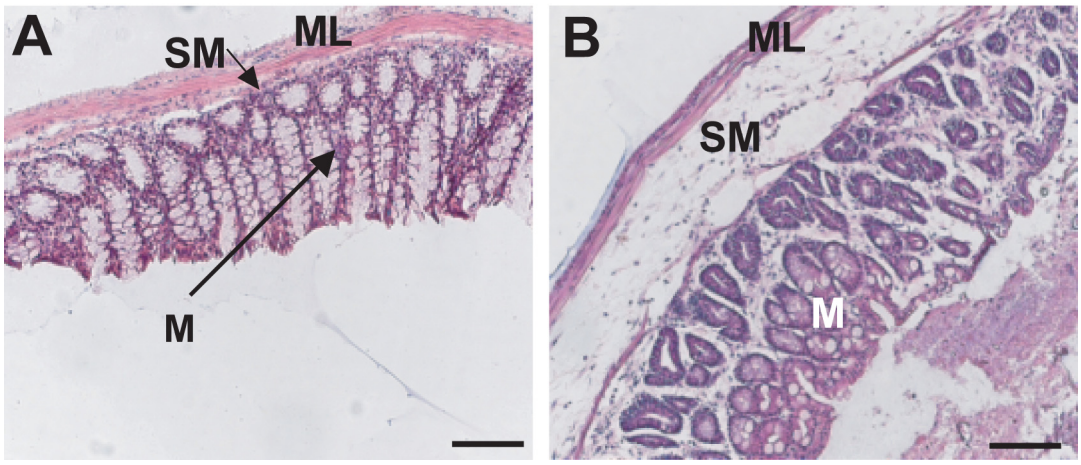


**Fig. 3-22: Histological micrographs showing haematoxylin/eosin stainings from transverse sections through the colon of naive and DNBS-treated C57BL/6N mice.**
(A) Colons from untreated C57BL/6N mice. (B) Colons from C57BL/6N mice 3 days after DNBS treatment. In particular, note the severe mucosal infiltration with inflammatory cells, severe submucosal edema and vascular alterations in treated mice. Abbreviations: muscular layer (ML), mucosa (M), submucosa (SM). Scale bars: 100 µm.

Intra-colonic infusion of DNBS induced stronger inflammation in CB1-deficient mice than in wild-type littermates indicating that CB1 receptors mediate protective physiological signals counteracting inflammatory responses (illustrated in detail in Massa et al., 2004). This might include an enhancement of cannabinoid signaling during colitis in neurons of the myenteric plexus. Therefore, the expression of CB1 mRNA was evaluated at single cell resolution using ISH on colon sections of C57BL/6N mice either in control conditions or 3 days after intrarectal administration of DNBS. In control colons, CB1 mRNA is predominantly expressed in neurons belonging to the myenteric plexus (Fig. 3-23A). After DNBS-induced inflammation, an increase in the number of CB1-expressing cells was observed (Fig. 3-23B). Counting of single CB1-expressing cell confirmed this observation, revealing an increase in the number of CB1-expressing cells in DNBS-treated colons as compared to untreated controls (untreated, 100% ± 17.8% vs. treated, 230.2 ± 25%, $P<0.05$; Fig. 3-23C).

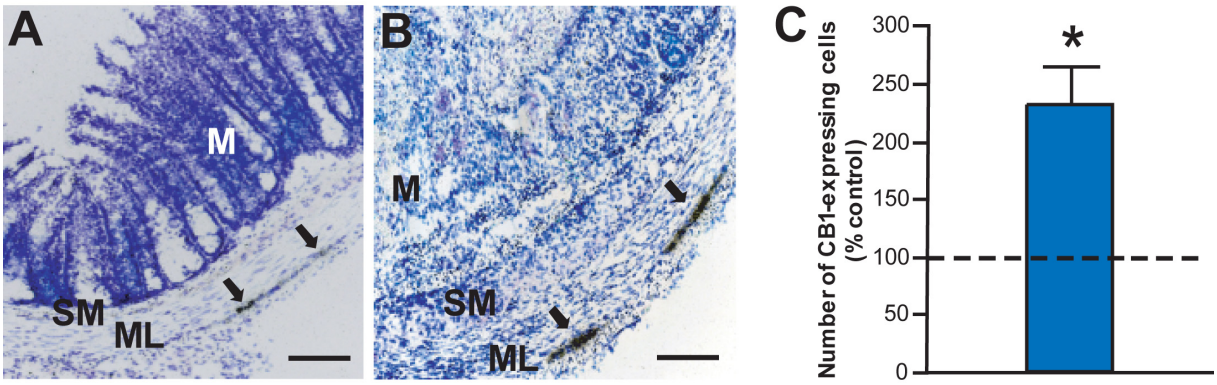


**Fig. 3-23: Levels of CB1 mRNA are increased in the colon of C57BL/6N mice 3 days after DNBS treatment.**
(A and B) Micrographs showing (A) CB1 mRNA detected by ISH in untreated mice and (B) in DNBS-treated mice. (C) Quantitative evaluation of CB1-expressing cells in the myenteric plexuses of DNBS-treated mice (bar, n=3) as compared to untreated controls (100%, dotted line, n=3). Values are means ± SEM; *, $P<0.05$. Abbreviations: muscular layer (ML), mucosa (M), submucosa (SM). Scale bars: 100 µm.

### *3.2.3.2 Preproenkephalin mRNA is upregulated in the colon after DNBS-induced inflammation*

The endogenous opioidergic system was recently suggested to exert anti-inflammatory properties in the DNBS model of colitis through the activation of µ-opioid receptors (Philippe et al., 2003). The opioid and cannabinoid system are known to functionally interact with each other. Therefore, protective functions of the endocannabinoid system might include regulation of opioids during inflammation. To test this notion, the expression of Enk mRNA in untreated and DNBS-treated colons derived from $CB1^{+/+}$ and $CB1^{-/-}$ mice, respectively, was analyzed by ISH. In untreated colons, the number of cells expressing Enk was not different between

genotypes ($CB1^{+/+}$, 100 ±19.3% vs. $CB1^{-/-}$, 106.4 ± 16.7%, P>0.05; Fig. 3-24A and B). After DNBS treatment, the number of cells expressing Enk was significantly increased in $CB1^{+/+}$ (300.9 ± 34.7%, P< 0.01 vs. $CB1^{+/+}$ untreated; Fig. 3-24 C, E) and in $CB1^{-/-}$ (211.6 ± 28.6 %, P<0.05 vs. $CB1^{-/-}$ untreated; Fig. 3-24D, E). A further comparison between genotypes revealed a trend pointing to a lower increase in Enk-expressing cells in $CB1^{-/-}$ as compared to $CB1^{+/+}$, which, however, failed to reach statistical significance (P = 0.07; n.s., not significant) (Fig. 3-24E).

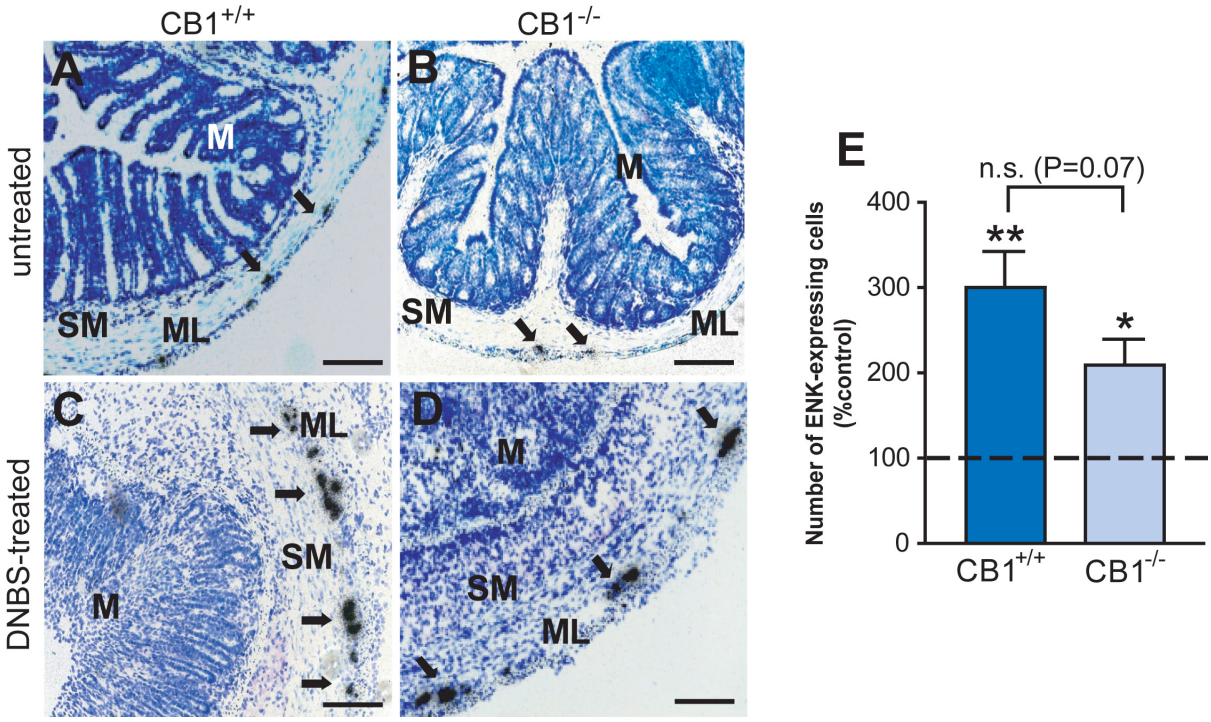


**Fig. 3-24: Levels of preproenkephalin mRNA are increased in the colon of $CB1^{+/+}$ and $CB1^{-/-}$ mice 3 days after DNBS treatment.**
Micrographs showing Enk mRNA detected by ISH in (A) untreated $CB1^{+/+}$ and (B) untreated $CB1^{-/-}$ colons and Enk mRNA in (D) DNBS-treated $CB1^{+/+}$ and (D) DNBS-treated $CB1^{-/-}$ mice. (E) Quantitative evaluation of Enk-expressing cells in the myenteric plexuses of DNBS-treated mice (bars, n=3) as compared to untreated controls (100%, dotted line, n=3). Values are means ± SEM; *, P<0.05; **, P<0.01; n.s., not significant. Abbreviations: muscular layer (ML), mucosa (M), submucosa (SM). Scale bars: 100 µm.

### *3.2.3.3 Number of neurons is unchanged between DNBS-treated and untreated colons*

To exclude the possibility that differences of expression levels of CB1 or Enk transcripts are due to an increased number of neurons in DNBS-treated animals, neurons were counted in sections parallel to the ones used for ISH. Neurons were visualized using cuprolinic blue staining as described in Karaosmanoglu et al. (1996) and neuronal counting did not reveal any change between DNBS-treated and untreated colons and between $CB1^{+/+}$ and $CB1^{-/-}$ mice ($CB1^{+/+}$, untreated, 100 ± 8.6%, $CB1^{+/+}$, treated, 113 ± 8.9%, $CB1^{-/-}$, untreated, 108 ± 5.8%,

$CB1^{-/-}$, treated, 118 ± 6.8%; P>0.1 for all comparisons), indicating that the increase in the number of cells expressing CB1 or Enk mRNA was not due to an increase in the total number of neurons.

### *Conclusion*

Using the DNBS-induced colitis model, this study shows that the endogenous cannabinoid system is physiologically involved in the protection against excessive inflammation by controlling cellular pathways leading to inflammatory responses. Enhanced signaling activity of the cannabinoid system can be proposed as the levels of CB1 receptor transcripts in the colon are upregulated in inflamed mice. Moreover, the expression of the opioid enkephalin was shown to be increased in these mice and seems to be reduced in CB1-deficient animals. These results suggest that modulation of the physiological activity of the endogenous cannabinoid system during colonic inflammation might be a promising therapeutic tool for the treatment of several diseases characterized by inflammation of the gastrointestinal tract.

# 4 DISCUSSION

## 4.1 Functional cross-talk of CB1 with other receptor systems

### 4.1.1 High coexpression levels of CB1 with dopamine and serotonin receptors indicate functional interactions of the cannabinoid system with these neurotransmitter systems

The first indication for possible interactions between different receptors is given, when both receptors are expressed within the same neuron. Therefore, the aim was to define in detail coexpression patterns of CB1 with dopamine and serotonin receptors in distinct neuronal subpopulations of the adult mouse forebrain. For this purpose double-ISH experiments were performed using a DIG-labeled CB1 riboprobe in combination with $^{35}$S-labeled riboprobes for D1, D2, 5-HT1B and 5-HT3, respectively.

Both, the significant extent of overlapping expression of CB1 with various dopamine and serotonin receptors and several investigations observing functional interactions between the cannabinoid system and other neurotransmitter systems gave reason to chose the markers mentioned above. Previous immunohistochemical and ISH studies carried out in rats observed high levels of D1 and D2 mRNA (Weiner et al., 1991; Levey et al., 1993) and 5-HT1B mRNA (Maroteaux et al., 1992) in the striatum. CB1 is also expressed in many neurons throughout the striatum (Tsou et al., 1998a; Marsicano and Lutz, 1999). These observations are in agreement with results observed here, which revealed a high density of hybridization signals of these probes in this area. Particularly in the dorsolateral caudate putamen, a high degree of colocalization of CB1 with D1, D2 and 5-HT1B, respectively, was detected. The striatum is a key component of the forebrain system that controls planning and execution of motor behaviors (reviewed in Nakano et al., 2000). CB1 agonists can markedly affect the function of these systems, producing alterations of locomotion and catalepsy (Howlett, 1995; Martellotta et al., 1998; see also chapter 1.1.5.3). Also dopamine stimulates motor activity in the basal ganglia (Alexander and Crutcher, 1990; Parent and Hazrati, 1995). A mechanism for influencing striatal function through cannabinoids and/or dopamine could be an interaction between the cannabinoid and dopamine system which is suggested by the high coexpression rates of CB1 and D2 shown here. Cell culture experiments on striatal neurons revealed clear evidence for an interaction between CB1 and D2. Activation of either CB1 or D2 resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an augmentation of cAMP accumulation (Glass and Felder, 1997). Also *in vivo*

studies with mice treated with different combinations of cannabinoid agonists and dopamine agonists/antagonists revealed that the two receptor systems appear to interact by exerting opposing influences in regulating motor activity. This hinted to a coexistence of CB1 and D1/D2 on the same striatal neurons (Meschler et al., 2000; Meschler and Howlett, 2001) as evidenced also in this study. Immunocytochemical investigations of Aizman et al. (2000) demonstrated the coexistence of D1- and D2-like receptors in all virtually striatal neurons which can be divided in two different populations responsible either for substance P release or enkephalin release (Graybiel, 1990). CB1 was also detected in these two neuronal subpopulations and is involved in the regulation of expression of these neuropeptides (Mailleux and Vanderhaeghen, 1992). CB1-deficient mice display significantly increased levels of substance P and enkephalin mRNAs in these striatal neurons (Steiner et al., 1999). Therefore, colocalization of CB1 with D1 and D2 in the caudate putamen suggests a putative regulation system of the two receptor systems in controlling expression of these neuropeptides. A recent study carried out by Julian et al. (2003) provided a neuroanatomical basis to explain functional interactions between the endocannabinoid and dopaminergic system in the basal ganglia. Immunhistochemical experiments showed that the majority of the striatal CB1 receptors are located presynaptically on inhibitory GABAergic terminals, in a position to modulate neurotransmitter release and influence the activity of substantia nigra dopaminergic neurons. In turn, afferent dopaminergic fibers from the substantia nigra innervate CB1 receptor-expressing striatal neurons that are known to also express dopamine receptors.

5-HT1B was also shown to be involved in motor behavior. Administration of the 5-HT1B agonist RU24969 to rats resulted in an increase of locomotor activity (Oberlander et al., 1987), whereas application of the 5-HT1B antagonists GR127935 could block the RU24969-induced hyperactivity in rodents (O'Neill et al., 1996). In our study, high coexpression levels of 5-HT1B and CB1 were detected in the striatum assuming a modulatory role for 5-HT1B together with CB1 in motor function.

CB1 is differentially coexpressed with all four markers in several cortical regions (hippocampus, neocortex, entorhinal/perirhinal cortex, amygdala) which contribute to important brain functions, e.g. learning and memory (Suzuki, 1996; Miller et al., 1998). Considering this, modulation of cognitive processes could be mediated through the interaction of CB1 with dopamine or serotonin receptors. A functional interaction between the cannabinoid and dopamine system im memory storage was shown by Castellano et al. (1997). They demonstrated that AEA-mediated impairment of consolidation in mice was antagonized

by pretreatment with either D1 or D2 agonists. Similarly, it was shown that $\Delta^9$-THC-induced impairment of working memory was reversed by a D2 antagonist and potentiated by a D2 agonist, concluding that this effect is mediated by the simultaneous activation of CB1 and D2 (Nava et al., 2000). The concurrent activation of both receptors might produce an accumulation of cellular cAMP in neurons where these receptors are colocalized.

A striking finding of this study was that the coexpression of CB1 with 5-HT3 in several forebrain regions (hippocampus, neocortex, anterior olfactory nucleus, amygdala) was mainly detected in high CB1-expressing cells, which are considered as GABAergic neurons belonging predominantly to the cholecystokinin (CCK)-positive type of interneurons (Tsou et al., 1998a; Marsicano and Lutz, 1999; Katona et al., 1999). In line with the observation made here, ISH and IHC experiments by Morales and Bloom (1997) showed that 5-HT3-expressing neurons in the neocortex, olfactory cortex, hippocampus and amygdala are mainly GABA-containing cells with CCK immunoreactivity. Specifically in the hippocampus, 5-HT3 was detected in CB1-expressing interneurons (Morales and Backman, 2002). The widespread colocalization of 5-HT3 with CB1 in GABAergic neurons suggests the participation of these two receptors in the modulation of inhibitory neurons. As these cells contain CCK, a role of 5-HT3 and CB1 in regulating CCK neurotransmission might be put forward.

CB1 and its colocalization with receptors for other neurotransmitters can contribute to understanding certain neurodegenerative diseases, caused by multiple neurotransmitter and receptor alterations. In reserpine-treated rats, an animal model for Parkinson's disease, increased levels of endocannabinoids in basal ganglia were found (Di Marzo et al., 2000), which might be correlated to significantly reduced levels of CB1 expression in striatum in this disease (Silverdale et al., 2001). Moreover, Di Marzo et al. (2000) could show that coadministration of a D2 agonist and a CB1 antagonist leads to full restoration of normal locomotor behavior in reserpine-treated rats suggesting a close functional relationship between the cannabinoid and the dopamine system. These data support the idea that modulation of the endocannabinoid signaling system provides a useful treatment for the symptoms of Parkinson's disease or other basal ganglia-related movement disorders.

In summary, CB1 is differentially coexpressed in the mouse forebrain with dopamine and serotonin receptors either in principal projecting neurons (mainly with D1, D2 and 5-HT1B) or in interneurons (mainly with 5-HT3). Together, these receptor systems might be involved in modulating excitatory circuits as well as inhibitory GABAergic circuits. Particularly in the striatum, high coexpression extent of CB1 with D1, D2 and 5-HT1B, respectively, were observed, suggesting putative cross-talks between the cannabinoid system

and other neurotransmitter systems regulating locomotor activity. High levels of coexpressing cells in cortical areas might be an indication for a functional interaction of CB1 with dopamine and serotonin receptors, respectively, having modulatory effects on cannabinoid-induced impairment of working memory and cognitive functions.

### 4.1.2 Expression levels of several marker genes are not affected in CB1-deficient mice

The cannabinoid system is involved in multiple signal transduction pathways and therefore participates in various cognitive and behavioral effects (summarized in chapters 1.1.2 and 1.1.5). However, its role in brain function has not been completely understood, yet. To identify genes involved in CB1-mediated signaling, CB1-deficient mice ($CB1^{-/-}$) were screened for differences in gene expression by ISH. Nine candidate genes, known to be linked to the cannabinoid system, were chosen for the analysis. These included:

- the dopamine receptors D1 and D2 and the serotonin receptors 5-HT1B and 5-HT3, all shown to be colocalized with CB1 in several regions of the mouse forebrain (see chapter 3.1.1),
- the enzymes GAD65 and GAD67 as markers for GABAergic neurons in which CB1 receptors are expressed to a high extent (Marsicano and Lutz, 1999),
- the enzyme FAAH, a key component of the endogenous cannabinoid system (reviewed in Deutsch et al., 2002),
- the peptide CCK which is coexpressed with CB1 in GABAergic interneurons of the forebrain (Marsicano and Lutz, 1999) and
- the enzyme nNOS. nNOS-deficient mice showed lower levels of CB1 transcripts in the hypothalamus and striatum suggesting a link between cannabinoid signaling and the NO pathway (Azad et al., 2001).

While the basis for an involvement of these particular genes in CB1-mediated functional processes within the cell is still not well understood, awareness that significant numbers of genes and presumably proteins are changed may provide new insights into cannabinoid-mediated signaling. However, none of these candidate genes showed altered levels of mRNA transcripts in $CB1^{-/-}$ animals compared to $CB1^{+/+}$ littermates. These results suggest that CB1 receptors have no effect on expression of these genes in basal conditions. Nevertheless, this does not necessarily exclude a regulation of these genes by the cannabinoid system. Several studies show that activation of CB1 with different cannabinoids regulates expression of different genes. Grigorenko et al. (2002) used gene microarrays to identify genes whose

expression is altered after chronic exposure of mice or cells to $\Delta^9$-THC or WIN55,212-2, respectively. Indeed, they found several genes that were altered in both circumstances, among them were genes associated with known CB1-coupled signaling pathways, while for other genes the basis for involvement in CB1-activated functional processes within the cell is still not well understood. However, Grigorenko et al. (2002) did not mention any expression difference of the genes examined in this present study. In chapter 3.2.1 of this work, it is also shown that CB1 only induces alterations in gene expression after its activation. Specifically, CB1, activated by enhanced levels of AEA, induced a protective signaling cascade against excitotoxicity which included increases of expression of the transcription factors c-fos and zif268 and the neurotrophin BDNF. This study clearly demonstrated that the expression of these genes is only upregulated when the endocannabinoid system is activated during certain physiological challenges.

Another publication reported an altered gene expression in basal conditions in CB1-deficient mice. These CB1 mutants display significantly increased levels of substance P, dynorphin, enkephalin, and GAD67 mRNAs in neurons of the two output pathways of the striatum that project to the substantia nigra and the globus pallidus (Steiner et al., 1999). These findings are in contrast to the observations obtained from this study where neither GAD65 nor GAD67 showed altered expression levels in $CB^{-/-}$ mice. These opposite findings could be due to the different genetic background of the two $CB1^{-/-}$ strains used in the two studies. $CB1^{-/-}$ mice generated in our lab were backcrossed in the C57BL/6N substrain whereas Steiner et al. (1999) backcrossed their mutant mice into the C57BL/6J substrain. These two substrains are known to differ in the physiology and in various behavioral paradigms (Wotjak, 2003) which might explain the variation in gene expression described above.

In conclusion, this study showed that CB1 receptors rather regulate expression of genes upon activation of the cannabinoid system than in basal conditions. Whether or not the genes investigated in this study are modulated by cannabinoid signaling remains an open question. Further investigations are necessary which should screen for altered expression of these genes after cannabinoid treatment *in vitro* or *in vivo* and/or in experimental animal models in which the endocannabinoid system is known to be activated.

### 4.1.3 CB1 regulates VR1 activity through modulation of multiple signaling pathways

VR1 and CB1 share AEA as their common endogenous ligand (Zygmunt et al., 1999; Smart et al., 2000) which gives reason to suggest a functional interaction between the vanilloid system and the cannabinoid system. Moreover, the two receptors are colocalized in many sensory C fibres, both at the level of the spinal cord and in DRGs (Ahluwalia et al., 2000) where they play opposite roles in the control of nociception. VR1 appears to be partly responsible for the transmission of pain during thermal and inflammatory hyperalgesia (Caterina et al., 1997; Davis et al., 2000), whereas CB1 was suggested to counteract hyperalgesia by inhibiting VR1-mediated nociception (Richardson et al., 1997; Kelly and Chapman, 2001). To get a first impression about putative interactions of VR1 and CB1 in the brain, IHC with an anti-VR1 antibody was performed on mouse brain sections. The staining revealed VR1-expressing neurons in several areas of the mouse brain such as the neocortex, hippocampus, ventromedial hypothalmus, and substantia nigra. CB1 receptors are known to be present in the same areas hinting to a cross-talk of both receptor systems not only in the perception of noxious stimuli, but also in the regulation of higher brain functions, such as learning and memory.

The prerequisite for an investigation of a putative cross-talk between CB1 and VR1, is the coexpression of both receptors within the same cell. Therefore, a transgenic HEK-293 cell line was established, coexpressing functionally active CB1 and VR1 receptors (CB1-VR1-HEK cells), which was subjected to different pharmacological treatments. Results gained from these experiments indicate that, at least in the *in vitro* model applied here, stimulation of cannabinoid CB1 receptors may exert a regulatory effect on VR1-induced biological responses. Interestingly, this effect depends on the state of activation of cAMP-mediated signaling. It was demonstrated that a 5-min pretreatment with the CB1 agonist HU210 of CB1-VR1-HEK cells leads to a significant enhancement of capsaicin-induced and VR1-mediated increase of $[Ca^{2+}]_i$. This effect was counteracted by the CB1-selective antagonist, SR141716A, and was not observed in HEK-293 cells expressing only VR1 receptors (VR1-HEK cells), thus conclusively demonstrating the involvement of CB1 receptors in HU210 action. Interestingly, simultaneous treatment of CB1-VR1-HEK cells with HU210 and capsaicin did not lead to a similar potentiation of the effect on $[Ca^{2+}]_i$ by the latter compound. This temporal dependence of the effect suggests that: i) CB1-coupled intracellular signaling events, rather than a direct interaction between the two receptors, are necessary to observe the enhancement of VR1-induced biological effects; ii) endogenous substances, like AEA or *N*-

arachidonoyl-ethanolamine (Huang et al., 2002), which are capable of activating both receptor types, might produce different overall biological effects depending on which of the two receptors they activate first.

In order to investigate the first of the above possibilities some pilot experiments were carried out. Based on the recent findings that VR1 activity can be enhanced by protein phosphorylation catalyzed by PKC (Premkumar and Ahern, 2000; De Petrocellis et al., 2001b; Vellani et al., 2001), and inhibited by PIP2 (Chuang et al., 2001), and bearing in mind that CB1 receptors have been recently found to be coupled to activation of PLC (possibly via the βγ subunits of $G_{i/o}$ proteins; Ho et al., 1999; Netzeband et al., 1999) and stimulation of PI-3-K (Gomez Del Pulgar et al., 2000; Gomez Del Pulgar et al., 2002), the effects of PLC and PI-3-K inhibitors on the enhancement by HU210 of capsaicin effect on $[Ca^{2+}]_i$ were tested. *Per se*, two PI-3-K inhibitors and two PI-PLC inhibitors significantly reduced the effect of capsaicin on $[Ca^{2+}]_i$ at the concentrations previously reported to inhibit PI-3-K and PLC, respectively. Furthermore, these four compounds, at concentrations *per se* inactive on capsaicin-induced responses, abolished the potentiation of capsaicin effect caused by pretreatment with HU210. PI-3-K is not only responsible for the formation of PIP2, but it also catalyzes its phosphorylation to phosphatidylinositol-tris-phosphate (PIP3), whereas PI-PLC catalyzes PIP2 hydrolysis. Therefore, on the basis of these experiments, it is possible to hypothesize that, when overexpressed in HEK-293 cells, VR1 is under the negative influence of PIP2 (Chuang et al., 2001), whose concentration and turnover are in turn controlled by tonic PI-PLC and PI-3-K activity, respectively. When these two enzymes are inhibited, PIP2 remains associated with VR1 and the effect of capsaicin on $[Ca^{2+}]_i$ is therefore reduced (Fig. 4-1). Conversely, stimulation of PI-PLC (Netzeband et al., 1999) and PI-3-K (Gomez Del Pulgar et al., 2000) by CB1 receptor activation leads to an enhanced turnover of PIP2, with subsequent release of VR1 from the tonic inhibitory action exerted by this lipid (Fig. 4-1). Moreover, PC-PLC inhibitor, D609, inhibited the potentiation of capsaicin effect caused by pretreatment with HU210. Thus, it is possible that CB1 stimulation also leads to the activation of PC-PLC (Ho et al., 1999). This enzyme, together with PI-PLC, causes the release of diacylglycerols (DAG) and the subsequent activation of PKC, which can then sensitize VR1 to capsaicin (Premkumar and Ahern, 2000; De Petrocellis et al., 2001b; Vellani et al., 2001; Fig. 4-1).

A second set of experiments was carried out with exogenous AEA which is capable to activate both CB1 and VR1 receptors. Indeed, since the binding sites of CB1 and VR1 for AEA are extra- and intracellular, respectively (Pertwee, 1997; De Petrocellis et al., 2001a; Jordt and Julius, 2002), and AEA can be transported into HEK cells (De Petrocellis et al.,

2001a), treatment of CB1-VR1-HEK cells with this lipid is very likely to produce the sequential stimulation of CB1 and VR1 receptors, which was found here to be necessary for the enhancement of VR1 activity. Indeed, AEA was significantly more efficacious on $[Ca^{2+}]_i$ in CB1-VR1-HEK cells than in VR1-HEK cells, and that its effect in the former cells was reduced by the CB1 antagonist SR141716A to an extent undistinguishable from that observed in VR1-HEK cells. These findings might open the possibility that extra-cellular AEA exerts a more efficacious action on VR1 in those cells that naturally coexpress this receptor with CB1 receptors, such as some DRG neurons in culture (Ahluwalia et al., 2000). Indeed, in sensory neurons, either a strong excitatory effect, or a weaker excitatory effect that is enhanced by CB1 antagonists, have been observed on VR1-mediated cation currents or neuropeptide release (Nemeth et al., 2003). Also in other cells and tissues, AEA was found to exhibit varying potency at VR1 receptors. In general, it can be hypothesized that when a strong VR1-mediated effect is observed, as in the case of mesenteric sensory neurons (Zygmunt et al., 1999), and hippocampal slices (Al Hayani et al., 2001), CB1 and VR1 receptors are coexpressed in the majority of the cells. Conversely, when both an inhibitory CB1-mediated effect (observed at low AEA doses) and an excitatory VR1-mediated action (observed at high AEA doses and strengthened by CB1 receptor antagonists) are seen (Gauldie et al., 2001; Morisset et al., 2001), the two receptor types might be coexpressed only in a minority of the neurons. Finally, when substances that selectively activate CB1 receptors, such as HU210, inhibit the biological effects of substances that selectively activate VR1 receptors, such as capsaicin (Millns et al., 2001), this might be due to the lack of coupling of CB1 receptors to those intracellular signaling pathways that facilitate the gating of VR1 (i.e. PI-PLC or PI3-K, see above), or to their inhibition of signaling events that instead lead to sensitization of VR1 activity (PKA).

Recent studies have indeed shown that the sensitivity of VR1 receptors to ligands can be enhanced by substances that stimulate AC and subsequently activate PKA, thus leading to VR1 phosphorylation (De Petrocellis et al., 2001b; Morisset et al., 2001; Rathee et al., 2002; Fig. 4-1). Since CB1 receptors are coupled to inhibition of AC via the $\alpha$ subunits of $G_{i/o}$ proteins (Pertwee, 1997), it is reasonable that, in CB1-VR1 HEK cells, where HU210 was found to inhibit the FRSK-stimulated formation of cAMP, the synthetic cannabinoid would inhibit, rather than enhance, the previously reported potentiation of FRSK on the capsaicin-mediated effect on $[Ca^{2+}]_i$. In fact, in agreement with previous studies carried out with VR1-HEK cells (De Petrocellis et al., 2001b), a 5 min pretreatment with FRSK enhanced the effect of capsaicin on $[Ca^{2+}]_i$ also in CB1-VR1-HEK cells, and that, when incubated together with

FRSK, HU210 totally abolished this enhancement of capsaicin activity. This finding might provide an explanation to the previously reported inhibition of capsaicin-induced thermal and inflammatory hyperalgesia by AEA or HU210 (Richardson et al., 1997; Kelly and Chapman, 2001; Millns et al., 2001). It is in fact possible that, during inflammation, cAMP levels are enhanced, PKA is activated, and VR1 phosphorylated and upregulated, and that HU210 inhibits the effects of capsaicin (or of inflammatory stimuli that indirectly gate the VR1 receptors) by inhibiting AC. In contrast, in other experimental systems, such as the electrically-stimulated mouse vas deferens (Ross et al., 2001), it is possible that VR1 is not over-activated by the cAMP-signaling cascade, and thus substances that stimulate both CB1 and VR1 receptors can exert a very strong effect on VR1.

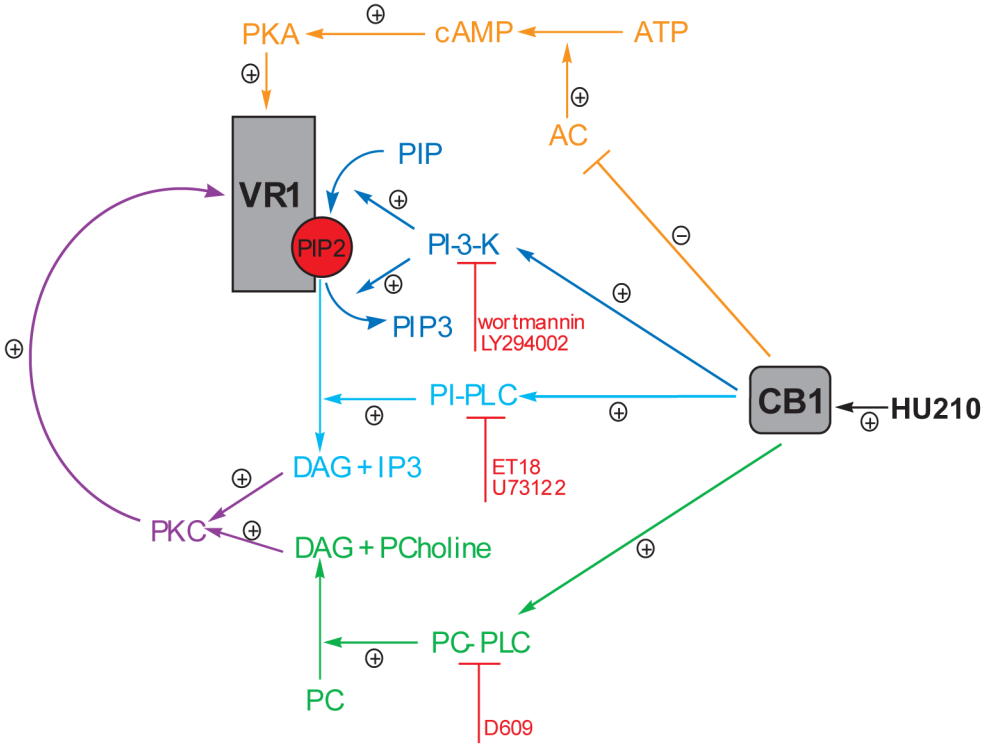


**Fig. 4-1: Schematic representation of the possible intracellular pathways underlying the CB1 receptor-mediated control of VR1 receptor activity.**
VR1 is tonically inhibited by PIP2, which in turn can be produced by the action of PI-3-K on PIP, and transformed by the same enzyme into PIP3, or into DAG and IP3 by PI-PLC. Thus, inhibitors of PI-3-K and of PI-PLC (whose action is indicated by blunt arrows) stabilize the VR1-PIP2 complex, thus leading to the inhibition of VR1 sensitivity to capsaicin, or to the inhibition of CB1-mediated activation of the two enzymes. The potentiation of capsaicin activity by HU210 observed in this study may involve these signaling pathways. Tonic or CB1-induced stimulation of VR1 by PC-PLC, and subsequent stimulation of DAG release and PKC activity, might also explain why a selective PC-PLC inhibitor (D609), as well as PI-PLC inhibitors (ET18 and U73122), attenuate both basal and HU210-enhanced activity of capsaicin at VR1. Finally, stimulation of adenylate cyclase (AC) and PKA by forskolin or during, e.g., inflammation, might lead to sensitization (or inhibition of desensitization) of VR1. In this case, activation of CB1 receptors by HU210 or other CB1 agonists would lead to VR1 inhibition by inhibiting AC.

In conclusion, this study demonstrated that when cannabinoid CB1 receptors and vanilloid VR1 receptors are coexpressed in the same cells, the sequential stimulation of the two receptors leads to a stronger stimulation of VR1 activity or to its inhibition depending on whether or not the cAMP-signaling pathway is activated. Sequential CB1-VR1 stimulation occurs *in vitro* when cells are treated first with HU210 and then with capsaicin, and might occur *in vivo* with extracellular AEA or other endogenous mediators, such as *N*-arachidonoyl-dopamine, that are capable of activating both receptors types (Huang et al., 2002). These findings provide an explanation to the often discrepant effects of AEA on sensory neurons, and strengthen the hypothesis that CB1 and VR1 receptors can be regarded as interacting metabotropic and ionotropic receptors for this endogenous compound and some of its congeners.

### 4.1.4 CB1 regulates BDNF expression via dampening of CRH-mediated signaling

This study demonstrates a functional cross-talk between the CRH system and the cannabinoid system regarding the regulation of the cAMP cascade and BDNF gene expression. A prerequisite for a possible cross-talk between the two receptor systems is their coexistance within the same neuron. In fact, ISH experiments on sections from adult mouse brain showed high levels of coexpression in several brain regions. As both receptors belong to the family of G protein-coupled receptors, the modulation of the cAMP signaling cascade after stimulation with both ligands and its effect on the putative downstream target gene BDNF were examined. Intracellular cAMP concentrations, levels of phospho-CREB and changes in BDNF gene expression were monitored in cerebellar primary cultures, a brain region with particularly high coexpression levels of both receptors. CRH was able to induce elevations of cAMP, phospho-CREB and BDNF levels which was inhibited by activation of CB1 receptors. These data showed both an important role of CRH in the induction of BDNF expression, but also the pivotal role of CB1 receptors in modulating this action of CRH.

CRHR1 is a seven transmembrane receptor linked to AC through $G_s$ protein activation. Subsequent cAMP production leads to the activation of PKA. Recently, neuroprotective effects associated with CRHR1 activation in response to a number of toxins have been demonstrated to be dependent on PKA activation (Pedersen et al., 2002; Bayatti et al., 2003). It is well-established that PKA activation leads to the phosphorylation and, hence, activation of CREB. Indeed, CRH-induced CREB activation in neurons was also shown to be PKA dependent, as application of H89, a PKA inhibitor, inhibited CREB phosphorylation (Bayatti

et al., 2003). As PKA is known to phosphorylate L-type $Ca^{2+}$ channels (Koob and Bloom, 1985), CRH may also activate this channel type resulting in an increased $Ca^{2+}$ influx (Somlyo and Somlyo, 1994). In fact, there are several examples of coupling of seven transmembrane receptors to L-type $Ca^{2+}$ channels via pertussis toxin-sensitive G proteins (Haws et al., 1993; Hescheler and Schultz, 1994; Somlyo and Somlyo, 1994). Some reports have suggested that the L-type $Ca^{2+}$ channel is also subject to direct inhibition by a pertussis toxin-sensitive G protein in neuronal cells (Dolphin and Scott, 1989; Haws et al., 1993). The regulation of BDNF expression by CREB has been studied extensively, and a majority of reports examined a $Ca^{2+}$-dependent mechanism of CREB phosphorylation and induction of BDNF expression (reviewed in West et al., 2001). In cortical neurons, a co-operation between FRSK-induced PKA activation and $Ca^{2+}$ influx triggers phosphorylation of CREB, followed by binding to the $Ca^{2+}$-dependent response element within the BDNF gene (Tao et al., 1998). Moreover, it was shown that FRSK induced increases in BDNF expression in raphe neurons in a PKA-dependent manner (Galter and Unsicker, 2000). Taking together, these data indicate that CRH is able to induce BDNF expression by activation of the cAMP signaling cascade and, possibly also by an increase of intracellular $Ca^{2+}$ concentration, due to an activation of L-type $Ca^{2+}$ channels.

Cannabinoids were found to inhibit N- and P/Q-type voltage-dependent $Ca^{2+}$ currents in several cell lines (Caulfield and Brown, 1992; Mackie and Hille, 1992; Mackie et al., 1995) and primary cultures of cerebellar granule neurons (Nogueron et al., 2001) via pertussis toxin-sensitive G proteins. In addition, another study using cerebral artery smooth muscle cells of the cat showed also an inhibition of L-type $Ca^{2+}$ channels upon activation of CB1 with WIN55,212-2 and the endocannabinoid AEA in a pertussis toxin-sensitive manner (Gebremedhin et al., 1999). On the other hand, the cannabinoid receptor agonist desacetly levonantradol is able to increase $Ca^{2+}$ influx into the neuroblastoma cell line N18TG2 at nanomolar concentrations (Rubovitch et al., 2002). This effect is mediated by $G_s$ GTP-binding proteins (Bash et al., 2003). Since CREB phosphorylation and activation of BDNF transcription are preferentially driven by $Ca^{2+}$ influx through L-type $Ca^{2+}$ channels, whereas they are poorly induced by $Ca^{2+}$ entering through N-methyl-D-aspartate (NMDA) receptors and non-L-type $Ca^{2+}$ channels (Dolphin and Scott, 1989; Haws et al., 1993), the possible role of L-type $Ca^{2+}$ channels in CB1-mediated inhibition of CRH-induced effects remains to be further investigated.

A main feature of CB1 effects is the inhibition of AC via $G_{i/o}$. This has been observed in a number of cell types including neuroblastoma cells (Howlett and Fleming, 1984), in CB1

transfected-cell lines (Matsuda et al., 1990), and in rat cerebellar granule cells (Pacheco et al., 1993). Cannabinoid-induced inhibition of AC results in the attenuation of PKA activity and a decrease in binding of transcription factors to cAMP response elements present in target genes (Koh et al., 1997). Additionally, independent of its activation state, CB1 is able to sequester G proteins required by other receptors linked with pertussis toxin-sensitive $G_{i/o}$ proteins (Vasquez and Lewis, 1999). CRH receptors are highly promiscuous as they can activate many different types of G proteins. In the rat cerebral cortex, CRH receptors can activate $G_s$, $G_i$, $G_q$, and $G_z$ (Grammatopoulos et al., 2001). Therefore, both inhibition of CRH-mediated cAMP augmentation, as well as sequestration of G proteins required by CRHR1 may be involved in the inhibitory action of CB1 on CRH-mediated signaling and induction of BDNF expression.

Activation of the mitogen-activated protein kinase (MAPK) pathway by excitatory actions of GABA is also able to increase BDNF expression via phosphorylation of CREB in developing neurons (Obrietan et al., 2002). In CHO cells transfected with CRHR1, application of a receptor agonist increased phosphorylation of MAPK and CREB (Rossant et al., 1999), providing a link between CRHR1 activation and control of changes in gene expression, e.g. of BDNF. Interestingly, activation of CB1 receptors in mice by a number of CB1 ligands has been shown to induce MAPK activation in transfected cell lines and in hippocampus (Bouaboula et al., 1995; Derkinderen et al., 2001; Derkinderen et al., 2003). Specifically, Derkinderen et al. (2003) showed that endocannabinoids and $\Delta^9$-THC activated extracellular signal-regulated kinase (ERK) in hippocampal slices and BDNF expression *in vivo*. The effects of cannabinoids were dependent on NMDA receptor activation *in vivo* but not in hippocampal slices, suggesting that multiple pathways lead to the initiation of CB1-mediated signaling pathways. In the present study, CB1 activation with the agonist WIN55,212-2 did not result in increases in BDNF expression in cultured cerebellar granule neurons. This may be due to region-specific coupling of signaling pathways, as it has been demonstrated in the case of CRH (Blank et al., 2003; Bayatti et al., 2003) or due to the advantage of the primary cell culture system in being able to analyze more closely the intracellular effects of receptor activation on specific cellular subtypes in isolation.

As CRH induced BDNF expression after 48 hours, physiological long-term changes such as synaptic plasticity might be the consequence of this altered gene expression. The neocortex and hippocampus are well-known to play a major role in such cognitive processes. Here, coexpression of CB1 and CRHR1 was shown in several cortical and hippocampal subregions hinting to a function of these receptors to modulate memory processing in concert with BDNF. Indeed, several publications underline this notion. Deprivation of BDNF leads to

an impairment of long-term potentiation (LTP), a form of synaptic plasticity, suggesting that BDNF is essential for certain forms of learning and memory (Korte et al., 1995). Also, CRH was shown to enhance memory in multiple learning tasks (Koob and Bloom, 1985; Liang and Lee, 1988; Behan et al., 1995; Heinrichs et al., 1997). For example, CRH, acting in a CRHR1-dependent manner, has been demonstrated to modulate hippocampus-dependent learning processes in stressful conditions when injected prior to training (Radulovic et al., 1999). In contrast to CRH, learning and memory impairments are among the most commonly reported behavioral effects of exogenous cannabinoids (reviewed in Sullivan, 2000). On the other hand, the temporally and spatially restricted release of endocannabinoids facilitates the induction of LTP in the hippocampus in single neurons (Carlson et al., 2002). As CB1 was activated in all cells by exogenously applied WIN55,212-2, and as CB1 receptors are located on excitatory glutamatergic synapses of granule neurons (Harvey and Napper, 1988; Harvey and Napper, 1991) in the model system used here, negative effects on LTP would be more plausible. Moreover, previous work has indicated that cerebellar LTP induction requires presynaptic $Ca^{2+}$ influx (Linden, 1998), cAMP production (Salin et al., 1996), and activation of PKA (Linden and Ahn, 1999), which all are negatively influenced by CB1 activation. Thus, exogenously applied cannabinoids might mediate their negative effects on learning and memory through dampening signaling pathway of other neurotransmitter systems which improve cognitive functions and therefore regulating the expression of downstream target genes.

Taken together, this study points to an important cross-talk between CB1 and CRHR1 in regulating BDNF expression. The function of CB1 in inhibiting the CRH signaling cascade is consistent with the role that CB1 ligands typically exert in this type of pathway (Howlett and Fleming, 1984; Koh et al., 1997). The general pharmacological stimulation of CB1 receptors in the whole tissue clearly has a negative effect on plasticity (Collins et al., 1995; Koh et al., 1997; Misner and Sullivan, 1999; Bohme et al., 2000) which might be mediated by inhibiting signaling pathways leading to expression changes of target genes. Further studies should therefore concentrate on the molecular and intracellular role of CRH not only in stress responses but also in memory processes with particular attention to the regulation of expression of neurotrophins, as well as the possible interplay with other neurotransmitter systems.

## 4.2 The cannabinoid system protects against neurotoxic insults and inflammation

### 4.2.1 CB1 in principal forebrain neurons activates a protective intracellular cascade after kainic acid-induced excitotoxicity

Excitotoxicity is a process in which glutamate or other excitatory amino acids, such as kainic acid (KA) induce neuronal cell death (Fig. 4-2). Accumulating evidence suggests that excitotoxicity may contribute to human neuronal cell loss caused by acute insults and chronic degeneration in the CNS (Coyle and Puttfarcken, 1993; Lee et al., 1999; McNamara, 1999). The results obtained from this study show that CB1 receptors in glutamatergic forebrain neurons of the mouse brain promote activation of IEG such as c-fos, zif268 and BDNF, all known to exert neuroprotective properties during excitotoxicity (Fig. 4-2). In particular, BDNF seems to be an important downstream target of this activated pathway as it completely restored the protective functions of the endogenous cannabinoid system impaired by the blockade of CB1 receptors in organotypic hippocampal cultures. On the other hand, CB1 receptors on GABAergic interneurons exert only a negligible function under these circumstances. Considering that in other behavioral paradigms, CB1 receptors on GABAergic interneurons were proposed to play a crucial role (Katona et al., 1999; Howlett et al., 2002; Marsicano et al., 2002b), these present data further underline the diverse functions of the endogenous cannabinoid system in different neuronal processes and the functional interaction with discrete neurotransmitter systems.

This study demonstrated that the cannabinoid system mediates protection against excitotoxicity not only by increased levels of AEA and dampening neuronal excitability of pyramidal neurons (see in detail in Marsicano et al., 2003), but also by the induction of intracellular cascades (Fig. 4-2). All subregions of the hippocampi derived from KA-treated $CB1^{f/f}$ mice showed dramatically increased levels of c-fos, zif268, and BDNF transcripts compared to saline-treated animals. In the hippocampi derived from KA-treated $CB1^{f/f;CaMKII\alpha Cre}$ mice, the induction of c-fos, zif268, and BDNF expression was abolished. c-fos is induced by neuronal activity, including KA-induced seizures (Popovici et al., 1990; Smeyne et al., 1992). Zhang et al. (2002a) showed that c-fos participates in key cellular mechanisms underlying both neuronal excitability and protection by selectively regulating gene expression in the brain, e.g. BDNF. Also zif268 is rapidly induced after KA administration (O'Donovan et al., 1999).

To further examine the relationship between CB1 and BDNF during excitotoxicity, organotypic hippocampal cultures were used as an *in vitro* model system, to investigate whether or not the KA-induced increase of BDNF levels is functionally involved in the CB1 receptor-dependent neuroprotection. KA application increased BDNF protein levels in hippocampal explants and administration of the CB1 receptor antagonist SR141716A blocked this effect. These findings are in agreement with the data obtained *in vivo* as described before. Moreover, exogenous BDNF was able to counteract the damaging effects of CB1 receptor inactivation and completely prevented the neuronal death induced by KA treatment. Several investigations indicate a pivotal role of BDNF in neuroprotection in different models of neurotoxicity, such as *in vivo* and *in vitro* ischemic neuronal damage (Tsukahara et al., 1994; Pringle et al., 1996; Endres et al., 2000; Pardridge, 2002; Mishra et al., 2003), glucose deprivation or glutamate-induced neuronal damage in neuronal cultures (Cheng and Mattson, 1994), and KA-induced excitotoxicity in the striatum (Gratacos et al., 2001). However, high doses of exogenous BDNF or genetic overexpression of BDNF were shown to exacerbate the injury caused by *in vivo* application of KA (Rudge et al., 1998; Croll et al., 1999), suggesting that BDNF levels need to be tightly regulated to exert the protective functions.

In the past decade, the intracellular mechanisms of CB1 and BDNF signaling have been extensively investigated and show some converging features that might cooperate in neuroprotection. *In vivo* cannabinoid application induces the expression of IEG products, including BDNF mRNA, and this induction depends on the activation of extracellular regulated kinases (ERKs; Derkinderen et al., 2003). Phosphorylation of ERKs has also been proposed to mediate the neuroprotective effects of BDNF (Hetman et al., 1999; Han and Holtzman, 2000). Notably, KA-induced increases of both ERK phosphorylation and BDNF expression have been observed in CB1 receptor-deficient mice (Marsicano et al., 2003). It is, therefore, tempting to speculate that ERK phosphorylation might represent an intracellular event functionally connecting activation of CB1 receptors, BDNF signaling and protection against excitotoxicity (Fig. 4-2). Therefore, BDNF is a molecular link between acute activation of the endogenous cannabinoid system during excitotoxicity and long-term protection.

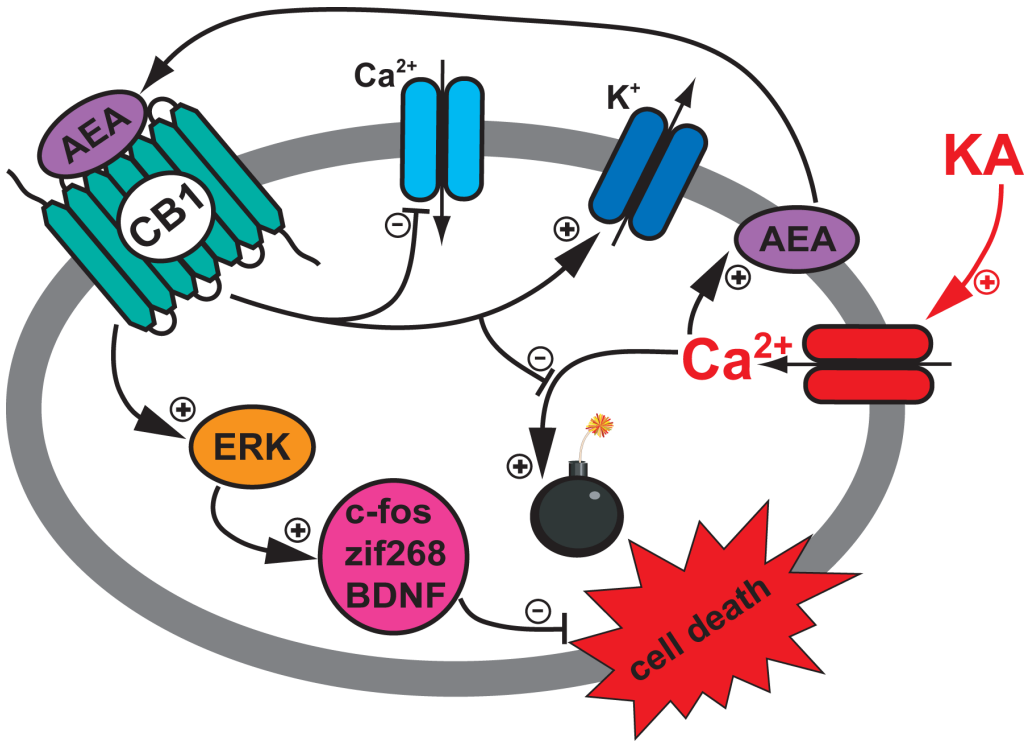


**Fig. 4-2: Schematic representation of the possible intracellular pathways underlying the CB1-mediated protection against kainic acid-induced excitotoxicity.**
Kainic acid (KA) binds to its receptor and leads to massive influx of $Ca^{2+}$, which triggers neuronal death. Elevation of intracellular $Ca^{2+}$ stimulates the biosynthesis of anandamide (AEA). Binding of AEA to CB1 leads not only to dampening of neuronal excitability via inhibition of $Ca^{2+}$ channels ($Ca^{2+}$) and activation of $K^+$ channels ($K^+$), but also induces a protective signaling cascade. Specifically, the expression of the immediate early genes c-fos, zif268 and BDNF is driven by the activation of extracellular regulated kinases (ERK) and counteracts against the damaging excitotoxic effects mediated by KA application.

There is evidence from different neuropathological models that the endogenous cannabinoid system can be differentially activated in a species- and age-dependent manner (Baker et al., 2001; Hansen et al., 2001; Panikashvili et al., 2001; van der Stelt et al., 2001b; Howlett et al., 2002; Marsicano et al., 2002b; Parmentier-Batteur et al., 2002; Wallace et al., 2002) or even via non-CB1 receptor-mediated mechanisms (Di Marzo et al., 2002). For instance, brain trauma induced an increase of 2-AG in adult mice (Panikashvili et al., 2001), whereas in a similar experimental model in neonatal rats AEA, but not 2-AG, increased (Hansen et al., 2001). In neonatal rats, blocking of CB1 receptors with SR141716A induced a "paradoxical" protection against NMDA-induced neurotoxicity (Hansen et al., 2002), whereas exogenous AEA was protective in a model of neurotoxicity in the same species at the same age (van der Stelt et al., 2001b; Veldhuis et al., 2003). The reasons of these apparent discrepancies are not clear at present. Different processing of endocannabinoids in different species and at different developmental stages (Moesgaard et al., 2003), different experimental conditions (e.g. the way to induce neurotoxicity and the parameters monitored) or differences in neuronal

circuitries at different ages (Ben Ari, 2002) may be responsible for some of these divergent findings.

The present results demonstrate a sophisticated network of signaling interactions of the cannabinoid system with other neuronal systems to protect against excitotoxicity in the mouse brain. CB1 receptors in glutamatergic neurons serve to rapidly activate a protective cascade with BDNF being an important downstream mediator which rescues neurons from excitotoxic insults. Therefore, the endogenous cannabinoid system might become a promising therapeutic target for the treatment of neurodegenerative diseases with excitotoxic events as their hallmarks (Choi, 1988; Coyle and Puttfarcken, 1993; McNamara, 1999; Ben Ari and Cossart, 2000).

### 4.2.2 Cannabinoids exert non-CB1-mediated antioxidant, neuroprotective effects

As shown in chapter 3.2.1, neuroprotection against excitotoxic insults is mediated by CB1 receptors. Although most of the actions of cannabinoids in the CNS appear to be exerted by CB1, there is also evidence for non-CB1 receptor-mediated protection against neurotoxicity (Di Marzo et al., 2002). To define the involvement of CB1 in cannabinoid-mediated neuroprotection in another model system than the KA model of excitotoxicity in mice (as used in chapter 3.2.1), the hippocampal cell line HT22 was used for oxidative stress assays. As it is necessary to test the protection potential of cannabinoids in identical systems that differ only in the expression of CB1, HT22 cells were stably transfected with an expression vector containing the CB1 cDNA. The functional expression of the receptor was confirmed by Northern blot experiments and cAMP accumulation assays. As CB1 is characterized by the inhibition of AC in response to agonists (Howlett and Fleming, 1984; Howlett, 1985), the concentration of intracellular cAMP should decrease. Indeed, the presence of WIN55,212-2 was able to decrease the FRSK-induced cAMP accumulation in CB1-expressing cells to an extent already reported in other heterologous expression systems (Song and Bonner, 1996). Afterwards, the neuroprotective properties of cannabinoids were analyzed in *in vitro* oxidative stress assays. No differences were observed in the neuroprotective activity of the tested drugs, each belonging to one group of cannabinoids, in presence or in absence of CB1, in the HT22 cell lines. These results correlate with the data from (Chen and Buck, 2000). They used a different model of oxidative stress in non-neuronal cell lines and found a CB1-independent protection caused by several cannabinoids. However, they showed the presence of CB1 in their cell lines by RT-PCR and did not provide any data using the same cell type with and

without CB1. The present results strongly extend the concept of the antioxidant action of cannabinoids, providing direct evidence of the independence of such activity from CB1.

Oxidative stress is one of the central events onto which many neurodegenerative cascades converge. Therefore, $H_2O_2$-induced oxidative cell death *in vitro* is a clear paradigm of neurodegeneration that can provide useful information about the neuroprotective aspects of certain pharmacological compounds. Indeed, $H_2O_2$ is known to be the mediator of oxidative apoptosis in neuronal cells (Behl et al., 1994; Maher and Davis, 1996; Chun et al., 2001). Drugs that are able to inhibit these oxidative processes are promising candidates for the treatment of such diseases. Many cannabinoids have structural features typical for phenolic antioxidants and could exert neuroprotective activities (Pertwee, 1997). Therefore, they possess very interesting therapeutic potential for the treatment of several neurodegenerative diseases. In addition to the direct antioxidant activity of the phenolic cannabinoids, these compounds may further affect membrane-associated and intracellular signaling mechanisms. For instance, due to their lipophilicity, these compounds could increase the membrane fluidity and may eventually lead to changes in the activity of membrane-bound receptor systems (e.g. neurotransmitter receptors). Moreover, molecular interactions with intracellular signaling processes are possibly similar to those that are known to be executed by 17β-estradiol, which is also a phenolic neuroprotective antioxidant acting independently of its cognate estrogen receptor (Moosmann and Behl, 1999). In addition to its wide range of estrogen receptor-dependent effects, estradiol performs various receptor-independent neuromodulatory activities including also the activation of the neuroprotective MAPK signaling (Behl and Holsboer, 1999), which is known to be also activated by cannabinoids, both in a CB1-dependent (Valjent et al., 2001) and CB1-independent manner (Jan and Kaminski, 2001).

In contrast to the phenolic moieties, which are the mediators of antioxidant neuroprotection of several exogenous cannabinoid drugs, neuroprotective activities of endocannabinoids appear to be CB1-mediated and do not involve antioxidant properties due to lack of the phenolic group. Endocannabinoids are increased in brain after closed-head injury (Panikashvili et al., 2001) and KA-induced excitotoxicity (Marsicano et al., 2003) where they exert neuroprotective properties by a CB1-dependent mechanism. These data suggest a general neuroprotective function of the endocannabinoid system. Therefore, it is tempting to propose potent therapeutic applications of drugs that are able to both sustain the "endogenous" CB1-mediated neuroprotective activity of endocannabinoids and to provide antioxidant protection. Good candidates are inhibitors of endocannabinoid uptake, such as AM404 and VDM11 (De Petrocellis et al., 2000), both of which contain a phenolic residue.

The uptake inhibitor UCM707 was shown to protect against excitotoxicity in KA treated mice (Marsicano et al., 2003). In addition, given the "on-demand" activation of the endocannabinoid system (Di Marzo et al., 1998; Piomelli et al., 2000), using endocannabinoid uptake inhibitors might diminish the undesirable psychotropic side effects generally observed after treatment with CB1 agonists.

In conclusion, the use of antioxidant cannabinoids or, in particular, the inhibition of endocannabinoid uptake by antioxidant drugs could provide promising avenues for the therapeutic targeting of different aspects of neurodegenerative diseases, by stimulating a self-protective endogenous system of the brain (via CB1-dependent mechanisms) and by counteracting oxidative stress (via CB1-independent mechanisms).

### 4.2.3 CB1 expression is important during the acute phase of inflammation

Upon inflammatory insults, several different cellular pathways are activated in the intestinal tract leading to a pathological state (Wood et al., 1999). However, simultaneous protective mechanisms are also activated, and the balance between pro- and anti-inflammatory responses determines the outcome of the pathological processes (Holzer, 2001). CB1 receptors have been detected on enteric nerves, and pharmacological effects of their activation include gastroprotection, reduction of gastric and intestinal motility and reduction of intestinal secretion. A pharmacological modulation of the endogenous cannabinoid system could provide new therapeutics for the treatment of a number of gastrointestinal diseases, including nausea and vomiting, gastric ulcers, irritable bowel syndrome, Crohn's disease, secretory diarrhea, paralytic ileus and gastroesophageal reflux disease (reviewed in Di Carlo and Izzo, 2003; Izzo et al., 2003).

In this study, the involvement of the endogenous cannabinoid system and its cross-talk with other receptor systems in the development of experimental colitis in mice, induced by intrarectal DNBS treatment, was analyzed. Genetic and pharmacological ablation of CB1 receptors rendered mice much more sensitive to DNBS, indicating a protective role of the CB1 receptors during inflammation (Massa et al., 2004) which was further supported by the increased levels of CB1 transcripts in wild-type mice after induction of inflammation. By ISH, CB1 mRNA was detected in the myenteric plexus of the colon, consistent with other studies investigating a detailed localization of CB1 in the mouse gastroenteric tract (Izzo et al., 2001b). An upregulation of CB1 receptors in the intestine was already shown in another model of intestinal inflammation (Izzo et al., 2001a) suggesting that inflammation of the gut increases the potency of cannabinoid agonists possibly by upregulating CB1 receptor

expression. Recently, in mice with induced secretory diarrhea, the endocannabinoid system was shown to inhibit fluid accumulation in the small intestine via enhanced levels of AEA and increased CB1 transcripts (Izzo et al., 2003). These observations suggest that the endocannabinoid system might in part protect against intestinal diseases by enhanced cannabinoid signaling through an increased number of CB1 receptors.

Enhanced cannabinoid signaling could lead to activation of other neuronal systems implicated in the protection of intestinal diseases. A protective role against colon inflammation was recently demonstrated for the endogenous opioidergic system by Philippe et al. (2003), showing that agonists of peripheral μ-opioid receptors significantly reduced inflammation in two experimental models of colitis. Interestingly, a cross-talk between the endogenous cannabinoid and opioid system has been established (reviewed in Manzanares et al., 1999). Several studies provide evidence that cannabinoids increase the synthesis and release of opioids. Chronic administration of $\Delta^9$-THC to rats markedly increased levels of the endogenous opioid preproenkephalin (Enk) in the spinal cord and several regions of the brain (Corchero et al., 1997; Manzanares et al., 1998). To see whether the cannabinoid system in the inflamed colon might also be connected to the opioid system, expression levels of Enk were investigated. DNBS treatment increased the expression of mRNA coding for Enk in the colon. This increase was present in DNBS-treated colons of both genotypes, but it appeared less pronounced in $CB1^{-/-}$ mice as compared to $CB1^{+/+}$ littermates, even though this difference did not reach complete statistical significance. These observations could indicate that the stimulation of Enk expression by CB1 receptors, might mediate part of the protective actions of the endogenous cannabinoid system during colon inflammation. However, further investigations are needed to substantiate this potentially interesting protective cross-talk between the two endogenous systems in the intestinal tract.

In conclusion, using the DNBS-induced colitis model, this study shows that the endogenous cannabinoid system is physiologically involved in the protection against excessive inflammation, possibly by interacting with the opioid system. These results strongly suggest that modulation of the physiological activity of the endogenous cannabinoid system during colonic inflammation might be a promising therapeutic tool for the treatment of several diseases characterized by inflammation of the gastrointestinal tract.

# 5 SUMMARY

The work described in this thesis was aimed to better understand some aspects of the physiological functions of the "brain-type" cannabinoid receptor CB1 and of the cannabinoid system in the murine nervous system, with special respect to interactions with other receptor systems. By means of different methological approaches it was shown that the cannabinoid system acts in concert with other receptor systems and is able to modulate many brain functions under both physiological and pathological conditions.

Neuroanatomical results showed that CB1 is coexpressed with several other receptors (D1, D2, 5-HT1B, 5-HT3, CRHR1) within the same neurons in many regions of the mouse brain, suggesting cross-talks with these signaling systems. Moreover, the expression analysis of nine different genes, known to be linked to the cannabinoid system, in CB1-deficient animals and wild-type littermates revealed no differences between genotypes. This does not exclude the involvement of these genes in CB1-mediated signaling but suggests that CB1 receptors rather regulate gene expression upon activation of the cannabinoid system than in basal conditions.

Functional analyses of putative interactions are presented for the vanilloid receptor VR1 and the corticotropine-releasing hormone receptor CRHR1. In HEK-293 cells coexpressing CB1 and VR1, the sequential stimulation of both receptors led to a stronger stimulation of VR1 activity. The pharmacological blockade of several key enzymes, known to be activated by CB1, totally abolished this effect, suggesting that these signaling pathways contribute to the potentiating effects of CB1 stimulation on VR1 activity. Interestingly, sequential stimulation of CB1 and VR1 led to a weaker stimulation of VR1 activity when the cAMP-signaling pathway was concomitantly activated. These data suggest that CB1 differentially influences VR1 gating depending on whether or not cAMP-mediated signaling has been activated.

In primary cerebellar granule neurons, where CB1 and CRHR1 are highly coexpressed, an important cross-talk between both receptors was shown in regulating the expression of the neurotrophin BDNF. Stimulation of CRHR1 with CRH led to increases in cAMP, phosphorylated CREB and finally BDNF, which might be considered as a consecutive signaling pathway. All these steps were inhibited by the simultaneous stimulation of CB1, which is consistent with the role that CB1 ligands typically exert in this type of pathway. The regulation of BDNF expression by the interaction of the cannabinoid and the CRH system might play a role in cognitive brain functions and synaptic plasticity.

Concerning physiological functions of the cannabinoid system, this work also focused on neuroprotection and inflammation. Two model systems were used to examine the involvement of CB1 during neurotoxicity. The *in vitro*- and *in vivo*-model of kainic acid-induced epileptiform seizures clearly demonstrated the physiological importance of CB1 in glutamatergic forebrain neurons to protect against excitotoxicity. Under these circumstances, CB1 receptor activation is a necessary step to induce a protective signaling cascade in the mouse brain. Especially BDNF seems to be an important downstream mediator to rescue neurons from excitotoxic insults. In contrast to these results are the findings from oxidative stress assays in CB1-transfected HT22 cell lines, which strongly suggest that CB1 is not involved in the cellular antioxidant properties of cannabinoids.

Finally, an *in vivo*-model of intestinal inflammation showed that the cannabinoid system is involved in the protection against excessive inflammation by controlling cellular pathways triggering this pathological condition. Enhanced signaling activity of CB1 is proposed as the levels of receptor transcripts are increased in the inflamed colon.

In conclusion, the results of these thesis demonstrate a sophisticated network of signaling interactions of the cannabinoid system with other neuronal systems in the murine nervous system which regulate different physiological and pathological processes in neurons. Therefore, the endogenous cannabinoid system might become a promising therapeutic target for the treatment of neurodegenerative and inflammatory diseases.

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# 8 APPENDIX

## 8.1 Abbreviations

| Abbreviation | Meaning |
|---|---|
| $\Delta^9$-THC | $\Delta^9$-tetrahydrocannabinol |
| µl | microliter |
| $[Ca^{2+}]_i$ | intracellular calcium |
| 2-AG | 2-arachidonoylglycerol |
| 5-HT1B | serotonin receptor type 1B |
| 5-HT3 | serotonin receptor type 3 |
| AC | adenylate cyclase |
| ACTH | adrenocorticotropic hormone |
| AEA | anandamide |
| AP | alkaline phosphatase |
| AT | anandamide transporter |
| ATP | adenosintriphosphate |
| BDNF | brain-derived neurotrophic factor |
| BLA | basolateral amygdaloid nucleus |
| BMA | basomedial amygdaloid nucleus |
| bp | base pair |
| BSA | bovine serum albumine |
| CA | Ammon's horn |
| CA1 | CA1 region of hippocampus |
| $Ca^{2+}$ | calcium ions |
| CA3 | CA3 region of hippocampus |
| CaMK IIα | calcium/calmodulin-dependent kinase IIα |
| cAMP | cyclic adenosine monophosphate |
| CB1 | cannabinoid receptor type 1 |
| $CB1^{-/-}$ | CB1-deficient mice |
| $CB1^{+/+}$ | CB1 wild-type littermates |
| $CB1^{f/f}$ | CB1 floxed mice |
| $CB1^{f/f;CaMKII\alpha Cre}$ | mutant mice lacking CB1 in all principal projecting forebrain (including glutamatergic) neurons |
| CB2 | cannabinoid receptor type 2 |
| CBP | CREB-binding protein |
| CCK | cholecystokinin |
| cDNA | complementary DNA |
| CNS | central nervous system |
| CP55,940 | c (-)-cis-3[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl)cycloexanol |
| cpm | counts per minute |
| Cpu | caudate putamen |
| Cre | causing recombination |
| CRE | cAMP response element |
| CREB | cAMP response element binding protein |
| CRH | corticotropine releasing hormone |
| CRHR1 | corticotropine releasing hormone receptor type 1 |
| CRHR2 | corticotropine releasing hormone receptor type 2 |
| D1 | dopamine receptor type 1 |
| D2 | dopamine receptor type 2 |
| DAB | diaminobenzidine |
| DAG | diacylglycerol |

| | |
|---|---|
| DEn | dorsal endopirifom nucleus |
| DEPC | diethylpyrocarbonate |
| DG | dentate gyrus |
| DIG | dioxygenin |
| DMEM | Dulbecco's Modified Eagles Medium |
| DMH | dorsomedial hypothalamic nucleus |
| DMSO | dimethyl sulfoxide |
| DNA | desoxyribonucleic acid |
| DNase | desoxyribonuclease |
| DNBS | dinitrobenzenesulphonic acid |
| dNTP | desoxynucleosidtriphosphate |
| DRG | dorsal root ganglion |
| DSE | depolarized-induced suppression of excitation |
| DSI | depolarized-induced suppression of inhibition |
| DTT | dithiothreitol |
| e.g. | for example |
| EDTA | ethylendiamintetracetate |
| ELISA | enzyme-linked immunosorbent assay |
| Enk | preproenkephalin |
| eNOS | endothelial nitric oxide synthase |
| ERK | extracellular signal-regulated kinase |
| FAAH | fatty acid amide hydrolase |
| FCS | fetal calf serum |
| FRSK | forskolin |
| g | gram |
| G418 | geneticin |
| GABA | γ-aminobutyric acid |
| GAD65 | glutamic acid decarboxylase 65 kDa |
| GAD67 | glutamic acid decarboxylase 67 kDa |
| GL | granular layer of cerebellum |
| GR | granular layer of dentate gyrus |
| h | hour(s) |
| HA | hemaglutinin |
| HEK-293 | human embryonic kidney cells 293 |
| HEPES | (N-2-Hydroxyethylpiperazine-N'-2-ethanesulfonic acid) |
| Hipp. | hippocampal region |
| HPA | hypothalamic-pituitary-adrenal |
| HPRT | hypoxanthine-guanine phosphoribosyltransferase |
| HU210 | (6aR)-trans-3-(1,1-Dimethylheptyl)-6a,7,10,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol |
| hVR1 | human VR1 |
| IBMX | 3-Isobutyl-1-methylxanthine |
| IEG | immediate early gene |
| iNOS | inducible nitric oxide synthase |
| $IP_3$ | inositol-1,4,4-trisphosphate |
| ISH | *in situ* hybridization |
| I-VI | layers of neocortex |
| $K^+$ | potassium ions |
| KA | kainic acid |
| kb | kilobase |
| kDa | kilo Dalton |
| Lat | lateral cerebellar nucleus |
| LEnt | lateral entorhinal cortex |
| loxP | location of crossover |
| LTD | long-term depression |
| LTP | long-term potentiation |

| | |
|---|---|
| M | molar |
| M1/M2 | primary and secondary motor cortex |
| mAChR | muscarinic acetylcholine receptor |
| MAPK | mitogen-activated protein kinase |
| MEM | minimal essential medium |
| mGluR | metabotropic glutamate receptor |
| min | minute(s) |
| ML | molecular layer of cerebellum |
| mM | millimolar |
| mRNA | messenger RNA |
| MW | molecular weight |
| n | number of experiments |
| nM | nanomolar |
| NMDA | N-methyl-D-aspartate |
| nNOS | neuronal nitric oxide synthase |
| NO | nitric oxide |
| P | probability value of a statistical hypothesis |
| PBS | phosphate buffer saline |
| PC-PLC | phosphatidyl-choline selective PLC |
| PCR | polymerase chain reaction |
| pCREB | phosphorylated CREB |
| PFA | paraformaldehyde |
| PI-3-K | phosphoinositide-3-kinase |
| PIP2 | phosphatidyl-inositol-bis-phosphate |
| PIP3 | phosphatidyl-inositol-tris-phosphate |
| PI-PLC | phosphatidyl-inositol-selective phospholipase |
| Pir | piriform cortex |
| pKS | pBlueScript KS (-) |
| PLC | phospholipase C |
| POD | horse radish peroxidase |
| pyr | pyramidal cell layer of hippocampus |
| rCB1 | rat CB1 |
| RNA | ribonucleic acid |
| RNase | ribonuclease |
| RNasin | ribonuclease inhibitor |
| rpm | rotations per minute |
| RT | reverse transcriptase |
| Rt | reticular thalamic nucleus |
| rVR1 | rat VR1 |
| SDS | sodium dodecylphosphate |
| SEM | standard error of the mean |
| SR141716A | N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2, 4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl |
| SSC | standard saline citrate |
| TAE | Tris Acetate EDTA buffer |
| Tu | olfactory tubercle |
| UTP | uridintriphosphate |
| V | volt |
| VMH | ventromedial hypothalamic area |
| VR1 | vanilloid receptor type 1 |
| $VR1^{-/-}$ | VR1-deficient mice |
| $VR1^{+/+}$ | VR1 wild-type littermates |
| vs | versus |
| WIN55,212-2 | (*R*)-(+)-[2,3-Dihydro-5-methyl-3-(4-morpholinylmethyl) pyrrolo [1,2,3-de]-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone |

## 8.2 Published articles of the thesis and data in preparation for publication

H. Hermann, G. Marsicano, and B. Lutz

# Coexpression of the cannabinoid receptor type 1 with dopamine and serotonin receptors in distinct neuronal subpopulations of the adult mouse forebrain

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# COEXPRESSION OF THE CANNABINOID RECEPTOR TYPE 1 WITH DOPAMINE AND SEROTONIN RECEPTORS IN DISTINCT NEURONAL SUBPOPULATIONS OF THE ADULT MOUSE FOREBRAIN

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**Abstract**—The cannabinoid receptor type 1 ($CB_1$) displays unusual properties, including the dual capacity to inhibit or stimulate adenylate cyclase and a brain density considerably higher than the majority of G protein-coupled receptors. Together with overlapping expression patterns of dopamine and serotonin receptors this suggests a potential of $CB_1$ to modulate the function of the dopamine and serotonin system. Indeed, pharmacological studies provide evidence for cross-talks between $CB_1$ and receptors of these neurotransmitter systems. In trying to obtain further insights into possible functional and/or structural interactions between $CB_1$ and the dopamine receptors and the serotonin receptors, we performed double-label *in situ* hybridization at the cellular level on mouse forebrain sections by combining a digoxigenin-labelled riboprobe for $CB_1$ with $^{35}S$-labelled riboprobes for dopamine receptors D1 and D2, and for serotonin receptors 5-HT1B and 5-HT3, respectively. As a general rule, we found that $CB_1$ colocalizes with D1, D2 and 5-HT1B only in low-$CB_1$-expressing cells which are principal projecting neurons, whereas $CB_1$ coexpression with 5-HT3 was also observed in high-$CB_1$-expressing cells which are considered to be mostly GABAergic. In striatum and olfactory tubercle, $CB_1$ is coexpressed to a high extent with D1, D2 and 5-HT1B. Throughout the hippocampal formation, $CB_1$ is coexpressed with D2, 5-HT1B and 5-HT3. In the neocortex, coexpression was detected only with 5-HT1B and 5-HT3. In summary a distinct pattern is emerging for the cannabinoid system with regard to its colocalization with dopamine and serotonin receptors and, therefore, it is likely that different mechanisms underlie its cross-talk with these neurotransmitter systems. © 2002 IBRO. Published by Elsevier Science Ltd. All rights reserved.

*Key words:* cannabinoids, colocalization, *in situ* hybridization, cross-talk, neurotransmitter systems.

The main psychoactive component of *Cannabis sativa*, $\Delta^9$-tetrahydrocannabinol, exerts most of its effects by interacting with cannabinoid receptors. At present, two G protein-coupled receptors have been identified; the cannabinoid receptor type 1 ($CB_1$) is preferentially expressed in the CNS, whereas the cannabinoid receptor type 2 ($CB_2$) is mainly present in immune cells (for review see Pertwee, 1997). Based on the finding of endogenous ligands (Devane et al., 1992; Sugiura et al., 1995; Mechoulam et al., 1998), the endocannabinoid system has emerged as an important neuromodulatory system in brain physiology (for review see Di Marzo et al., 1998). Recent analyses of $CB_1$-deficient mice have further underlined the importance of the cannabinoid system in various behaviors such as learning and memory (Reibaud et al., 1999), drug withdrawal response (Ledent et al., 1999) and locomotor activity (Zimmer et al., 1999).

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*Abbreviations:* $CB_1$, cannabinoid receptor type 1; $CB_2$, cannabinoid receptor type 2; CCK, cholecystokinin; D1, dopamine receptor 1; D2, dopamine receptor 2; DIG, digoxigenin; DTT, dithiothreitol; EDTA, ethylene diaminetetra-acetate; PBS, phoshate-buffered saline; RT-PCR, reverse transcription-polymerase chain reaction; SSC, saline sodium citrate; 5-HT1B, serotonin receptor 1B; 5-HT3, serotonin receptor 3.

$CB_1$ displays unusual properties, including the dual capacity to inhibit or stimulate adenylate cyclase via $G_{i/o}$ or $G_s$ proteins (Bonhaus et al., 1998; Shire et al., 1999) and a brain density considerably higher than any other known G protein-coupled receptor (Herkenham et al., 1990). Implicit in these properties is the potential of $CB_1$ to modulate the function of other receptor systems such as the dopamine and serotonin system, and evidence to support this notion is mounting, as the following observations exemplify. Glass and Felder (1997) found that the activation of either $CB_1$ or dopamine receptor 2 (D2) in rat primary striatal cells resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an increase of cAMP accumulation. Pharmacological experiments in mice by Meschler et al. (2000) showed that the application of a D2 agonist was able to attenuate the motor dysfunction caused by the $CB_1$ agonist levonantradol. Similarly, a dopamine receptor 1 (D1) agonist attenuated the effect of levonantradol, while a D1 antagonist enhanced the effects of levonantradol. A functional interaction of the cannabinoid and the dopamine system was also suggested in memory storage (Castellano et al., 1997; Nava et al., 2000). Regarding the serotonin system, it was shown that low concentrations of cannabinoid agonists inhibit the function of the serotonin receptor 3 (5-HT3) (Fan, 1995). Similar results were shown later by another group revealing that the pharma-



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cological activity of the endocannabinoid anandamide might be partially mediated through serotonin receptors (Kimura et al., 1998). Such observations lead to the notion that there are allosteric binding sites for cannabinoids on the 5-HT3 receptor or that there is a cooperative interaction between these two receptors.

The first indication to possible interactions between different receptors is given, when both receptors are expressed within the same neuron. Indeed, $CB_1$ in rodent forebrain structures (Marsicano and Lutz, 1999; Tsou et al., 1998; Egertová and Elphick, 2000) displays a significant extent of overlapping expression with various dopamine and serotonin receptors, among which D1 (Mansour et al., 1991), D2 (Meador-Woodruff et al., 1989), serotonin receptor 1B (5-HT1B) (Maroteaux et al., 1992) and 5-HT3 (Tecott et al., 1993) are the focus of the present work. This study provides a comparative coexpression analysis at single-cell level of the $CB_1$ receptor with dopamine and serotonin receptors for the first time using double-label *in situ* hybridization experiments on mouse forebrain sections, by combining $^{35}S$-labelled riboprobes for D1, D2, 5-HT1B, and 5-HT3, respectively, with a digoxigenin (DIG)-labelled riboprobe for $CB_1$.

## EXPERIMENTAL PROCEDURES

### *Animals and tissue preparation*

Animals were housed in a temperature- and humidity-controlled room with a 12 h light-dark cycle (light from 07:00–19:00) and with access to food and water *ad libitum*. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

Adult mice (3–5 months old; C57BL/6) were killed by cervical dislocation. Brains were removed, snap-frozen on dry-ice and stored at -80°C prior to sectioning. Brains were mounted on Tissue Tek (Polysciences, PA, USA), and 14-µm-thick coronal sections in consecutive series were cut from the forebrain on a cryostat Microtome HM560 (Microm, Germany). Sections were mounted onto frozen SuperFrost/Plus slides (Fisher Scientific, Germany), dried on a 42°C warming plate and stored at −20°C until used.

### *Synthesis of probes*

Both radioactive ($^{35}S$) and non-radioactive (DIG)-labelled riboprobes were used. Probes were generated by reverse transcription-polymerase chain reaction (RT-PCR) from cDNA derived from total mouse brain RNA. For each probe, GenBank accession number, length and sequence of the primers are listed below; nucleotide positions are identical to those used in deposited sequences in GenBank: $CB_1$, accession number U22948, 1530 bp from 152 to 1682 (primers as described in Marsicano and Lutz, 1999); D1, accession number S46131, 560 bp from 4237 to 4792 (mouse probe cloned from the homologous rat sequence was a gift from Dr. Thomas Lemberger, DKFZ Heidelberg, Germany); D2, accession number X55674, 837 bp from 302 to 1139 (forward primer 5′-CTG TAT CAC GAG AGA AGG CTT; reverse primer 5′-CTG GGA TTG ACA ATC TTG GCA); 5-HT1B, accession number Z11597, 763 bp from 443 to 1206 (forward primer 5′-GCC AAA GGA GAC AAG CCT ATA, reverse primer 5′-GAG CAG GGT GGG TAA ATA GAA), and 5-HT3, accession number M74425, 1121 bp from 458 to 1579 (forward primer 5′-GGA AGT CTC CGA ACA TTC CTT, reverse primer 5′-CCC CCA TAC TTA TCC TAA CCA). PCR products were cloned into pBluescript $KS^-$ (Stratagene, CA, USA) and used as templates for riboprobe synthesis. The identity of all fragments was checked by sequencing. Linearized template DNA was phenol-extracted, precipitated, resuspended in diethyl pyrocarbonate-treated $H_2O$ at a concentration of 1 µg/µl, and stored at −20°C until used. For $^{35}S$-labelled riboprobes, *in vitro* transcription was carried out for 3 h at 37°C in a total volume of 30 µl containing 1.5 µg of linearized DNA, 1× transcription buffer, 1 mM of rATP/rCTP/rGTP each, 16.7 mM dithiothreitol (DTT), 40 units RNasin (Promega, WI, USA), 10 µl of [α-$^{35}S$]UTP (NEN, MA, USA; 1250 Ci/mmol), and 30 units of T7 or T3 RNA polymerase (Roche Molecular Diagnostics, Mannheim, Germany). For DIG-labelled riboprobes, *in vitro* transcription was carried out for 3 h at 37°C in a total volume of 50 µl containing 1.5 µg of linearized DNA, 1× transcription buffer, 5 µl of DIG RNA labelling mix (Roche Molecular Diagnostics), 80 units RNasin (Promega), and 100 units of T7 or T3 RNA polymerase. Reactions were treated with 20 units of RNase-free DNaseI (Roche Molecular Diagnostics) for 15 min at 37°C, and labelled probes were purified by ammonium acetate precipitation. Restriction enzymes (New England Biolabs, MA, USA) used for linearization and RNA polymerases used for each probe were as follows: $CB_1$ sense, *Pst*I, T7; $CB_1$ antisense, *Bam*HI, T3; 5-HT1B sense, *Xba*I, T3; 5-HT1B antisense, *Eco*RI, T7; 5-HT3 sense *Eco*RI, T7; 5-HT3 antisense, *Xba*I, T3; D1 sense, *Sac*I, T3, D1 antisense, *Sal*I, T7; D2 sense, *Eco*RI, T7; D2 antisense *Bam*HI, T3. Using these probes in *in situ* hybridization experiments, sense controls did not give any detectable signals (data not shown), and antisense probes gave distribution patterns identical to those already published in rat or mouse (D2: Meador-Woodruff et al., 1989; D1: Mansour et al., 1991; 5-HT1B: Maroteaux et al., 1992; 5-HT3: Tecott et al., 1993; $CB_1$: Marsicano and Lutz, 1999).

### *In situ hybridization*

Slides were warmed up for 30 min at RT and selected in such a manner that represents the whole mouse forebrain. They were fixed in ice-cold 4% paraformaldehyde in phosphate-buffered saline (PBS, containing, in mM: NaCl, 136; KCl, 2.7; $Na_2HPO_4$, 10; $KH_2PO_4$, 1.8, pH 7.4) for 20 min, rinsed twice in PBS, quenched for 15 min in 1% $H_2O_2$ in 100% methanol, rinsed twice in PBS, quenched for 8 min in 0.2 M HCl, rinsed twice with PBS, treated with proteinase K 20 µg/ml (Roche Molecular Diagnostics) in 50 mM Tris–HCl, 5 mM EDTA (pH 8.0) for 10 min, rinsed once with PBS, fixed in ice-cold 4% paraformaldehyde in PBS, incubated for 10 min in 0.1 M triethanolamine (pH 8.0) to which 1.2 ml acetic anhydride was added dropwise, rinsed once with PBS, washed with 0.9% NaCl for 5 min, dehydrated in graded series of ethanol (30, 50, 70, 80, 95, 100%), and air-dried. Hybridization was carried out in 100 µl of hybridization buffer containing $^{35}S$-labelled riboprobe (70 000–100 000 c.p.m./µl) and DIG-labelled riboprobe (0.2 µg/ml). Hybridization buffer consisted of 50% deionized formamide, 20 mM Tris–HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA (pH 8.0), 10% dextran sulfate (D8906, Sigma, Germany), 0.02% Ficoll 400 (F2637, Sigma), 0.02% polyvinylpyrrolidone (MW 40 000, PVP 40, Sigma), 0.02% bovine serum albumin (A6793, Sigma), 0.5 mg/ml tRNA (Roche Molecular Diagnostics), 0.2 mg/ml fragmented herring sperm DNA and 200 mM dithiothreitol. Before applying to the tissue, hybridization cocktail was denatured for 2 min at 95°C. Slides were incubated overnight at 54°C in a humidified chamber.

Four high-stringency washes were carried out at 62°C with 5× saline sodium citrate (SSC) (1× SSC contains 150 mM NaCl, 15 mM $Na_3$ citrate, pH 7.4)/0.05% Tween-20 (P7949, Sigma), then with 50% formamide/2× SSC/0.05% Tween-20, with 50% formamide/1× SSC/0.05% Tween-20, and finally with 0.1× SSC/0.05% Tween-20. All of the following post-hybridization washes and incubations were carried out at 30°C. Slides were washed with 0.5 M NaCl, 10 mM Tris–HCl (pH 8.0), 5 mM EDTA (NTE)/0.05% Tween-20, incubated with 15 mM iodoacetamide in NTE/0.05% Tween-20 for destruction of intracellular alkaline phosphatase, and washed twice with NTE/

0.05% Tween-20. Slides were blocked with 4% heat-inactivated sheep serum in 100 mM Tris–HCl (pH 7.6)/150 mM NaCl/0.05% Tween-20 (TNT), which was filtered through a 0.45-μm filter, washed three times with TNT, incubated for 30 min in blocking buffer (TSA Biotin System, NEN Life Science Products, Boston, MA, USA), incubated 1.5 h with anti-DIG antibody (Roche Molecular Diagnostics) diluted 1:1200 in blocking buffer, and washed three times in TNT. After antibody treatment, slides were incubated for 12 min with biotin-labelled tyramide (TSA Biotin System, NEN Life Science Products), washed with 100 mM maleic acid/150 mM NaCl/0.05% Tween-20 (wash buffer), incubated for 1 h with streptavidin–alkaline phosphatase (Roche Molecular Diagnostics) diluted 1:1000 with 1% blocking reagent (Roche Molecular Diagnostics) in wash buffer, and washed three times with Wash buffer. Chromogenic reaction was carried out with Vector Red kit (Vector Laboratories, CA, USA) at RT for 10–30 min. The reaction was stopped with a 10-min incubation in PBS, followed by 30 min in 2.5% glutaraldehyde in PBS and three washes for 10 min in 0.1× SSC. Slides were dehydrated in graded ethanol series, air-dried and exposed to Biomax MR film (Scientific Imaging Systems, NY, USA). On the next day, slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled $H_2O$). After exposure for 3–6 weeks at 4°C, slides were developed for 3 min (D-19, Kodak), fixed for 6 min (Kodak fixer), rinsed for 30 min in tap water and air-dried. Counterstaining was carried out for 10 s in 0.1% aqueous Toluidine Blue. Slides were mounted in Histofluid (Marienfeld, Lauda-Königshofen, Germany).

### *Numerical evaluation of coexpression*

In double-label *in situ* hybridization experiments $CB_1$ mRNA was detected with a DIG-labelled riboprobe. As $CB_1$ is expressed at various levels, stained cells were classified according to the following criteria. Cells expressing $CB_1$ at high levels (termed high-$CB_1$-expressing cells described as GABAergic interneurons; Marsicano and Lutz, 1999) were considered to be those showing a round-shaped and intense red staining surrounding the nucleus or even covering the entire nucleus. Cells expressing $CB_1$ at low levels (termed low-$CB_1$-expressing cells described as mainly projecting principal neurons; Marsicano and Lutz, 1999) were defined as cells clearly stained above background levels and in a discontinuous shape and/or at uniform and low intensity of staining. The absolute intensity of staining varied in different *in situ* hybridization experiments, but the relative proportion of staining intensity of high to low-$CB_1$-expressing cells was the same. Different regions were chosen for numerical evaluation of coexpression based on the published distribution patterns of $CB_1$ and D1, D2, 5-HT1B or 5-HT3, which indicate a high extent of overlapping expression throughout the murine brain. Cells positive for $CB_1$ and one of the four markers were counted by choosing particular fields in these regions on at least three different brain sections and coexpression values were calculated as percentages. Double-label *in situ* hybridization experiments were carried out on three animals for each marker combination, showing always the same expression patterns. Our data exemplify the results from one experiment for each marker combination. Sections were analyzed on a Leica DMRB microscope.

## RESULTS

### *Coexpression of cannabinoid CB1 receptor with dopamine receptors*

*CB1 and D1 receptors.* The highest levels of D1 transcripts are observed in the basal ganglia (Mansour et al., 1991; Weiner et al., 1991), including caudate putamen, nucleus accumbens, and olfactory tubercle. High levels of low-$CB_1$-expressing cells are detected in the dorsolateral caudate putamen, while the nucleus accumbens contains only few low-$CB_1$-expressing cells. The olfactory tubercle shows an intense staining due to a high density of low-$CB_1$-expressing cells. Coexpressing cells were counted at a single-cell resolution in the caudate putamen (Table 1, Fig. 1A) and the olfactory tubercle, but not in the nucleus accumbens due to too weak signals of $CB_1$-positive cells. 46% of the medium-sized, $CB_1$-positive neurons in the dorsolateral caudate putamen coexpressed D1. Considering all D1-positive cells containing $CB_1$, the percentage reached 81% (Table 1). In the olfactory tubercle, D1 mRNA is present in 90% of the $CB_1$-expressing cells. The fraction of D1-positive cells containing $CB_1$ was 76%.

D1 transcripts were observed in two regions of the neocortex, but at much lower levels than in the striatum. A striking finding was that none of the high-$CB_1$-expressing cells in these cortical areas described below contained D1. The highest levels of D1 transcripts were observed in the piriform cortex. This cortical region showed also a high number of low-$CB_1$-expressing cells and a rather low number of high-$CB_1$-expressing cells. Due to the uniform distribution of cells expressing low levels of D1 in this area, it was not feasible to count coexpressing cells at a single-cell resolution. Thus, the numbers reflect an estimate only. 90% of $CB_1$-positive cells in the piriform cortex contain D1 mRNA, whereas 70% of D1-expressing cells showed also signals for $CB_1$ (Table 1). Coexpression of $CB_1$ with D1 was also observed in the dorsal endopiriform nucleus where 89% of $CB_1$-positive cells contain D1 mRNA and 69% of D1-positive cells coexpress $CB_1$ (Table 1). In other cortical areas such as the neocortex, entorhinal/perirhinal cortex and amygdala as well as in non-cortical areas such as the hypothalamus no signals for D1 transcripts were detected.

*CB1 and D2 receptors.* Similarly to D1, the strongest signals of D2 transcripts were detected in the caudate putamen, nucleus accumbens, and olfactory tubercle

Table 1. Coexpression of D1 receptor in low-cannabinoid-CB1-receptor-expressing neurons of the adult mouse forebrain

| | Coexpression in cells expressing low $CB_1$ | | |
|---|---|---|---|
| | $CB_1$ cells with D1 (%) | D1 cells with $CB_1$ (%) | (*n*) |
| Dorsolateral caudate putamen | 46 | 81 | (3798) |
| Olfactory tubercle | 90 | 76 | (102) |
| Piriform cortex | 90* | 70* | (n.c.) |
| Dorsal endopiriform nucleus | 89 | 69 | (94) |

n.c., not counted; *estimated percentages.

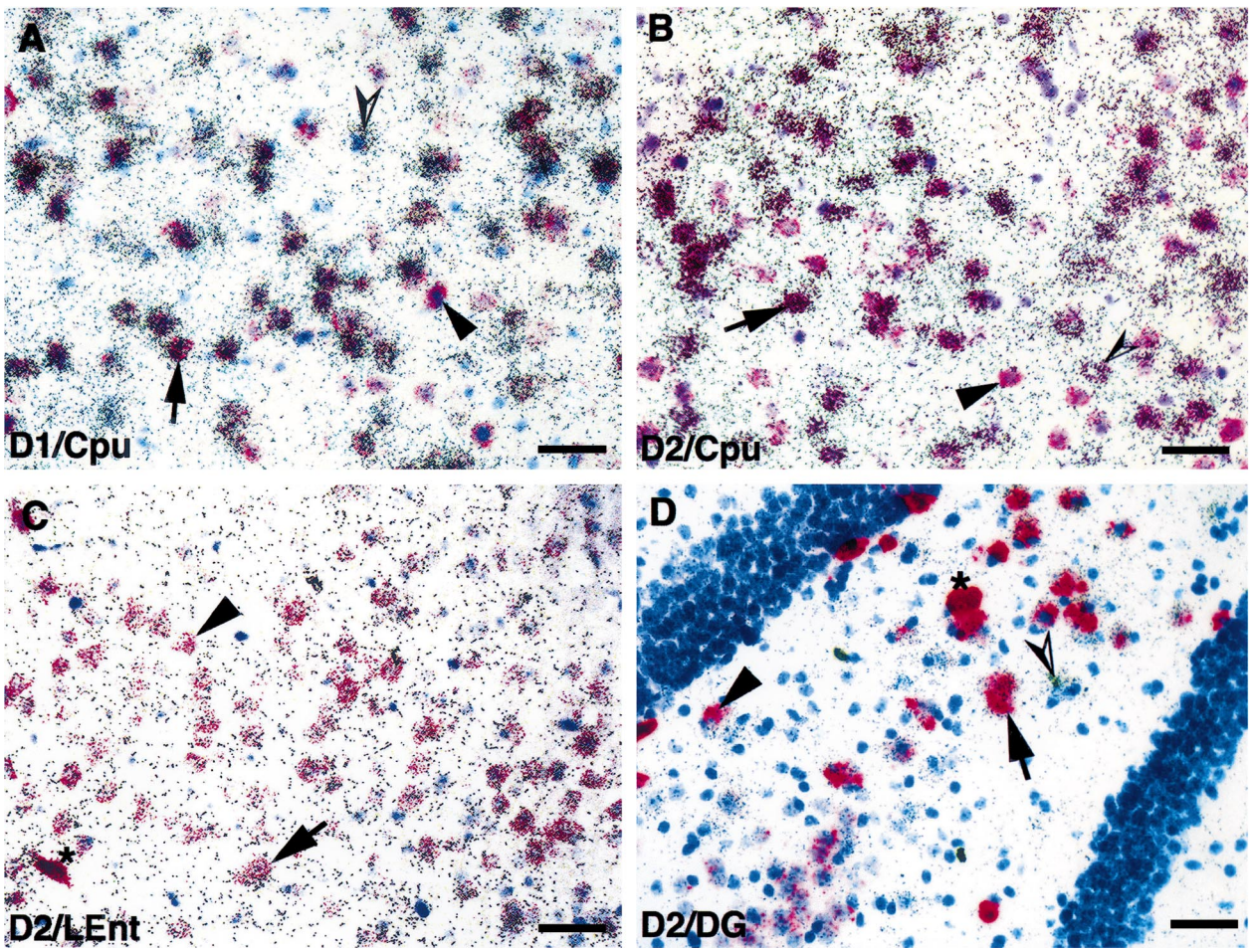


Fig. 1. Bright field micrographs of coronal sections showing examples of coexpression of $CB_1$ (red staining) with D1 and D2 (silver grains), respectively, as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A, B) Coexpression of $CB_1$ with D1 and D2, respectively, in the caudate putamen (Cpu). (C) Coexpression of $CB_1$ with D2 in the lateral entorhinal cortex (LEnt). (D) Coexpression of $CB_1$ with D2 in the dentate gyrus (DG). Filled arrow, low-$CB_1$-expressing cell that coexpresses D1 and D2, respectively; filled arrowhead, low-$CB_1$-expressing cell; open arrowhead, D1- or D2-expressing cell; *high-$CB_1$-expressing cell. Scale bars = 200 μm.

(Meador-Woodruff et al., 1989; Weiner et al., 1991). In the dorsolateral caudate putamen and in the olfactory tubercle, 38% of the $CB_1$-positive neurons coexpress D2 (Table 2, Fig. 1B). Higher percentages of coexpression in these two areas were evaluated considering all D2-positive cells containing $CB_1$ with values of 73% in the striatum and 74% in the olfactory tubercle (Table 2).

Compared to the basal ganglia, the level of D2 transcripts in cortical areas is much lower. D2-$CB_1$ coexpression was detected only in low-$CB_1$-expressing cells. Rather high levels of D2 mRNA were observed in the piriform cortex. Due to the uniform distribution of D2-expressing cells in this area, the coexpression with $CB_1$ was estimated to 90% considering all $CB_1$-positive cells and 70% considering all D2-positive cells, respectively (Table 2). In the entorhinal/perirhinal cortex area,

Table 2. Coexpression of dopamine D2 receptor in low-cannabinoid-CB1-receptor-expressing neurons of the adult mouse forebrain

| | Coexpression in cells expressing low $CB_1$ | | |
|---|---|---|---|
| | $CB_1$ cells with D2 (%) | D2 cells with $CB_1$ (%) | (*n*) |
| Dorsolateral caudate putamen | 38 | 73 | (4260) |
| Olfactory tubercle | 38 | 74 | (96) |
| Piriform cortex | 90* | 70* | (n.c.) |
| Entorhinal/perirhinal cortex area | 80 | 77 | (104) |
| Dentate gyrus (polymorph layer) | 88 | 48 | (376) |

n.c., not counted; *estimated percentages.

which contains high numbers of low- and high-$CB_1$-expressing cells, neurons could be counted at a single-cell resolution. 80% of $CB_1$-positive cells coexpress D2, and 77% of D2-positive cells express $CB_1$ (Fig. 1C, Table 2).

In the hippocampus, $CB_1$ signals with intensities ranging from low to very high were observed in all layers. Coexpression with D2 was detected in the polymorph layer of the dentate gyrus (Fig. 1D), where D2 hybridization signals were detected in 88% of the low-$CB_1$-expressing cells, but only 48% of all D2-positive cells do coexpress $CB_1$ (Table 2). In other cortical areas such as the neocortex and amygdala D2 signals were not detected.

*Coexpression of cannabinoid CB1 receptors with serotonin receptors*

*CB1 and 5-HT1B receptors.* High expression levels of 5-HT1B mRNA were detected in striatum and olfactory tubercle, in agreement with the described expression pattern (Maroteaux et al., 1992). Percentages of coexpression in these regions are illustrated in Table 3. Evidently, the majority of $CB_1$- and 5-HT1B-expressing cells in the dorsolateral part of the caudate putamen (Fig. 2A) and the olfactory tubercle show coexpression. Intense signals for 5-HT1B were also observed in the nucleus accumbens, where most of the cells express 5-HT1B (data not shown). Due to the low expression levels of $CB_1$ in this area, coexpression could not be numerically evaluated by double-label *in situ* hybridization, but $CB_1$ is expressed in approximately 20% of the cells (Moldrich and Wenger, 2000; B. Lutz, data not shown). Thus, also in the nucleus accumbens, an estimate of 90% $CB_1$-expressing cells coexpress 5-HT1B.

Weaker signals for 5-HT1B were detected in the hippocampus, neocortex and hypothalamus, consistent with the known expression pattern (Maroteaux et al., 1992). In the pyramidal cells of hippocampal CA1 region, which express low levels of $CB_1$ mRNA (Marsicano and Lutz, 1999), 100% coexpression was observed. 5-HT1B mRNA was observed in a scattered manner throughout layers II–III of the neocortex, whereas both low- and high-$CB_1$-expressing cells were located primarily in layers II–III and V–VI. In layers II–III, at least 70% of all low-$CB_1$- and 5-HT1B-expressing cells show coexpression (Table 3, Fig. 2B), whereas high-$CB_1$-expressing neurons never express 5-HT1B. The ventromedial hypothalamic nuclei showed the presence of low-$CB_1$- and 5-HT1B-expressing cells that are uniformly distributed at high cell density. The coexpression rate was estimated to be more than 90% (Fig. 2C).

*CB1 and 5-HT3 receptors.* Coexpression of $CB_1$ and 5-HT3 was observed in several cortical regions for both low- and high-$CB_1$-expressing cells. As compared to $CB_1$ and all other markers described above, the number of 5-HT3-expressing cells in the mouse forebrain is rather low (Tecott et al., 1993). Therefore, the percentages of coexpression considering low-$CB_1$-expressing cells that coexpress 5-HT3 are very low, in the range of 0.9–3.6%, for all described regions except for the hippocampal formation (Table 4). In the hippocampal CA1 and CA3 areas (excluding the pyramidal cells, which do express $CB_1$ but not 5-HT3), the majority of both high and low-$CB_1$-expressing cells shows coexpression with 5-HT3, the extent being higher in CA3 (Fig. 3A). In the dentate gyrus, coexpression of 5-HT3 with low-$CB_1$-expressing cells is only 17% (Fig. 3B). In all parts of hippocampus, the fraction of 5-HT3-expressing cells containing $CB_1$ is between 35% and 39% for high-$CB_1$-expressing cells, thus, it is much higher than for low-$CB_1$-expressing cells (9–14%). This characteristic is also observed in all other forebrain regions analyzed, including neocortex (Fig. 3C), anterior olfactory nucleus, piriform cortex and entorhinal/perirhinal cortex. Regarding this feature, the basolateral amygdaloid nucleus is peculiar, as the extent of 5-HT3-expressing cells containing $CB_1$ was approximately the same for high (21%) and low (24%) $CB_1$-expressing cells (Table 4, Fig. 3D).

## DISCUSSION

Increasing evidence indicates that a single receptor subtype may be linked to the formation of multiple, intracellular signals. However, it is unlikely that all signals driven by a single receptor subtype are equally operative under all circumstances, but it seems that the functional weight of one pathway relative to another can be altered by interactions with other receptors. The first indication for possible interactions between different receptors is given, when both receptors are expressed within the same neuron. Therefore, the aim of this study was to define in detail coexpression patterns of $CB_1$ with dopamine and serotonin receptors in distinct neuronal subpopulations of the adult mouse forebrain. We thus performed double-label *in situ* hybridization

Table 3. Coexpression of 5-HT1B receptor in low-$CB_1$-expressing neurons of the adult mouse forebrain

| | Coexpression in cells expressing low $CB_1$ | | |
|---|---|---|---|
| | $CB_1$ cells with 5-HT1B (%) | 5-HT1B cells with $CB_1$ (%) | (*n*) |
| Dorsolateral caudate putamen | 72 | 81 | (2983) |
| Olfactory tubercle | 70 | 77 | (226) |
| Hippocampal CA1 area** | 100* | 100* | (n.c.) |
| Layers II–III of neocortex | 70 | 74 | (1084) |
| Ventromedial hypothalamic nuclei | $>90$* | $>90$* | (n.c.) |

n.c., not counted; *estimated percentages; **principal neurons only.

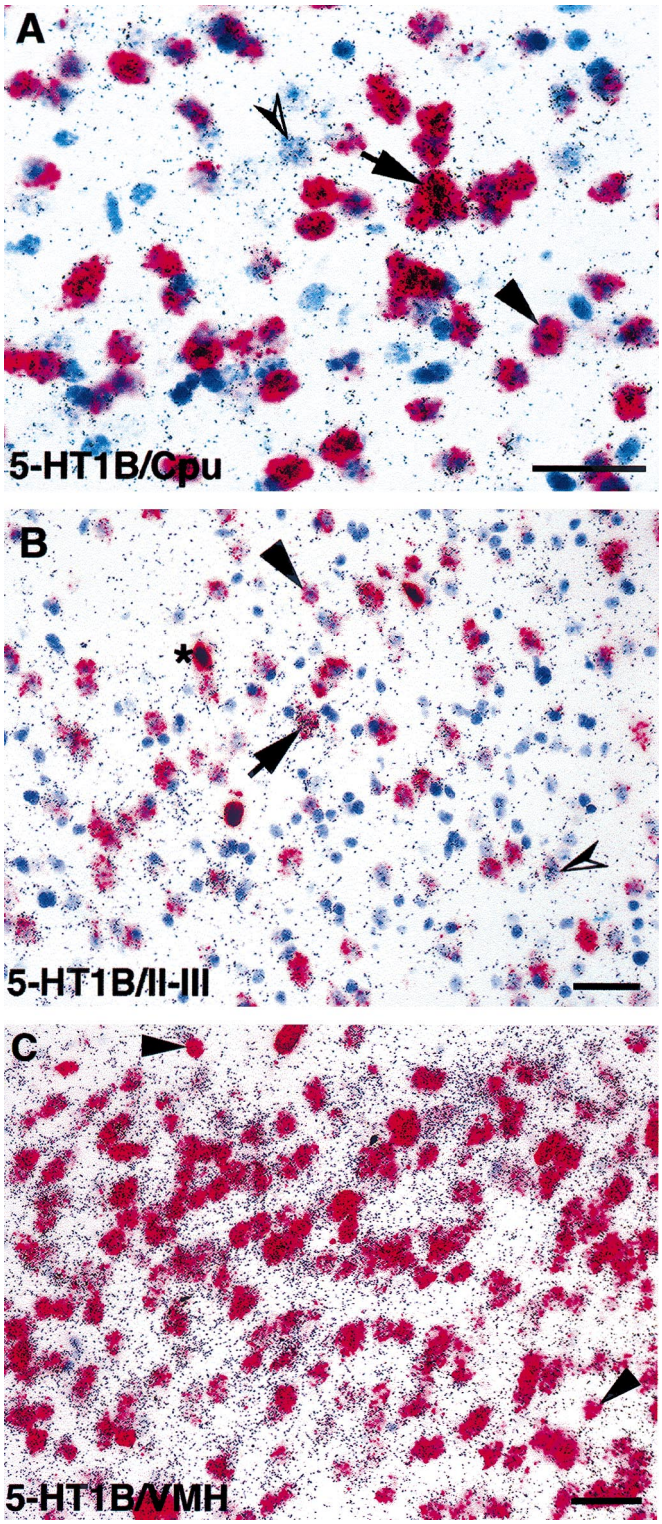


Fig. 2. Bright field micrographs of coronal sections showing examples of coexpression of $CB_1$ (red staining) with 5-HT1B (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A) Coexpression of $CB_1$ with 5-HT1B in the caudate putamen (Cpu). (B) Coexpression of $CB_1$ with 5-HT1B in layers II and III of neocortex (II–III). (C) Coexpression of $CB_1$ with 5-HT1B in the ventromedial hypothalamic nuclei (VMH). Filled arrow, low-$CB_1$-expressing cell that coexpresses 5-HT1B; filled arrowhead, low-$CB_1$-expressing cell; open arrowhead, 5-HT1B-expressing cell; *high-$CB_1$-expressing cell. Scale bars = 200 μm.

Table 4. Coexpression of 5-HT3 receptor in cannabinoid-CB1-receptor-expressing cells of the adult mouse forebrain

| | Coexpression in cells expressing low $CB_1$ | | | Coexpression in cells expressing high $CB_1$ | | |
|---|---|---|---|---|---|---|
| | $CB_1$ cells with 5-HT3 (%) | 5-HT3 cells with $CB_1$ (%) | (*n*) | $CB_1$ cells with 5-HT3 (%) | 5-HT3 cells with $CB_1$ (%) | (*n*) |
| Anterior olfactory nucleus | 1.0 | 19 | (1134) | 42 | 36 | (97) |
| Piriform cortex | 2.1 | 26 | (1056) | 41 | 32 | (117) |
| Entorhinal/perirhinal cortex area | 1.0 | 20 | (1715) | 40 | 31 | (165) |
| Neocortex, layers I–II | 2.2 | 22 | (1691) | 46 | 30 | (336) |
| Neocortex, layers III–IV | 0.9 | 20 | (1573) | 40 | 35 | (160) |
| Neocortex, layers V–VI | 3.6 | 18 | (609) | 41 | 35 | (187) |
| Hippocampus, CA1* | 77 | 13 | (313) | 73 | 35 | (480) |
| Hippocampus, CA3* | 96 | 9 | (275) | 82 | 35 | (409) |
| Dentate gyrus | 17 | 14 | (151) | 77 | 39 | (134) |
| Basolateral amygdaloid nuclei (anterior) | 2.8 | 24 | (767) | 31 | 21 | (129) |

*Excluding principal neurons, which do not coexpress 5-HT3.

experiments using a DIG-labelled $CB_1$ riboprobe in combination with $^{35}$S-labelled riboprobes for D1, D2, 5-HT1B and 5-HT3, respectively.

Both the significant extent of overlapping expression of $CB_1$ with various dopamine and serotonin receptors and several investigations observing functional interactions between the cannabinoid system and other neurotransmitter systems gave reason to chose the markers mentioned above. Previous immunohistochemical and *in situ* hybridization studies carried out in rats observed high levels of D1 and D2 mRNA (Weiner et al., 1991; Levey et al., 1993) and 5-HT1B mRNA (Maroteaux et al., 1992) in the striatum. $CB_1$ is also expressed in many neurons throughout the striatum (Herkenham et al., 1991; Egertová and Elphick, 2000; Tsou et al., 1998; Marsicano and Lutz, 1999). These observations are in agreement with our results which revealed a high density of hybridization signals of these probes. Particularly in the dorsolateral caudate putamen, a high degree of colocalization of $CB_1$ with D1, D2 and 5-HT1B, respectively, was detected. The striatum is a key component of the forebrain system that controls planning and execution of motor behaviors (for a review see Nakano et al., 2000). $CB_1$ agonists can markedly affect the function of these systems, producing alterations of locomotion and catalepsy (Howlett, 1995; Martellotta et al., 1998). Also dopamine stimulates motor activity in the basal ganglia (Alexander and Crutcher, 1990; Parent and Hazrati, 1995). A mechanism for influencing striatal function through cannabinoids and/or dopamine could be an interaction between the cannabinoid and dopamine system which is suggested by the high coexpression rates of $CB_1$ and D2 shown in this study. Cell culture experiments on striatal neurons revealed clear evidence for an interaction between $CB_1$ and D2. Activation of either $CB_1$ or D2 resulted in an inhibition of cAMP accumulation, whereas simultaneous activation of both receptors resulted in an augmentation of cAMP accumulation (Glass and Felder, 1997). Also *in vivo* studies with mice treated with different combinations of cannabinoid agonists and dopamine agonists/antagonists revealed that the two receptor systems appear to interact by exerting opposing influences in regulating motor activity which also hinted to a coexistence of $CB_1$ and D1/D2 on the same striatal neurons (Meschler et al., 2000) as evidenced also in our study.

Immunocytochemical investigations of Aizman et al. (2000) demonstrate the coexistence of D1- and D2-like receptors in all virtually striatal neurons which can be divided in two different populations responsible either for substance P release or enkephalin release (Graybiel, 1990). $CB_1$ was also detected in these two neuronal subpopulations and is involved in the regulation of expression of these neuropeptides (Mailleux and Vanderhaeghen, 1992). $CB_1$-deficient mice display significantly increased levels of substance P and enkephalin mRNAs in these striatal neurons (Steiner et al., 1999). Therefore, colocalization of $CB_1$ with D1 and D2 in the caudate putamen suggests a putative regulation system of the two receptor systems in controlling expression of these neuropeptides.

5-HT1B was also shown to be involved in motor behavior. Administration of the 5-HT1B agonist RU 24969 to rats resulted in an increase of locomotor activity (Oberlander et al., 1987), whereas application of the 5-HT1B antagonists GR 127935 could block the RU 24969-induced hyperactivity in rodents (O'Neill et al. et al., 1996). In our study, high coexpression levels of 5-HT1B and $CB_1$ were detected in the striatum assuming a modulatory role for 5-HT1B together with $CB_1$ in motor function.

$CB_1$ is differentially coexpressed with all four markers in several cortical regions (hippocampus, neocortex, entorhinal/perirhinal cortex, amygdala) which contribute to important brain functions, e.g. learning and memory (Suzuki, 1996; Miller et al., 1998). Considering this, modulation of cognitive processes could be mediated through the interaction of $CB_1$ with dopamine or serotonin receptors. Recently, it was shown that $\Delta^9$-tetrahydrocannabinol-induced impairment of working memory was reversed by a D2 antagonist and potentiated by a D2 agonist, concluding that this effect is mediated by the simultaneous activation of $CB_1$ and D2 (Nava et al., 2000). The concurrent activation of both receptors might produce an accumulation of cellular cyclic AMP in neurons where these receptors are colocalized.

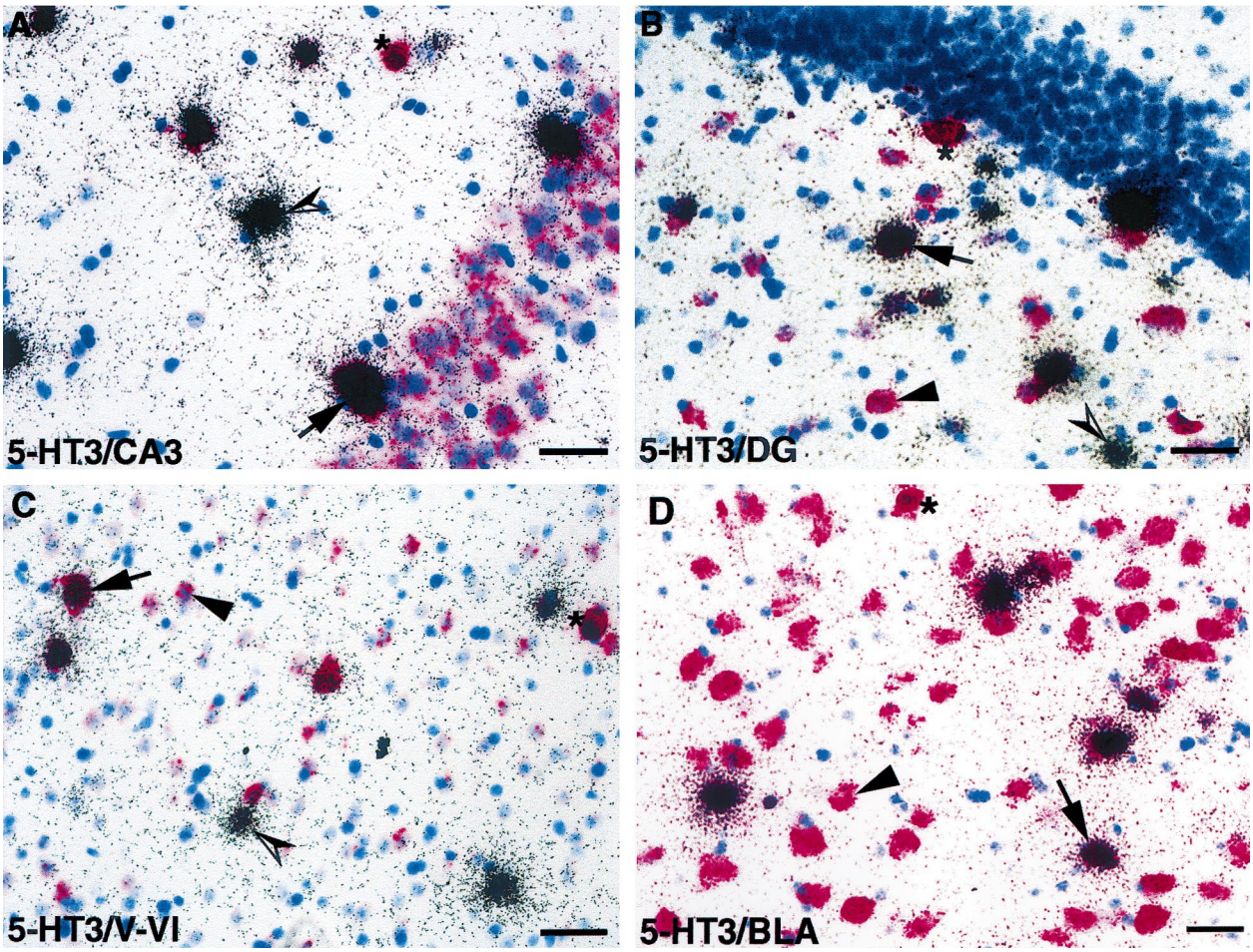


Fig. 3. Bright field micrographs of coronal sections showing examples of coexpression of $CB_1$ (red staining) with 5-HT3 (silver grains) as detected by double-*in situ* hybridization. All sections were counterstained with Toluidine Blue. (A) Coexpression of $CB_1$ with 5-HT3 in the CA3 area of hippocampus (CA3). (B) Coexpression of $CB_1$ with 5-HT1B in the dentate gyrus (DG). (C) Coexpression of $CB_1$ with 5-HT1B in layers V and VI of neocortex (V–VI). (D) Coexpression of $CB_1$ with 5-HT3 in the basolateral amygdala (BLA). Filled arrow, high-$CB_1$-expressing cell that coexpresses 5-HT3; filled arrowhead, low-$CB_1$-expressing cell; open arrowhead, 5-HT3-expressing cell; *high-$CB_1$-expressing cell. Scale bars = 200 µm.

A striking finding of our study was that the coexpression of $CB_1$ with 5-HT3 in several forebrain regions (hippocampus, neocortex, anterior olfactory nucleus, amygdala) was mainly detected in high-$CB_1$-expressing cells, which are considered as GABAergic neurons belonging predominantly to the cholecystokinin (CCK)-positive type of interneurons (Marsicano and Lutz, 1999; Tsou et al., 1999; Katona et al., 1999). In line with our observation, *in situ* hybridization and immunocytochemistry experiments by Morales and Bloom (1997) showed that 5-HT3-expressing neurons in the neocortex, olfactory cortex, hippocampus and amygdala are mainly GABA-containing cells with CCK immunoreactivity. The widespread colocalization of 5-HT3 with $CB_1$ in GABAergic neurons suggests the participation of these two receptors in the modulation of inhibitory neurons. As these cells contain CCK, a role of 5-HT3 and $CB_1$ in regulating CCK neurotransmission might be put forward.

The mechanism of such interactions remains to be investigated, but recently, direct evidences for heterodimerization between different neurotransmitter receptors have been reported (for review see Bouvier, 2001). However, interaction could also take place at the level of the intracellular signalling pathways. Considering the high levels of expression of $CB_1$ in mammalian brain (Herkenham et al., 1990), it is therefore plausible to hypothesize a putative mechanism explaining the functional interaction of the cannabinoid system with other receptor systems. Coexpression of $CB_1$ and other receptors in the same neurons provides the first condition for such direct, functional interactions.

$CB_1$ and its colocalization with receptors for other neurotransmitters can contribute to understanding certain neurodegenerative diseases, caused by multiple neurotransmitter and receptor alterations. In reserpine-treated rats, an animal model for Parkinson's disease, increased levels of endocannabinoids in basal ganglia were found (Di Marzo et al., 2000), which might be correlated to significantly reduced levels of $CB_1$ expression in striatum in this disease (Silverdale et al., 2001). Moreover, Di Marzo et al. (2000) could show that coadministration of a D2 agonist and a $CB_1$ antagonist leads to full restoration of normal locomotor behavior in

reserpine-treated rats suggesting a close functional relationship between the cannabinoid and the dopamine system. These data support the idea that modulation of the endocannabinoid signalling system provide a useful treatment for the symptoms of Parkinson's disease or other basal ganglia-related movement disorders.

In summary, $CB_1$ is differentially coexpressed in the mouse forebrain with dopamine and serotonin receptors either in principal projecting neurons (mainly with D1, D2 and 5-HT1B) or in interneurons (mainly with 5-HT3). Together, these receptor systems might be involved in modulating excitatory circuits as well as inhibitory GABAergic circuits. Particularly in the striatum, high coexpression extent of $CB_1$ with D1, D2 and 5-HT1B, respectively, were observed, suggesting putative cross-talks between the cannabinoid system and other neurotransmitter systems regulating locomotor activity. High levels of coexpressing cells in cortical areas might be an indication for a functional interaction of $CB_1$ with dopamine and serotonin receptors, respectively, having modulatory effects on cannabinoid-induced impairment of working memory and cognitive functions.

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Research Article

# Dual effect of cannabinoid $CB_1$ receptor stimulation on a vanilloid VR1 receptor-mediated response

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**Abstract.** Cannabinoid $CB_1$ receptors and vanilloid VR1 receptors are co-localized to some extent in sensory neurons of the spinal cord and dorsal root ganglia. In this study, we over-expressed both receptor types in human embryonic kidney (HEK)-293 cells and investigated the effect of the $CB_1$ agonist HU-210 on the VR1-mediated increase in intracellular $Ca^{2+}$ ($[Ca^{2+}]_i$), a well-known response of the prototypical VR1 agonist capsaicin. After a 5-min pre-treatment, HU-210 (0.1 μM) significantly enhanced the effect of several concentrations of capsaicin on $[Ca^{2+}]_i$ in HEK-293 cells over-expressing both rat $CB_1$ and human VR1 ($CB_1$-VR1-HEK cells), but not in cells over-expressing only human VR1 (VR1-HEK cells). This effect was blocked by the $CB_1$ receptor antagonist SR141716A (0.5 μM), and by phosphoinositide-3-kinase and phospholipase C inhibitors. The endogenous agonist of $CB_1$ and VR1 receptors, anandamide, was more efficacious in inducing a VR1-mediated stimulation of $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells than in VR1-HEK cells, and part of its effect on the former cells was blocked by SR141716A (0.5 μM). Pre-treatment of $CB_1$-VR1-HEK cells with forskolin, an adenylate cyclase activator, enhanced the capsaicin effect on $[Ca^{2+}]_i$. HU-210, which in the same cells inhibits forskolin-induced enhancement of cAMP levels, blocked the stimulatory effect of forskolin on capsaicin. Our data suggest that in cells co-expressing both $CB_1$ and VR1 receptors, pre-treatment with $CB_1$ agonists inhibits or stimulates VR1 gating by capsaicin depending on whether or not cAMP-mediated signalling has been concomitantly activated.

**Key words.** Anandamide; capsaicin; cannabinoid; vanilloid; receptor; signalling; pain.

Recent evidence points to the existence of functional relationships between the brain G protein-coupled receptor for the psychoactive principle of marijuana, $\Delta^9$-tetrahydrocannabinol (THC), i.e. the cannabinoid $CB_1$ receptor [1, 2], and the membrane cation channel gated by heat, protons and the pungent hot chilli pepper ingredient, capsaicin, i.e. the vanilloid VR1 receptor [3]. The two receptors are co-localized in many, although not all, small-diameter, non-myelinated sensory C fibres, both at the level of the spinal cord, and in dorsal root ganglia (DRGs) as well as, apparently, in the peripheral terminals of C fibres [4]. In these neurons, $CB_1$ and VR1 receptors play opposite roles in the control of nociception. VR1 appears to be partly responsible for the transmission of pain during thermal and inflammatory hyperalgesia [5, 6], whereas $CB_1$ receptors were suggested to counteract hyperalgesia, at least in part by inhibiting VR1-mediated nociception [7, 8]. However, VR1 activation by potent synthetic agonists is immediately followed by desensitization, thereby leading to powerful analgesic effects in vivo [3]. There is now evidence for the co-existence of $CB_1$ and VR1 re-

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ceptors also in brain nuclei and areas involved in the control of motor (substantia nigra, striatum, cerebellum), cognitive and mnemonic (hippocampus, cortex), emotional (amygdala) and nociceptive (periaqueductal grey) functions [9, 10].

Several connections have also been identified between the endogenous ligands of $CB_1$ and VR1 receptors in the brain. Anandamide (N-arachidonoyl-ethanolamine, AEA), the first endogenous cannabinoid receptor ligand discovered [11], acts as a full agonist at VR1 receptors [12, 13; for a review see ref. 14] at concentrations that are normally higher than those required to activate $CB_1$, but that can be significantly decreased under certain conditions [15, 16; for a review see ref. 17]. Furthermore, some long-chain homologues of capsaicin, and synthetic VR1 agonists, can indirectly activate $CB_1$ receptors either by retarding the cellular uptake and inactivation of endogenous AEA [via inhibition of the AEA membrane transporter (AMT)] [18, 19], or by triggering AEA formation [20].

Stimulation of $CB_1$ receptors on sensory neurons with $CB_1$-selective agonists can induce inhibition of VR1-mediated thermal hyperalgesia [7, 8, 21]. However, agents capable of activating both $CB_1$ and VR1 receptors, such as the AEA/capsaicin structural 'hybrid' arvanil [22] and its analogues [23], are more potent analgesics [23, 24], and produce a much stronger response in the mouse vas deferens assay [25] than 'pure' $CB_1$ and VR1 agonists. Therefore, functional cross-talk between $CB_1$ and VR1 receptors, localized in the same or neighbouring neurons, might explain the different impact that $CB_1$ receptor stimulation has so far been found to have on VR1-mediated signalling.

Here we investigated the effect of $CB_1$ receptor stimulation on the VR1-induced increase in intracellular calcium concentration ($[Ca^{2+}]_i$) by using human embryonic kidney (HEK)-293 cells stably transfected with cDNAs encoding the $CB_1$ and VR1 receptors, and therefore co-expressing both receptor types. We report that, depending on whether or not the cAMP cascade is activated, $CB_1$ receptor stimulation may either inhibit or enhance inhibit VR1-mediated biological responses.

## Materials and methods

### Drugs

HU-210 and SR141716A were kind gifts from Prof. R. Mechoulam, Hebrew University of Jerusalem and from Sanofi Recherche, respectively. 3-Isobutyl-1-methylxanthine (IBMX) and forskolin were purchased from Sigma (Deisenhofen, Germany) and WIN 55,212-2 was purchased from Tocris (Cologne, Germany). These compounds were prepared as 10 mM stock solutions in 100% dimethylsulfoxide (DMSO; in the case of HU-210, SR141716A, WIN 55,212-2 and forskolin) or in phosphate-buffered saline (PBS) (136.8 mM NaCl, 2.7 mM KCl, 1.5 mM $KH_2PO_4$, 10.2 mM $Na_2HPO_4$, pH 7.4: in the case of IBMX). Ionomycin was purchased from Sigma. AEA was synthesized as previously described [19]. The phosphatidyl-inositol-3-kinase (PI-3-K) inhibitors, wortmannin and LY294002 were purchased from Alexis Biochemicals (Lausen, Switzerland). The phosphatidyl-inositol-selective phospholipase C (PI-PLC) inhibitors ET-18 and U73122, and the phosphatidyl-choline-selective PLC (PC-PLC) inhibitor D609 were obtained from Biomol Research Laboratories (Plymouth Meeting, Pa, USA).

### Construction of the pZeoSV-$CB_1$ plasmid

The pcDNA3 plasmid containing the N-terminal haemagglutinin (HA)-tagged cDNA of rat $CB_1$ (pcDNA3-$CB_1$) was a kind gift from Dr. K. Mackie. pcDNA3-$CB_1$ was linearized with *Xho*I and overhangs were blunted with Klenow. $CB_1$ was released with *Acc*65I and subcloned into pZeoSV containing resistence against zeocin (Invitrogen, Karlsruhe, Germany) linearized with *Acc*65I and *Pvu*II to obtain pZeoSV-$CB_1$. The plasmid was checked by sequencing. For transfection into HEK-293 cells, pZeoSV-$CB_1$ was linearized with *Not*I [all molecular biology methods were performed as described in ref. 26].

### Cell culture and transfection

HEK-293 cells stably expressing human VR1 (hVR1) were obtained from J. Davis (GlaxoSmithKline, Harlow, UK). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal calf serum, 2 mM glutamine and, to prevent bacterial and fungal contamination, 1% antibiotic-antimycotic (penicillin/streptomycin/amphotericin; Gibco BRL, Karlsruhe, Germany), at 37°C in a humidified 5% $CO_2$ incubator. Cell lines were generated by transfection of linearized pZeoSV-$CB_1$ into HEK-293 cells already stably expressing hVR1 by electroporation as described elsewhere [26]. Stable transfectants were selected in medium containing zeocin (0.6 mg/ml) for $CB_1$ selection and geneticin (G418, 2 mg/ml) for VR1 selection. Zeocin is an antibiotic that causes cell death by cleaving DNA, and resistance to it is conferred by the Sh ble gene product, which binds the antibiotic and prevents its action. Geneticin is instead an antibiotic that interferes with 80S ribosomes, thus blocking protein synthesis, and resistance to it is conferred by the Tn5 or Tn601 aminoglycoside phosphotransferase. Colonies of about 500 cells were picked (about 2 weeks after transfection) and allowed to expand, then tested for expression of $CB_1$ mRNA and protein by Northern and Western blot, respectively. $CB_1$-VR1-HEK clones containing high levels of $CB_1$ mRNA and protein were tested for functional receptor properties by measurement of a forskolin-stimulated decrease in cAMP [27]. $CB_1$-VR1-

HEK cells were maintained under selection by adding antibiotics to culture medium every third passage. No difference in the levels of hVR1 mRNA transcripts, assessed by reverse transcriptase-polymerase chain reaction, between cells expressing only hVR1 and cells expressing both hVR1 and rat $CB_1$ was observed (data not shown).

**Northern blot analysis**

Standard Northern blotting protocols were used [27]. Briefly, total RNA (20 µg) was loaded onto formaldehyde-containing 1% agarose gels, blotted onto nylon membranes (Hybond NX; Amersham, Freiburg, Germany), and immobilized by UV cross-linking (UV Stratalinker 2400; Stratagen, Amsterdam, The Netherlands). Blots were pre-hybridized in rapid-hyb buffer (Amersham) and hybridized in the same solution containing [$^{32}$P]dCTP-labelled probe at 70 °C according to the manufacturer's instructions. Probe labelling of the full-length cDNA of $CB_1$ was carried out with a random primer DNA labelling system (Gibco BRL). Blots were exposed at –80 °C for 1–2 days to Kodak Biomax films with intensifying screens.

**Western blot**

For detection of the $CB_1$ receptor protein we used the Western immunoblotting technique, by exploiting the tag with the short HA epitope (corresponding to an internal 9-amino-acid sequence of the influenza HA) attached to the N terminus of the $CB_1$ receptor (see Construction of the pZeoSV-$CB_1$ plasmid), and hence using an anti-HA monoclonal antibody. Transfected HEK-293 cells were solubilized in a glass homogenizer with 20 mM Tris-HCl, pH 7.4, containing protease inhibitors (Complete Mini tablets; Roche, Basel, Switzerland). The lysate was centrifuged for 5 min at 1000 g, and the supernatant collected and assayed for protein content (Bio-Rad, Munich, Germany). Loading buffer (Roti-load 1; Roth, Karlsruhe, Germany) was added to protein samples which were denatured for 5 min at 95 °C, centrifuged and loaded (20 µg/lane) on a 7.5% polyacrylamide gel. After electrophoresis, proteins were transferred overnight at 4 °C onto a cellulose nitrate membrane (Schleicher & Schüll, Dassel, Germany) with transfer buffer (48 mM Tris, 390 mM glycine, 0.1% SDS, 20% methanol), using a Bio-Rad Blot apparatus. The membrane was blocked for 1 h with blocking buffer (10% non-fat milk powder, 20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween-20, pH 7.6). To detect the HA-$CB_1$ fusion protein, blots were incubated with an anti-HA monoclonal antibody (Santa Cruz, Heidelberg, Germany), diluted 1:200 in blocking buffer overnight at 4 °C. After incubation with anti-mouse IgG-horseradish peroxidase as secondary antibody (Dako, Glostrup, Denmark) diluted 1:2000 in blocking buffer, chemiluminescence was performed using the Lumi GLO reagent (Cell Signaling, Frankfurt, Germany) according to the manufacturer's instructions, and the blots were exposed to Biomax films for 1–10 min. Preadsorption of the anti-HA antibody with the corresponding immunizing peptide (Santa Cruz) was carried out to test the specificity of the antibody, and produced no band on the gel.

**cAMP accumulation assay**

The cAMP assay was performed as described elsewhere [27] with slight modifications. One day before the experiment, $CB_1$-VR1-HEK cells were plated into 48-well plates in 500 µl of complete DMEM at a density of $4 \times 10^5$ cells/ml. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Reaction was initiated by adding stimulation buffer containing 20 mM HEPES, 0.1 mg/ml BSA, 5 µM forskolin, 0.5 mM IBMX and the $CB_1$ agonists WIN 55,212-2 and HU-210. Forskolin, WIN 55,212-2 and HU-210 were dissolved in DMSO. DMSO alone served as a vehicle control and had no effect on cAMP accumulation (data not shown). Reactions were terminated 10 min later by aspiration of the medium and the addition of 500 µl ice-cold 6% trichloroacetic acid followed by incubation overnight at 4 °C. To remove the trichloroacetic acid, the extracts were treated twice with 3 ml diethylether, dried overnight in a lyophilizator and reconstituted in DMEM. Intracellular cAMP levels were measured with a competitive protein-binding assay (non-acetylated procedure; Perkin Elmer, Boston, MA, USA). Data obtained in the cAMP accumulation assay were expressed as the percentage of forskolin-stimulated cAMP accumulation. Samples were measured in triplicate and data are given with the standard error of the mean (SE).

**$[Ca^{2+}]_i$ assays**

The effect of test substances on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK and VR1-HEK cells was determined using Fluo-3 methylester (Molecular Probes, Leiden, The Netherlands), a selective intracellular fluorescent probe for $Ca^{2+}$. Cells were prepared and loaded as described previously [19]. Experiments were carried out by measuring cell fluorescence at 25 °C ($\lambda_{EX}$ = 488 nm, $\lambda_{EM}$ = 540 nm) before and after the addition of the test compounds at various concentrations. HU-210 (100 nM) or forskolin (5 µM) were added, alone or together, 5 min before capsaicin. SR141716A (0.5 µM) was also added 5 min before HU-210 or AEA. The PI-3-K and PLC inhibitors were added 5 min before HU-210. The efficacy of the effect of each treatment was determined by normalizing it to the analogous effect observed with 4 µM ionomycin in each single experiment. A typical experiment consisted in suspending in a quartz cuvette the cells pre-loaded with Fluo-3, followed by measuring cell fluorescence for 5 min while the response became stable. This was followed by addition of capsaicin or AEA (the 'stimulant'). In the case of

pre-treatments, HU-210 or forskolin or SR141716A or the phospholipase and/or kinase inhibitors, or their combinations, were added 5 min prior to incubation with the stimulant. During this pre-treatment, fluorescence was measured so that the effect, if any, of the pre-treatment on basal $[Ca^{2+}]_i$ could be observed. After the addition of the stimulant, fluorescence was measured for 10–20 min, after which ionomycin (4 μM) was always added to calculate the maximal inducible $[Ca^{2+}]_i$ in those conditions. The effect of the stimulant was then normalized to the effect of ionomycin, which in turn depends almost uniquely on the amount of viable cells present in each incubation. Data for the compounds tested at varying concentrations were expressed as the concentration exerting a half-maximal effect ($EC_{50}$), calculated using GraphPad software.

## Results

### $CB_1$-VR1-HEK cells express functional $CB_1$ receptors

Northern blots of $CB_1$-VR1-HEK clones produced single discrete bands of the same, expected size (1.6 kb), while no band was observed using RNA of cells transfected only with hVR1 (fig. 1A). To test whether receptor mRNA is effectively translated into receptor protein, Western blot analysis was carried out and showed a band of the expected size of 80 kDa for the HA-$CB_1$ fusion protein (fig. 1B), which was not observed by blocking of the antigen recognition site of the antibody with the immunizing peptide (data not shown). Two clones of $CB_1$-VR1-HEK cells (no. 10 and no. 15) expressing high levels of mRNA and protein were tested for functional receptor properties. Both clones exhibited functional coupling of $CB_1$ receptors to $G_i$ proteins, as demonstrated by the inhibition of forskolin-stimulated intracellular cAMP accumulation by HU-210 and WIN 55,212-2 (fig. 1C, D),

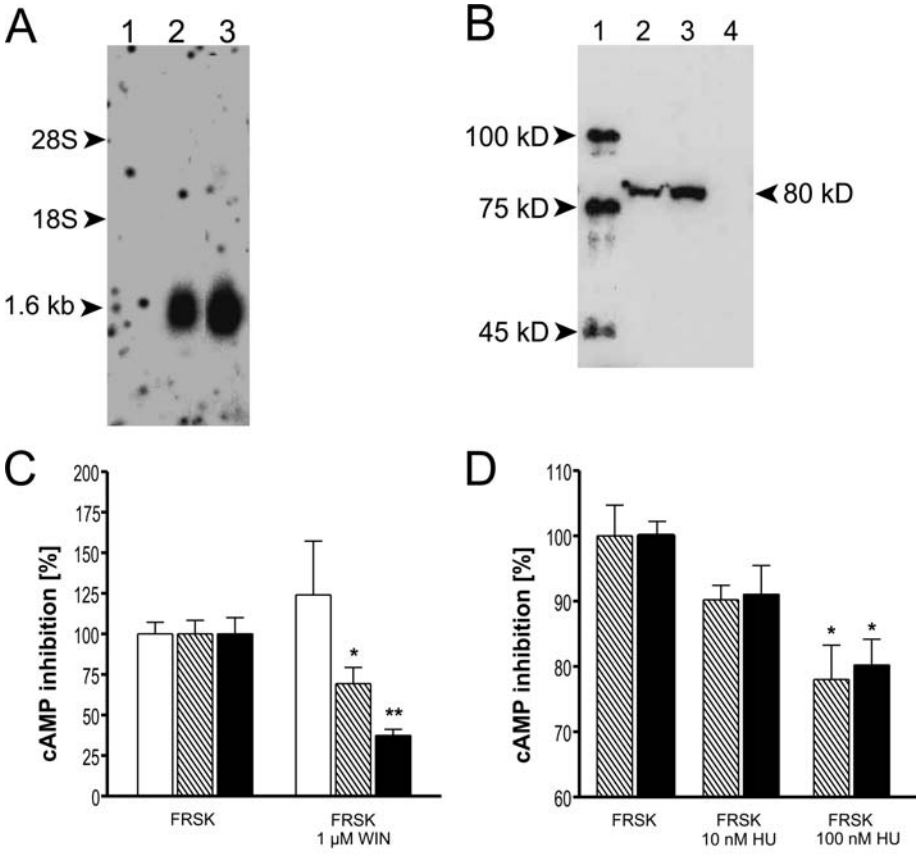


Figure 1. Analysis of $CB_1$ expression in $CB_1$-VR1 double-transfected HEK-293 cells. (*A*) Northern blot showing $CB_1$ mRNA in the two different clones of $CB_1$-VR1-HEK cells (lane 2, clone no. 10; lane 3, clone no. 15); VR1-HEK cells served as a negative control (lane 1). (*B*) Western blot showing $CB_1$ protein in the same CB1-VR1-HEK clones (lane 2, clone no. 10; lane 3, clone no. 15); VR1-HEK cells served as negative control (lane 4); HA-tagged protein as molecular-weight standard (lane 1). (*C*) Effect of WIN 55,212-2 (WIN) on forskolin-induced cAMP accumulation in clone no. 10 (striped bars) and clone no. 15 (black bars) of $CB_1$-VR1-HEK cells, and in cells expressing only VR1 (white bars). (*D*) HU-210 (HU)-induced inhibition of cAMP accumulation in clone no. 10 (striped bars) and clone no. 15 (black bars) of $CB_1$-VR1-HEK cells. Data are expressed as percentages of the effect of forskolin (FRSK) and are means ± SE of n = 3 experiments. *$p < 0.05$ vs FRSK only clone no.10; **$p < 0.01$ vs FRSK only clone no.15, calculated by ANOVA followed by the Bonferroni test.

whereas VR1-HEK cells did not show any response upon stimulation with WIN 55,212-2 (fig. 1C). The two $CB_1$-VR1-HEK clones were subsequently used for the experiments carried out in this study.

### Effect of HU-210 on capsaicin response in $CB_1$-VR1-HEK cells

The effect of capsaicin, the prototypical VR1 agonist, on $[Ca^{2+}]_i$ in $CB_1$-VR1 HEK cells (clone no. 10) is shown in figure 2A. The compound enhanced $[Ca^{2+}]_i$ in a dose-dependent manner, with an $EC_{50}$ = 35.0 ± 4.0 nM (mean ± SE, n = 3) that was indistinguishable from that observed in HEK cells over-expressing only VR1 ($EC_{50}$ = 32.1 ± 5.0 nM, n = 3). The $CB_1$ receptor agonist HU-210, at a concentration (100 nM) previously shown to be fully effective on $CB_1$ receptors [1], and shown here to inhibit forskolin-induced cAMP formation in the same cells (fig. 1C), significantly enhanced the effect on $[Ca^{2+}]_i$ of 10–50 nM capsaicin (fig. 2A), without having any effect per se on basal $[Ca^{2+}]_i$ (data not shown). The $EC_{50}$ for the effect of capsaicin was lowered from 35.0 ± 4.0 to 17.0 ± 2.1 nM, n = 6, $p < 0.05$ by ANOVA). This effect was antagonized by the $CB_1$ receptor antagonist, SR141716A (fig. 2B), at a dose (0.5 µM) selective for $CB_1$ receptors and devoid per se of any effect on $[Ca^{2+}]_i$ (data not shown). The effect of HU-210 was not observed in VR1-HEK cells (data not shown). The HU-210 effect was also observed in a second clone (clone no. 15) of $CB_1$-VR1 HEK cells, which again responded to capsaicin to the same extent as VR1-HEK cells ($EC_{50}$ = 27.7 ± 4.3 nM, n = 3). In these cells, the $CB_1$ agonist decreased the $EC_{50}$ for the effect of capsaicin to 14.5 ± 1.5 nM, (n = 3, $p < 0.05$ by ANOVA). Interestingly, simultaneous treatment of $CB_1$-VR1-HEK cells (clone no. 10) with HU-210 and capsaicin did not lead to a potentiation of the effect on $[Ca^{2+}]_i$ of the latter compound (data not shown).

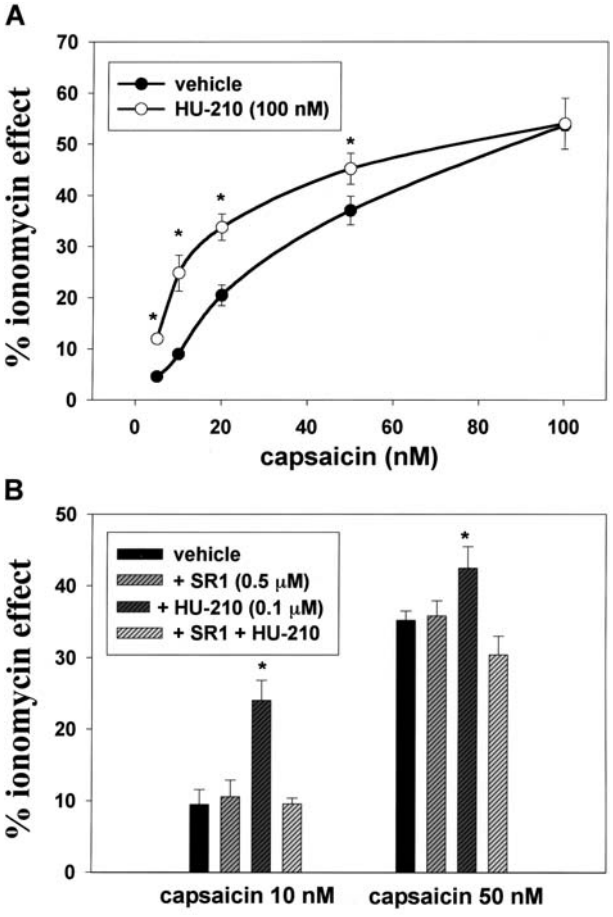


Figure 2. HU-210 enhances the VR1-mediated capsaicin effect on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells via a $CB_1$ receptor-mediated mechanism. (*A*) Dose-response for the VR1-mediated effect of capsaicin on $[Ca^{2+}]_i$ with (○) or without (●) pre-treatment of cells (clone no. 10) with HU-210 (100 nM). (*B*) Reversal of HU-210-induced potentiation of the VR1-mediated capsaicin effect on $[Ca^{2+}]_i$ by the $CB_1$ antagonist SR141716A (SR1, 0.5 µM). Data are expressed as percent of the effect of ionomycin (4 µM) and are means ± SE of at least n = 3 independent experiments carried out in duplicate. *$p < 0.05$ by ANOVA followed by the Bonferroni test. Neither HU-210 nor SR141716A per se caused any significant change in basal intracellular calcium (data not shown).

### Effect of various inhibitors on HU-210 potentiation of the capsaicin response

The two selective inhibitors of PI-3-K, wortmannin (10 µM) and LY294002 (20 µM), the two selective inhibitors of PI-PLC, ET-18 (20 µM) and U73122 (10 µM), and the selective PC-PLC inhibitor D609 (20 µM) strongly attenuated the effect of capsaicin (20 nM) on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells (clone no. 10) (fig. 3), while exhibiting no effect per se on basal $[Ca^{2+}]_i$ (not shown). Moreover, when cells were pre-incubated with HU-210 (100 nM), and the inhibitors were tested at concentrations (1–10 µM) that were inactive per se on the response induced by capsaicin alone, a complete blockade of HU-210 potentiation of the capsaicin effect was observed (fig. 3).

### Effect of AEA on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK and VR1-HEK cells

We compared the effect of the endogenous agonist of $CB_1$ and VR1 receptors, AEA, on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK and VR1-HEK cells. Unlike capsaicin, AEA was significantly more efficacious in $CB_1$-VR1 HEK cells (clone no. 10) than in VR1-HEK cells at the two highest concentrations tested (fig. 4). Importantly, after pre-treatment of $CB_1$-VR1-HEK cells with a concentration of SR141716A (0.5 µM) selective for $CB_1$ versus VR1 receptors [15], the effect of AEA became identical to that observed in VR1-HEK cells (fig. 4). AEA was also more potent and efficacious in clone no. 15 of $CB_1$-VR1-HEK cells than in VR1-HEK cells (data not shown).

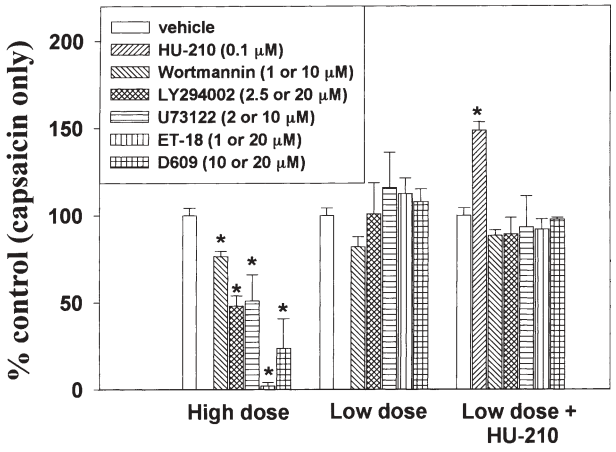


Figure 3. Effect of various inhibitors of PI-3-K (wortmannin, LY294002), PI-PLC (U73122, ET-18) and PC-PLC (D609) on capsaicin action on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells. Clone no. 10 of $CB_1$-VR1-HEK cells was used in these experiments. The dose of capsaicin used was 20 nM and led to a stimulation of $[Ca^{2+}]_i$ of 22.9+1.1% (mean ± SE, n = 12) of the effect of ionomycin (4 μM). The effects of the inhibitors, which were given to cells 5 min before capsaicin, are expressed as a percent of the effect of capsaicin alone and are means ± SE of at least n = 3 independent experiments carried out in duplicate. The high dose of the inhibitors was tested only on capsaicin alone and was 10 μM for wortmannin and U73122, and 20 μM for LY294002, ET-18 and D609. The low dose of the inhibitors was tested both on capsaicin alone and on capsaicin + HU-210 (100 nM), and was 1 μM for wortmannin and ET-18, 2 μM for U73122, 2.5 μM for LY294002 and 10 μM for D609. The effect of HU-210 (100 nM, 5 min pre-treatment) on capsaicin is also shown as a percent of the effect of capsaicin alone. *$p < 0.05$ vs. vehicle (i.e. capsaicin only), calculated by ANOVA followed by the Bonferroni test, and using the raw data (i.e. expressed as percent of the ionomycin effect and not as percent of capsaicin alone). None of the inhibitors per se caused any significant change in basal $[Ca^{2+}]_i$.

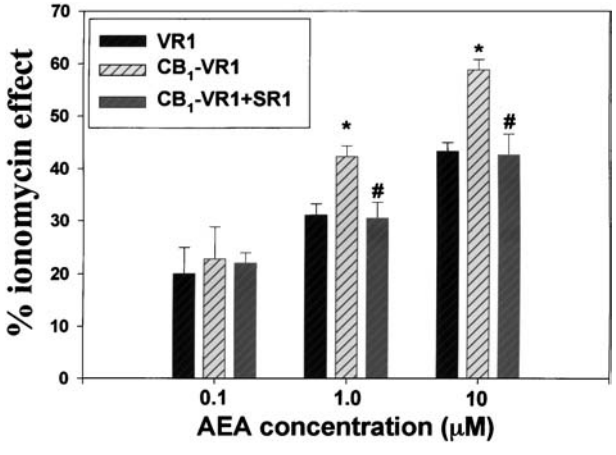


Figure 4. Dose-dependent effect of anandamide (AEA) on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK and VR1-HEK cells. The effect on $[Ca^{2+}]_i$ was expressed as a percent of the effect of ionomycin (4 μM) and, in $CB_1$-VR1-HEK cells (clone no. 10), measured after 5 min pre-treatment with SR141716A (SR1, 0.5 μM). Data are means ± SE of at least n = 3 independent experiments carried out in duplicate. *$p < 0.05$ vs VR1-HEK cells; #$p<0.05$ vs CB1-VR1-HEK cells without SR1, as calculated by ANOVA followed by the Bonferroni test.

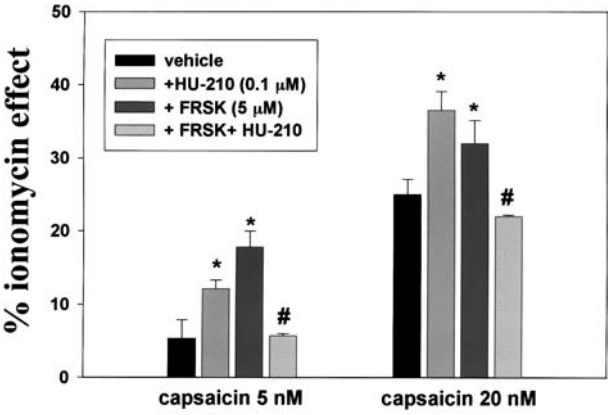


Figure 5. HU-210 inhibits the effect of capsaicin on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells pre-treated with forskolin. Cells (clone no. 10) were treated with vehicle, forskolin (FRSK, 5 μM), HU-210 (100 nM) or with both HU-210 and FRSK 5 min prior to stimulation with capsaicin (5 or 20 nM). FRSK or HU-210+FRSK caused no significant change in basal $[Ca^{2+}]_i$. Data are expressed as percent of the effect of ionomycin (4 μM) and are means ± SE of at least n = 3 independent experiments carried out in duplicate. *$p < 0.05$ vs control; #$p < 0.05$ vs FRSK, calculated by ANOVA followed by the Bonferroni test.

### Effect of HU-210 on forskolin-induced potentiation of the capsaicin response in $CB_1$-VR1-HEK cells

In agreement with a previous study carried out with VR1-HEK cells [16], we found that 5 min pre-treatment with forskolin, at a dose (5 μM) inactive per se on basal $[Ca^{2+}]_i$, led to a significantly enhanced effect of capsaicin on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells (clone no. 10) (fig. 5). When cells were pre-treated with both forskolin and HU-210 (100 nM), however, the overall response on $[Ca^{2+}]_i$ was not significantly different from that observed with capsaicin alone (fig. 5).

## Discussion

The results reported here indicate that, at least in our in vitro model, stimulation of cannabinoid $CB_1$ receptors exerts a dual regulatory effect on VR1-induced biological responses, and that the final outcome of this effect depends on the state of activation of cAMP-mediated signalling. We found that a 5-min pre-treatment with the $CB_1$ agonist, HU-210, of HEK-293 cells co-expressing functionally active $CB_1$ and VR1 receptors ($CB_1$-VR1-HEK cells) significantly enhances the capsaicin-induced, and VR1-mediated, increase in $[Ca^{2+}]_i$. When using a 100 nM concentration of HU-210, the $EC_{50}$ for the capsaicin effect in these cells was decreased twofold. This effect was counteracted by the $CB_1$-selective antagonist, SR141716A, and was not observed in HEK-293 cells expressing only VR1 receptors (VR1-HEK cells), thus conclusively demonstrating the involvement of $CB_1$ receptors

in HU-210 action. Interestingly, simultaneous treatment of $CB_1$-VR1-HEK cells with HU-210 and capsaicin did not lead to a similar potentiation of the effect on $[Ca^{2+}]_i$ by the latter compound. The time dependency of the effect suggests that (i) $CB_1$-coupled intracellular signalling events, rather than a direct interaction between the two receptors, might be necessary to observe the enhancement of VR1-induced biological effects and (ii) endogenous substances, like AEA or N-arachidonoyl-dopamine [28], which are capable of activating both receptor types, might produce different overall biological effects depending on which of the two receptors they activate first.

To investigate the first of the above possibilities, we carried out some pilot experiments. We started from the recent findings that VR1 activity can be enhanced by protein phosphorylation catalysed by protein kinase C (PKC) [16, 29, 30], and inhibited by phosphatidyl-inositol-bis phosphate (PIP2) [31], and that $CB_1$ receptors are coupled to activation of PLC (possibly via the $\beta\gamma$ subunits of $G_{i/o}$ proteins [32, 33]) and stimulation of PI-3-K [34, 35]. Therefore, we tested the effects of PLC and PI-3-K inhibitors on the enhancement by HU-210 of the capsaicin effect on $[Ca^{2+}]_i$. We found that, per se, two PI-3-K inhibitors and two PI-PLC inhibitors significantly reduced the effect of capsaicin on $[Ca^{2+}]_i$ at the concentrations previously reported to inhibit PI-3-K and PLC, respectively. Furthermore, these four compounds, at concentrations per se inactive on the capsaicin-induced response, abolished the potentiation of the capsaicin effect caused by pre-treatment with HU-210. PI-3-K is not only responsible for the formation of PIP2, but it also catalyses its phosphorylation to phosphatidyl-inositol-tris-phosphate, whereas PI-PLC catalyses PIP2 hydrolysis. Therefore, on the basis of these experiments, one can hypothesize that, when over-expressed in HEK-293 cells, VR1 is under the negative influence of PIP2 [31], whose concentration and turnover are in turn controlled by tonic PI-PLC and PI-3-K activity, respectively. When these two enzymes are inhibited, PIP2 remains associated with VR1 and the effect of capsaicin on $[Ca^{2+}]_i$ is therefore reduced. Conversely, further stimulation of PI-PLC [32] and PI-3-K [34] by $CB_1$ receptors leads to an enhanced turnover of PIP2, with subsequent release of VR1 from the tonic inhibitory action exerted by this lipid (fig. 6). We also found that the PC-PLC inhibitor, D609, inhibited the potentiation of the capsaicin effect caused by pre-treatment with HU-210. Thus, $CB_1$ stimulation may also lead to the activation of PC-PLC [33]. This enzyme, together with PI-PLC, causes the release of diacylglycerols and the subsequent activation of PKC, which can then sensitise VR1 to capsaicin [16, 29, 30].

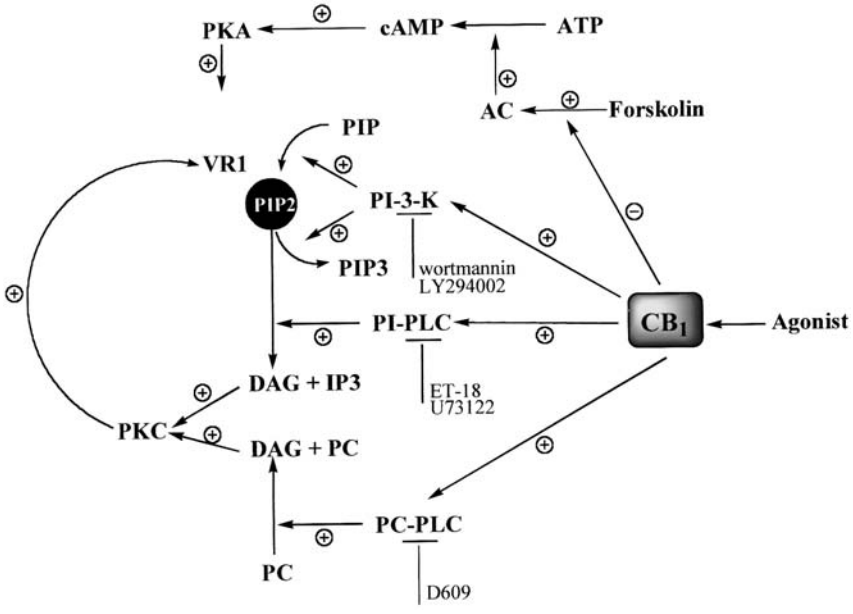


Figure 6. Schematic representation of the possible intracellular pathways underlying the $CB_1$ receptor-mediated control of VR1 activity. VR1 is tonically inhibited by PIP2, which in turn can be produced by the action of PI-3-K on phosphatidyl-inositol-mono-phosphate (PIP), and transformed by the same enzyme into phosphatidyl-inositol-tris-phosphate (PIP3), or into diacylglycerols (DAG) and inositol-tris-phosphate (IP3) by PI-PLC. Thus, inhibitors of PI-3-K and PI-PLC (whose action is indicated by blunt arrows) stabilize the VR1-PIP2 complex, leading to the inhibition of VR1 sensitivity to capsaicin, or to the inhibition of $CB_1$-mediated activation of the two enzymes. The potentiation of capsaicin activity by HU-210 observed in this study may involve this signalling pathway. Tonic, or $CB_1$-induced, stimulation of VR1 by PC-PLC, and subsequent stimulation of DAG release and PKC activity, might also explain why a selective PC-PLC inhibitor (D609), as well as PI-PLC inhibitors, attenuate both basal and HU-210-enhanced activity of capsaicin at VR1. Finally, stimulation of adenylate cyclase (AC) and protein kinase A (PKA) by forskolin, or during e.g. inflammation, might lead to sensitization (or inhibition of desensitization) of VR1. In this case, activation of $CB_1$ receptors by agonists, by leading to inhibition of AC, would lead to VR1 inhibition.

A second set of experiments was carried out with exogenous AEA, an endogenous mediator capable of activating both $CB_1$ and VR1 receptors. Indeed, since the binding sites of $CB_1$ and VR1 for AEA are extra- and intracellular, respectively [1, 15, 36], and AEA can be rapidly transported into HEK cells [15], treatment of $CB_1$-VR1-HEK cells with this lipid is likely to produce the sequential stimulation of $CB_1$ and VR1 receptors, which we found here to be necessary for the enhancement of VR1 activity. Indeed, we observed that AEA was significantly more efficacious on $[Ca^{2+}]_i$ in $CB_1$-VR1-HEK cells than in VR1-HEK cells, and that its effect in the former cells was reduced by the $CB_1$ antagonist SR141716A to an extent indistinguishable from that observed in VR1-HEK cells. These findings might open the possibility that extracellular AEA exerts a more efficacious action on VR1 in those cells that naturally co-express this receptor together with $CB_1$ receptors, such as some DRG neurons in culture [4]. Indeed, in sensory neurons, either a strong excitatory effect, or a weaker excitatory effect that is enhanced by $CB_1$ antagonists have been observed on VR1-mediated cation currents or neuropeptide release [37–39]. In other cells and tissues also, AEA was found to exhibit varying potency at VR1 receptors. In general, one can hypothesize that when a strong VR1-mediated effect is observed, as in the case of mesenteric sensory neurons [12], some DRG preparations [38] and hippocampal slices [40], $CB_1$ and VR1 receptors are co-expressed in the majority of the cells. Conversely, when both an inhibitory, $CB_1$-mediated effect (observed at low AEA doses) and an excitatory, VR1-mediated action (observed at high AEA doses and strengthened by $CB_1$ receptor antagonists) are seen [37, 41, 42], the two receptor types might be co-expressed only in a minority of neurons. Finally, when substances that selectively activate $CB_1$ receptors, such as HU-210, inhibit the biological effects of substances that selectively activate VR1 receptors, such as capsaicin [21], this might be due to the lack of coupling of $CB_1$ receptors to those intracellular signalling pathways that facilitate the gating of VR1 (i.e. PI-PLC or PI-3-K; see above), or to their inhibition of signalling events that instead lead to sensitization of VR1 activity [i.e. protein kinase A (PKA); see below].

Recent studies have in fact shown that the sensitivity of VR1 receptors to ligands can be enhanced by substances that stimulate adenylate cyclase and subsequently activate the cAMP-dependent protein kinase (PKA), thus leading to VR1 phosphorylation [16, 43, 44]. Since $CB_1$ receptors are coupled to inhibition of adenylate cyclase via the $\alpha$ subunits of $G_{i/o}$ proteins [1], we reasoned that, in $CB_1$-VR1-HEK cells, where we found here that stimulation with two distinct $CB_1$ receptor agonists inhibits the forskolin-induced formation of cAMP, HU-210 would inhibit, rather than enhance the previously reported enhancement of capsaicin VR1-mediated effect on $[Ca^{2+}]_i$ by forskolin. In fact, we found that, in agreement with previous studies carried out with VR1-HEK cells [16], a 5-min pre-treatment with forskolin enhanced the effect of capsaicin on $[Ca^{2+}]_i$ also in $CB_1$-VR1-HEK cells, and that, when incubated together with forskolin, HU-210 totally abolished this enhancement of capsaicin activity. This finding might provide an explanation for the previously reported inhibition of capsaicin-induced thermal and inflammatory hyperalgesia by prior $CB_1$ receptor stimulation [7, 8, 21]. It is in fact possible that during inflammation, cAMP levels are enhanced, PKA is activated, and VR1 phosphorylated and up-regulated, and that $CB_1$ receptor agonists inhibit the effects of capsaicin (or of inflammatory stimuli that indirectly gate the VR1 receptors) by inhibiting adenylate cyclase. By contrast, in other experimental systems, such as the electrically stimulated mouse vas deferens [25], VR1 is possibly not over-activated by the cAMP-signalling cascade, and thus substances that stimulate both $CB_1$ and VR1 receptors can exert a very strong effect on VR1.

In conclusion, we have demonstrated for the first time that when cannabinoid $CB_1$ receptors and vanilloid VR1 receptors are co-expressed in the same cells, pre-treatment of cells with $CB_1$ receptor agonists leads to inhibition of VR1 activity or to its enhanced stimulation depending on whether or not the cAMP-signalling pathway is concomitantly activated. Sequential $CB_1$-VR1 stimulation occurs in vitro when cells are treated first with HU-210 and then with capsaicin, and might occur in vivo with extracellular AEA or other endogenous mediators, such as N-arachidonoyl-dopamine, that are capable of activating both receptor types [28]. These findings provide an explanation for the often discrepant effects of AEA on sensory neurons, and strengthen the hypothesis that $CB_1$ and VR1 receptors can be regarded as interacting metabotropic and ionotropic receptors for this endogenous compound and some of its congeners [45].

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# Corticotropine-releasing hormone increases BDNF expression in cerebellar neurons: inhibition by cannabinoid receptor type 1 activation

*In preparation*

# CORTICOTROPIN-RELEASING HORMONE INCREASES BDNF EXPRESSION IN CEREBELLAR NEURONS: INHIBITION BY CANNABINOID RECEPTOR TYPE 1 ACTIVATION

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**ABSTRACT**

Both the corticotropin-releasing hormone (CRH) as well as the cannabinoid systems have been demonstrated to affect synaptic plasticity. Activation of CRH receptor type 1 (CRHR1) leads to increases in cAMP production, and subsequent phosphorylation of the transcription factor cAMP response element-binding protein (CREB). The cannabinoid receptor type 1 (CB1) however, is negatively coupled to the cAMP signaling cascade. In this study we analyzed a putative cross-talk between these two systems focussing on the regulation of the expression of brain-derived neurotrophic factor (BDNF), a CREB-regulated gene that also affects neuronal survival and synaptic plasticity. *In situ* hybridization revealed a high degree of coexpression of CRHR1 and CB1 receptors in cerebellar granule cells, a necessary prerequisite for any cross-talk. We analyzed the effects of CRH and the CB1 agonist WIN-55,212-2 on BDNF expression in primary cerebellar neurons. BDNF mRNA and protein levels were increased 48 hours after application of CRH ($10^{-8}$ M). This effect was inhibited by simultaneous administration of WIN-55,212-2 ($10^{-6}$ M). WIN-55,212-2 alone had no effect on BDNF expression. Moreover at the level of intracellular signalling, short-term application of WIN-55,212-2 inhibited CRH-induced cAMP accumulation and CREB phosphorylation. These data highlight a cross-talk between the CRH and the cannabinoid system in the regulation of BDNF expression.

## INTRODUCTION

Corticotropin-releasing hormone (CRH), the major mediator of the stress response in the central nervous system (CNS) (Vale et al., 1981; Reul and Holsboer 2002) also affects other central processes, such as memory and learning, synaptic plasticity, and neuroprotection (Radulovic et al, 1999; Wang et al., 1998; Lezoualc'h et al., 2000). These effects are mediated by CRH binding and activation of two distinct CRH receptors, CRHR1 and CRHR2, that are found throughout the CNS and periphery (De Souza, 1995). CRH has a higher affinity for CRHR1 than for CRHR2, and in the brain CRHR1 is expressed at high levels in the hippocampus, cortex, and cerebellum (Van Pett et al., 2000). CRH binding to CRHR1 typically activates adenylate cyclase (AC), which leads to increased intracellular concentrations of cAMP and activation of protein kinase A (PKA) (Eckart et al., 2002). The neuroprotective action of CRH is mediated through this CRHR1/cAMP/PKA-dependent signaling mechanism (Bayatti et al., 2003), but downstream target genes of CRH have not yet been investigated. One putative target is the brain-derived neurotrophic factor (BDNF) whose expression is controlled by cAMP-elevating agents in neurons (Galter and Unsicker, 2000). In addition to its role as a classical target-derived growth factor during neuronal development (Lewin & Barde, 1996), BDNF is an essential autocrine factor, released and acting locally after neuronal depolarization (Ghosh et al., 1994).

Cannabinoids predominantly exert their effects through activation of G protein-coupled receptors by inhibition of AC through $G_{i/o}$ proteins (Ameri, 1999). To date, two cannabinoid receptors have been identified; the cannabinoid receptor type 1 (CB1) is expressed throughout the nervous system (Freund et al., 2003) and is localized densely in the developing and adult cerebellum (Berrendero et al., 1999; Egertova & Elphick 2000) whereas the cannabinoid receptor type 2 (CB2) is mainly present in immune cells (Munro et al., 1993). The cannabinoid system modulates various neurotransmitter systems, mainly by decreasing synaptic release (Schlicker & Kathmann, 2001). The signal transduction pathways regulated by $G_i/_o$-coupled CB1 in the cerebellum are poorly characterized. In non-neuronal cell lines, stimulation of CB1 activates signaling pathways leading to the expression of immediate-early genes including *c-fos* and *zif268* (Mailleux et al., 1994). CB1 activation by exogenous ligands and its endogenous ligand anandamide leads to increased CRH-dependent adrenocorticotrophic hormone release within the hypothalamic-pituitary-adrenal axis (Weidenfeld et al., 1994). Nevertheless, signaling interactions between the corresponding receptors and effects on downstream transcriptional targets are unclear.

Therefore, we examined a putative cross-talk mechanism between the cannabinoid and CRH systems in cerebellar granular neurons, cells that express both CB1 and CRHR1 and focused on the modulation of the expression levels of BDNF as well as the cAMP signaling cascade and the phosphorylation status of the transcription factor cAMP response element-binding protein (CREB).

## MATERIAL AND METHODS

*Drugs*

IBMX (3-isobutyl-1-methylxanthine; Sigma, Deisenhofen, Germany), WIN-55,212-2 (Tocris, Cologne, Germany) and CRH (Calbiochem, Schwalbach, Germany) were prepared as 10 mM stock solutions in phosphate buffered saline (PBS; IBMX), 100% dimethylsulfoxide (DMSO; WIN-55,212-2) or in 2% acetic acid (CRH).

*Animals*

C57BL/6N mice and Sprague Dawley rats (Charles River, Sulzfeld, Germany) were housed with a 12 h: 12 h light-dark cycle and allowed to access food and water *ad libitum*. Adult mice (3-5 months old) were killed by cervical dislocation, and newborn rats were decapitated.

*In situ hybridization*

Single-*in situ* hybridization with $^{35}$S-labelled riboprobes for CB1 and CRHR1 was carried out as described in detail in Marsicano and Lutz (1999). Double-*in situ* hybridization was carried out as described in detail in Hermann et al. (2002). The TSA Biotin System (NEN Life Science Products, Boston, USA) was used for detection of the DIG-labelled CB1 probe, and the chromogenic reaction was carried out with Vector Red kit (Vector Laboratories, Gruenberg, Germany). Slides were dipped in photographic emulsion (NTB-2 from Kodak, diluted 1:1 in distilled $H_2O$) for detection of the $^{35}$S-labelled, CRHR1. After exposition for 4 weeks at 4°C, slides were developed (D-19, Kodak) and fixed (Kodak fixer). Counterstaining was carried out in 0.1 % aqueous toluidine blue solution. Slides were mounted in histofluid (Marienfeld, Lauda-Königshofen, Germany).

*Cell culture*

Rat cerebellar granular neuronal cultures were prepared as described previously (Franke et al., 2000). Cells were seeded in minimal essential medium (MEM; Invitrogen) supplemented with 10% horse serum into either 6- or 24-well plates (150,000 cells/cm$^2$; TPP, Trasadingen, Switzerland) coated with poly-L-ornithine (0.1 mg/ml; molecular weight 100-200 kDa; Sigma). Culture medium was changed to serum-free N2-supplemented MEM/F12 (Invitrogen) medium after 24 h. Cells were used for experiments after another 24 h.

*cAMP accumulation assay*

The cAMP assay was performed as described previously (Marsicano et al., 2002) with slight modifications. IBMX was added to cultures 5 min before addition of CRH ($10^{-8}$ M) and/or WIN-55,212-2 ($10^{-6}$ M), to prevent degradation of accumulated cAMP. Cells were incubated for 10 min with the drugs, and reactions were terminated by aspiration of the medium and addition of 1 ml ice-cold 6% trichloroacetic acid followed by incubation overnight at 4°C. DMSO and 2% acetic acid vehicle controls had no effect on cAMP accumulation. Extracts were treated twice with 4 ml diethylether, dried overnight and reconstituted in serum-free culture medium. Intracellular cAMP levels were measured with a competitive protein binding assay (NEN Life Science Products). Data is expressed as percentage of basal cAMP levels. Extracts from 2 wells were pooled and samples were measured in triplicates. Data includes the standard error of mean (SEM).

*Immunoblot analysis*

CREB Western blotting was carried out as described previously (Bayatti et al., 2003). The following antibodies were used: anti-CREB (1:500; Calbiochem, Darmstadt, Germany) and anti-pCREB (1:500; Upsate Biotechnologies, Lake Placid, NY). Optical densities of bands were calculated with Scion software (Scion Corp., Frederick, MA). Phosphorylated proteins levels were normalized to total unphosphorylated levels and depicted as percentage-increase of control with SEM using data pooled from 3 independent experiments.

*Semi-quantitative RT-PCR*

Total RNA was isolated from cultures with peqGOLD RNAPure (Peqlab, Erlangen, Germany) according to manufacturer's instructions. Residual genomic DNA was removed with RNase-free DNase I. 5 µg of RNA was used for Superscript II (BRL Gibco) reverse transcriptase (RT)-mediated synthesis of oligo$(dT)_{12\text{-}18}$-primed (Roche) cDNA. PCR was carried out as follows: 94°C for 1 min; 55°C for hypoxanthineguanine phosphoribosyl transferase (HPRT) or 63°C for BDNF for 1 min; 72°C for 1 min, with a 10 min extension at 72°C during the last cycle. PCR was carried out with 28 and 32 cycles for HPRT and BDNF respectively. Primer sequences: BDNF (Gibbs, 1999): sense 5'-AGC CTC CTC TGC TCT TTC TGC TGG A-3', antisense 5'-CTT TTG TCT ATG CCC CTG CAG CCT T-3';. HPRT: sense 5'-CCT GCT GGA TTA CAT TAA AGC ACT G-3'; antisense 5'-GTC AAG GGC ATA TCC AAC AAA C-3'. Specific PCR products of 297 and 351 bp for BDNF and HPRT, respectively, were amplified. Negative RNA controls ensured a lack of genomic DNA

contamination. Optical densities of PCR bands were measured with the Kodak-1D software. Results were calculated as ratios of optical density of the BDNF vs. HPRT bands, and depicted with standard error of mean (SEM).

*ELISA for BDNF expression*

The Emax$^{TM}$ immunoassay system (Promega, Mannheim, Germany) was used to quantify the levels of BDNF protein in primary neuronal cultures. DMSO and 2% vehicle controls had no effect on BDNF expression. After stimulation, cells were lysed in 137 mM NaCl; 20 mM Tris-HCl, pH 8.0; 1% NP-40; 10% glycerol; 0.5 mM sodium vanadate; protease inhibitor (complete mini tablets, Roche, Basel, Switzerland). Lysates were briefly sonicated and total protein content was measured using the DC protein assay (Bio-Rad, Munich, Germany). Prior to each assay, lysates were acid-treated to increase the detectable amount of free protein in solution by dissociating it from their proforms or receptors (Okragly & Haak-Frendscho, 1997). Maxisorp 96-well plates (Nunc, Neerijse, Belgium) were used for antibody coating, and ELISA was carried out according to manufacturer's instructions. Samples from 3 pooled wells were measured in duplicates and depicted with standard error of mean (SEM).

*Statistical analysis*

Data were analyzed by one way ANOVA using GraphPad software. Significance between groups was further analyzed using the *post hoc* Tukey test. $P<0.05$ was considered as statistically significant.

## RESULTS

*Co-localization of CB1 and CRHR1 in the mouse cerebellum*

The expression of both CB1 and CRHR1 within the same cells must be demonstrated to put forward a putative cross-talk between these two receptor systems. Therefore, double-*in situ* hybridization analysis was carried out in coronal sections of the adult mouse cerebellum. We estimated a 100% co-expression of CB1 and CRHR1 receptors in all layers of granule cells (data not shown). As CB1 positive cells are barely visible in the granule layer due to intense toluidine blue counterstaining, we show two sections of the cerbellum which are hybridized with radioactive labelled riboprobes either for CB1 (Fig. 1A) or CRHR1 (Fig. 1B) and indicate the high expression levels of both receptors in this layer. The molecular layer showed a high density of CB1 signals and low levels of CRHR1 mRNA (Fig. 1A, C). Co-expression could only be detected in few cells (Fig. 1C). Moreover, moderate to high levels of co-expression could be detected in the deep nuclei of the brainstem such as the lateral cerebellar nucleus (Lat) (Fig. 1D), spinal vestibular nucleus, and medial vestibular nucleus (data not shown).

*CRH-mediated increases of BDNF expression are inhibited by CB1 activation*

Semi-quantitative RT-PCR analysis using BDNF-specific primers was carried out with total RNA extracted from cultures of rat cerebellar neurons treated with CRH ($10^{-8}$ M), WIN-55,212-2 ($10^{-6}$ M), or in combination. Relative expression was determined by normalizing to the levels of the housekeeping gene hypoxanthineguanine phosphoribosyl transferase (HPRT) mRNA (Fig. 2A). Treatment of neurons with CRH alone had no effect on levels of BDNF mRNA after 24 hours (data not shown), but resulted in an increase in BDNF mRNA transcripts to 200 ± 40.76% ($P<0.05$ vs. vehicle; n=4) as compared to untreated controls (100%) after 48 hours. Simultaneous treatment with WIN-55,212-2 significantly reduced the stimulatory effect of CRH (CRH and WIN-55,212-2, 128.3 ± 10.61%; $P<0.05$ vs. CRH; n=4). WIN-55,212-2 alone had no observable effects on BDNF expression as compared to controls (Fig. 2A). Using ELISA, it was observed that BDNF protein levels were unchanged after 24 hours stimulation with CRH (data not shown), but significantly increased BDNF protein levels were observed after 48 hours (149.5 ± 13.08%; $P<0.05$ vs. vehicle; n=3) as compared to untreated controls (100%) (Fig. 2B). This increase was inhibited by addition of WIN-55,212-2 (CRH and WIN-55,212-2 86.86 ± 16.17%; $P<0.05$ vs. CRH; n=3). Application of

WIN-55,212-2 alone did not show any significant changes of BDNF protein levels as compared to controls (Fig. 2B).

*Inhibition of CRH-mediated signaling by the CB1 agonist WIN-55,212-2*

As CRH-induced activation of CRHR1 leads to the production of cAMP and CB1 has conversely been reported to inhibit cAMP production, we analyzed the effects of the CB1 agonist WIN-55,212-2 on CRH-induced cAMP accumulation in cultures from postnatal cerebellar granular neurons. While application of CRH ($10^{-8}$ M) for 10 min induced a significant increase in intracellular cAMP levels to 259.6 ± 17.04% ($P<0.05$ vs. vehicle; n=6) as compared to basal levels (100%), simultaneous incubation for 10 min with the CB1 agonist WIN-55,212-2 ($10^{-6}$ M) reduced CRH-mediated cAMP production, but did not reach significant difference as compared to CRH alone (CRH and WIN-55,212-2, 193.6 ± 17.92%; CRH, 259.6 ± 17.04%; $P<0.05$ vs. CRH, n=6). WIN-55,212-2 alone had no effect on intracellular cAMP levels (Fig. 3A).

Increases in intracellular cAMP concentrations lead to the activation of PKA, which in turn promotes the phosphorylation of CREB. In order to monitor interactions between CB1 activation and CRH-mediated signaling, Western blot analysis was carried out using phospho-specific antibodies directed against the activated form of CREB (pCREB). Treatment of cerebellar neurons with CRH ($10^{-8}$ M) for 30 min led to a 469% ± 153% ($P<0.05$ vs. vehicle control; n=3) increase in pCREB levels as compared to untreated controls (100%). However, co-treatment of cell cultures with WIN-55,212-2 ($10^{-6}$ M) for 30 min inhibited CRH-mediated CREB phosphorylation (128% ± 27% of control, n=3; $P<0.05$ vs. CRH; n=3, Fig. 3B).

**DISCUSSION**

Our results demonstrate a functional cross-talk between the CRH and the cannabinoid systems regarding the regulation of CREB activity and BDNF gene expression. *In situ* hybridization experiments on sections from mouse cerebellum showed high levels of co-expression in granular neurons, a necessary prerequisite for any direct cross-talk. CRH was demonstrated to induce elevations in BDNF transcripts and protein, as well as increasing cAMP and phospho-CREB levels. These effects were inhibited by activation of CB1 receptors. This study reveals an important role of CRH in the induction of BDNF expression, and the pivotal role of CB1 receptors in modulating this action.

CRHR1, a seven transmembrane receptor linked to AC through $G\alpha_s$ protein activation, activates PKA, leading to the phosphorylation and, hence, activation of CREB. Additionally, PKA is known to phosphorylate L-type $Ca^{2+}$ channels (Mundina-Weilenmann, et al. 1991). In a manner similar to other seven transmembrane receptors, CRHRs may also activate this channel type resulting in an increased $Ca^{2+}$ influx (Haws et al., 1993). The regulation of BDNF expression by CREB has been studied extensively, and a majority of reports demonstrated a $Ca^{2+}$-dependent mechanism of CREB phosphorylation and induction of BDNF expression (West et al., 2001). Our data therefore indicate that CRH is able to induce BDNF expression by activation of the cAMP signaling cascade, possibly involving an increase of intracellular $Ca^{2+}$ concentration due to an activation of L-type $Ca^{2+}$ channels.

Cannabinoids were found to inhibit N- and P/Q-type voltage-dependent calcium currents in primary cultures of cerebellar granule neurons via pertussis toxin-sensitive G proteins (Nogueron et al, 2001). An inhibition of L-type $Ca^{2+}$ channels upon activation of CB1 with WIN-55,212-2 and the endocannabinoid anandamide has been reported in cat cerebral artery smooth muscle cells (Gebremedhin et al., 1999). Since CREB phosphorylation and BDNF transcription are preferentially driven by calcium influx through L-type $Ca^{2+}$ channels, whereas they are poorly induced by calcium entering through N-methyl-D-aspartate (NMDA) receptors and non-L-type $Ca^{2+}$ channels (Ghosh et al., 1994; Westenbroek et al., 1992), the possible role of L-type $Ca^{2+}$ channels in CB1-mediated inhibition of CRH-induced effects remains to be further investigated.

CB1-mediated inhibition of AC via $G\alpha_{i/o}$ has been observed in CB1 transfected-cell lines (Matsuda et al., 1990), and in rat cerebellar granule cells (Nogueron et al., 2001) and results in the attenuation of PKA activity and a decrease in transcription factor binding to CREs present in target gene promoters (Koh et al., 1997). Additionally, independent of its activation state, CB1 receptors are able to sequester $G\alpha$,-$\beta$, and, -$\gamma$ proteins required by other

receptors linked with pertussis toxin-sensitive $G\alpha_{i/o}$ proteins (Vasquez & Lewis, 1999). In the rat cerebral cortex, CRH receptors can activate $G\alpha_s$, $G\alpha_i$, $G\alpha_q$, and $G\alpha_z$ (Gammatopoulos et al., 2001). Therefore, both inhibition of CRH-mediated cAMP augmentation, as well as sequestration of G proteins required by CRHR1, may be involved in the inhibitory action of CB1 on CRH-mediated signaling and induction of BDNF expression.

Endocannabinoids and $\Delta^9$-tetrahydrocannabinol have been demonstrated to activate extracellular signal-regulated kinase in hippocampal slices and BDNF expression *in vivo* (Derkinderen et al., 2003). These effects were dependent on NMDA receptor activation *in vivo,* but not in hippocampal slices, suggesting that multiple pathways lead to the initiation of CB1-mediated signaling pathways. In the present study, CB1 activation with the agonist WIN-55,212-2 did not result in an increase in BDNF expression in cultured cerebellar granule neurons. This may be due to region-specific coupling of signaling pathways, as it has been demonstrated for CRH (Bayatti et al., 2003), or due to differences in cellular subtype specificities.

As we were able to show CRH-induced BDNF expression after 48 hours, changes in synaptic plasticity might be the consequence of this altered gene expression. Deprivation of BDNF leads to an impairment of long-term potentiation (LTP), indicating that BDNF is essential for certain forms of learning and memory (Korte et al., 1995). CRH is also involved in learning processes, acting in a CRHR1-dependent manner. For example, CRH has been demonstrated to promote hippocampus-dependent learning processes in an immobilization stress model when injected prior to training (Radulovic et al., 1999). Considering that mRNA levels of neurotrophins, including BDNF, are reduced in the brain after immobilization stress (Ueyama et al., 1997), a potential role emerges for CRH in regulating neurotrophin levels under these circumstances.

This study highlights an important role of the stress response peptide CRH in regulating BDNF expression and a counter-regulatory role of CB1 activation. Further studies should therefore concentrate on the molecular and intracellular role of CRH in memory processes with particular attention to the regulation of expression of neurotrophins, as well as the possible interplay with other neurotransmitter systems.

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**FIGURE LEGENDS**

Figure 1

Bright and dark field micrographs of coronal cerebellar sections showing examples of co-expression of CB1 with CRHR1 as detected by single- or double-*in situ* hybridization, respectively. All sections were counterstained with toluidine blue. Expression of (A) CB1 and (B) CRHR1 in the cerebellum as detected with $^{35}$S-labelled riboprobes. (C) Co-expression of CB1 (red staining) and CRHR1 (silver grains) in the molecular layer. (D) Coexpression of CB1 (red staining) and CRHR1 (silver grains) in the lateral cerebellar nucleus nucleus. Filled arrow, CB1-expressing cell that coexpresses CRHR1; filled arrowhead, CB1-expressing cell; open arrowhead, CRHR1-expressing cell. Scale bars (A) and (B) 1 mm; (C) and (D) 200 μm. Abbrevations ML, molecular layer; GL, granular layer; Lat, lateral cerebellar nucleus.

Figure 2

Effects of WIN-55,212-2 on CRH-induced BDNF expression in cerebellar granular neurons. (A) Semi-quantitative RT-PCR for BDNF, using HPRT as an internal standard (one representative gel is shown). Neurons were treated with CRH ($10^{-8}$ M) and/or WIN-55,212-2 ($10^{-6}$ M) as indicated for 48 h. Results were calculated as ratios of optical density of the BDNF band vs. the HPRT band and expressed as the mean ± S.E.M. of the percentage of control (considered as 100%). *, $P<0.05$ vs. vehicle; #, $P<0.05$ vs. CRH (n=4). (B) ELISA for BDNF; Neurons were treated with CRH ($10^{-8}$ M) and/or WIN-55,212-2 ($10^{-6}$ M) as indicated for 48 h. Samples were measured in duplicates and data were expressed as the mean ± S.E.M. of the percentage of basal BDNF levels (considered as 100%). *, $P<0.05$ vs. vehicle; #, $P<0.05$ vs. CRH (n=3).

Figure 3

Modulation of CRH-induced signaling by WIN-55,212-2 in cerebellar granular neurons. (A) cAMP accumulation assays with neurons treated for 10 min with CRH ($10^{-8}$ M) and/or WIN-55,212-2 ($10^{-6}$ M) as indicated. Samples were measured in triplicates, and data is expressed as the mean ± S.E.M. of the percentage of basal cAMP levels (considered as 100%). *, $P<0.05$ vs. vehicle (n=6). (B) Western blot analyzing the phosphorylation status of CREB (one representative blot is shown). Neurons were treated for 30 min with CRH ($10^{-8}$ M) and/or WIN-55,212-2 ($10^{-6}$ M) as indicated. Phosphorylated proteins levels were normalized to total unphosphorylated levels and depicted as percentage-increase ± SEM of vehicle controls (considered as 100%). *, $P<0.05$ vs. control (n=3).

# Figure 1

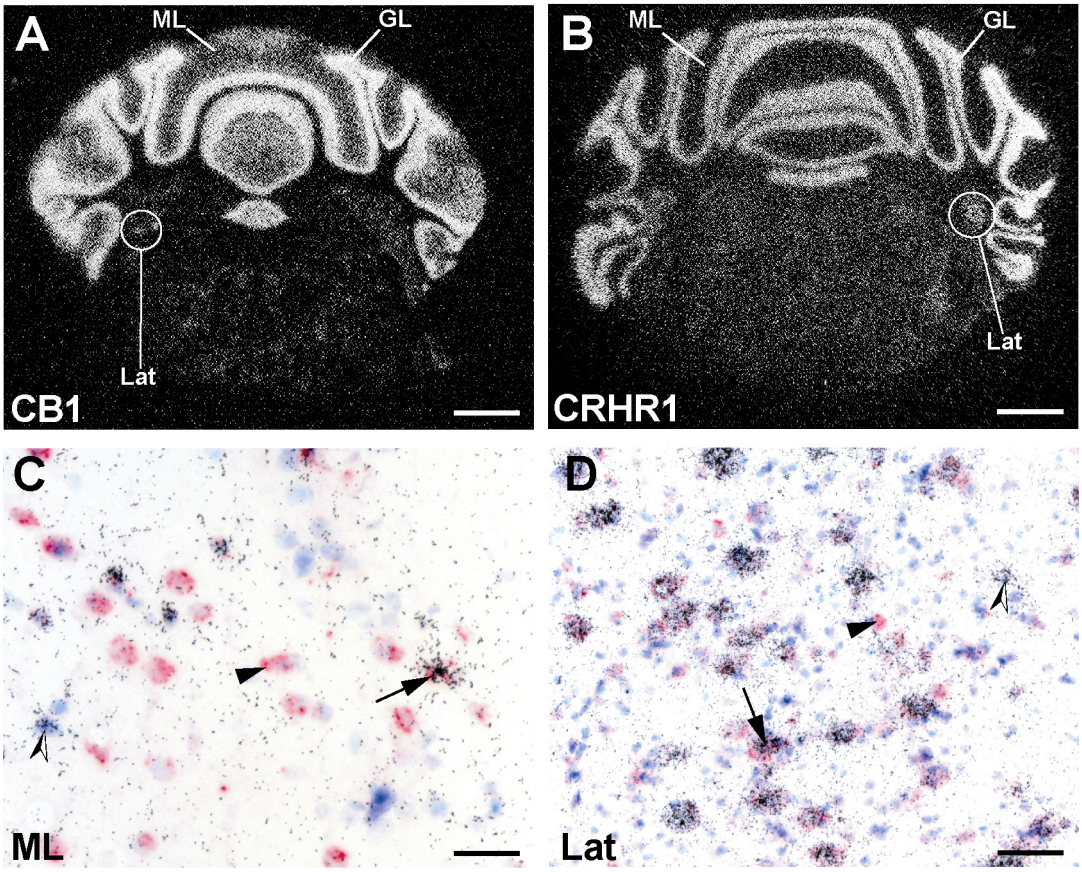
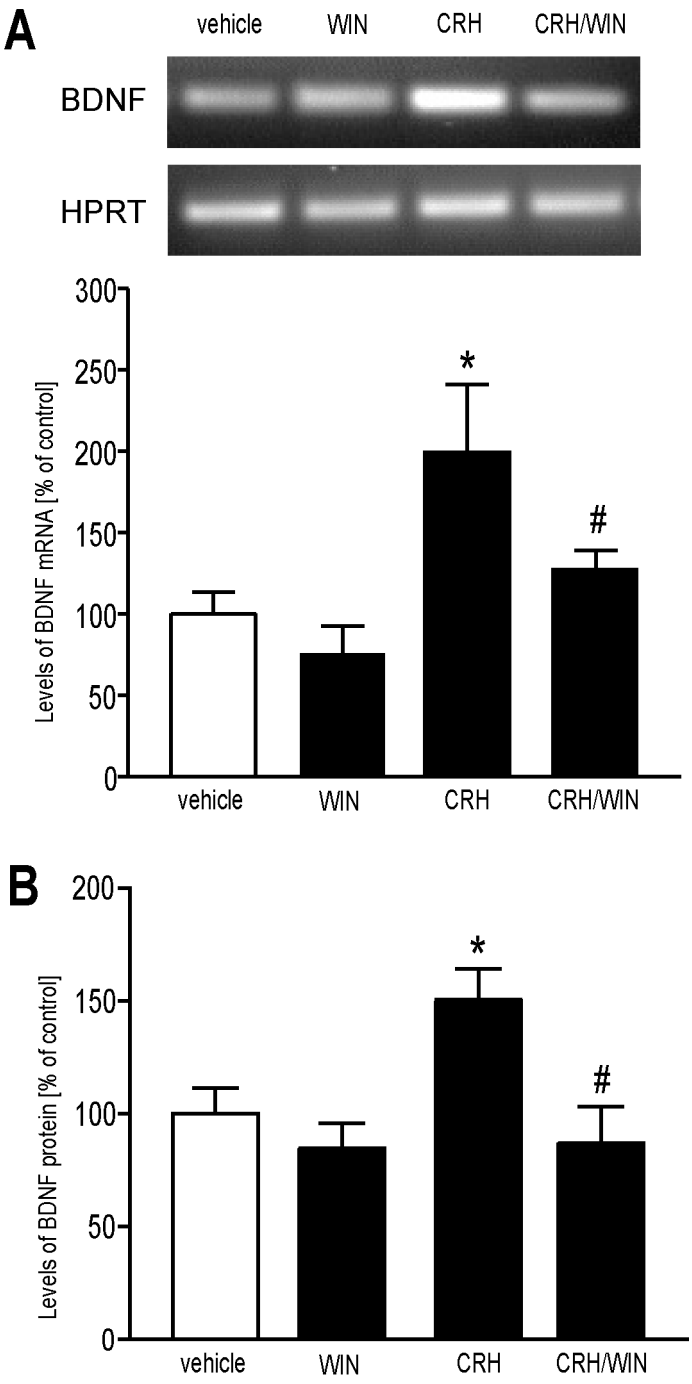


**Figure 2**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vehicle
WIN
CRH
CRH/WIN
BDNF
HPRT
Levels of BDNF mRNA [% of control]
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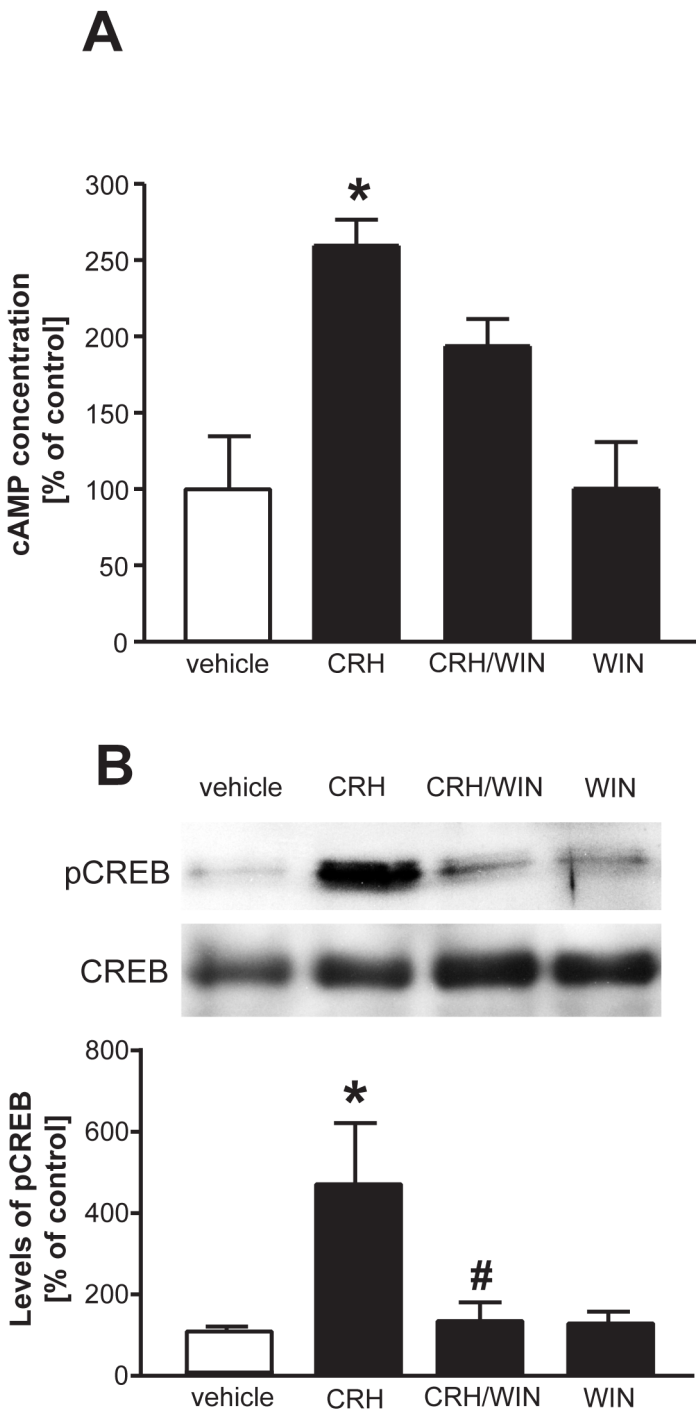
250
200
150
100
50
0
*
#
vehicle
WIN
CRH
CRH/WIN
B
Levels of BDNF protein [% of control]
200
150
100
50
0
*
#
vehicle
WIN
CRH
CRH/WIN

**Figure 3**


A
cAMP concentration [% of control]
300
250
200
150
100
50
0
*
vehicle
CRH
CRH/WIN
WIN
B
vehicle
CRH
CRH/WIN
WIN
pCREB
CREB
Levels of pCREB [% of control]
800
600
400
200
0
*
#
vehicle
CRH
CRH/WIN
WIN

G. Marsicano, S. Goodenough, K. Monory, H. Hermann, M. Eder, A. Cannich, S.C. Azad, M.G. Cascio, S.O. Gutierrez, M. van der Stelt, M.L. Lopez-Rodriguez, E. Casanova, G. Schütz, W. Zieglgänsberger, V. Di Marzo, C. Behl, and B. Lutz

# CB1 Cannabinoid Receptors and On-Demand Defense Against Excitotoxicity

*Science (2003); 302, 84-88*

it was also responsible for the instability in *dw3*.

Because direct duplications are apt to undergo unequal crossing-over (*18*), could this be the mechanism by which *dw3* reverts back to *Dw3*? One way of answering this question is by identifying one or more recombinants that contain at least three copies of the duplicated region. To find such a recombinant, DNA from another 200 dwarf plants was subjected to Southern analysis (*6*). We identified a single plant that displayed a restriction pattern indicative of three copies of the duplicated region (Fig. 4E). Subsequent cloning and sequencing of this restriction fragment confirmed its triplicate nature, thereby demonstrating that *dw3* reverts back to *Dw3* by unequal crossing-over.

Interestingly, a dwarf plant with a restriction band diagnostic of wild-type revertants was also found among these 200 plants (Fig. 4E). PCR amplification and subsequent sequencing of its product indicated that unequal recombination had removed the duplicated part of the gene but introduced a number of simple nucleotide changes in the copy that was left behind (fig. S4). These changes disrupted the reading frame of DW3 and also truncated the protein by about 200 amino acids, thereby explaining the mutant nature of this new allele. Because this allele, designated *dw3-sd1*, lacks the duplication, it is expected to confer a stable mutant phenotype. This was determined by generating progeny that were homozygous for the *dw3-sd1* allele. We screened more than 2400 such plants in the field and found that none reverted back to the tall type, confirming the stable dwarf nature of this mutant derivative. To determine whether imprecise recombination at *dw3* was common enough to be practically useful, we analyzed another 500 dwarf plants by PCR (*6*). One plant was identified that yielded a product indicative of a loss of duplication. Its sequence revealed that it had undergone mutational changes similar to that of *dw3-sd1* (*19*).

**Concluding remarks.** These findings not only resolve a long-standing puzzle in sorghum genetics and breeding but also provide a simple strategy for effectively correcting *dw3* in the sorghum germplasm. Moreover, new mutant alleles of sorghum *dw3* or of corresponding genes in other cereals may be generated by conventional mutagenesis approaches. There is also the prospect of inciting a renewed interest in this locus for maize breeding by generating new and improved alleles of *br2*. A key advantage of the dwarfing mechanism described here is its synergistic effect on stalk quality, a trait considered to be of utmost importance for enhancing crop yields beyond those that have already been achieved (*1*).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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/302/5642/81/DC1
Materials and Methods
Figs. S1 to S4
References and Notes


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# CB1 Cannabinoid Receptors and On-Demand Defense Against Excitotoxicity


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**Abnormally high spiking activity can damage neurons. Signaling systems to protect neurons from the consequences of abnormal discharge activity have been postulated. We generated conditional mutant mice that lack expression of the cannabinoid receptor type 1 in principal forebrain neurons but not in adjacent inhibitory interneurons. In mutant mice, the excitotoxin kainic acid (KA) induced excessive seizures in vivo. The threshold to KA-induced neuronal excitation in vitro was severely reduced in hippocampal pyramidal neurons of mutants. KA administration rapidly raised hippocampal levels of anandamide and induced protective mechanisms in wild-type principal hippocampal neurons. These protective mechanisms could not be triggered in mutant mice. The endogenous cannabinoid system thus provides on-demand protection against acute excitotoxicity in central nervous system neurons.**


Mnemonic processes and normal functioning of the brain require elevated neuronal activity. However, neuronal systems need to protect themselves against the risk of excessive activity, which could lead to pathological processes known as excitotoxicity (*1*). Therefore, it is conceivable that protective signaling systems exist that are able to provide on-demand defense in case of abnormally high spiking activity. The endogenous cannabinoid system in the brain is a neuromodulatory system comprising the cannabinoid receptor type 1 (CB1), its endogenous ligands (endocannabinoids), and the machinery for their synthesis and degradation (*2*, *3*). Exogenous natural and synthetic cannabinoids have been shown to exert neuroprotective functions in several models of neurotoxicity (*4*–*7*), and neuronal depolarization increases the production of endocannabinoids (*2*–*4*, *8*). However, the involvement of the endogenous cannabinoid system in physiological protection against the consequences of excessive


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neuronal activity is still a matter of debate (*4*), and even CB1 receptor–mediated neurotoxic effects have been reported (*9*–*11*).

**CB1 receptors and KA-induced seizures.** To test the role of the endogenous cannabinoid system in the control of excessive neuronal activity in the brain, we first compared CB1-null mutant mice ($CB1^{-/-}$) (*12*) and their $CB1^{+/+}$ control littermates in the kainic acid (KA) model of excitotoxic epileptiform seizures (*1*, *13*). In this model, the hippocampus appears as the brain region most susceptible to KA-induced effects (*1*). Injection of KA (30 mg/kg) into $CB1^{-/-}$ mice induced clearly more severe seizures than injection into $CB1^{+/+}$ littermates (genotype: $F_{1,13} = 8.8, P < 0.05$) (*13*) (Fig. 1A), and more than 75% of $CB1^{-/-}$ mice died within 1 hour after KA injection (fig. S1A). At lower doses of KA, the death rate was still significantly higher (fig. S1A) and behavioral responses were more pronounced (fig. S1B) in $CB1^{-/-}$ than in $CB1^{+/+}$ and $CB1^{+/-}$ mice (15 mg/kg, genotype: $F_{2,15} = 4.3$, $P < 0.05$; 20 mg/kg, genotype: $F_{2,15} = 4.0$, $P < 0.05$), indicating that genetic ablation of the CB1 receptor lowers the threshold for KA-induced seizures.

If CB1 receptor activation is involved in endogenous protection against KA-induced excitotoxicity, administration of KA should induce a rapid increase in the production of endocannabinoids for CB1 receptors. We therefore measured the levels of endocannabinoids in the hippocampi of wild-type mice from the C57BL/6N line, isolated at different time points after KA treatment (30 mg/kg) (*13*). Whereas the levels of the endocannabinoid 2-arachidonoyl-glycerol and of palmitoyl-ethanolamide (an endocannabinoid-related compound) remained unaltered at any time point analyzed (*14*), the tissue concentrations of anandamide (arachidonoyl-ethanolamide) markedly increased, peaked 20 min after KA injection, and returned to basal levels within 1 hour (Fig. 1B). These findings suggest a specific involvement of the endogenous cannabinoid system in acute protection against excitotoxicity induced by KA.

To substantiate the relationship between elevated levels of anandamide and activation of CB1 receptors, we tested the acute requirement of CB1 receptor activation by treating wild-type C57BL/6N mice with the specific CB1 receptor antagonist SR141716A (3 mg/kg) 30 min before KA injection (20 mg/kg) (*13*). SR141716A-treated mice experienced more severe seizures than vehicle-treated mice (treatment: $F_{1,10} = 5.0, P < 0.05$) (Fig. 1C). This effect of the antagonist was significantly more pronounced when heterozygous CB1-null ($CB1^{+/-}$) mutants, known to possess about half the density of CB1 receptors in the hippocampus (*15*), were treated with the same dose of the antagonist (treatment in $CB1^{+/-}$ mice: $F_{1,8} = 8.5, P < 0.05$; comparison C57BL/6N mice versus $CB1^{+/-}$ mice: behavioral scores of C57BL/6N: $2.9 \pm 0.5$ and of $CB1^{+/-}$: $5.2 \pm 1.1$, $P < 0.05$) (Fig. 1C). Consistently, preadministration of the selective and potent inhibitor of endocannabinoid uptake UCM707 (*16*) (3 mg/kg) significantly protected C57BL/6N mice against KA-induced seizures (35 mg/kg; treatment: $F_{1,21} = 4.8$, $P < 0.05$) (Fig. 1D), indicating that the endogenous cannabinoid system provides on-demand protection.

**Role of forebrain principal neurons.** In cortical areas, the CB1 receptor is highly expressed in interneurons that contain γ-aminobutyric acid (GABAergic interneurons) (*17*, *18*), but evidence exists for its presence also in principal neurons of, for example, the hippocampus (*17*, *19*). Thus, we generated a mouse line in which the CB1 coding region is flanked by two *loxP* sites (CB1-floxed mice, $CB1^{f/f}$) (Fig. 2A). By crossing this mouse line with mice that express Cre recombinase under the control of the regulatory sequences of the *$Ca^{2+}$/calmodulin-dependent kinase IIα* gene ($CB1^{CaMKII\alpha Cre}$ mice) (*20*), we obtained $CB1^{f/f;CaMKII\alpha Cre}$ mice (*13*) in which the CB1 receptor is deleted in all principal neurons of the forebrain but maintains its expression in cortical GABAergic interneurons (including those in the hippocampus) (Fig. 2, B to E) and in cerebellar neurons (*14*). Injection of 30 mg/kg of KA induced clearly more severe seizures in $CB1^{f/f;CaMKII\alpha Cre}$ mice than in $CB1^{f/f}$ littermates (genotype: $F_{1,16} = 14.9, P < 0.01$) (Fig. 2F) and decreased their survival rate ($P < 0.01$) (fig. S2A). Mice expressing only the transgenic Cre protein ($CB1^{CaMKII\alpha Cre}$ mice) and their wild-type littermates did not show any differences between genotypes after injection of 30 mg/kg of KA (genotype: $F_{1,18} = 0.7, P = 0.4$), thus precluding the expression of Cre recombinase as the cause of the phenotype in $CB1^{f/f;CaMKII\alpha Cre}$ mice. A comparison of behavioral scores of $CB1^{-/-}$ and $CB1^{f/f;CaMKII\alpha Cre}$ mice, and of their respective littermate controls, revealed that the development of seizures did not differ between the CB1-null mutants and the conditional CB1 knockouts (fig. S2B). Moreover, pretreatment with 3 mg/kg of UCM707 significantly protected $CB1^{f/f}$ mice against seizures induced by 30 mg/kg of KA. However, the same treatment was ineffective in $CB1^{f/f;CaMKII\alpha Cre}$ littermates (genotype and treatment: $F_{3,28} = 14.0, P < 0.001$; comparison $CB1^{f/f}$-vehicle versus $CB1^{f/f}$-UCM707, $P < 0.05$; comparison $CB1^{f/f;CaMKII\alpha Cre}$-vehicle versus $CB1^{f/f;CaMKII\alpha Cre}$-UCM707, $P = 0.95$) (Fig. 2G), thus indicating that the effects of the drug are specifically mediated by CB1 receptors on glutamatergic neurons. In addition, the blockade of CB1 receptors by treatment with 3 mg/kg of SR141716A was without any effect on seizures induced by 20 mg/kg of KA in $CB1^{f/f;CaMKII\alpha Cre}$ mice (Fig. 2H). Thus, GABAergic interneurons endowed with CB1 receptors apparently do not confer substantial protection against KA-induced acute excitotoxicity. We therefore suggest that the endogenous cannabinoid system exerts its neuroprotective action through CB1 receptors on principal glutamatergic neurons.

**Dampening of KA-induced excitation.** Injection of KA activates the endogenous cannabinoid system, which, in turn, protects neurons from the excitotoxic effects of this drug through the activation of CB1 receptors. How does CB1 receptor activation reduce excitotoxicity? Exogenously applied cannabinoids most commonly decrease neuronal excitability and inhibit glutamatergic transmission (*2*–*4*). It is thus conceivable to assume that an endogenously released ligand of the CB1 receptor, such as anandamide, might prevent excitotoxicity by a CB1 receptor–mediated inhibition of glutamatergic transmission. To test this hypothesis, we gauged glutamatergic excitation of CA1 pyramidal neurons in an in vitro hippocampal slice prep-

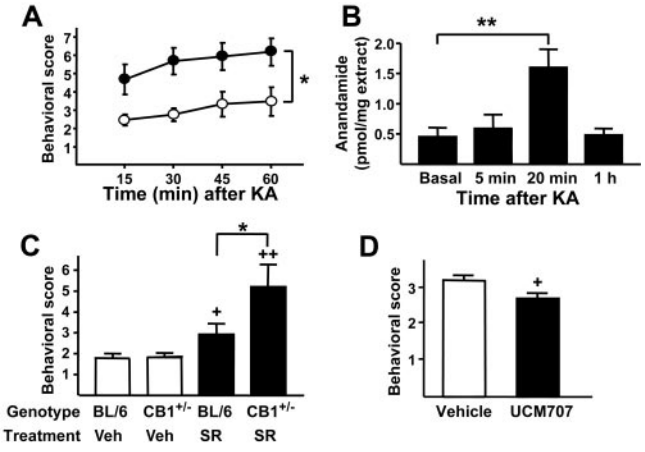


**Fig. 1.** The endogenous cannabinoid system is activated by KA and protects against seizures. (**A**) Seizure scoring (30 mg/kg of KA) of $CB1^{+/+}$ mice (open circles, $n = 7$) and $CB1^{-/-}$ mice (solid circles, $n = 8$). Higher scores indicate more severe seizures. (**B**) Levels of hippocampal anandamide at different time points after KA injection into C57BL/6N mice (30 mg/kg, $n = 5$ mice per group). (**C**) Effects of the CB1 receptor antagonist SR141716A (SR, solid bars) and the vehicle (Veh, open bars) on seizure scoring (20 mg/kg of KA) in C57BL/6N mice (BL/6, $n = 6$ per group) and in $CB1^{+/-}$ mice ($n = 6$ per group). (**D**) Effects of the anandamide uptake inhibitor UCM707 (solid bar) and the vehicle (open bar) on seizure scoring in C57BL/6N mice (35 mg/kg of KA, $n = 23$ to 24 per group). Means ± SEM; *, $P < 0.05$; **, $P < 0.01$; +, $P < 0.05$; ++, $P < 0.01$ versus respective vehicle-treated groups.

aration from CB1$^{f/f;CaMKII\alpha Cre}$ and CB1$^{f/f}$ littermates before (Fig. 3A) and after (Fig. 3B) bath application of 150 nM KA (*13*). At this concentration, KA did not significantly change the excitation of neurons obtained from CB1$^{f/f}$ mice. We monitored neuronal excitation as the spontaneous excitatory postsynaptic currents (EPSCs, relative excitation: 4 ± 2, $P > 0.05$, versus the baseline) (Fig. 3C). In contrast, neurons obtained from CB1$^{f/f;CaMKII\alpha Cre}$ mice showed strong excitation under these conditions (relative excitation: 17 ± 4, $P < 0.05$, versus the baseline) (Fig. 3C), which was accompanied by an increase in the frequency of EPSCs (frequency: 4.5 ± 0.5 Hz versus a baseline of 1.0 ± 0.1 Hz, $P < 0.01$).

**KA-induced intracellular events.** Several intracellular pathways have been implicated in the development of KA-induced excitotoxicity (*21*). In the hippocampus, injection of KA activates various kinases, including extracellular-regulated kinases (ERKs) (*21*), at different time points. Because CB1 receptor agonists stimulate the phosphorylation of ERKs (*2*), we isolated hippocampi derived from CB1$^{f/f;CaMKII\alpha Cre}$ and CB1$^{f/f}$ littermates 75 min after injection of KA (15 mg/kg) or saline, then quantified the levels of CB1 receptor–mediated activation of ERKs by Western blotting (*13*). Administration of KA induced a significant increase in phosphorylation of both p42 (phospho-p42) and p44 (phospho-p44) ERKs in CB1$^{f/f}$ mice (phospho-p42: to 173.0 ± 21.2%, $P < 0.05$; phospho-p44: to 220.1 ± 36.1%, $P < 0.01$) (Fig. 4, A and B), whereas there was no significant difference between KA- and saline-treated CB1$^{f/f;CaMKII\alpha Cre}$ mice (phospho-p42: to 101.1 ± 9.8%, $P > 0.05$; phospho-p44: to 144.0 ± 36.9%, $P > 0.05$) (Fig. 4, A and B).

KA administration rapidly induces expression of immediate early genes (IEGs) such as c-*fos* or *zif268* (*22*). This induction depends, at least in part, on the activation of ERKs (*23*). In particular, the activation of the c-*fos* gene plays a central role in protection against KA-induced excitotoxicity (*24*). Because the pharmacological stimulation of CB1 receptors induces the expression of these IEGs (*2*, *25*), we analyzed by in situ hybridization (*13*) the levels of c-*fos* and *zif268* transcripts in hippocampi from CB1$^{f/f;CaMKII\alpha Cre}$ and CB1$^{f/f}$ littermates 75 min after KA or saline injection. In saline-injected mice, the hippocampal levels of c-*fos* (Fig. 4, C, E, and O) and *zif268* transcripts (Fig. 4, G, I, and P) were similar between genotypes. However, all subregions of the hippocampi derived from KA-treated CB1$^{f/f}$ mice showed markedly increased levels of both c-*fos* (Fig. 4, D and O) and *zif268* transcripts (Fig. 4, H and P). In the hippocampi derived from KA-treated CB1$^{f/f;CaMKII\alpha Cre}$ mice, the induction of

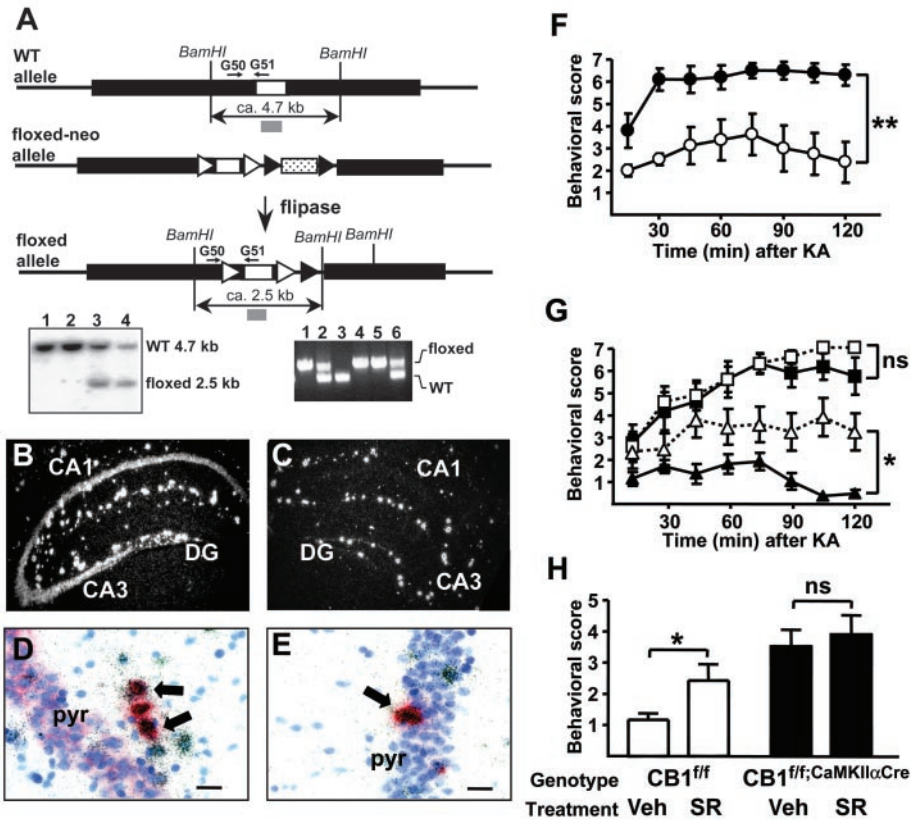


**Fig. 2.** Activation of CB1 receptors on principal forebrain neurons mediates protection from seizures. (**A**) Generation of the CB1$^{f/f}$ mouse line. Open box, the *CB1* open reading frame; dotted box, the phosphoglycerate kinase–neomycin phosphotransferase (*PGK-Neo*) selection cassette; open triangles, *loxP* sites; solid triangles, FLP recombinase recognition target (*FRT*) sites; gray box, the probe for Southern blot analysis; small arrows, primers for polymerase chain reaction (PCR) genotyping. Bottom left: Southern blot analysis showing CB1$^{+/+}$ mice (lanes 1 and 2) and CB1$^{f/+}$ mice (lines 3 and 4) obtained after FLP recombinase–mediated excision of *PGK-Neo* cassette. Bottom right: PCR analysis of CB1$^{f/f}$ (lanes 1, 4, and 5), CB1$^{f/+}$ (lanes 2 and 6), and CB1$^{+/+}$ (lane 3) mice. WT, wild-type; BamHI, endonuclease recognition site; G50 and G51, PCR primers (*12*, *13*); flipase, FLP recombinase. (**B** and **C**) Expression of *CB1* mRNA (dark-field) in hippocampi from (B) CB1$^{f/f}$ and (C) CB1$^{f/f;CaMKII\alpha Cre}$ mice. The CA1, CA3, and DG regions of the hippocampus are marked. (**D** and **E**) Expression of *CB1* mRNA (red staining), in combination with the GABAergic-specific marker *GAD65* (silver grains) in the CA3 region of the hippocampus in (D) CB1$^{f/f}$ and (E) CB1$^{f/f;CaMKII\alpha Cre}$ mice. *CB1* mRNA is present in pyramidal neurons in CB1$^{f/f}$ but not in CB1$^{f/f;CaMKII\alpha Cre}$ mice. Pyr, the CA3 pyramidal layer; arrows, interneurons co-expressing *CB1* and *GAD65*; blue stain, toluidine-blue counterstaining. Scale bars, 20 μm. (**F**) Seizure scoring (30 mg/kg of KA) of CB1$^{f/f}$ mice (open circles, $n = 8$) and CB1$^{f/f;CaMKII\alpha Cre}$ mice (solid circles, $n = 10$). (**G**) Effects of the anandamide uptake inhibitor UCM707 (3 mg/kg, solid symbols) and the vehicle (open symbols) on seizure scoring (30 mg/kg of KA) of CB1$^{f/f}$ mice (triangles, $n = 9$ per group) and CB1$^{f/f;CaMKII\alpha Cre}$ mice (squares, $n = 7$ per group). (**H**) Effects of the CB1 receptor antagonist SR141716A (3 mg/kg) on seizure scoring (20 mg/kg of KA) of CB1$^{f/f}$ mice (open bars, $n = 12$ to 14 per group) and of CB1$^{f/f;CaMKII\alpha Cre}$ mice (solid bars, $n = 11$ per group). Means ± SEM; *, $P < 0.05$; **, $P < 0.01$; ns, not significant.

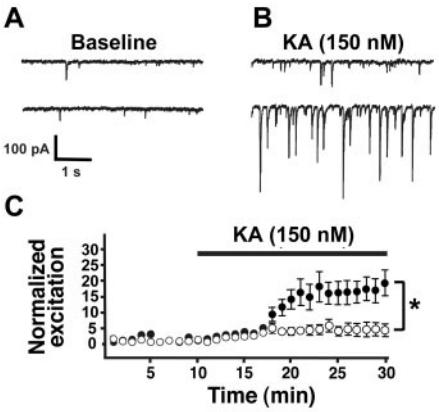


**Fig. 3**. On-demand activation of the endogenous cannabinoid system dampens KA-induced excitation of CA1 hippocampal pyramidal neurons. (**A**) Representative traces of CB1$^{f/f}$ (upper) and CB1$^{f/f;CaMKII\alpha Cre}$ (lower) neurons, before KA application. (**B**) Representative traces of the same neurons 20 min after KA application. (**C**) Normalized excitation values over the course of the experiments. Open circles, CB1$^{f/f}$ (7 cells from 2 mice); solid circles, CB1$^{f/f;CaMKII\alpha Cre}$ (6 cells from 2 mice). Bar represents duration of bath application of KA. Means ± SEM; *, $P < 0.05$.

c-*fos* (Fig. 4, F and O) and *zif268* expression (Fig. 4, J and P) was abolished.

The brain-derived neurotrophic factor (BDNF) exerts neuroprotective functions (*26*, *27*) and participates in c-*fos*–dependent neuronal protection against KA-induced excitotoxicity (*24*). We measured *BDNF* messenger RNA (mRNA) levels by in situ hybridization in the hippocampi of the same mice used for the analysis of c-*fos* and *zif268* expression (*13*). In saline-treated mice, *BDNF* mRNA was expressed at moderate levels in all subregions of the hippocampus (Fig. 4, K, M, and Q). Slightly but significantly lower levels of *BDNF* were observed in the CA3 region of $CB1^{f/f;CaMKII\alpha Cre}$ mice, possibly indicating a role of CB1 receptors in the basal control of *BDNF* expression (Fig. 4Q). In KA-treated $CB1^{f/f}$ mice, *BDNF* expression was strongly enhanced compared to that of saline-treated littermates in all hippocampal subregions (Fig. 4, L and Q). However, as with c-*fos* and *zif268*, no increase of *BDNF* expression was observed in KA-treated $CB1^{f/f;CaMKII\alpha Cre}$ mice as compared to saline-treated controls (Fig. 4, N and Q).

**Long-term effects.** Excitotoxic stimuli lead to neuronal cell death through the activation of several molecular pathways (*28*). To test the involvement of the endogenous cannabinoid system in protection against the long-term effects of KA, surviving $CB1^{f/f}$ and $CB1^{f/f;CaMKII\alpha Cre}$ mice were killed 4 days after the injection of 20 mg/kg of KA. The degree of neuronal damage in their hippocampi was evaluated by staining with terminal deoxynucleotide transferase–mediated deoxyuridine triphosphate nick end labeling (TUNEL) (*13*). KA-treated $CB1^{f/f;CaMKII\alpha Cre}$ mice showed significantly higher levels of TUNEL staining in the CA1 and CA3 regions of the hippocampus ($P < 0.05$) (fig. S3, A to C), indicating higher levels of neuronal damage. Immunostaining for glial fibrillary acidic protein (*13*) in the same hippocampi revealed increased levels of gliosis in KA-treated mutants ($P < 0.05$) (fig. S3, D to F).

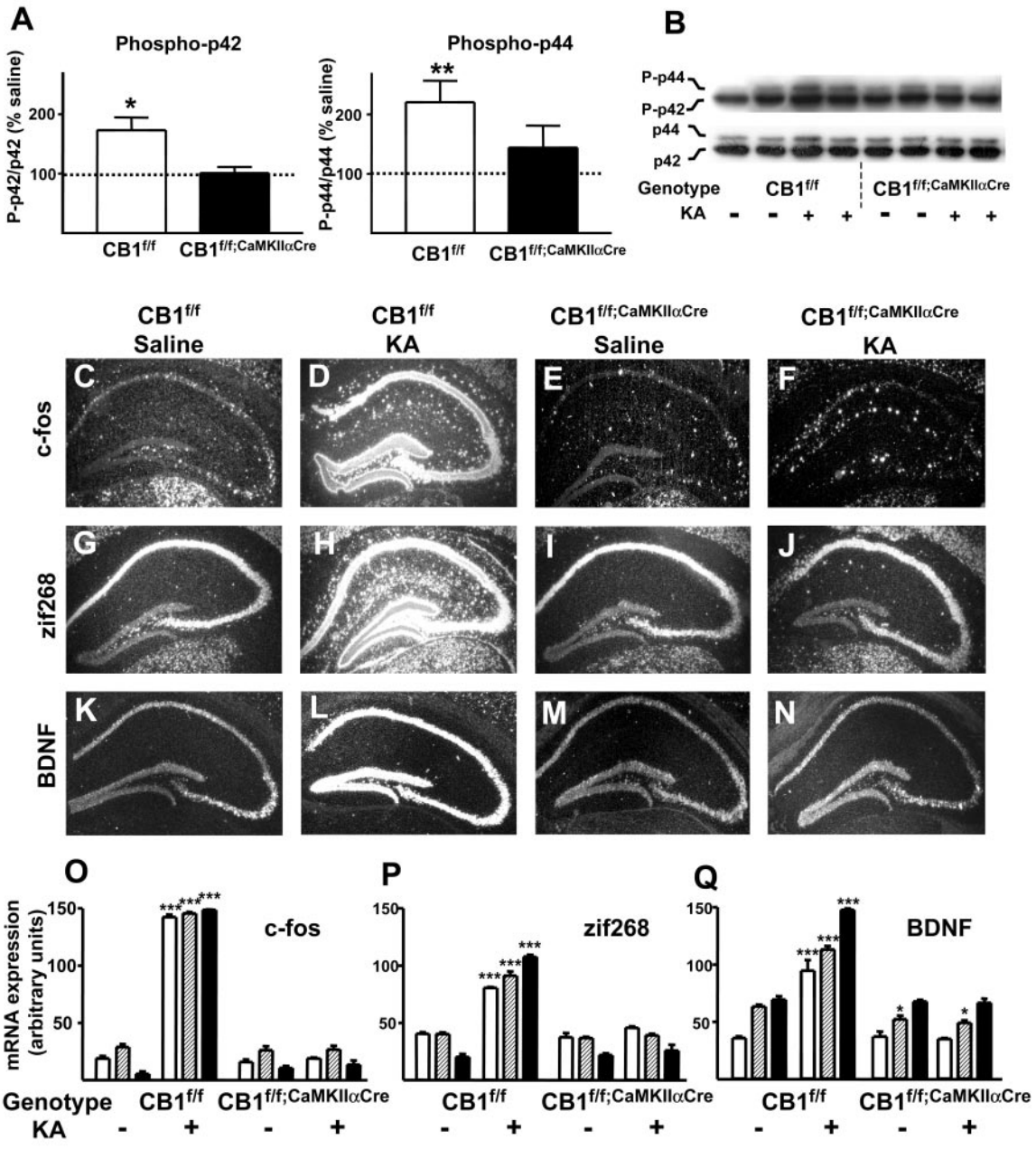


**Fig. 4.** On-demand activation of the endogenous cannabinoid system in principal hippocampal neurons is required to induce protective molecular cascades. (**A**) Densitometric quantification of KA-induced ERK phosphorylation in $CB1^{f/f}$ (open bars) and $CB1^{f/f;CaMKII\alpha Cre}$ (solid bars) mice, relative to saline-treated littermates (100%, dotted lines); *, $P < 0.05$; **, $P < 0.01$ versus respective controls; $n = 5$ to 6 mice per group. P-p42 and P-p44, phospho-p42 and phospho-p44. (**B**) Representative Western blots of phosphorylated ERKs (P-p42 and P-p44) and total ERKs (p42 and p44). (**C** to **N**) Representative dark-field micrographs showing expression of [(C) to (F)] c-*fos*, [(G) to (J)] *zif268*, and [(K) to (N)] *BDNF* mRNA in $CB1^{f/f}$ and $CB1^{f/f;CaMKII\alpha Cre}$ mice, 75 min after injection of KA (15 mg/kg) or saline. The dark halos in (D) and (H) are artifacts due to the excessive presence of silver grains. (**O** to **Q**) Densitometric quantification from autoradiographic films for mRNA expression of (O) c-*fos*, (P) *zif268*, and (Q) *BDNF* in the CA1 (open bars), CA3 (hatched bars), and DG (solid bars) regions of the hippocampus ($n = 5$ to 6 mice per group). Means ± SEM; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ versus saline-treated $CB1^{f/f}$.

**Discussion.** Taken together, these results show that endogenous activation of CB1 receptors in principal forebrain neurons promotes neuronal survival during excitotoxicity. Activation of CB1 receptors on principal forebrain neurons mediates the prominent protective role, whereas CB1 receptors on GABAergic interneurons exert only a negligible function. Considering that in other behavioral paradigms, CB1 receptors on GABAergic interneurons have been proposed to play a crucial role (*2*, *12*, *18*), our data further underline the diverse functions of the endogenous cannabinoid system in different neuronal processes.

Anandamide levels rapidly increase after KA administration and protect against excitotoxicity. The mechanisms inducing this rise in anandamide levels in the adult mouse brain are still to be determined, but they are more likely to rely on enhanced production and/or decreased degradation of this endocannabinoid than on enhanced synthesis of its biosynthetic precursors (*29*).

Cell-type specificity and dynamic regulation appear to be fundamental features of this highly efficient physiological protection system. It has been reported that pharmacological treatment of mice with CB1 receptor agonists and genetic enhancement of endocannabinoid tissue concentrations can increase susceptibility to KA-induced seizures (*10*). Some of these findings may be attributed to the lack of spatial and temporal specificity of CB1 receptor activation (i.e., CB1 receptors on both GABAergic and glutamatergic neurons are probably activated simultaneously by pharmacological application of agonists or by genetic enhancement of anandamide levels). We were able to observe significant protection induced by the anandamide uptake inhibitor UCM707 in wild-type animals but not in $CB1^{f/f;CaMKII\alpha Cre}$ mice, indicating that an enhancement of anandamide concentration at sites of synthesis is pivotal for physiological protection. The increased ability of KA to induce neuronal excitation mediated by spontaneous EPSCs in $CB1^{f/f;CaMKII\alpha Cre}$ hippocampal slices indicates a presumable CB1 receptor–mediated control of the presynaptic release of L-glutamate. CB1 receptor activation is known to induce hyperpolarization of neuronal membranes, mainly by increasing $K^+$ and decreasing $Ca^{2+}$ conductance (*2*). Such a hyperpolarization, caused by an autocrine or paracrine activation of CB1 receptors by endocannabinoids (presumably anandamide), would also decrease the L-glutamate release evoked during excitotoxicity, as indicated by the higher frequency of EPSCs in $CB1^{f/f;CaMKII\alpha Cre}$ hippocampal principal neurons. Previous immunohistochemical experiments in rodent hippocampus could not detect CB1 protein associated with glutamatergic synapses (*18*). Thus, it remains to be clarified in which compartment of the projecting neurons the endogenous cannabinoid system acts. An additional postsynaptic site of action of the endocannabinoid system cannot be excluded.

CB1 receptors mediate protection against excitotoxicity not only by dampening the neuronal excitability of pyramidal neurons but also by inducing intracellular cascades, including ERK phosphorylation and the expression of IEGs that code for transcription factors (c-*fos* and *zif268*) and neurotrophins (such as BDNF). The two separate mechanisms may act in concert to provide protection against the consequences of excessive neuronal activity. Whereas lowering neuronal excitability by hyperpolarization provides rapidly available protection, the activation of the intracellular cascades might contribute to long-term adaptive cellular changes in response to the excitotoxic insult in neuronal circuits (*24*). Nevertheless, rapid effects of ERK activation or IEG expression after KA application might also contribute to the early adaptive reactions.

There is evidence from different neuropathological models that the endogenous cannabinoid system can be differentially activated in a species- and age-dependent manner (*30*–*35*) or even through non-CB1 receptor–mediated mechanisms (*36*). For instance, brain trauma induced an increase of 2-arachidonoyl-glycerol levels in adult mice (*31*), whereas in a similar experimental model in neonatal rats, the tissue concentrations of anandamide but not of 2-arachidonoyl-glycerol were increased (*37*). In neonatal rats, blocking of CB1 receptors with SR141716A induced a "paradoxical" protection against *N*-methyl-D-aspartate–induced neurotoxicity (*11*), whereas exogenous anandamide was protective in a model of ouabain-induced neurotoxicity in the same species at the same age (*7*, *34*). The reasons for these apparent discrepancies are not clear. Different processing of endocannabinoids in different species and at different developmental stages (*29*), different experimental conditions (such as the method of inducing neurotoxicity and the parameters monitored), or differences in neuronal circuitries at different ages (*38*) may be responsible for some of these divergent findings.

Our results establish the CB1 receptor–dependent activation of the endogenous cannabinoid system as a rapidly activated early step in a protective cascade against excitotoxicity in the adult mouse brain. The endogenous cannabinoid system might become a promising therapeutic target for the treatment of neurodegenerative diseases with excitotoxic events as their hallmarks (*1*, *39*–*41*).

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**Supporting Online Material**

www.sciencemag.org/cgi/content/full/302/5642/84/DC1
Materials and Methods
Figs. S1 to S3
References and Notes

19 June 2003; accepted 15 August 2003

**Supporting Online Material**

## Material and Methods

**Animals.** Male adult (8-16 weeks) mice were used for all experiments. C57BL/6N mice were purchased from Charles River (Germany). $CB1^{-/-}$ and $CB1^{+/+}$ littermates were described previously (*1*). $CB1^{f/f}$ mice were obtained by crossing mice carrying the CB1-floxed-neo allele (*1*) with flipase-deleter mice (*2*), carrying the germ-line expression of the recombinase flipase (*2*) in order to delete the FRT-PGK-Neo selection cassette. $CB1^{f/f;CaMKII\alpha Cre}$ mice were obtained by crossing $CB1^{f/f}$ mice with mice expressing the improved Cre recombinase from a bacterial artificial chromosome containing the regulatory sequences of $Ca^{2+}$/calmodulin-dependent kinase II$\alpha$ (CaMKII$\alpha$ iCre BAC) (ref. [*3*], here named $CB1^{CaMKII\alpha Cre}$), in order to obtain $CB1^{f/f}$ x $CB1^{f/f;CaMKII\alpha Cre}$ breeding pairs. Littermates were used for each experiment. All lines were in mixed genetic background, with a predominant C57BL/6N contribution (6-7 backcrossings). Genotyping was performed as described for Cre transgene (*3*) and by PCR using the primers G50 and G51 described in ref. (*1*). All experimental procedures were approved by the Committee on Animal Health and Care of local Government.

**Seizure scoring and pharmacology.** Kainic acid (KA; Sigma) was dissolved in saline and administered intraperitoneally at 10 ml/kg body weight. SR141716A (NIMH Chemical Synthesis and Drug Supply Program, U.S.A.) and UCM707 (*4, 5*) were dissolved in vehicle solution (1 drop of Tween-80 in 3 ml of 2.5% dimethylsulfoxide in saline) and injected subcutaneously at 3 mg/20 ml/kg body weight under light isoflurane anesthesia 30 min and 1 h before KA injection, respectively. Mice were monitored for 2 h and behavioral scores were recorded every 15 min. Scores were quantified by trained observers blind to genotype and drug treatment according to ref. (*6*), with stage 7 indicating death. Scores were analyzed by 2-way repeated measures ANOVA with genotype or treatment as between subjects factors and time as within subject factor (followed by Tukey's test, when applicable), and death rates by Fisher Exact test.

**Measurement of endocannabinoids.** C57BL/6N mice were sacrificed at different time points after the injection of 30 mg/kg KA. Hippocampi were rapidly dissected, snap-

frozen in liquid nitrogen and stored at –80°C. Endocannabinoids were extracted, and their levels were measured by isotope-dilution liquid chromatography-mass spectrometry as described (*1*). Data were analyzed with ANOVA, followed by Bonferroni's *post-hoc* test.

**Electrophysiology.** Preparation of coronal brain slices (400 µm thick) and whole-cell voltage-clamp recording (-70 mV holding potential) was performed essentially as described (*7*). The pipette solution consisted of (in mM): K-gluconate, 105; KCl, 30; Mg-ATP, 4; phosphocreatine, 10; GTP, 0.3; HEPES, 10; QX314, 5 (pH 7.3). All experiments were performed at room temperature (22-24°C). As a measure of the excitation of the recorded neuron by spontaneous synaptic activity, the charge transfer (in Coulomb) across the neuronal membrane mediated by spontaneous excitatory postsynaptic currents (EPSCs) was calculated. For statistical analysis, values from each neuron were collected 15 to 20 min after KA application, averaged and normalized to baseline (last 5 min before KA application). Frequencies of spontaneous EPSCs were calculated for 1 minute immediately before and 20 minutes after KA application. Data are expressed as means ± s.e.m. Significance was tested using the Student`s *t*-test.

***In situ* hybridization.** Brains were isolated and snap-frozen on dry ice. Single and double *in situ* hybridization were performed as described (*8, 9*). Sources of cDNA clones, restriction enzymes for linearization and RNA polymerases (NEB) for synthesis of antisense riboprobes are listed: CB1, as described (*8*); glutamic acid decarboxylase 65kD (GAD65), as described (*8*); c-fos, I.M.A.G.E. clone 2647069 (Research Genetics) *EcoRV*, T7; zif268, 2370 bp corresponding to 3' region, *EcoRV*, T7; BDNF, I.M.A.G.E. clone 1397218 (Research Genetics), *XhoI*, T3. Identity of all cDNA clones was checked by sequencing. Densitometric analyses were performed on autoradiographic films using the NIH Image software (http://rsb.info.nih.gov/nih-image/Default.html). Data were analyzed with ANOVA, followed by Bonferroni's *post-hoc* test.

**Western blotting.** Hippocampi were dissected and homogenized by sonication in protease inhibitor solution (Roche) containing phosphatase inhibitors (phosphatase inhibitor cocktails I and II; Sigma). After determination of protein content, 15 µg protein samples were electrophoresed on a 10% SDS-polyacrylamide mini-gel and blotted

electrophoretically to Immobilon-P (Millipore), following standard procedures. Immunodetection was performed by incubating membranes with the polyclonal antibody anti-phospho-Erk1/2 (Thr202/Tyr204; 1:1000; #9101; Cell Signaling) overnight at 4°C. After washing, blots were incubated at room temperature for 1-2 h with secondary antibody conjugated to horseradish peroxidase (1:2000; DAKO) and developed using the enhanced chemoluminescence method (ECLplus; Amersham). After stripping (in 2% SDS, 50 mM DTT, 50 mM Tris/HCl pH 7.0 for 30 min at 70°C), blots were incubated with the polyclonal antibody anti-Erk1/2 (1:1000; #9102; Cell Signaling). Densitometric analysis of bands was performed using the Quantity One software (BioRad). Normalized values were analyzed by Student's *t*-test.

**TUNEL staining and immunohistochemistry.** Cell damage was detected on frozen sections by terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling of DNA double strand breaks (TUNEL method) using an *in situ* cell death detection kit (POD; Roche) following manufacturer's instructions. TUNEL staining was evaluated by densitometric analysis. Gliosis was evaluated by immunohistochemistry on frozen sections by overnight incubation at 4°C with a polyclonal anti-GFAP antibody (1:1000; DAKO) followed by immunoperoxidase staining (Vectastain ABC kit, Vector Laboratories). Staining was evaluated by subjective scoring by 3 independent observers blind of genotype and treatment. Student's *t*-test was used for statistical analysis.

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**Supporting Online Figures**

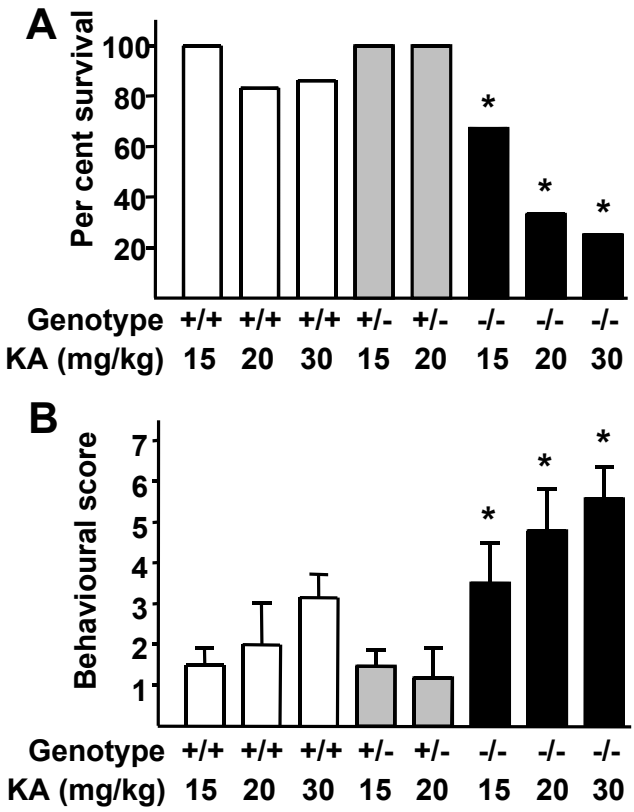


**Fig. S1.** Responses of $CB1^{+/+}$, $CB1^{+/-}$ and $CB1^{-/-}$ mice to the administration of different doses of KA. (**A**) Survival after injection of 15, 20 and 30 mg/kg KA (n=6-8/group), and (**B**) averaged seizure scoring of $CB1^{+/+}$, $CB1^{+/-}$, and $CB1^{-/-}$ 1 h after injection of KA. Means ± s.e.m.; *, $p<0.05$ versus $CB1^{+/+}$.

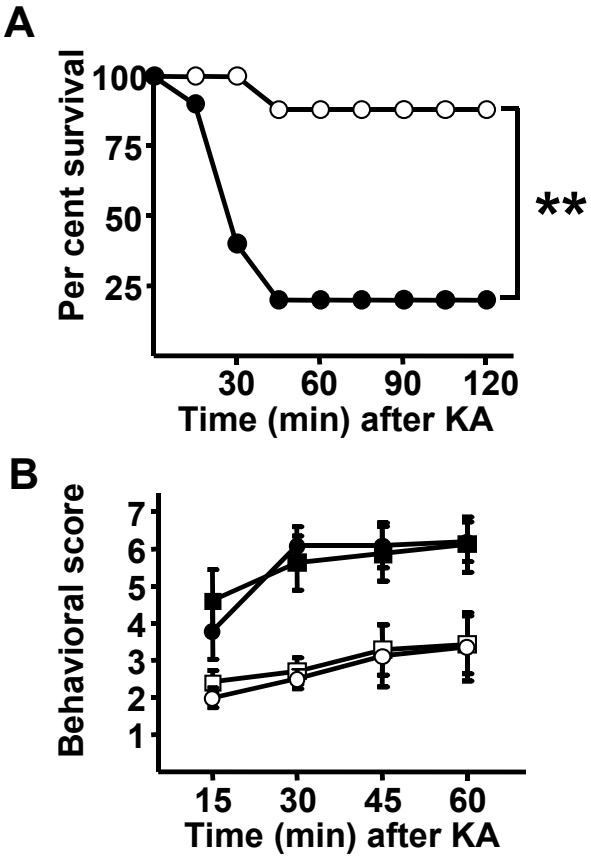


**Fig. S2.** (**A**) Survival of $CB1^{f/f}$ and $CB1^{f/f;CaMKII\alpha Cre}$ littermates over the course of the experiment depicted in Fig. 2F of main text. (**B**) Comparison between seizure scoring (30 mg/kg) of $CB1^{-/-}$ (filled squares) and $CB1^{f/f;CaMKII\alpha Cre}$ (filled circles) and respective control littermates ($CB1^{+/+}$, open squares, and $CB1^{f/f,}$ open circles). Same data as in Fig. 1A and 2F of main text. Means ± s.e.m.; **, $p<0.01$.

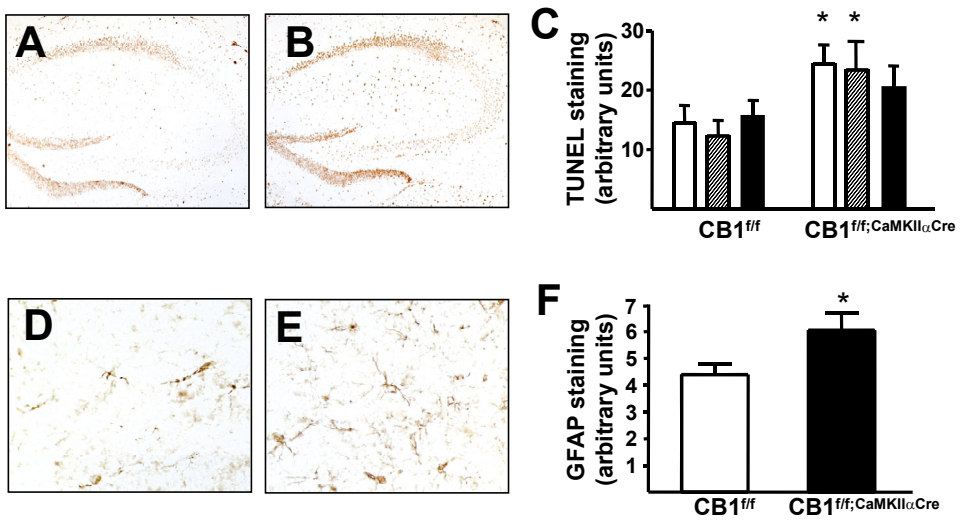


**Fig. S3.** (**A-C**) Increased degree of KA-induced neuronal death in CB1$^{f/f;CaMKII\alpha Cre}$ hippocampi as compared to CB1$^{f/f}$ littermates. (**A, B**) Representative TUNEL staining of hippocampi from CB1$^{f/f}$ (**A**) and CB1$^{f/f;CaMKII\alpha Cre}$ (**B**), 4 days after KA injection (20 mg/kg). (**C**) Densitometric quantification of TUNEL staining in CA1 (open bars), CA3 (hatched bars) and dentate gyrus (filled bars) of hippocampus in KA-treated CB1$^{f/f}$ (n=7 mice) and CB1$^{f/f;CaMKII\alpha Cre}$ (n=4 mice). *, $p<0.05$ versus respective region of CB1$^{f/f}$ mice. **(D-F)** Increased degree of KA-induced gliosis in CB1$^{f/f;CaMKII\alpha Cre}$ hippocampi as compared to CB1$^{f/f}$ littermates. (**D, E**) Representative GFAP immunostaining of hippocampi from CB1$^{f/f}$ (**D**) and CB1$^{f/f;CaMKII\alpha Cre}$ (**E**), 4 days after KA injection (20 mg/kg). (**F**) Quantification of GFAP staining in hippocampi from KA-treated CB1$^{f/f}$ (open bar, n=7) and CB1$^{f/f;CaMKII\alpha Cre}$ (filled bar, n=4). Means ± s.e.m.; *, $p<0.05$ versus CB1$^{f/f}$.

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# Involvement of brain-derived neurotrophic factor in cannabinoid receptor-dependent protection against excitotoxicity

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# Involvement of brain-derived neurotrophic factor in cannabinoid receptor-dependent protection against excitotoxicity

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*Keywords*: CB1 knockout mouse, hippocampus, kainic acid, organotypic culture, SR141716A

## Abstract

Cannabinoid type 1 (CB1) receptors play a central role in the protection against excitotoxicity induced by treatment of mice with kainic acid (KA). As inactivation of CB1 receptor function in mice blocks KA-induced increase of brain-derived neurotrophic factor (BDNF) mRNA levels in hippocampus, the notion was put forward that BDNF might be a mediator, at least in part, of CB1 receptor-dependent neuroprotection [Marsicano *et al.* (2003) *Science*, 302, 84–88]. To assess this signalling cascade in more detail, organotypic hippocampal slice cultures were used, as this *in vitro* system conserves morphological and functional properties of the hippocampus. Here, we show that both genetic ablation of CB1 receptors and pharmacological blockade with the specific CB1 receptor antagonist SR141716A increased the susceptibility of the *in vitro* cultures to KA-induced excitotoxicity, leading to extensive neuronal death. Next, we found that the application of SR141716A to hippocampal cultures from wild-type mice abolished the KA-induced increase in BDNF protein levels. Therefore, we tried to rescue these organotypic cultures from neuronal death by exogenously applied BDNF. Indeed, BDNF was sufficient to prevent KA-induced neuronal death after blockade of CB1 receptor signalling. In conclusion, our results strongly suggest that BDNF is a key mediator in CB1 receptor-dependent protection against excitotoxicity, and further underline the physiological importance of the endogenous cannabinoid system in neuroprotection.

## Introduction

The endogenous cannabinoid system comprises two G protein-coupled cannabinoid receptors (CB1 and CB2 receptors), their endogenous ligands (endocannabinoids), and synthesizing and degrading enzymes for endocannabinoids, and is involved in the regulation of a number of physiological functions in the nervous system (reviewed in Di Marzo *et al*., 1998; Howlett *et al*., 2002; Lutz, 2002).

CB1 receptor activation is able to mitigate neuronal damage in several experimental paradigms of neurotoxicity, e.g. in global and focal ischaemia *in vivo* (Parmentier-Batteur *et al*., 2002), after head trauma (Panikashvili *et al*., 2001), and after excitotoxic damage *in vitro* and *in vivo* (Shen & Thayer, 1998; Abood *et al*., 2001; Marsicano *et al*., 2003; Pryce *et al*., 2003; Veldhuis *et al*., 2003). Increase of endocannabinoid levels under neurotoxic conditions appears to mediate the CB1 receptor-dependent protection (Hansen *et al*., 1999, 2001; Panikashvili *et al*., 2001; Franklin *et al*., 2003; Marsicano *et al*., 2003). Possible mechanisms underlying this action include a decrease of intracellular calcium ion concentration as a result of blockade of voltage-sensitive calcium channels, an inhibition of presynaptic glutamate release and an activation of protective signalling pathways (reviewed in Van der Stelt *et al*., 2002).

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Using the kainic acid (KA) model of epileptiform seizures (Ben-Ari & Cossart, 2000), we have recently shown that the endogenous cannabinoid system is activated in the hippocampus of adult mice upon systemic KA treatment, leading to a protection of neurons against this excitotoxic insult *in vivo* (Marsicano *et al*., 2003). Acute dampening of neuronal excitation and activation of protective cellular pathways in hippocampal glutamatergic neurons were proposed to underlie CB1 receptor-mediated neuroprotection. KA treatment increased levels of transcripts encoding brain-derived neurotrophic factor (BDNF) in a CB1 receptor-dependent manner (Marsicano *et al*., 2003). However, the function of BDNF in the CB1 receptor-dependent protection against excitotoxicity has remained elusive. BDNF plays essential roles in development, survival and maintenance of neurons (Huang & Reichardt, 2001). It has been described that experimentally induced seizures resulted in a rapid but transient increase in BDNF transcript and protein levels in hippocampal and cortical neurons (Isackson *et al*., 1991; Dugich-Djordjevic *et al*., 1992). Therefore, endocannabinoid-induced stimulation of BDNF synthesis might be important for CB1 receptor-mediated neuroprotection against KA-induced excitotoxicity.

To substantiate this notion, we performed a series of experiments using hippocampal organotypic cultures. This technique represents an adequate model to study mechanisms of excitotoxicity because it reproduces *in vitro* the basic morphological and functional properties of the hippocampal neuronal network and allows to perform experiments using distinct pharmacological treatments and to circumvent

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some of the technical limitations that may occur in *in vivo* approaches (Stoppini *et al*., 1991; Kristensen *et al*., 2001). Using explants from CB1 receptor-deficient mice and from wild-type mice treated with CB1 receptors antagonists, we first established that protection against KA-induced excitotoxicity in these cultures is CB1 receptor-dependent. Then, a time-course of BDNF protein levels after KA treatment and its CB1 receptor dependency were determined, to finally ask whether or not BDNF is sufficient to rescue the KA-treated explants from excitotoxicity after blockade of CB1 receptor function.

## Materials and methods

### Animals

All experimental procedures were carried out in accordance with the Committee of Animal Health and Care of local Government. The generation of $CB1^{-/-}$ and $CB1^{+/+}$ mice was described previously (Marsicano *et al*., 2002). These mice were in a mixed 129/Ola and C57BL/6N genetic background with a predominance of C57BL/6N (five backcrosses). Wild-type C57BL/6N mice were purchased from Charles River (Sulzfeld, Germany).

### Organotypic hippocampal slice cultures

Hippocampal slice cultures were prepared using the static interface culture method of Stoppini *et al*. (1991). Briefly, 7–8-day-old $CB1^{+/+}$ and $CB1^{-/-}$ mice or wild-type C57BL/6N mice were narcotized with chloroform, brains were removed, hippocampi were dissected and transverse slices (250–300 μm in thickness) were prepared with a McIlwain tissue chopper (The Mickle Laboratory Engineering, Guildford, UK). Slices were placed for 1–2 h at 4 °C into Gey's balanced salt solution (GBSS; Sigma) supplemented with 6.5 mg/mL D-glucose and were then transferred onto porous (0.4 μm) transparent membrane inserts (30-mm in diameter; Millipore) with five–six slices on each insert. Inserts were placed into six-well culture plates. Each well contained 1 mL of nutrient medium, composed of 25% heat-inactivated horse serum, 25% Hank's balanced salt solution, 50% Opti-MEM (all from GibcoBRL), supplemented with 25 mM D-glucose and 1 mM glutamine (Sigma). Neither antibiotics nor antimitotics were used. Plates were kept in a humidified $CO_2$ incubator (5% $CO_2$, 95% atmospheric air) at 35.5 °C, and cultures were maintained for 2–3 weeks before experiments were performed. Medium was changed every 2 or 3 days.

### Pharmacological treatments

Prior to exposure to KA (Sigma), the conditioned nutrient medium was collected and stored, the cultures were washed with GBSS and then exposed for 3 h to KA, which was dissolved in GBSS. Control cultures were treated similarly, but kept for 3 h in GBSS without KA. The KA exposure was according to previous reports (e.g. Kristensen *et al*., 2001), and the duration of the excitotoxin treatment was chosen as described by Liu *et al*. (2001).

The CB1 antagonist SR141716A (NIHM Chemical Synthesis and Drug Supply Program, USA) was dissolved in vehicle solution (GBSS containing 1 : 1000 dimethylsulphoxide). Explants were preincubated for 30 min with SR141716A (5 μM) or vehicle followed by exposure to KA for 3 h in the presence of the same concentration of SR141716A or vehicle. Other explants were treated for 3.5 h with vehicle or with SR141716A (5 μM) in GBSS without KA.

For BDNF treatment, explants were incubated in recombinant BDNF (Sigma; 100 ng/mL dissolved in nutrient medium) 24 h prior to KA exposure (Pringle *et al*., 1996) and at the same concentration in GBSS during KA exposure, in the presence of SR141716A or vehicle as described above.

After treatment, cultures were washed in GBSS, returned to the original conditioned nutrient medium and kept in $CO_2$ incubator for 20–24 h before assessment of neuronal injury or BDNF protein quantification.

### Assessment of KA neurotoxicity

Propidium iodide (PI; Sigma) was used as an indicator of neuronal membrane integrity and cell damage. As a polar compound, PI only enters dead or dying cells with damaged or leaky cell membrane and then interacts with DNA to yield strong fluorescence (Kristensen *et al*., 2001). Three–four hours before experimental treatment, cultures were incubated in nutrient medium supplemented with PI (5 μg/mL). PI fluorescence was examined with an inverted fluorescence microscope (Olympus IX 50, Japan) using a standard rhodamine filter, and digital images were acquired with Camera DP 50 (MTV 3), Program Olympus DP Soft. Slices were imaged prior to and 20–24 h after treatment. Cultures displaying nuclear fluorescence before experiment were excluded from analysis. The intensity of PI fluorescence in pyramidal layer of the hippocampal explants was used as an index of cell death and was measured by semi-quantitative densitometric analysis using the NIH IMAGE program (http://rsb.info.nih.gov/nih-image/Default.html). After background subtraction, densitometric values before KA treatment were subtracted from the values after KA treatment for each slice, and data were expressed as relative fluorescence of each slice. Each experiment was repeated at least twice, giving similar results.

At the end of the experiments, cultures were fixed for 3–4 h in modified Tellyesnicky–Lillie fixative (consisting of 70% ethanol, 20% formalin and 10% acetic acid) and stained with cresyl violet (Lillie, 1965) for morphological evaluation.

### Assessment of BDNF protein levels

BDNF protein levels were assessed in untreated C57BL/6N slices and 1, 9 and 21 h after 3 h exposure to KA (6 μM). To test the effect of CB1 receptor blockade on BDNF protein levels, slices were treated with SR141716A or vehicle as described above, and BDNF levels were measured 9 h after exposure to KA (6 μM). The Emax™ immunoassay system (Promega, Mannheim, Germany) was used to quantify the levels of BDNF protein in explants. After treatment, cultures from each well (three–five explants) were lysed in 100 μL lysis buffer (137 mM NaCl; 20 mM Tris–HCl, pH 8.0; 1% NP-40; 10% glycerol; 0.5 mM sodium vanadate; protease inhibitor complete Mini tablets, Roche, Basel, Switzerland). Lysates were briefly sonicated and stored at −80 °C. Prior to each assay, lysates were acid-treated. This process increases the detectable amount of free BDNF in solution by dissociating it from its proforms or receptors (Okragly & Haak-Frendscho, 1997). Briefly, samples were acidified with 1 μL 1 M HCl/50 μL sample to obtain a pH less than 3.0. After 15 min incubation at room temperature, samples were neutralized with the same amount of 1 M NaOH to reach a pH of approximately 7.6. Maxisorp 96-well plates (Nunc, Neerijse, Belgium) were used for antibody coating, and ELISA was carried out according to manufacturer's instructions. BDNF levels were normalized to the total amount of protein from each experimental group, measured using the DC protein assay (Bio-Rad, Munich, Germany). Samples were measured in triplicates. Data were calculated as percentage of respective control and were expressed as means ± SEM of three independent experiments.

### Statistical analyses

Data were analysed by Student's *t*-test or by one-way ANOVA, followed by post-hoc Newman–Keuls multiple comparison test, where it was appropriate. $P < 0.05$ was considered statistically significant.

## Results

### *Neuromorphological features of explants from CB1$^{+/+}$ and CB1$^{-/-}$ mice*

Hippocampal explants from CB1$^{+/+}$ mice contained all characteristic structures observed in hippocampus *in vivo* as shown by Cresyl violet staining on day 14–16 *in vitro* (Fig. 1A and C). They comprise Ammon's horn (CA), containing pyramidal cell layer, formed by densely packed pyramidal neurons, and dentate gyrus (DG), containing the hilus with large sparse neurons and a layer of smaller granule cells. The hippocampal explants from CB1$^{-/-}$ mice showed the same structures (Fig. 1B and D), including pyramidal layer CA, hilus and granular layer of DG. Observable differences were present neither in localization and orientation of cellular layers of CA and DG, nor in the overall morphology of neurons between the hippocampal explants from CB1$^{+/+}$ and CB1$^{-/-}$ mice.

### *Excitotoxic effects of KA on CB1$^{+/+}$ and CB1$^{-/-}$ explants*

Addition of 3 μM KA for 3 h did not induce any observable destructive changes in neurons of hippocampal explants from either genotypes (data not shown). At doses of 6 μM and 25 μM KA, significant neuronal damage was observed in CB1$^{+/+}$ explants as compared with untreated CB1$^{+/+}$ explants ($P < 0.001$; relative PI fluorescence, untreated CB1$^{+/+}$, $13.2 \pm 3.2$; 6 μM KA-treated CB1$^{+/+}$, $49.0 \pm 11.1$; 25 μM KA-treated CB1$^{+/+}$, $72.6 \pm 19.5$; Fig. 2G). Concomitantly, the susceptibility of neurons to the excitotoxic action was different in CB1$^{+/+}$ and CB1$^{-/-}$ explants. KA at a concentration of 6 μM exerted stronger neurotoxic effects in cultures from CB1$^{-/-}$ than from CB1$^{+/+}$ mice (Fig. 2A and B). At this concentration, we could only observe a small number of both pycnotic (Fig. 2C) and PI fluorescent (Fig. 2D) nuclei in the pyramidal layer of CB1$^{+/+}$ explants, corresponding to dying or dead neurons, whereas the granule cells of DG remained largely

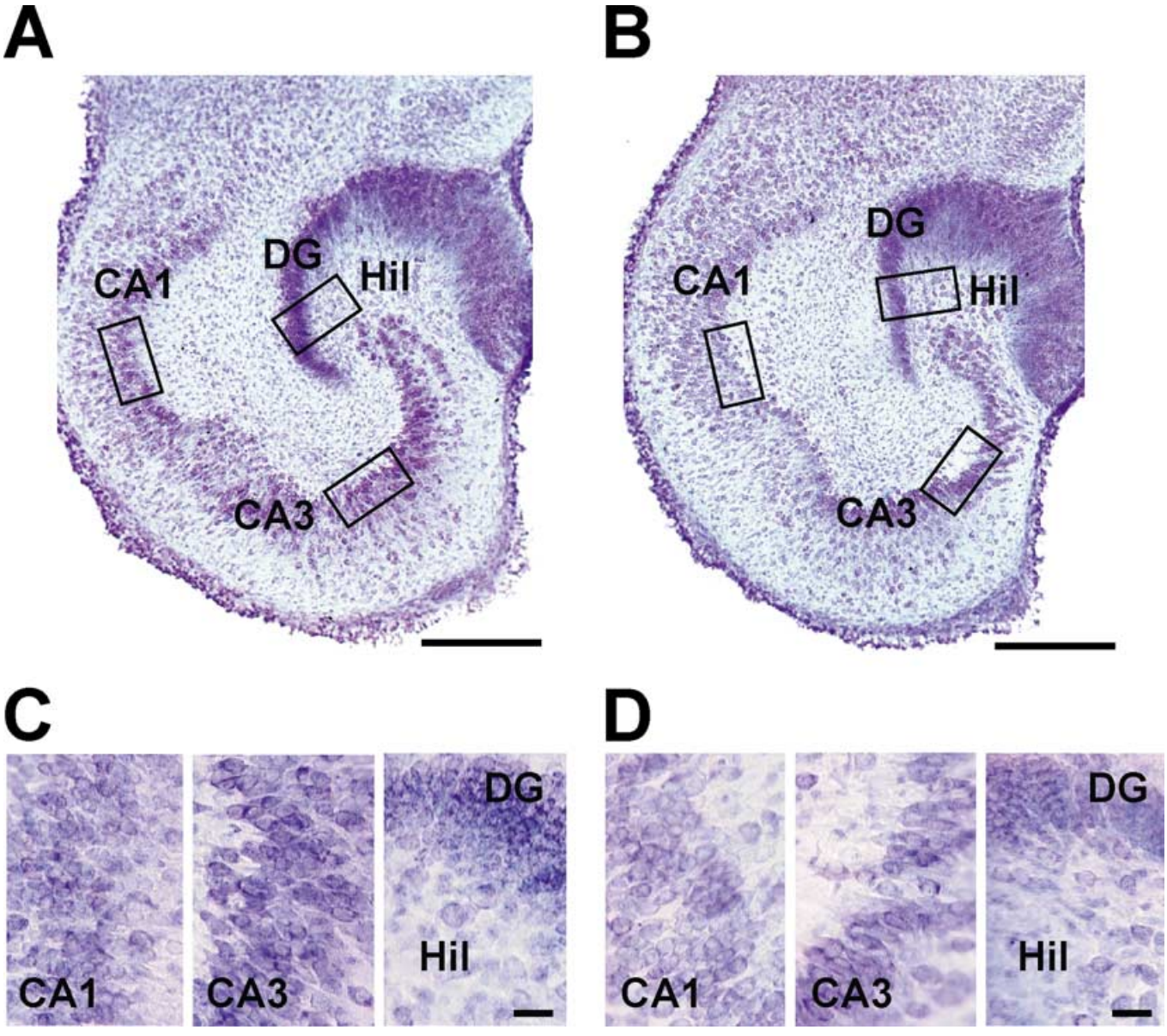


FIG. 1. Cresyl violet-stained hippocampal explants cultured for 16 days *in vitro* from CB1$^{+/+}$ (A and C) and CB1$^{-/-}$ (B and D) mice. CA1 and CA3 pyramidal layers of Ammon's horn as well as hilus (Hil) and granular layer of dentate gyrus (DG) are well preserved in cultures obtained from both CB1$^{+/+}$ (C) and CB1$^{-/-}$ (D) mice. Scale bars: 300 μm (A and B); 25 μm (C and D).

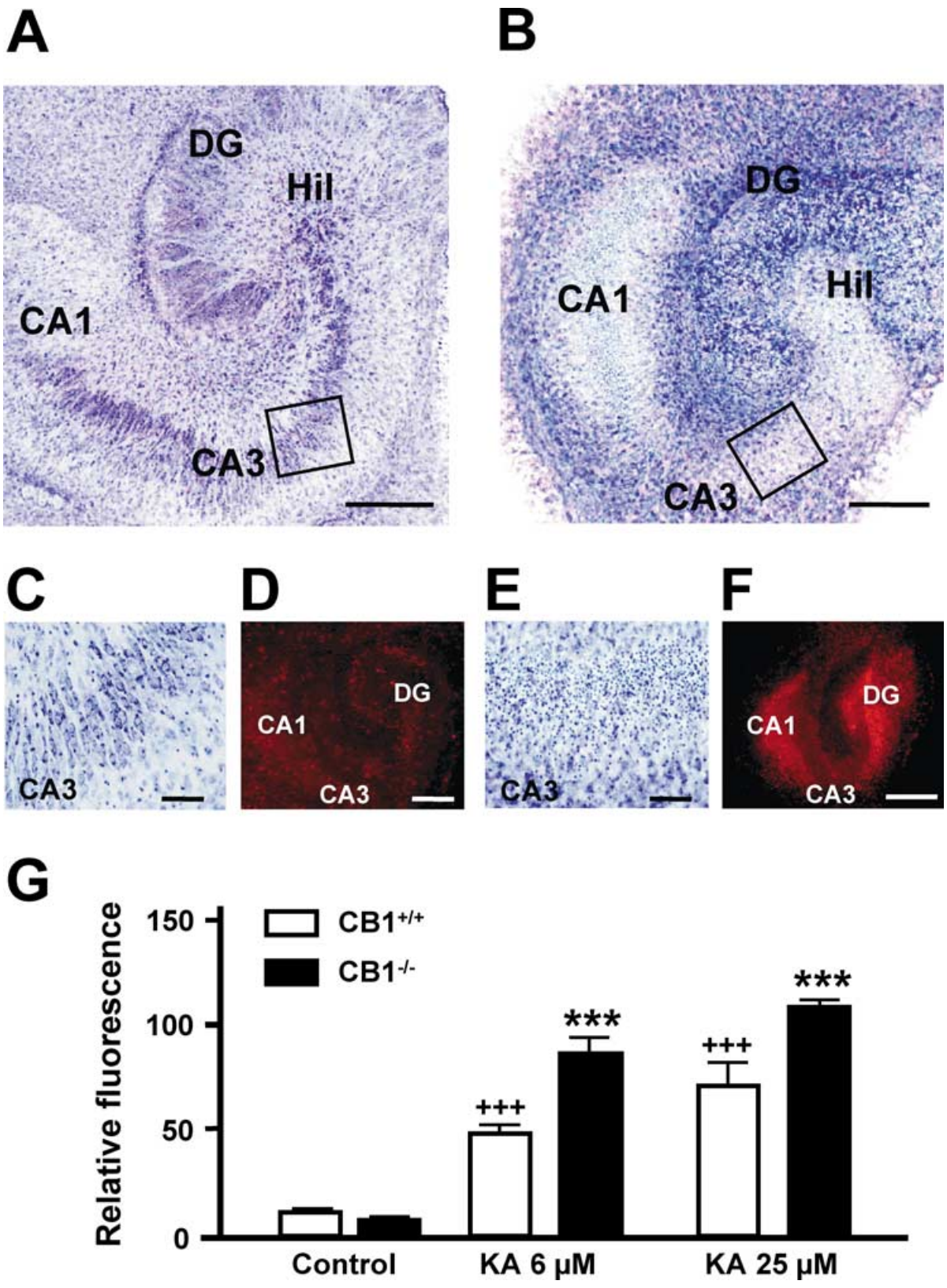


FIG. 2. Genetic deletion of cannabinoid type 1 (CB1) receptor increases vulnerability to kainic acid (KA)-induced neurotoxicity in organotypic hippocampal cultures. (A–F) Histology and PI staining of $CB1^{+/+}$ (A, C, D) and $CB1^{-/-}$ (B, E, F) cultures 21 h after KA treatment (6 μM). Note the mild neuronal damage in pyramidal layer of $CB1^{+/+}$ explants (C and D) as compared with the extensive neuronal death in $CB1^{-/-}$ explants (E and F), as visualized by Cresyl violet staining (C and E) and PI fluorescence (D and F). Scale bars: 300 μm (A, B, D and F); 25 μm (C and E). (G) Densitometric evaluation of relative PI staining in $CB1^{+/+}$ and $CB1^{-/-}$ hippocampal cultures 21 h after vehicle (control) or KA treatments (6 and 25 μM). $n = 3$–7 explants per group; $^{***}P < 0.001$ vs. treated $CB1^{+/+}$; $^{+++}P < 0.001$ vs. untreated $CB1^{+/+}$ explants. DG, dentate gyrus; Hil, hilus.

undamaged (fig. 2D, and data not shown). On the other hand, the same concentration of KA in hippocampal explants from $CB1^{-/-}$ mice caused considerable damage of neurons of both CA pyramidal layer and DG granular layer (Fig. 2E and F). By evaluation of PI fluorescence intensity, we observed that the level of neuronal damage in $CB1^{-/-}$ explants was almost two times higher than in $CB1^{+/+}$ explants (relative PI fluorescence, 6 μM KA, $CB1^{+/+}$, $49.0 \pm 11.1$; $CB1^{-/-}$, $89.3 \pm 7.6$; $P < 0.001$; Fig. 2G). Treatment with 25 μM KA also resulted in significantly stronger neuronal destruction in $CB1^{-/-}$ explants than in $CB1^{+/+}$ (relative PI fluorescence, 25 μM KA, $CB1^{+/+}$, $72.6 \pm 19.5$; $CB1^{-/-}$, $111.2 \pm 6.0$, $P < 0.001$; Fig. 2G). The increase of KA concentration up to 50 μM resulted in a complete destruction of neurons in pyramidal layer of CA and granular layer of DG in both $CB1^{+/+}$ and $CB1^{-/-}$ hippocampal explants (data not shown).

### *Pharmacological blockade of CB1 receptors increases vulnerability to KA exposure*

The stronger sensitivity to KA-induced neurotoxicity of hippocampal explants from $CB1^{-/-}$ mice might be due to developmental defects caused by the life-long absence of CB1 receptors in the mutant mice. Therefore, in order to verify the acute activation of CB1 receptors after KA treatment, wild-type explants from C57BL/6N mice were exposed to a low dose of KA (6 μM) in the presence of vehicle (1 : 1000 dimethylsulphoxide) or of the specific CB1 antagonist SR141716A. As evaluated by PI fluorescence intensity, treatment of slices with KA alone or SR141716A alone did not increase significantly neuronal death as compared with control vehicle-treated slices (Fig. 3). However, the combination of KA and SR141716A treatment induced a very strong increase in KA-induced neuronal death (relative PI fluorescence, KA treatment, $8.9 \pm 2.5$; KA + SR treatment, $46.5 \pm 3.4$; $P < 0.001$; Fig. 3). These results show that pharmacological blockade of CB1 receptors strongly increases the sensitivity of neurons to KA-induced toxicity, thus indicating that acute activation of the endogenous cannabinoid system participates in physiological protective mechanisms in hippocampal organotypic cultures.

### *BDNF prevents KA-induced toxicity resulting from blockade of CB1 receptors*

Using an ELISA method, the amount of intracellular BDNF protein was determined at different time points after KA treatment (6 μM) in organotypic hippocampal cultures. The levels of BDNF protein significantly increased 9 h after KA treatment and decreased after 21 h (BDNF levels, untreated, $100 \pm 17.1\%$; 1 h, $82.5 \pm 20.9\%$; 9 h, $246.7 \pm 38.3\%$; 21 h, $153.2 \pm 75.3\%$; Fig. 4A), indicating that KA

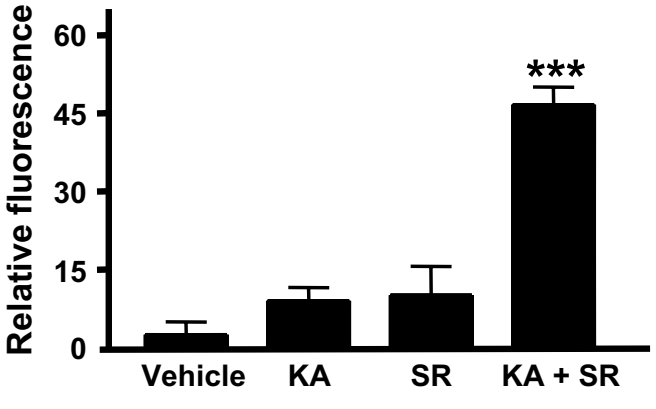


FIG. 3. Pharmacological blockade of CB1 receptors increases vulnerability to kainic acid (KA)-induced neurotoxicity in organotypic hippocampal cultures from wild-type C57BL/6N mice. Densitometric evaluation of relative PI fluorescence of explants treated with the specific CB1 antagonist SR141716A (SR; 5 μM) or vehicle, in presence or absence of KA (6 μM) 21 h after KA treatment. $n = 5$–13 explants per group; $^{***}P < 0.001$ vs. all other groups.

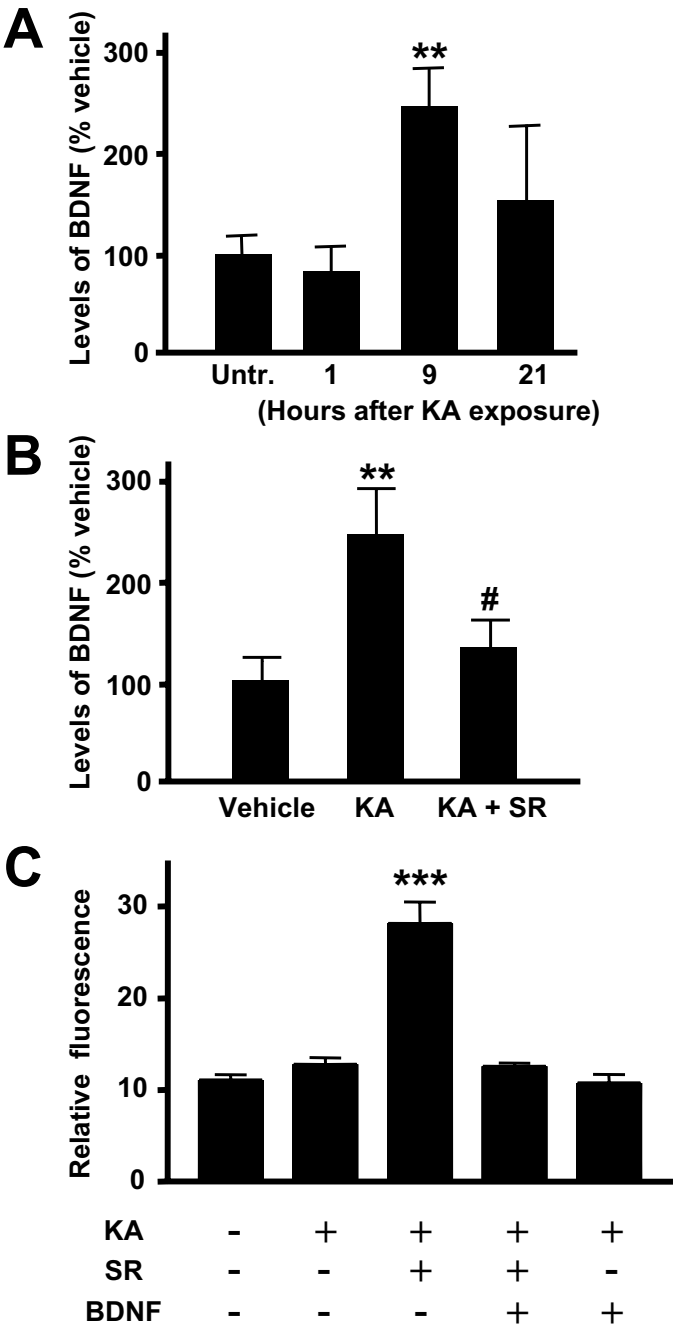


FIG. 4. Brain-derived neurotrophic factor (BDNF) mediates CB1 receptor-dependent neuroprotection. (A) Kainic acid (KA) treatment (6 μM) increases BDNF protein levels in organotypic hippocampal cultures 9 h after KA application. Untr., untreated cultures. $^{**}P < 0.01$ vs. untreated group ($n = 3$). (B) Pharmacological blockade of CB1 receptors prevents the increase of BDNF protein levels induced by KA (6 μM). Cultures were treated with vehicle or SR141716A (SR; 5 μM), and BDNF protein levels were evaluated 9 h after KA exposure. $^{**}P < 0.01$ vs. vehicle; $^{\#}P < 0.05$ vs. KA ($n = 3$). (C) BDNF treatment (100 ng/mL) completely prevents the increased vulnerability to KA neurotoxicity (6 μM) induced by SR141716A (SR; 5 μM). Cultures were treated as depicted and relative PI staining was evaluated 21 h after KA treatment. $n = 15$–24 explants per group; $^{***}P < 0.001$ vs. all other groups.

treatment induces a transient increase of BDNF protein in organotypic hippocampal cultures. To test whether this increase in BDNF levels was dependent on the acute activation of CB1 receptors, wild-type explants were exposed to the same dose of KA in the presence of vehicle or SR141716A (5 μM), and BDNF protein levels were evaluated 9 h later. As expected, KA treatment induced an increase in BDNF levels as compared with vehicle-treated slices ($P < 0.01$, Fig. 4B). Interestingly, treatment of the slices with 5 μM SR141716A abolished the induction of BDNF by KA (BDNF levels, percent of vehicle; KA, $240.4 \pm 42.0\%$; KA + SR, $131.2 \pm 22.8\%$, $P < 0.05$, Fig. 4B). These results show that KA-induced activation of CB1 receptors not only inhibits neuronal damage (Fig. 3), but also mediates the increase of BDNF protein induced by the excitotoxin. To test the causal relationship between CB1 receptor-mediated neuroprotection and enhancement of BDNF levels, we treated hippocampal cultures with SR141716A or vehicle in the presence or absence of exogenous BDNF protein and challenged them with a low dose of KA (6 μM). In these experiments, 6 μM KA did not induce significant neuronal damage, whereas 5 μM SR141716A strongly increased the effect of the excitotoxin ($P < 0.001$, Fig. 4C). Intriguingly, this effect of the CB1 receptor antagonist was abolished by the presence of BDNF (100 mg/mL; $P < 0.001$, Fig. 4C). BDNF had no effect on the viability of the slices when SR141716A treatment was absent (Fig. 4C). These results show that exogenous BDNF is sufficient to prevent the increased sensitivity of organotypic hippocampal cultures to KA induced by blockade of CB1 receptors, indicating that this neurotrophin mediates, at least in part, the on-demand protection of the endogenous cannabinoid system against excitotoxicity.

## Discussion

Using an *in vitro* model of excitotoxicity, we investigated the mechanisms underlying the neuroprotective activity of the endogenous cannabinoid system. Interestingly, the relationship between the dose of KA used and the neurotoxic outcome appeared to depend on the genetic background of the slices, i.e. 6 μM KA induced significant neuronal damage in control $CB1^{+/+}$ slices, derived from mice with a predominant C57BL/6N background, but still containing some 129 contribution (Fig. 2G), whereas no significant effect was detected in pure C57BL/6N slices (Figs 3 and 4C). These observations appear to be consistent with data on the higher susceptibility of 129 mice to KA-induced damage as compared with C57BL/6 mice (Schauwecker & Steward, 1997). However, independently of these differences in basal sensitivity to KA, both genetic and pharmacological blockade of CB1 receptors increased the susceptibility of neurons to the excitotoxin. We studied the intracellular events following KA stimulation both in wild-type explants and upon CB1 receptor inactivation in order to uncover important mediators of CB1 receptor-dependent neuroprotection. We found that KA induced an increase in BDNF protein levels in hippocampal explants, and that the application of the CB1 receptor antagonist SR141716A blocked this effect. These findings are in agreement with recent data reporting a CB1 receptor-dependent increase of BDNF mRNA levels following KA administration *in vivo* (Marsicano *et al*., 2003).

In the present work, we took advantage of organotypic hippocampal cultures as an *in vitro* model system, allowing us to investigate whether or not the KA-induced increase of BDNF levels is functionally involved in the CB1 receptor-dependent neuroprotection. Exogenous BDNF was able to counteract the damaging effects of CB1 receptor inactivation and completely prevented the neuronal death induced by KA treatment. This strongly suggests that the activation of BDNF signalling represents a downstream event in the on-demand protection against excitotoxicity mediated by the endogenous cannabinoid system.

Several investigations indicate a pivotal role of BDNF in neuroprotection in different models of neurotoxicity, such as *in vivo* and *in vitro* ischaemic neuronal damage (Tsukahara *et al*., 1994; Pringle *et al*., 1996; Endres *et al*., 2000; Pardridge, 2002; Mishra *et al*., 2003), glucose deprivation or glutamate-induced neuronal damage in neuronal cultures (Cheng & Mattson, 1994), KA-induced excitotoxicity in the striatum (Gratacos *et al*., 2001) and hippocampal kindling (Larmet *et al*., 1995). However, high doses of exogenous BDNF or genetic overexpression of BDNF were shown to increase neuronal excitability and severity of symptoms in animal models of epilepsy (Rudge *et al*., 1998; Croll *et al*., 1999; reviewed in Binder *et al*., 2001), suggesting that BDNF levels need to be tightly regulated to exert the protective functions.

Previous work (Marsicano *et al*., 2003) and the present results show that the on-demand activation of the endogenous cannabinoid system after excitotoxic insults might provide such a precise physiological regulation of the levels of BDNF mRNA and protein. Moreover, a dose of BDNF, which *per se* did not change the susceptibility of organotypic cultures to KA, completely restored the protective functions of the endogenous cannabinoid system impaired by the blockade of CB1 receptors. Altogether, these data show that the endogenous cannabinoid system reacts to excitotoxic insults by raising cellular BDNF to physiologically protective levels.

In the past decade, the intracellular mechanisms of CB1 and BDNF signalling have been extensively investigated and show some converging features that might cooperate in neuroprotection. *In vivo* cannabinoid application induces the expression of immediate-early gene products, including BDNF mRNA, and this induction depends on the activation of extracellular regulated kinases (ERKs, Derkinderen *et al*., 2003). Notably, KA-induced increases of both ERK phosphorylation and BDNF expression are absent in CB1 receptor-deficient mice (Marsicano *et al*., 2003). Consistently, the present results showed impaired KA-induced production of BDNF protein after pharmacological blockade of CB1 receptors. It is therefore tempting to speculate that ERK phosphorylation might represent an intracellular event functionally connecting activation of CB1 receptors and BDNF gene transcription in response to KA treatment. Neuronal activity with a concomitant increase of intracellular $Ca^{2+}$ leads to BDNF gene expression through several pathways, including ERK activation (reviewed in West *et al*., 2001). However, the current view points to the requirement for a cooperativity of these intracellular pathways to finally induce BDNF gene transcription when appropriate and needed (West *et al*., 2001). Although the mechanisms need still to be clarified, the on-demand activation of CB1 receptors during excitotoxicity might represent one of these coinciding events to guarantee BDNF gene activation after excitotoxic insults.

Neuroprotective effects of BDNF were shown to be mediated by both ERK (Hetman *et al*., 1999; Han & Holtzman, 2000) and the phosphoinositol-3-kinase-protein kinase B/AKT pathways (Nakazawa *et al*., 2002; Cheng *et al*., 2003). Interestingly, the latter pathway can also be activated by CB1 receptors (Gomez del Pulgar *et al*., 2000). AKT in turn is able to engage the pro-survival protein Bcl-2 (Brunet *et al*., 2001), providing a likely mechanism for BDNF-dependent protection against neuronal death (Allsopp *et al*., 1995). Therefore, BDNF might represent a molecular link between acute activation of the endogenous cannabinoid system during excitotoxicity and long-term protection.

In conclusion, our results clearly demonstrate the physiological importance of the endogenous cannabinoid system in neuroprotection against excitotoxicity and provide strong evidence for BDNF being an

important downstream mediator of this function. The functional cross-talk between CB1 receptors and BDNF signalling might represent a promising target for treatment of neurodegenerative disorders characterized by the occurrence of excitotoxic events.

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## Abbreviations

BDNF, brain-derived neurotrophic factor; CA, pyramidal layer of Ammon's horn; CB1 receptor, cannabinoid type 1 receptor; DG, dentate gyrus; ERK, extracellular regulated kinase; KA, kainic acid; GBSS, Gey's balanced salt solution; PI, propidium iodide; SR, SR141716A [*N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide].

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# Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1

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# Neuroprotective properties of cannabinoids against oxidative stress: role of the cannabinoid receptor CB1

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**Abstract**

Neuroprotective effects have been described for many cannabinoids in several neurotoxicity models. However, the exact mechanisms have not been clearly understood yet. In the present study, antioxidant neuroprotective effects of cannabinoids and the involvement of the cannabinoid receptor 1 (CB1) were analysed in detail employing cell-free biochemical assays and cultured cells. As it was reported for oestrogens that the phenolic group is a lead structure for antioxidant neuroprotective effects, eight compounds were classified into three groups. Group A: phenolic compounds that do not bind to CB1. Group B: non-phenolic compounds that bind to CB1. Group C: phenolic compounds that bind to CB1. In the biochemical assays employed, a requirement of the phenolic lead structure for antioxidant activity was shown. The effects paralleled the protective potential of group A and C compounds against oxidative neuronal cell death using the mouse hippocampal HT22 cell line and rat primary cerebellar cell cultures. To elucidate the role of CB1 in neuroprotection, we established stably transfected HT22 cells containing CB1 and compared the protective potential of cannabinoids with that observed in the control transfected HT22 cell line. Furthermore, oxidative stress experiments were performed in cultured cerebellar granule cells, which were derived either from CB1 knock-out mice or from control wild-type littermates. The results strongly suggest that CB1 is not involved in the cellular antioxidant neuroprotective effects of cannabinoids.

**Keywords:** CB1 knock-out, cell lines, oxidative stress, primary neuronal cultures.

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Neuroprotective effects have been proposed for natural, synthetic and endogenous cannabinoids in several *in vitro* and *in vivo* neurotoxicity models (e.g. Hampson *et al*. 1998; Nagayama *et al*. 1999; Sinor *et al*. 2000). Although most of the actions of cannabinoids in the central nervous system appear to be exerted by the 'brain type' cannabinoid receptor 1, CB1 (Matsuda *et al*. 1990; Ledent *et al*. 1999; Zimmer *et al*. 1999), some cannabinoids such as the non-CB1-binding components of *Cannabis sativa*, cannabinol and cannabidiol, were also shown to protect cells from oxidative stress (Hampson *et al*. 1998; Chen and Buck 2000). These observations would indicate a completely CB1-independent mechanism of neuroprotection of cannabinoids. Indeed, for many natural and synthetic phenolic compounds, a protective activity against oxidative stress, independent of any specific receptor-mediated action, has recently been proposed, e.g. for oestrogens (Moosmann and Behl 1999). However, CB1 has been implicated in some neuroprotective mechanisms. WIN 55,212-2, a potent CB1 agonist belonging to the family of aminoalkylindoles, exerted potent neuroprotection in *in vivo* rat models of global and focal ischaemia (Nagayama *et al*. 1999), which was blocked by the pre-administration of the specific CB1 antagonist SR 141716A, suggesting an involvement of CB1 in this particular paradigm.

In the present study, the antioxidant neuroprotective effects of cannabinoid compounds and the involvement of CB1 in

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*Abbreviations used*: AIBN, 2,2′-azo-bis-(2-methylpropionamidine); AM 404, all-Z Eicosa-5,8,11,14-tetraenoic acid (4-hydroxy-phenyl)-amide; CB1, cannabinoid receptor 1; CBN, CB1-null mutant mouse line; CP 55,940, (-)-cis-3-[2-hydroxy-4-(1,1-dimethylheptyl)phenyl]-trans-4-(3-hydroxypropyl) cyclohexanol; $\Delta^9$-THC, $\Delta^9$-tetrahydrocannabinol; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; FSK, forskolin; HU 210, (6aR)-trans-3-(1,1-dimethylheptyl)-6a,7,10a-tetrahydro-1-hydroxy-6,6-dimethyl-6H-dibenzo[b,d]pyran-9-methanol; IBMX, 3-isobutyl-1-methylxanthine; LDL, low-density lipoprotein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; SR 141716A, N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-1H-pyrazole-3-carboxamide HCl; WIN 55,212-2, (R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinylmethyl)pyrrolo[1,2,3-de]-1,4-benzoxazin-6-yl]-naphtalenylmethanone.

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these effects were analysed in detail, employing cell-free biochemical assays and cell culture models of oxidative stress and neurodegeneration. First, several cannabinoid compounds were classified on the basis of their chemical structure and according to their ability to bind to CB1, and a structure–activity relationship analysis was performed in different cell-free biochemical assays to test their chemical antioxidant potential. Their neuroprotective effects were then tested in cellular models of oxidative stress, i.e. in neuronal cell lines and in rat primary cerebellar granule cells. Subsequently, in order to analyse the involvement of CB1 in the neuroprotective effects of various cannabinoid compounds, two genetic approaches were adopted. First, cannabinoids belonging to different subgroups (classed on their ability to activate CB1 and their chemical antioxidant properties) were tested in oxidative cell death assays, using cell lines stably transfected with CB1 cDNA and control-transfected cells lacking CB1. Second, a potent CB1 agonist (CP 55,940) was tested as neuroprotective agent in primary cerebellar granule cell cultures derived either from CB1 knock-out mice or from wild-type littermate controls. We found that CB1 is not directly involved in the mechanism(s) by which antioxidant cannabinoids protect cells from oxidative stress *in vitro*.

## Materials and methods

### Chemicals

Cannabinoids ($\Delta^9$-THC, cannabinol and cannabidiol) were purchased from Sigma (Deisenhofen, Germany), CP 55,940, WIN 55,212-2, methanandamide, HU 210 and AM 404 were purchased from Tocris (Cologne, Germany) and SR 141716A was from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program. These compounds were prepared as 10 mM stock solutions in 100% ethanol ($\Delta^9$-THC, cannabinol, cannabidiol and methanandamide) or in 100% dimethylsulfoxide (DMSO; CP 55,940, WIN 55,212-2, HU 210, AM 404 and SR 141716A). IBMX and forskolin (FSK), were purchased from Sigma and were prepared as 200 mM and 10 mM stock solutions in DMSO, respectively.

### Animals

Sprague–Dawley rats and mice with a deficiency in the CB1 gene were used for the study. The generation of the CB1-deficient mouse line CBN/CBN, lacking the entire CB1 open reading frame, will be described elsewhere (G. Marsicano *et al.* in preparation). For genotyping of the CBN allele, PCR was performed with DNA derived from tails of 1–2-day-old-pups. Wild-type (WT) and homozygous CBN/CBN mice (CB1 KO) were used for the experiments. The experimental protocols were approved by the Ethical Committee on Animal Care and Use of the Government of Bavaria, Germany.

### Biochemical oxidation assays

*Brain lipid oxidation assays*

For the preparation of native brain lipids, dissected cerebral cortex of adult Sprague–Dawley rats was homogenized in 3 volumes of degassed lipid buffer (20 mM Tris–HCl, 1 mM $MgCl_2$, 5 mM KCl, pH 7.4) with a Kontes glass homogenizer (all preparative steps were performed at 4°C). After centrifugation (3000 *g*, 5 min), the pellet was solubilized by sonication in 3 volumes of lipid buffer supplemented with 0.5 M NaCl, incubated for 10 min and centrifuged (100 000 *g*, 20 min). This step was repeated and followed by three washing steps using 3 volumes of degassed water instead of lipid buffer. Finally, the pellet was resuspended in water at a concentration of 5 mg/mL protein, snap-frozen in liquid nitrogen and stored at −80°C.

For the oxidation assay, the rat brain membrane preparation was diluted with phosphate-buffered saline (PBS) to a concentration of 0.6 mg/mL protein and sonicated. Cannabinoids to be tested were added to the 1-mL aliquots at various concentrations and the oxidative chain reaction was started by adding 50 μM ascorbate and incubation at 37°C. Six hours later, single photon counting was done for 1 min in a Beckman scintillation counter set in the visible light range. Data were corrected for the baseline photocurrent and normalized to control values.

*Low-density lipoprotein oxidation assays*

The oxidation of human blood plasma low-density lipoprotein (LDL) was essentially performed as described (Moosmann and Behl 1999). In brief, fresh human LDL (0.1 mg/mL protein, diluted in PBS supplemented with 0.5 mM $MgCl_2$) was oxidized catalytically by the addition of 10 μM $CuSO_4$ at 37°C with or without the concomitant addition of the cannabinoids. The reaction products of LDL decomposition (conjugated dienes) were measured photometrically as an increase in UV absorption at 234 nm. Results were expressed as percentage (mean ± SEM) of the increase in absorption after 1 h of the cannabinoid-treated samples versus the vehicle-treated samples; the absorption of the blank samples (without oxidant) at 234 nm did not change during the assay time; the same applied to all the measured samples at a wavelength of 600 nm, indicating a stable LDL sample solution.

*Peroxyl radical scavenging assays*

To establish the direct radical scavenging properties of the cannabinoids in aqueous medium, they were challenged by a hydrophilic azo-initiator of free peroxyl radicals, 2,2′-azo-bis-(2-methylpropionamidine), AIBN. The carbon-centred radicals initially formed by the thermal decomposition of this compound react very fast with dissolved aqueous oxygen to form peroxyl radicals which in turn attack nucleophilic sites on biomolecules. As a biological indicator of the free peroxyl radical load, the phycobilinosomal fluorescent protein *Porphyrium cruentum* B-phycoerythrin was employed, and the temporal decrease in protein fluorescence was measured as a consequence of the free radical-induced structural decomposition of the protein. 10 nM B-phycoerythrin in PBS were oxidized employing 500 μM AIBN at 37°C; the temporal decrease in intrinsic protein fluorescence was quantified by a flash fluorimetry setting (excitation window 340 ± 50 nm, emission window 572 ± 6 nm, counting delay 25 μs, counting window 30 μs, number of repeats per sample 1000). Under the employed conditions, no significant photobleaching occurred, and the control protein kept stable for several hours. Results were calculated by comparing the decrease in fluorescence of the cannabinoid-treated samples versus the not treated samples (AIBN only) at three measuring times, after

190, 200 and 210 min. Data are presented as the grand mean ± SEM of all individual measurements (quadruplicate determinations at each measuring time).

### Cell cultures

*Cell lines*

The neuronal cell lines PC12 and HT22 (kind gift of Dr Dave Schubert, Salk Institute, San Diego, CA, USA) were used for oxidative stress experiments. Cells were cultured in complete DMEM at 37°C, 5% $CO_2$ in humidified atmosphere. Complete DMEM consists of Dulbecco's modified Eagle's medium (Life Technologies Gibco, Karlsruhe, Germany), supplemented with 15% (PC12) or 10% (HT22) fetal calf serum (FCS, Life Technologies Gibco) and 100 U/mL penicillin and 100 μg/mL streptomycin (100× penicillin/streptomycin solution, Life Technologies Gibco). Cells were kept on 10-cm cell culture dishes. One day prior to the experiment, cells were plated onto 96-well plates at a density of $10^4$ cells/well (PC12 cell line), $10^3$ cells/well (HT22 cell line), each well containing 100 μL of complete DMEM.

*Primary cerebellar granule cells*

Primary cerebellar granule cells were obtained from newborn Sprague–Dawley rats (3 days old) and from newborn mice (3–6 days old). The procedures for isolation and culture of primary cerebellar granule cells were identical for rats and mice. Newborn animals were decapitated, and cerebella were isolated and put into ice-cold, sterile DMEM containing 100 U/mL penicillin/streptomycin. Cerebella were dissected free of meninges and put into a 50-mL Falcon tube containing 10 mL of cold DMEM. Then, 10 mL of 0.1% Trypsin/1 mM EDTA were added to the tube. Cerebella were incubated with gentle shaking for 10–20 min at 37°C and then pipetted up and down first with a 5-mL plastic pipette and then with a Pasteur glass pipette. Tissues were centrifuged at 500 *g* for 4 min and then resuspended in 10 mL complete DMEM medium. After a new centrifugation and resuspension in 1 mL of complete DMEM, 100 μl of cells were plated onto poly-L-lysine-treated (Sigma) 96-wells plates, at a density of about $10^5$ cells/well. After 2 days of incubation, the cytostatic drug cytosine arabinofuranoside (Sigma) was added to each well at a final concentration of 10 μM, in order to block the growth of fibroblasts and glial cells. After 10–15 days of incubation, the cultures appeared to contain > 90% neurons and were used in oxidative stress experiments.

### Establishment of CB1-expressing cell lines

The mouse CB1 cDNA was subcloned into the eukaryotic expression vector pcDNA3 (Invitrogen, Groningen, the Netherlands), which was then used for the stable transfection of CB1 into HT22 cells. Cells were electroporated with 1 μg/μL of linearized plasmid and selected with 3.5 mg/mL geneticin (G418, Life Technologies Gibco), following standard procedures.

*Northern blot analysis*

RNA extraction from transfected clonal cells was performed using the PeqGold RNApure kit (PeqLab, Erlangen, Germany), following the manufacturer's instructions. RNA samples were electrophoresed in a 1% agarose/6% formaldehyde gel under standard conditions (Sambrook *et al*. 1989). After electrophoresis, capillary blotting was performed in 10 × saline–sodium citrate buffer (SSC), as described in Sambrook *et al*. (1989) to transfer RNA onto Hybond NX membranes (Amersham/Pharmacia, Freiburg, Germany). Hybridization was performed using a mouse CB1-specific probe.

*cAMP accumulation assay*

One day before the experiment, cell clones expressing CB1 mRNA were plated onto 48-well plates in 500 μL of complete DMEM at the density of $2 \times 10^5$ cells/mL. On the next day, cells were washed twice with DMEM to remove serum, and incubated for 1 h. Then, 0.5 mM IBMX (Sigma, Deisenhofen, Germany) was added 5 min before the initiation of the reaction to prevent the degradation of accumulated cAMP. Cells were pre-incubated with WIN 55,212-2 alone or in combination with the CB1 antagonist SR 141716A for 1 h. Then, 5 μM forskolin (FSK, Sigma) were added. The reaction was terminated 1 h later by aspiration of the medium and addition of 500 μL ice-cold 6% trichloroacetic acid followed by an incubation overnight at 4°C. CB1 receptor ligands were dissolved in DMSO. DMSO alone served as a vehicle control and had no effect on cAMP accumulation (data not shown). To remove the trichloroacetic acid, the extracts were treated twice with 3 mL diethylether, dried overnight in a lyophilisator and reconstituted in DMEM. Intracellular cAMP levels were measured with a competitive protein binding assay following the manufacturer's recommendations (non-acetylated procedure; NEN Life Science Products, Inc., Boston, MA, USA). Data obtained in the cAMP accumulation assays were expressed as percentage of FSK-stimulated cAMP accumulation. Samples were measured in duplicate in two independent experiments. Data are given as mean percentages with standard error of mean (SEM).

### Oxidative stress toxicity assays

The day before the experiment, cell lines were plated onto 96-well plates as described above. Cannabinoids were prepared as predilutions in ethanol or DMSO and added to the wells. All wells contained a final concentration of 1% ethanol or 1% DMSO. Experiments were performed in triplicate or quadruplicate and were repeated at least twice for each cell clone or primary cell culture. After adding the drug or vehicle, cells were incubated overnight, and then different concentrations of $H_2O_2$ were added to obtain final concentrations ranging from 60 to 250 μM. After overnight incubation, 10 μL of 5 mg/mL of dimethylthiazolyl-diphenyl-tetrazolium bromide in $H_2O$ (MTT, Sigma) were added to each well, and the plates were incubated at 37°C for 4 h. Then, 100 μL of cell lysis solution [45% dimethylformamide, 10% sodium dodecyl sulfate (SDS), pH 4.2] were added to each well. Lysis was done overnight at room temperature, and then the plates were read with a Dynatec microplate reader (Dynatec, El Paso, TX, USA) set at 570 nm. Decreased cell survival was indicated by a decreased MTT reduction, and thus, by a decreased absorption at 570 nm. Data were calculated as relative protection and are given as averages of the triplicate or quadruplicate experiments with SEM.

## Results

### Cannabinoids as antioxidant neuroprotective agents

Eight compounds were tested in biochemical oxidation experiments: four 'classical cannabinoids' ($\Delta^9$-THC,

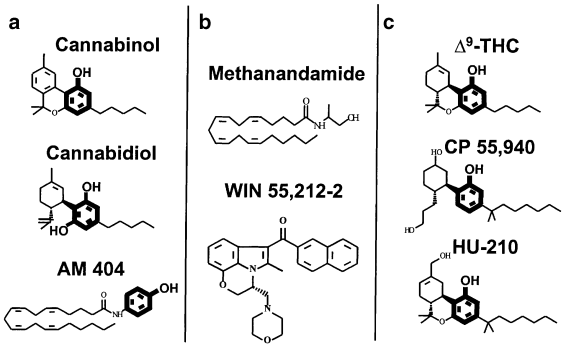


**Fig. 1** Classification of cannabinoids on the basis of CB1 binding and the presence of phenolic moieties (in bold). (a) Phenolic cannabinoids with no or very low affinity for CB1. (b) Non-phenolic compounds with high affinity for CB1. (c) Phenolic cannabinoids with high affinity for CB1.

cannabinol and cannabidiol as *Cannabis sativa* derivatives, and the synthetic HU 210); one synthetic 'non-classical cannabinoid' (CP 55,940); one aminoalkylindole (WIN 55,212-2); one metabolically stable synthetic analogue of anandamide (methanandamide); one inhibitor of 'anandamide transporter' (AM 404). These compounds can be classified into three groups, based on whether or not they have a phenolic structure and whether or not they bind to cannabinoid receptors: phenolic compounds that do not bind to CB1 (Fig. 1a); non-phenolic compounds that bind to CB1 (Fig. 1b); and phenolic compounds that bind to CB1 (Fig. 1c).

The antioxidant properties of these compounds were evaluated in various biochemical assays (Fig. 2a–c). Compounds containing a phenolic group, such as cannabinol, cannabidiol and AM 404 (not binding to CB1, Fig. 2a1), and $\Delta^9$-THC, CP 55,940 and HU 210 (binding to CB1, Fig. 2a3) were shown to be potent antioxidants in the brain lipid oxidation assay. However, cannabinoids not containing a phenolic ring, such as methanandamide and WIN 55,212-2 did not show any antioxidant activity in the same assay (Fig. 2a2). Similar results were obtained in the blood plasma LDL oxidation assay (Fig. 2b), with one exception: cannabinol was not protective. This discrepancy could be due to intrinsic molecular characteristics of cannabinol, which contains a biphenyl moiety that confers rigidity to the molecule and may prevent the penetration into the LDL particles. Figure 2(c) shows that all compounds were unable to protect globular proteins from oxidative destruction in aqueous medium, as it was expected from the lipophilic character of the compounds.

Chemical antioxidant properties often reflect the protective potential of the tested compounds in oxidative toxicity experiments in HT22 cells, as shown for oestrogenic drugs (Moosmann and Behl 1999). Therefore, this notion was tested here for cannabinoids. Indeed, the same compounds that showed biochemical antioxidant properties (Fig. 2a1 and a3) also exhibited potent cytoprotection from $H_2O_2$-induced oxidative cell death in HT22 cells (Fig. 2d1 and d3), whereas the non-phenolic compounds were not able to protect the cells from oxidative cell death (Fig. 2d2). Similar results were obtained in oxidative stress assays conducted on PC12 cells (data not shown). Thus, phenolic cannabinoids are antioxidant, cytoprotective drugs.

## Cannabinoids are neuroprotective in rat cerebellar granule cells

As HT22 and PC12 cells do not express CB1 (see below, Fig. 4 and data not shown), it is not possible to analyse the participation of CB1 to the neuroprotective activity of cannabinoids by experiments conducted using these cell lines. Therefore, as a source of CB1-expressing primary neurons, rat cerebellar granule cell cultures, which are known to contain CB1 protein (e.g. Hillard *et al*. 1999), were assessed and tested in similar oxidative stress paradigms. Three CB1 agonists were chosen for these tests, the non-phenolic compound methanandamide, and the two phenolic compounds CP 55,940 and HU 210. Figure 3(a) shows that CP 55,940 and HU 210 possess similar neuroprotective potentials in cerebellar neurons as in HT22 cells (Fig. 2d3). These results indicate that phenolic cannabinoids are able to effectively protect primary granule cells against oxidative nerve cell death. In contrast, methanandamide did not have any neuroprotective effect on granular neurones at any of the concentrations tested (Fig. 3a).

## Cannabinoid-mediated neuroprotection in a neuronal cell line expressing CB1

Antioxidant CB1 agonists protect cells that express CB1 (primary cerebellar cultures) or that lack CB1 (neuronal cell lines), suggesting a purely chemical antioxidant mechanism of action. However, to rule out definitely the participation of the receptor to the cannabinoid-induced neuroprotection, it is necessary to test the protection potential of the drugs in identical cellular model systems that differ only in the expression of CB1 and to compare the pharmacological effects in its presence or absence.

Therefore, HT22 cells were stably transfected with an expression vector coding for the mouse CB1, and G418-resistant clones were analysed by northern blot. Hybridization signals were detected at approximately 6.0 kb for cortex RNA (used as positive control), as previously described (Matsuda 1997) and at approximately 1.6 kb for many G418-resistant CB1-transfected clones (Fig. 4a). cAMP accumulation assays revealed that some of the clones expressing CB1 mRNA also expressed a functional receptor, as the presence of the potent CB1 agonist WIN 55,212-2 was able to decrease the forskolin (FSK)-induced cAMP accumulation in CB1-expressing cells (HT22 CB1), but not in cells

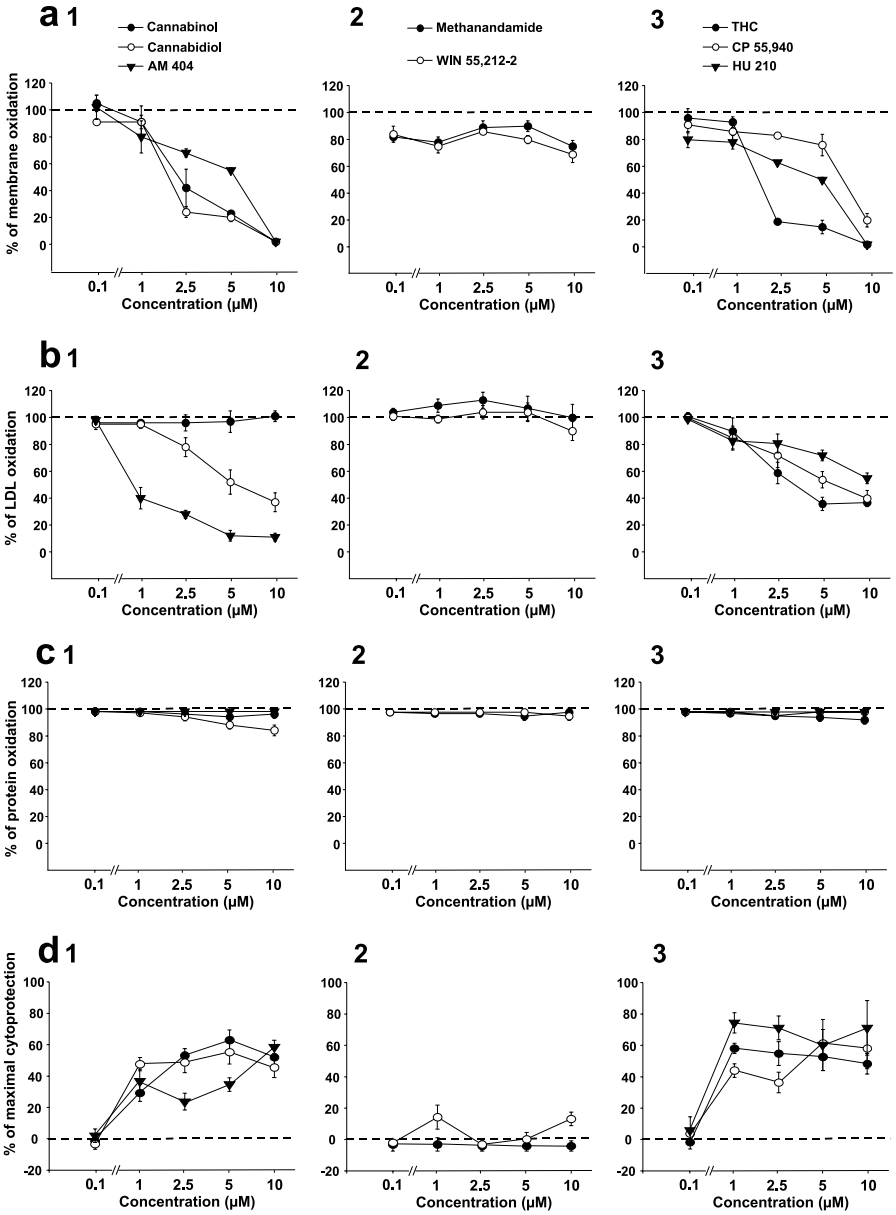


**Fig. 2** Antioxidant properties of cannabinoids and protection against oxidative stress in HT22 cells. (a) Percentages of oxidation of rat brain lipids as induced by 50 μM ascorbate. (b) Percentages of oxidation of human blood plasma LDL as induced by 10 μM copper sulfate. (c) Percentage of oxidative destruction of globular proteins by peroxyl radicals deliberated from an azo compound (AIBN). (d) Percentages of protection against oxidative stress in HT22 cells, induced by 120 μM of $H_2O_2$. In (a–c), 100% indicates maximal oxidation in the absence of any cannabinoid. In (d), 0% and 100% indicate the value in the absence of cannabinoids (only $H_2O_2$) and the value for untreated cells (i.e. without cannabinoids and without $H_2O_2$), respectively. Data are indicated as means ± SEM.

transfected with an empty vector (HT22 WT) or in parental cells (Fig. 4b and data not shown). The extent of reduction of FSK-induced cAMP accumulation was approximately 30%, consistent with reported values in other heterologous CB1 expression systems (Song and Bonner 1996). After confirming that the clonal cells expressed a functional CB1, HT22 CB1 and HT22 WT were used for oxidative stress assays in the presence of cannabinoids. The four CB1 agonists to be tested were chosen in light of their previously determined effects in the antioxidant assays and in the oxidative stress assays in parental HT22 cells and primary cerebellar granule cell cultures (Figs 2d and 3). $\Delta^9$-THC and CP 55,940 were used as prototypes of the phenolic antioxidant group, while methanandamide and WIN 55,212-2 represented non-phenolic and non-antioxidant compounds. As shown in Fig. 5(a and b), the two phenolic

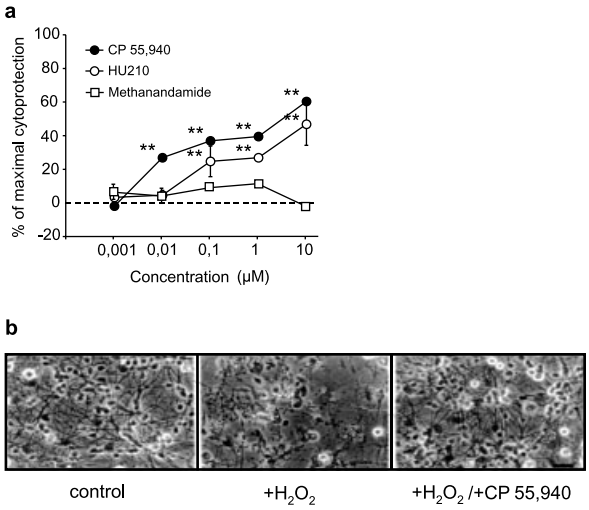


**Fig. 3** Cannabinoid-mediated neuroprotection in oxidative stress assays on cultured primary rat cerebellar granule cells. (a) Neuroprotective effects of CP 55,940 and HU 210 (phenolic antioxidant CB1 agonists) and methanandamide (non-phenolic CB1 agonist). (b) Phase-contrast micrographs showing examples of CP 55,940-mediated neuroprotection in cultured primary rat cerebellar granule cells. In (a), results are presented as in Fig. 2(d). $^{**}p < 0.01$, as compared to control (Student's *t*-test; $n = 3$).

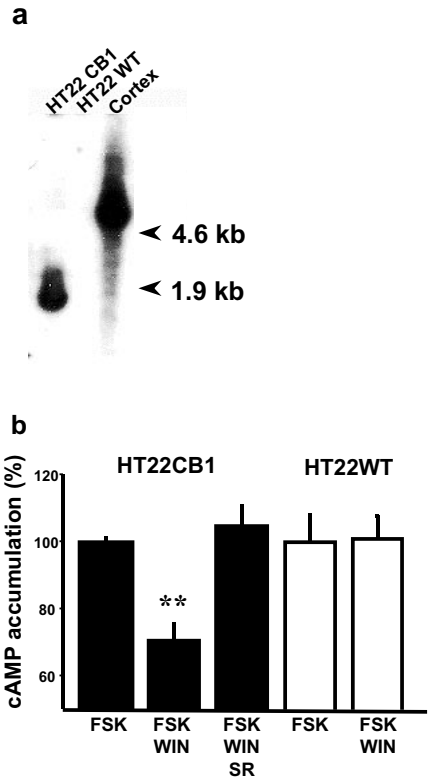


**Fig. 4** Heterologous expression of mouse CB1 in HT22 cells. (a) Northern blot analysis of total RNA from HT22 cell clones after stable transfection with an expression vector containing the mouse CB1 cDNA (HT22 CB1) and an empty control vector (HT22 WT). Ribosomal RNA was used as molecular weight marker (28S, approximately 4.6 kb; 18 S, approximately 1.9 kb). The CB1 open reading frame was used as hybridization probe. Mouse cortex RNA, containing high levels of CB1 mRNA (about 6.0 kb; Matsuda 1997), was used as positive control. The positive signal in clone HT22 CB1 corresponds to a band of about 1.6–1.7 kb, consistent with the expected size. (b) cAMP accumulation assays using the same clonal cells as in (a). Results are presented as percentages of the control stimulus induced by 5 μM forskolin (FSK). WIN is the CB1 agonist WIN 55,212-2 (1 μM), SR is the CB1 antagonist SR 141716A (1 μM). (■) Clone HT22 CB1; (□) clone HT22 WT. Data are means ± SEM $^{**}p < 0.01$, as compared to FSK control (Student's *t*-test, $n = 4$).

compounds $\Delta^9$-THC and CP 55,940 were able to protect HT22 WT cells up to values of approximately 70% and 60%, respectively. However, no differences were observed between the HT22 WT and the CB1-expressing cells HT22 CB1. The dose–response curves were basically identical, thus indicating that the presence of CB1 was altering neither the efficacy nor the potency of the drugs. Once again, methanandamide (Fig. 5c) showed no ability to protect cells from oxidative stress, neither in the absence nor in the presence of CB1. Also WIN 55,212-2 (Fig. 5d) did not show any significant protective effect even at concentrations as high as 10 μM. These observations indicate that CB1 is not required for the protective activity of cannabinoids in *in vitro* oxidative stress toxicity paradigms in neuronal cell lines.

### Cannabinoid-mediated neuroprotection in primary cerebellar granule cells from CB1-deficient mice

Neuronal cell lines, such as HT22, are of clonal origin and represent good models for neurotoxicity studies. Nevertheless, they share only a limited amount of characteristics with primary neurones. Therefore, we wondered whether the presence of CB1 in freshly prepared primary neurones could influence the neuroprotective actions of CB1 agonists. In order to address this notion, primary cerebellar granule cell cultures were assessed from WT and CB1 knock-out mice (CB1-KO). CP 55,940 was used as phenolic antioxidant CB1 agonist, and its effects were compared between WT and CB1-KO primary cerebellar cultures. Figure 6 shows that CP 55,940 was able to protect both WT and CB1-KO neurons. However, no differences were observed between WT and CB1-KO cells. These results clearly show that CB1 is not necessary for the neuroprotective activity of a potent CB1 agonist in oxidative stress assays using $H_2O_2$.

## Discussion

In this study, the neuroprotective properties of cannabinoids were analysed in *in vitro* oxidative stress assays. Oxidative stress represents one of the major events that occur during neurodegeneration in many neurological diseases such as

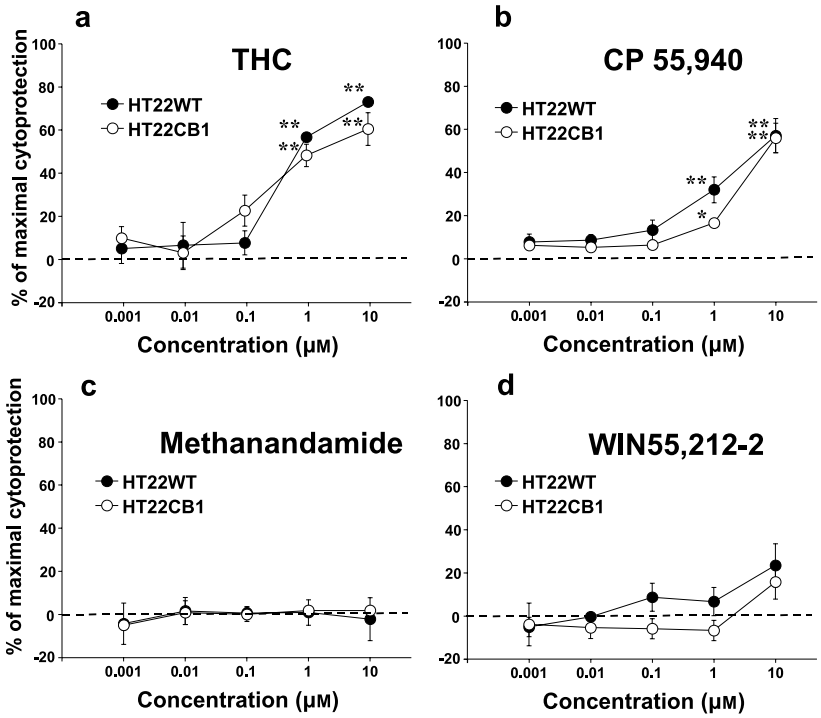


**Fig. 5** Cannabinoid-mediated neuroprotection in oxidative stress assays on HT22 cells expressing CB1 (HT22 CB1) and not expressing CB1 (HT22 WT). Results are presented as in Fig. 2(d). Data are means ± SEM $^{*}p < 0.05$; $^{**}p < 0.01$, as compared to control (Student's *t*-test, $n = 4$). No significant differences were observed between the two genotypes.

Alzheimer's disease (Simonian and Coyle 1996; Behl 1999a; Markesbery and Carney 1999). Oxidative neurodegeneration can be mimicked *in vitro* through the toxicity of $H_2O_2$ which is a mediator of various disease-related neurotoxins (Behl *et al*. 1994). Therefore, drugs that are able to inhibit these oxidative processes are promising candidates for the treatment of such diseases. Chemical antioxidants, e.g. vitamin E (Behl 1999b), are examples for these neuroprotective drugs. Many cannabinoids have structural features typical for phenolic antioxidants and, on the other hand, through CB1 activation, they are able to inhibit the excitability of the cells, by increasing $K^+$ and decreasing $Ca^{2+}$ permeability (for review see Pertwee 1997). Therefore, they could potentially exert neuroprotective activities through different mechanisms and, thus, possess very interesting therapeutic potential for the treatment of several neurodegenerative diseases.

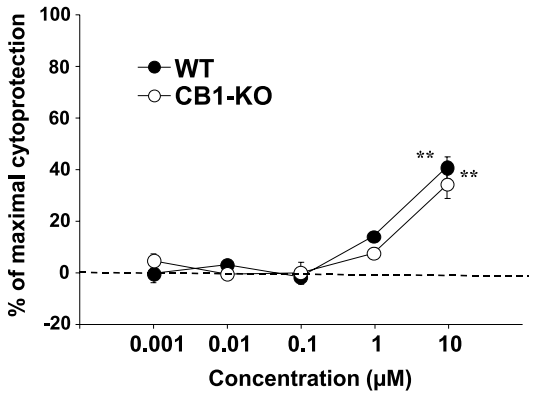


**Fig. 6** Cannabinoid-mediated neuroprotection (CP 55,940) in oxidative stress assays on primary cerebellar granule cell cultures derived from wild-type mice (WT) and homozygous CBN/CBN littermates (CB1-KO). Results are presented as in Fig. 2(d). Data are expressed as mean ± SEM. $^{**}p < 0.01$, as compared to control (Student's *t*-test, $n = 4$). Note the lack of significant differences between the two genotypes.

Several cannabinoids were tested in cell-free biochemical antioxidant assays and, as expected, phenolic compounds ($\Delta^9$-THC, cannabinol, cannabidiol, CP 55,940, HU 210 and AM 404) were found to be potent lipophilic antioxidants. These investigations were extended by *in vitro* oxidative stress toxicity experiments, using clonal neuronal cell lines (HT22 and PC12) and rat primary cerebellar granule cells: antioxidant phenolic cannabinoids were also protective against oxidative stress in the cells. These experiments did not completely rule out the putative participation of CB1 to the neuroprotective effects of cannabinoid compounds. One possibility to analyse the involvement of CB1 in the neuroprotective antioxidant effects of CB1 agonists would be to use the specific CB1 antagonist SR 141716A in the same neurotoxicity assays (Nagayama *et al*. 1999). However, SR 141716A was shown to exert, both *in vivo* and *in vitro*, more complex actions than a simple antagonistic effect at CB1 receptors. In CB1-transfected cells and in endogenously CB1-expressing neuronal cell lines, SR 141716A was shown to act as an inverse agonist (Shire *et al*. 1999; Meschler *et al*.

2000). Furthermore, in primary cerebellar granule cells, SR 141716A was recently shown to have different effects on cannabinoid-mediated inhibition of $Ca^{2+}$ mobility in different subcellular portions of the neurones. A mere antagonistic effect was observed at CB1 receptors located in the neurites, whereas a paradoxical 'agonist-like' inhibition of $Ca^{2+}$ influx was observed in the soma of the neurons (Hillard *et al*. 2000). Therefore, the involvement of CB1 in cannabinoid-mediated neuroprotection was checked by two genetic approaches, i.e. in heterologously CB1-expressing cell lines as compared to control-transfected lines and in primary cerebellar neurones derived from wild-type and CB1-deficient mice (CBN mouse line; G. Marsicano *et al.*, manuscript in preparation). No differences were observed in the neuroprotective activity of the tested drugs in presence or in absence of CB1, neither in the cell line nor in primary cerebellar cultures. These results correlate with the data from Chen and Buck (2000). These authors used a different model of oxidative stress in non-neuronal cell lines and found a CB1-independent protection caused by several cannabinoids. However, they showed the presence of CB1 in their cell lines by RT-PCR and did not provide any data using the same cell type with and without CB1. The present results strongly extend the concept of the antioxidant action of cannabinoids, providing direct evidence for the independence of such activity from CB1.

Oxidative stress is one of the central events onto which many neurodegenerative cascades converge. Therefore, $H_2O_2$-induced oxidative cell death *in vitro* is a clear paradigm of neurodegeneration that can provide useful information about the neuroprotective aspects of certain pharmacological compounds. Indeed, $H_2O_2$ is known to be a mediator of oxidative apoptosis in neuronal cells (Behl *et al*. 1994; Maher and Davis 1996; Chun *et al*. 2001). Here, we clearly show that cannabinoids possess protective antioxidant properties that are independent of the presence of the cannabinoid receptor CB1. In addition to the direct antioxidant activity of the phenolic cannabinoids, these compounds may further affect membrane-associated and intracellular signalling mechanisms. For instance, due to their lipophilicity, these compounds could increase the membrane fluidity and may eventually lead to changes in the activity of membrane-bound receptor systems (e.g. neurotransmitter receptors). Moreover, molecular interactions with intracellular signalling processes could be similar to those that are known to be executed by 17β-estradiol, which is another phenolic neuroprotective antioxidant acting independently of its cognate oestrogen receptors (Moosmann and Behl 1999). In addition to its wide range of oestrogen receptor-dependent effects, oestradiol performs various receptor-independent neuromodulatory activities including also the activation of the neuroprotective mitogen-activated protein kinase signalling (Behl and Holsboer 1999), which is known to be also activated by cannabinoids, both in a CB1-dependent (Valjent *et al*. 2001) and CB1-independent manner (Jan and Kaminski 2001). Finally, CB1-dependent effects of cannabinoids could be involved in upstream events that eventually could affect intracellular oxidative pathways. As an example, it is likely that activation of CB1 might inhibit glutamate toxicity by its counteracting hyperpolarizing action (Di Marzo *et al*. 1998; Hampson *et al*. 1998; Piomelli *et al*. 2000; Hampson and Grimaldi 2001). Indeed, CB1 was shown to be involved in some neuroprotective actions of cannabinoids *in vivo* (Nagayama *et al*. 1999) and is up-regulated in particular brain areas during experimental ischaemia in rats (Jin *et al*. 2000). Endocannabinoids are also increased in brain after closed-head injury and can mediate neuroprotective properties in the same paradigm by a CB1-dependent mechanism (Panikashvili *et al*. 2001). These data suggest a general neuroprotective function of the endocannabinoid system. Neuroprotective activities of endocannabinoids appear to be CB1-mediated and do not involve antioxidant properties due to their lack of phenolic moieties, which are the mediators of antioxidant neuroprotection of several exogenous cannabinoid drugs. Therefore, it is tempting to propose potent therapeutic applications for drugs that are able to both sustain the 'endogenous' CB1-mediated neuroprotective activity of endocannabinoids and to provide antioxidant protection. Good candidates are inhibitors of endocannabinoid uptake, such as AM 404 and the newly developed compound VDM 11 (De Petrocellis *et al*. 2000), both of which contain a phenolic residue. In addition, given the 'upon demand' activation of the endocannabinoid system (Di Marzo *et al*. 1998; Piomelli *et al*. 2000), using endocannabinoid uptake inhibitors might diminish the undesirable psychotropic side-effects generally observed after treatment with CB1 agonists. In conclusion, the use of antioxidant cannabinoids or, in particular, the inhibition of endocannabinoid uptake by antioxidant drugs could provide promising avenues for the therapeutic targeting of different aspects of neurodegenerative diseases, by stimulating a self-protective endogenous system of the brain and by counteracting oxidative stress.

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# The endogenous cannabinoid system protects against colonic inflammation

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# The endogenous cannabinoid system protects against colonic inflammation

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**Excessive inflammatory responses can emerge as a potential danger for organisms' health. Physiological balance between pro- and anti-inflammatory processes constitutes an important feature of responses against harmful events. Here, we show that cannabinoid receptors type 1 (CB1) mediate intrinsic protective signals that counteract proinflammatory responses. Both intrarectal infusion of 2,4-dinitrobenzene sulfonic acid (DNBS) and oral administration of dextrane sulfate sodium induced stronger inflammation in CB1-deficient mice (*CB1*$^{-/-}$) than in wild-type littermates (*CB1*$^{+/+}$). Treatment of wild-type mice with the specific CB1 antagonist *N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716A) mimicked the phenotype of *CB1*$^{-/-}$ mice, showing an acute requirement of CB1 receptors for protection from inflammation. Consistently, treatment with the cannabinoid receptor agonist *R*(-)-7-hydroxy-Δ$^6$-tetra-hydrocannabinol-dimethylheptyl (HU210) or genetic ablation of the endocannabinoid-degrading enzyme fatty acid amide hydrolase (FAAH) resulted in protection against DNBS-induced colitis. Electrophysiological recordings from circular smooth muscle cells, performed 8 hours after DNBS treatment, revealed spontaneous oscillatory action potentials in *CB1*$^{-/-}$ but not in *CB1*$^{+/+}$ colons, indicating an early CB1-mediated control of inflammation-induced irritation of smooth muscle cells. DNBS treatment increased the percentage of myenteric neurons expressing CB1 receptors, suggesting an enhancement of cannabinoid signaling during colitis. Our results indicate that the endogenous cannabinoid system represents a promising therapeutic target for the treatment of intestinal disease conditions characterized by excessive inflammatory responses.**

## Introduction

Colon pathologies span a wide range of different conditions, including frankly inflammatory bowel diseases (ulcerative colitis and Crohn disease) and so-called functional bowel diseases (e.g., irritable bowel syndrome), and represent an important and widespread health problem in modern society (1, 2). The occurrence of an enteric infection, trauma, or inflammation has been suggested to be related to the initiation of these diseases (2, 3). During the last decade, several experimental animal models of inflammatory bowel diseases have been developed to define the different components of the pathophysiological processes that characterize these disorders. Among these models, the intrarectal administration of 2,4-dinitrobenzene sulfonic acid (DNBS) and the oral administration of dextrane sulfate sodium (DSS) have been extensively used to study the mechanisms of colonic inflammation and to test anti-inflammatory drugs (4, 5). Infections, traumata, or chemical insults are believed to induce several cellular reactions, which eventually lead to an inflammatory status of the colon. At the same time, however, protective mechanisms aimed at preventing the pathological outcome of proinflammatory insults are also induced. Hence, the overall balance between pro- and anti-inflammatory mechanisms is likely to determine the progression and severity of colitis. A better understanding of intrinsic mechanisms that protect against inflammation would provide an effective starting point for development of novel therapeutic treatments.

The major active constituent of the plant *Cannabis sativa* (marijuana), Δ$^9$-tetrahydrocannabinol, and a variety of natural and synthetic cannabinoids have been shown to possess antinociceptive and anti-inflammatory activities (6–8). For millennia, *Cannabis* preparations have been used in folk medicine for the treatment of a wide variety of disorders, including those affecting the gastrointestinal tract (9). A century ago, extracts of *Cannabis* were used in the US to treat gastrointestinal pain of different origins, gastroenteritis, and diarrhea. There are also anecdotal reports suggesting that marijuana may be effective in alleviating symptoms of Crohn disease (10). Most of the biological actions of cannabinoids are mediated by two types of cannabinoid receptors, CB1 and CB2, both coupled to G proteins (11–13). Endogenous ligands for cannabinoid receptors (endocannabinoids) such as the agonists anandamide and 2-arachidonoyl-glycerol (2-AG) are lipidic messengers derived from arachidonic acid (11–13). After synthesis in the cell membrane, release into extracellular space, and action at cannabinoid receptors, endocannabinoids are

**Nonstandard abbreviations used:** 2-arachidonoyl-glycerol (2-AG); cannabinoid receptor type 1 (CB1); cannabinoid receptor type 2 (CB2); dextrane sulfate sodium (DSS); 2,4-dinitrobenzene sulfonic acid (DNBS); fatty acid amide hydrolase (FAAH); hexa-decyl-trimethyl-ammonium bromide (HTAB); in situ hybridization (ISH); myeloperoxidase (MPO); *N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716A); preproenkephalin (Enk); resting membrane potential (RMP); *R*(-)-7-hydroxy-Δ$^6$-tetra-hydrocannabinol-dimethylheptyl (HU210).

**Conflict of interest:** The authors have declared that no conflict of interest exists.

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rapidly inactivated by cellular uptake and degradation mediated by specific enzymes, among which the best-characterized is fatty acid amide hydrolase (FAAH) (12–14). The discovery of endocannabinoids and the characterization of the mechanisms for their synthesis and degradation evidenced the existence of the endogenous cannabinoid system, which exerts many physiological functions. Functional CB1 receptors are present on enteric neurons (15–20), and the gastrointestinal tract produces at least two endocannabinoids, namely anandamide and 2-AG (8, 20, 21). In fact, the endogenous cannabinoid system plays a role in the control of various functions, including gastroprotection, intestinal motility, and secretion (8). In the present investigation, we examined the role of the endogenous cannabinoid system after inflammatory insults in the colon and show that this system provides intrinsic protection against colonic inflammation.

## Methods

*Animals*. Eight- to ten-week-old CB1-deficient ($CB1^{-/-}$) and FAAH-deficient ($FAAH^{-/-}$) female mice and corresponding wild-type littermates were used for experiments (22, 23). Mice were in mixed genetic background, with a predominant C57BL/6N contribution (five backcrosses for both mutant lines). Eight- to ten-week-old female C57BL/6N mice were purchased from Charles River Wiga GmbH (Sulzfeld, Germany). Mice were housed under standard conditions and supplied with drinking water and food ad libitum. All animal procedures complied with the guidelines for care and use of laboratory animals of the government of the state of Bavaria, Germany.

*Materials*. DNBS was purchased from ICN Biomedicals GmbH (Eschwege, Germany), DSS from Sigma-Aldrich (Taufkirchen, Germany), and *R*(-)-7-hydroxy-$\Delta^6$-tetra-hydrocannabinol-dimethylheptyl (HU210) from Tocris Cookson Ltd. (Bristol, United Kingdom). *N*-(piperidino-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR141716A) was a gift from the National Institute of Mental Health's Chemical Synthesis and Drug Supply Program (USA).

*Induction of colitis*. After 36 hours of starving, colitis was induced by intrarectal administration of DNBS (5 mg per mouse) using a modification (24) of the method first described in rats (25). In pilot experiments, this dose of DNBS was found to induce reproducible colitis without mortality in heterozygous $CB1^{+/-}$ mice (data not shown). Briefly, mice were lightly anesthetized with isoflurane, and DNBS (5 mg in 100 μl of 50% ethanol) was infused into the rectum through a catheter (outer diameter 0.8 mm), inserted 4–5 cm proximally to the anus. Solvent alone (100 μl of 50% ethanol) was administered in control experiments. Colitis was also induced by oral administration of DSS (5% in drinking water for 7 days) as described in ref. 26.

*Pharmacological treatments*. Drugs were injected subcutaneously (20 ml/kg body weight) under light isoflurane anesthesia. SR141716A (3 mg/kg body weight) and HU210 (0.05 mg/kg body weight) were dissolved in vehicle solution (one drop of Tween-80 in 3 ml 2.5% dimethylsulfoxide in saline) and injected 30 minutes before and 24 hours and 48 hours after DNBS infusion. Vehicle solution was used in control experiments.

*Evaluation of colonic damage*. Mice were killed by cervical dislocation 3 days after DNBS treatment, unless otherwise specified, or 7 days after continuous DSS treatment. The colon was removed and rinsed gently with saline solution, then opened by longitudinal incision and examined immediately. Colonic damage was assessed by a semiquantitative scoring system originally established in rats (27) and adapted to mice for the present study. Morphology was scored according to the following scale: 0, no damage; 1, localized hyperemia without ulcers; 2, linear ulcers without significant inflammation; 3, one site of ulceration or inflammation; 4, two or more sites of ulceration or inflammation with a total extension less than 1 cm; 5, two or more sites of ulceration or inflammation with a total extension more than 1 cm longitudinally; 6–10, if damage covered more than 1 cm longitudinally, the score is increased by 1 for each additional 0.5 cm of damage. In the presence of diarrhea, the score is increased by 1.

*Determination of tissue myeloperoxidase activity*. Samples of colon were weighed, frozen, and processed for determination of myeloperoxidase (MPO) activity. MPO activity represents an index of neutrophil accumulation (28, 29). Tissue was suspended in hexadecyl-trimethyl-ammonium bromide (HTAB) buffer (0.5% HTAB [Sigma-Aldrich] in 50 mM potassium phosphate buffer, pH 6.0; 50 mg of tissue/ml) and disrupted using a homogenizer. HTAB is a detergent that releases MPO from the primary granules of neutrophils. After one freezing/thawing cycle of the homogenate and centrifugation (15–30 minutes, 16,000 *g*, 4°C), 0.1 ml of supernatant was added to 2.9 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml of *O*-dianisidine hydrochloride and 0.05% $H_2O_2$. Absorbance was measured at 460 nm (Beckman 640U photometer; Beckman Coulter GmbH, Unterschleißheim, Germany). MPO was expressed in milliunits per gram of wet tissue, 1 unit being the quantity of enzyme able to convert 1 μmol of $H_2O_2$ to water in 1 minute at room temperature. Units of MPO activity per minute were calculated from a standard curve using purified peroxidase enzyme (Sigma-Aldrich). As considerable variations in the magnitude of the MPO values were observed between batches of animals studied during a 12-month period, data for each experiment were normalized to the value obtained in control groups.

*In situ hybridization and neuron counting*. After isolation, the colons were snap-frozen on dry ice and stored at –80°C until sectioning. The colons were mounted on Tissue-Tek (Polysciences Europe GmbH, Eppelheim, Germany), and 20-μm-thick transverse sections were cut on a cryostat Microtome HM 560 (Microm International GmbH, Braunschweig, Germany). Sections were mounted onto frozen Superfrost Plus slides (Fisher Scientific GmbH, Hannover, Germany), dried on a 42°C warming plate, and stored at –20°C until use. $^{35}$S-labeled riboprobe for CB1 and preproenkephalin (Enk) were used for in situ hybridization (ISH). The probe for CB1 was generated as described previously (30, 31). The cDNA for the generation of the Enk riboprobe was synthesized by RT-PCR on whole mouse-brain RNA preparation, using the primers 5′-TGCACACTGGAATGTGAAGGA and 5′-CACAGACCCTAAAATCACAGC, corresponding to bp 57–77 and 887–867, respectively, of GenBank accession no. M13227. ISH and subsequent exposure to photographic emulsion (Kodak NTB-2 [Sigma-Aldrich] diluted 1:1 in distilled water) were carried out as described in detail in ref. 30.

Parallel sections, adjacent to the ones used for ISH, were fixed for 1 hour in Carnoy solution and were stained with cuprolinic blue (32) for total counts of myenteric neurons. The staining solution (0.3% cuprolinic acid and 1 M $MgCl_2$ in 0.025 M sodium acetate buffer, pH 5.6) was applied for 60 minutes at room temperature, and the sections were then rinsed in distilled water and placed in sodium acetate buffer (pH 5.6) containing 1 M $MgCl_2$. Finally, sections were rinsed in distilled water, placed in ethanol and xylene, and then mounted in DPX

(BDH Chemicals Ltd., Poole, United Kingdom). CB1-positive, Enk-positive, and cuprolinic blue–positive neurons (total population) were counted on four to six randomly chosen sections for each animal (three animals per group) by two independent observers blinded to animals' treatment and genotype. On each section, the length of serosa was measured, and the average number of cells per millimeter of serosal length was calculated for each mouse. Data were expressed as percentage of control groups (untreated *CB1*$^{+/+}$ mice).

*Electrophysiological experiments*. The colon was exposed by an abdominal midline incision. The complete large bowel was removed and placed into oxygenated Krebs solution of the following composition (in mM): NaCl 120.5, KCl 5.9, $MgCl_2$ 2.5, $NaH_2PO_4$ 1.2, $NaHCO_3$ 15.5, $CaCl_2$ 2.5, glucose 11.5, pH 7.4. The colon was opened along the mesenteric border, washed of remaining fecal material, and pinned out in a Sylgard-lined dissecting dish (Dow Corning Corp., Midland, Michigan, USA) containing oxygenated Krebs solution. The distal colon was separated, and mucosa and submucosa were removed, resulting in sheets of tissue consisting of circular and longitudinal muscle layers, together with the attached myenteric plexus. The sheets of distal colon were pinned using 150–200 wolfram wire micropins (15–25 μm in thickness) to the Sylgard-based electrophysiological chamber with the circular muscle layer uppermost. Experiments with *CB1*$^{-/-}$ and wild-type littermates at the same time point after induction of inflammation were performed simultaneously in one electrophysiological chamber. The chamber was perfused (5 ml/min; Kwik-peristaltic-pump, World Precision Instruments Inc., Sarasota, Florida, USA) with prewarmed (37°C) oxygenated (95% $O_2$/5% $CO_2$) Krebs solution. Tissues were allowed to equilibrate for 90–120 minutes before the start of the experiments. Capillary glass microelectrodes (borosilicate glass capillaries, 1.0 mm outer diameter × 0.58 mm inner diameter; Clark Electromedical Instruments, Edenbridge, United Kingdom) were made using a microelectrode puller (model P-97, 3-mm-wide filament; Sutter Instrument Co., Novato, California, USA), were filled with KCl (3 mM), and had resistances in the range 80–120 MΩ. Resting membrane potentials (RMPs) of circular smooth muscle cells were recorded against a ground Ag-AgCl electrode placed in the bath medium as described in detail in ref. 33. Membrane potentials were amplified (DUO 733 microelectrode amplifier; World Precision Instruments Inc.) and digitalized with an analogue-to-digital converter (SCB-68 interface; National Instruments Corp., Austin, Texas, USA). Permanent recordings of membrane potentials were made on a personal computer running the LabVIEW 5.0 program (National Instruments Corp.).

*Statistical analysis*. Results are expressed as mean ± SEM and were compared using Student's *t* test. *P* values less than 0.05 were considered statistically significant.

## Results

*Genetic and pharmacological blockade of CB1 signaling increases severity of induced colitis*. In order to study the involvement of CB1 and the endogenous cannabinoid system in colon inflammation, we first used CB1-deficient mice in the DNBS model of colitis. Intrarectal administration of 100 μl of 50% ethanol did not induce detectable inflammation in *CB1*$^{-/-}$ nor in *CB1*$^{+/+}$ mice, as macroscopically evaluated (data not shown). Conversely, after intrarectal administration of DNBS (5 mg per mouse), macroscopic evaluation of *CB1*$^{-/-}$ colons revealed stronger inflammation as compared with *CB1*$^{+/+}$ colons (Figure 1A). Macroscopic score in *CB1*$^{-/-}$ mice was 2.2-fold higher than in *CB1*$^{+/+}$ mice (*CB1*$^{+/+}$, 1.7 ± 0.2, vs. *CB1*$^{-/-}$, 3.7 ± 0.3, $P < 0.05$; Figure 1A). MPO assay revealed that the degree of inflammation was higher in *CB1*$^{-/-}$ than in *CB1*$^{+/+}$ mice (*CB1*$^{+/+}$, 100% ± 37%, vs. *CB1*$^{-/-}$, 230% ± 54%, $P < 0.05$; Figure 1B). Histological analysis confirmed these observations, showing no differ-

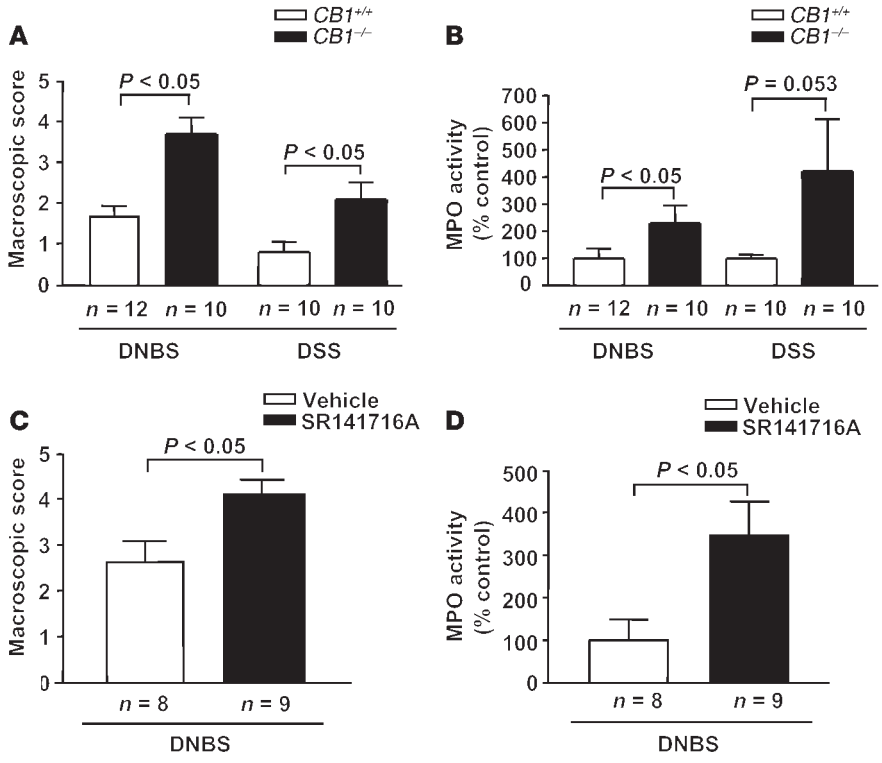


**Figure 1**
DNBS- and DSS-induced colitis is worsened in *CB1*$^{-/-}$ and in SR141716A-treated (3 mg/kg) C57BL/6N mice. (**A** and **B**) Macroscopic score of colonic inflammation (**A**) and levels of MPO activity (**B**) in *CB1*$^{+/+}$ and *CB1*$^{-/-}$ littermates. (**C** and **D**) Macroscopic score of colonic inflammation (**C**) and levels of MPO activity (**D**) in vehicle- and SR141716A-treated C57BL/6N mice. Data are means ± SEM.

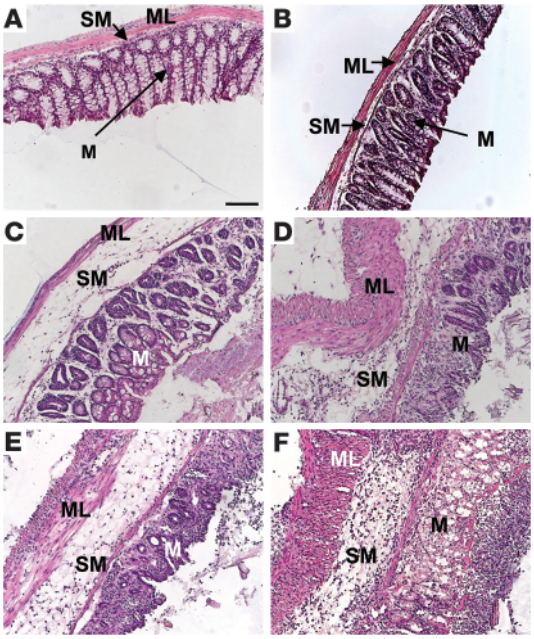


**Figure 2**
Histological micrographs showing H&E staining from transverse sections of the colon. (**A** and **B**) Colons from *CB1*$^{+/+}$ (**A**) and *CB1*$^{-/-}$ (**B**) mice without DNBS treatment. (**C** and **D**) Colons from *CB1*$^{+/+}$ (**C**) and *CB1*$^{-/-}$ (**D**) mice 3 days after DNBS treatment. (**E** and **F**) Colons from vehicle-treated (**E**) and SR141716A-treated (3 mg/kg) (**F**) C57BL/6N mice 3 days after DNBS treatment. In particular, note the severe mucosal infiltration with inflammatory cells, severe submucosal edema, and vascular alterations in *CB1*$^{-/-}$ and SR141716A-treated mice. ML, muscular layer; M, mucosa; SM, submucosa. Scale bar: 100 μm.

ences between genotypes in untreated colons (Figure 2, A and B) and dramatically increased inflammatory signs in DNBS-treated *CB1*$^{-/-}$ as compared with *CB1*$^{+/+}$ colons (Figure 2, C and D). In particular, a notable disruption of the epithelial structure with extensive hemorrhagic necrosis and infiltration of neutrophils into the mucosa was detected in *CB1*$^{-/-}$ colons, with acute inflammation extending into the submucosa and the muscle layer.

To further substantiate the general involvement of CB1 signaling in colon inflammation, we characterized the development of colitis in CB1-deficient mice subjected to oral DSS treatment (5% in drinking water) for 7 days. Macroscopic evaluation of *CB1*$^{-/-}$ colons revealed stronger inflammation as compared with *CB1*$^{+/+}$ colons (Figure 1A). Macroscopic score in *CB1*$^{-/-}$ was more than twofold higher than in *CB1*$^{+/+}$ mice (*CB1*$^{+/+}$, 0.8 ± 0.2, vs. *CB1*$^{-/-}$, 2.1 ± 0.4, $P < 0.05$). MPO assay showed that the degree of inflammation was higher in *CB1*$^{-/-}$ than in *CB1*$^{+/+}$ mice, although the difference did not reach statistical significance (*CB1*$^{+/+}$, 100% ± 14.9%, vs. *CB1*$^{-/-}$, 423.5% ± 189.8%, $P = 0.053$; Figure 1B).

In order to check for the acute involvement of CB1 in protection against inflammation, we treated wild-type C57BL/6N mice with the specific CB1 antagonist SR141716A (3 mg/kg) 30 minutes before and 24 and 48 hours after intrarectal administration of DNBS. Treatment with SR141716A induced stronger inflammation than treatment with vehicle. This was shown both by macroscopic scoring of inflammatory signs (vehicle, 2.6 ± 0.5, vs. SR141716A, 4.1 ± 0.3, $P < 0.05$; Figure 1C) and by MPO analysis (vehicle, 100% ± 48.4%, vs. SR141716A, 346.6% ± 79.5%, $P < 0.05$; Figure 1D). Histological analysis of inflamed colons confirmed these observations, revealing a more severe transmural colitis in SR141716A-treated specimens, with thickening of the bowel wall, inflammatory infiltrates, and stronger increase in lymphoid-follicle size, associated with adherence to surrounding tissues, as compared with vehicle-treated controls (Figure 2, E and F).

*DNBS-induced colitis is reduced by administration of the potent cannabinoid agonist HU210 as well as in FAAH-deficient mice.* If the endogenous cannabinoid system plays an important role in the pathophysiological protection from DNBS-induced colitis, it is conceivable that stimulation of cannabinoid receptors during inflammatory processes might decrease the level of inflammation. Therefore, we treated C57BL/6N wild-type mice with the potent cannabinoid agonist HU210 (0.05 mg/kg) 30 minutes before and 24 and 48 hours after intrarectal administration of DNBS. Treatment with HU210 markedly reduced the levels of inflammation as compared with treatment with vehicle, as revealed by macroscopic scoring (vehicle, 3.7 ± 0.5, vs. HU210, 1.8 ± 0.3, $P < 0.05$; Figure 3A) and by MPO assay (vehicle, 100% ± 39%, vs. HU210, 5.4% ± 2.7%, $P < 0.05$; Figure 3B). Moreover, we analyzed the response to DNBS-induced inflammation in FAAH-deficient (*FAAH*$^{-/-}$) mice, which are profoundly impaired in their ability to degrade anandamide (23). The macroscopic score in *FAAH*$^{-/-}$ mice was 2.0-fold lower than in *FAAH*$^{+/+}$ littermates (*FAAH*$^{+/+}$, 3.8 ± 0.8, vs. *FAAH*$^{-/-}$, 1.9 ± 0.5, $P < 0.05$; Figure 3C). MPO assay confirmed that the degree of inflammation was higher in *FAAH*$^{+/+}$ than in *FAAH*$^{-/-}$ mice (*FAAH*$^{+/+}$, 100% ± 32%, vs. *FAAH*$^{-/-}$, 22% ± 16%, $P < 0.05$; Figure 3D).

*CB1 and Enk mRNA levels are upregulated in the colon after DNBS-induced inflammation.* Using ISH, the levels of CB1 transcripts were evaluated at a single-cell resolution in colons of *CB1*$^{+/+}$ mice either in control conditions or 3 days after intrarectal administration of DNBS. In control colons, CB1 mRNA was predominantly expressed

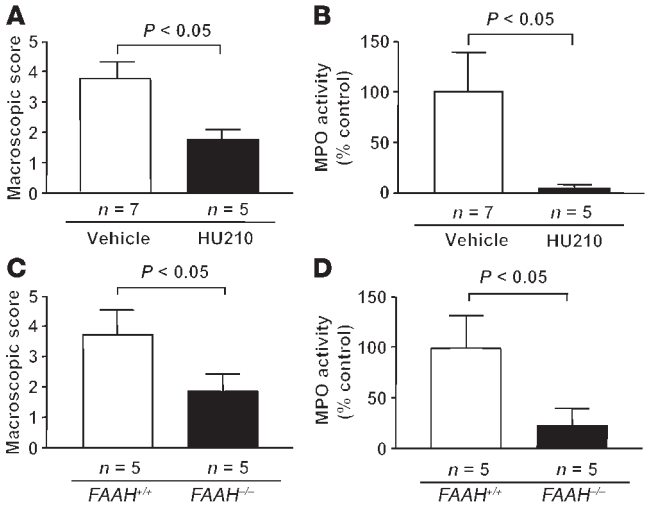


**Figure 3**
Pharmacological stimulation of cannabinoid receptors and genetic enhancement of endocannabinoid levels protect against DNBS-induced colitis. (**A** and **B**) Macroscopic score of colonic inflammation (**A**) and levels of MPO activity (**B**) in vehicle-treated and HU210-treated (0.05 mg/kg) C57BL/6N mice. (**C** and **D**) Macroscopic score of colonic inflammation (**C**) and levels of MPO activity (**D**) in *FAAH*$^{+/+}$ and *FAAH*$^{-/-}$ mice. Data are means ± SEM.

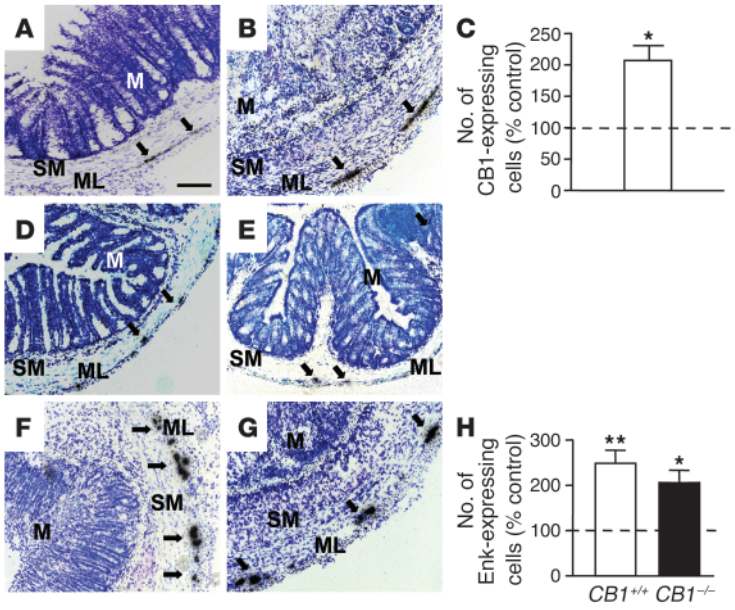


**Figure 4**
Relative numbers of CB1- and Enk-expressing myenteric neurons are increased 3 days after DNBS treatment, as detected by ISH. (**A** and **B**) Micrographs showing CB1 mRNA in untreated (**A**) and in DNBS-treated (**B**) *CB1*$^{+/+}$ mice. (**C**) Quantitative evaluation of CB1-expressing cells in the myenteric plexuses of DNBS-treated *CB1*$^{+/+}$ mice (white bar, $n = 3$). (**D**–**G**) Micrographs showing Enk mRNA in untreated *CB1*$^{+/+}$ (**D**), untreated CB1$^{-/-}$ (**E**), DNBS-treated *CB1*$^{+/+}$ (**F**), and DNBS-treated *CB1*$^{-/-}$ (**G**) colons. (**H**) Quantitative evaluation of Enk-expressing cells in the myenteric plexuses of DNBS-treated *CB1*$^{+/+}$ (white bar, $n = 3$) and *CB1*$^{-/-}$ (black bar, $n = 3$) mice. Values are means ± SEM. *$P < 0.05$, **$P < 0.01$ vs. respective untreated controls. Dotted lines, 100%. $n = 3$ per group. Arrows: CB1- and Enk-expressing cells. Scale bar: 100 μm.

in neurons belonging to the myenteric plexus (Figure 4A). After DNBS-induced inflammation, an increase in the number of CB1-expressing cells was observed (Figure 4B). Counting of single CB1-expressing cells confirmed this observation (untreated, 100% ± 17.5%, vs. treated, 205.2% ± 22.9%, $P < 0.05$; Figure 4C).

The endogenous opioidergic system was recently shown to participate in the protection against inflammation in the TNBS model of colitis through the activation of μ-opioid receptors (34). To test whether the protective functions of the endogenous cannabinoid system involve the activation of endogenous opioid signaling, we analyzed Enk mRNA–expressing neurons in untreated and DNBS-treated colons derived from both *CB1*$^{+/+}$ and *CB1*$^{-/-}$ mice. In untreated colons, the number of cells expressing Enk was not different between genotypes (*CB1*$^{+/+}$, 100% ± 18.9%, vs. *CB1*$^{-/-}$, 109.3% ± 17.1%, $P > 0.05$; Figure 4, D and E). After DNBS treatment, the number of Enk-expressing cells was significantly increased in both *CB1*$^{+/+}$ colons (249.0% ± 28.7%, $P < 0.01$ vs. untreated *CB1*$^{+/+}$; Figure 4H) and *CB1*$^{-/-}$ colons (205.7% ± 27.9%, $P < 0.05$ vs. untreated *CB1*$^{-/-}$; Figure 4H). After DNBS treatment, the number of Enk-expressing cells was significantly increased both in *CB1*$^{+/+}$ colons (249.0% ± 28.7%, $P < 0.01$ vs. untreated *CB1*$^{+/+}$; Figure 4, F and H) and in *CB1*$^{-/-}$ colons (205.7% ± 27.9%, $P < 0.05$ vs. untreated *CB1*$^{-/-}$; Figure 4, G and H).

Counts of myenteric neurons were carried out in parallel, on cuprolinic blue–stained sections adjacent to the ones used for ISH. Comparable numbers of neurons were shown per unit length of serosa in DNBS-treated versus untreated colons, as well as in *CB1*$^{+/+}$ versus *CB1*$^{-/-}$ mice (untreated *CB1*$^{+/+}$, 100% ± 5.1%; treated *CB1*$^{+/+}$, 120.7% ± 11.6%; untreated *CB1*$^{-/-}$, 97.8% ± 5.7%; treated *CB1*$^{-/-}$, 109.2% ± 6.4%; $P > 0.1$ for all comparisons); thus, no overt changes were shown in overall neuronal populations.

*Time-course analysis of DNBS-induced colitis.* Three days after intrarectal administration of DNBS, inflammatory responses appear to be modulated by the endogenous cannabinoid system. This time point was chosen because maximal acute DNBS-induced inflammation has been reported in mice after 3 days (35). However, the involvement of CB1 at different stages of the inflammatory process is still an open question. Macroscopic scoring, MPO activ-

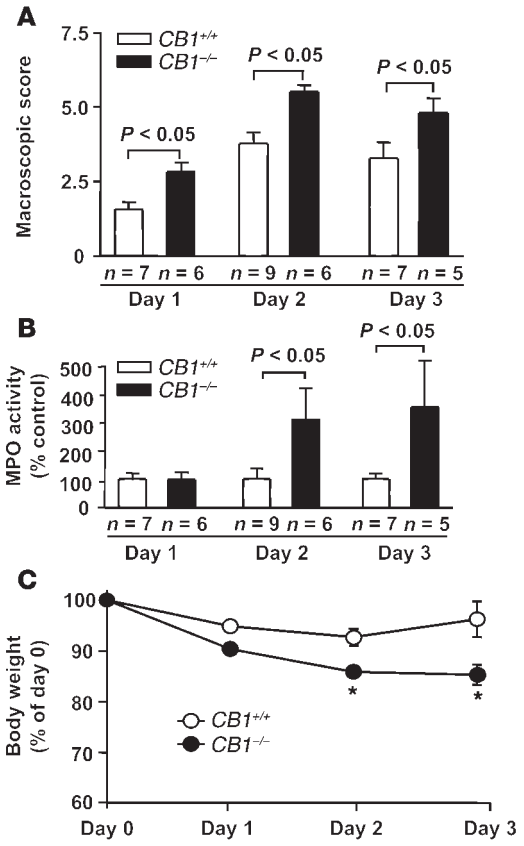


**Figure 5**
Temporal development of DNBS-induced colitis in *CB1*$^{-/-}$ and *CB1*$^{+/+}$ mice. (**A**) Macroscopic score, (**B**) MPO activity, and (**C**) body-weight changes at different time points after induction of colitis. Values are means ± SEM. *$P < 0.05$.

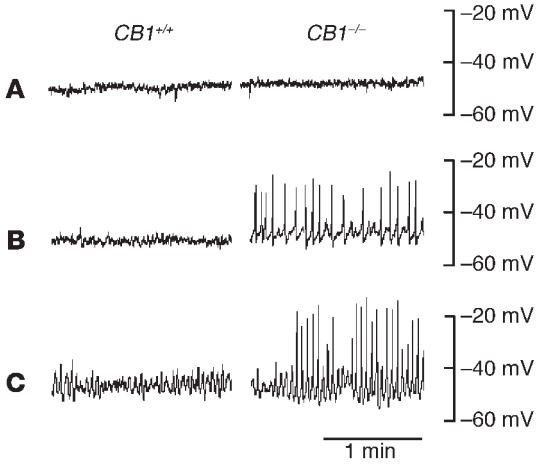


**Figure 6**
Intracellular recordings from circular smooth muscles in distal colon of *CB1*$^{+/+}$ and *CB1*$^{-/-}$ mice to monitor RMPs. Representative traces are shown for mice before (**A**), 8 hours after (**B**), and 24 hours after (**C**) induction of colitis. In **B** and **C**, note the occurrence of oscillatory action potentials in *CB1*$^{-/-}$ colons.

ity, and body-weight loss were analyzed during a 3-day time course in *CB1*$^{+/+}$ and *CB1*$^{-/-}$ littermates (Figure 5). On day 1, macroscopic evaluation revealed a significant difference between genotypes (*CB1*$^{+/+}$, 1.6 ± 0.2, vs. *CB1*$^{-/-}$, 2.8 ± 0.3, $P < 0.05$; Figure 5A), whereas no significant difference was found in colonic MPO levels (Figure 5B) and body-weight loss (Figure 5C) between genotypes. By contrast, on day 2 and day 3, significant differences were found in the analysis of macroscopic score (day 2: *CB1*$^{+/+}$, 3.7 ± 0.3, vs. *CB1*$^{-/-}$, 5.5 ± 0.2, $P < 0.05$; day 3: *CB1*$^{+/+}$, 3.2 ± 0.5, vs. *CB1*$^{-/-}$, 4.8 ± 0.5, $P < 0.05$; Figure 5A), in MPO activity (day 2: *CB1*$^{+/+}$, 100% ± 36%, vs. *CB1*$^{-/-}$, 314% ± 105%, $P < 0.05$; day 3: *CB1*$^{+/+}$, 100% ± 20%, vs. *CB1*$^{-/-}$, 354% ± 163%, $P < 0.05$; Figure 5B), and in body-weight loss (day 2: *CB1*$^{+/+}$, –7.3% ± 1.6%, vs. *CB1*$^{-/-}$, –14.1% ± 0.7%, $P < 0.05$; day 3: *CB1*$^{+/+}$, –3.8% ± 3.5%, vs. *CB1*$^{-/-}$, –14.7% ± 2.0%, $P < 0.05$; Figure 5C). Therefore, macroscopic inflammatory signs were stronger in *CB1*$^{-/-}$ already 24 hours after the inflammatory insult, whereas the effects of the lack of CB1 on MPO levels and body-weight loss required a longer period of time to become evident. Additionally, the regaining of body weight of *CB1*$^{+/+}$ mice on day 3 was absent in *CB1*$^{-/-}$ mice (Figure 5C), suggesting that CB1 might play a role in the recovery from colonic inflammation to reconstitute general health.

*Electrophysiological analysis shows spontaneous oscillatory activities in CB1*$^{-/-}$ *mice at early stages of colonic inflammation*. Circular smooth muscle cells of uninflamed *CB1*$^{+/+}$ mice displayed a stable RMP of –51.6 ± 0.9 mV ($n = 4$), which was not different from the RMP measured in *CB1*$^{-/-}$ mice (–51.1 ± 0.6 mV, $n = 4$, $P > 0.05$; Figure 6A). Additionally, 8 hours and 24 hours after DNBS treatment, RMP was not different between genotypes (8 hours: *CB1*$^{+/+}$, –50.0 ± 1.4 mV, vs. *CB1*$^{-/-}$, –51.3 ± 0.3 mV, $n = 4$, $P > 0.05$; Figure 6B; 24 hours: *CB1*$^{+/+}$, –55.0 ± 1.2 mV, vs. *CB1*$^{-/-}$, –53.4 ± 1.5 mV, $n = 3$, $P > 0.05$; Figure 6C). In both genotypes, RMP was not changed in the presence of atropine (1 μM), an inhibitor of cholinergic transmission (data not shown). After DNBS treatment, however, *CB1*$^{-/-}$ mice displayed spontaneous action potentials with a frequency of 26.8 ± 2.9 min$^{-1}$ and an amplitude of 28.8 ± 3.9 mV 8 hours after initiation of inflammation ($n = 5$), and a frequency of 40.4 ± 1.2 min$^{-1}$ and an amplitude of 29.3 ± 2.6 mV 24 hours after initiation of inflammation ($n = 5$). These spontaneous action potentials were not observed in *CB1*$^{+/+}$ mice at any time point after DNBS treatment or in untreated mice (Figure 6). The rhythmic action potentials were unchanged in the presence of atropine (1 μM) (data not shown).

## Discussion

Upon inflammatory insults, several different cellular pathways are activated in the intestinal tract, leading to a pathological state (36). However, simultaneous protective mechanisms are also activated, and the balance between pro- and anti-inflammatory responses determines the outcome of the pathological processes (37).

In this study, we analyzed the involvement of the endogenous cannabinoid system in the development of experimental colitis in mice, induced by intrarectal DNBS treatment and oral DSS application. Genetic ablation of CB1 receptors rendered mice more sensitive to inflammatory insults, indicating a protective role of the CB1 receptors during inflammation. This protection was mediated by an acute activation of the endogenous cannabinoid system rather than being caused by developmental defects due to the lifelong absence of CB1 receptors, since pharmacological blockade of CB1 with the specific antagonist SR141716A led to a worsening of colitis that was similar to that observed in CB1-deficient mice. The involvement of the endogenous cannabinoid system in the modulation of the acute phase of DNBS-induced colitis is further supported by the increased levels of transcripts coding for CB1 in wild-type mice after induction of inflammation. By ISH experiments, the number of CB1-expressing cells was found to be significantly increased after inflammation, without any simultaneous significant increase of the total number of neurons. Thus, it appears that neurons that express undetectable or very low levels of CB1 receptor in basal conditions, start to express this receptor to enhance endocannabinoid signaling.

The protective role of the endogenous cannabinoid system was observed 24 hours after DNBS treatment and became more evident on day 2 and day 3. However, increased spontaneous spiking activity of smooth muscle cell membrane of DNBS-treated colons from *CB1*$^{-/-}$ mice was already visible 8 hours after DNBS treatment, indicating that inflammation-induced irritation of smooth muscle occurs at an earlier stage than in wild-type mice. This gives further support to the notion that the endogenous cannabinoid system is protective against inflammatory changes. These data indicate that the activation of CB1 and of the endogenous cannabinoid system is an early and important physiological step during self-protection of the colon against inflammation.

The occurrence of dysmotility in inflammatory diseases of the small or the large intestine is widely accepted. Contractility of smooth muscle to cholinergic or tachykinergic stimuli is increased in the early stages and decreased in the later stages of inflammation (38, 39), and contractility following electrical neuronal stimulation in the inflamed gut is increased (40). The electrophysiological changes in the distal colon, as used in this study, show increased smooth muscular excitability, leading to the occurrence of spontaneous spiking activity. This kind of irritation was recently described for guinea pig distal colonic neurons, leading to the suggestion that dysmotility in inflamed colon is due to a disruption of the afferent limb of the intrinsic motor reflexes (41). In these changes, the release of tachykinins from capsaicin-sensitive neurons might play a major role (42), whereas cholinergic mechanisms seem not to be involved. These observations are in good agreement with our results, suggesting a cholinergic-independent mechanism, since the spiking activi-

ty was not changed after atropine application. Consistent with this hypothesis, CB1 receptors, identified by immunohistochemistry on substance P–containing neurons (43), have been shown to be involved in the noncholinergic control of intestinal motility (44).

In the small intestine, an involvement of CB1 receptors in the control of intestinal motility during croton oil–induced inflammation was recently evidenced. Izzo et al. showed that pharmacological administration of cannabinoids is able to delay gastrointestinal transit in croton oil–treated mice (45). Increased levels of CB1 receptor expression in inflamed jejuna may contribute to this protective effect (45). However, this work was not able to reveal a physiological protective action of the endogenous cannabinoid system against enteritis, since administration of the CB1 antagonist SR141716A alone failed to specifically worsen inflammation-induced gut hypermotility. By applying genetic and pharmacological approaches, our present investigation extended the observations by Izzo et al. (45) to the large intestine and, using morphological criteria, MPO measurements, and electrophysiological recordings, included the determination of inflammatory responses in the absence of a functional endogenous cannabinoid system. Most importantly, we were able to clearly show a physiological protective role of the endogenous cannabinoid system against intestinal inflammation.

A protective role against colon inflammation was recently shown for the endogenous opioidergic system (34), and a cross-talk between endogenous cannabinoid and opioid systems has been proposed (46). We found that DNBS treatment increased the levels of mRNA encoding the endogenous opioid Enk in the colon. This increase was present in DNBS-treated colons of both genotypes, indicating that CB1 receptors are not necessary to induce the increase in Enk mRNA levels during inflammatory processes. However, these results do not exclude a possible cross-talk between the two protective endogenous systems, and further investigations are needed to clarify this issue.

FAAH is the major enzyme involved in the degradation of several bioactive fatty amides, in particular of anandamide (47), and its genetic deletion in mice leads to strongly decreased ability to degrade this endocannabinoid and to an increase of anandamide levels in several tissues (23). FAAH-deficient mice showed significant protection against DNBS treatment. Moreover, pharmacological stimulation of cannabinoid receptors with the potent agonist HU210 also induced a reduction of experimental colitis. Anandamide is believed to act not only through CB1 receptors, but also through other targets, including the peripheral cannabinoid receptor CB2 and the vanilloid receptor TRPV1 (48). On the other hand, HU210 is also able to stimulate CB2 (13). Therefore, it cannot be excluded that decreased inflammation in *FAAH*$^{-/-}$ mice and the anti-inflammatory actions of HU210 are due to the activation of other targets than CB1 receptors. However, our data from *CB1*$^{-/-}$ and SR141716A-treated mice clearly point to a central role of CB1 in the physiological control of colonic inflammation. A recent investigation showed that cholera toxin–induced accumulation of intestinal fluid in mice is modulated by activation of the endogenous cannabinoid system acting through CB1, but not through TRPV1 nor CB2, receptors (49). Moreover, CB1 receptors were also shown to modulate gastrointestinal motility during croton oil–induced inflammation in mice (45). However, conclusive results regarding the involvement of TRPV1 in protective signaling cascades after DNBS treatment should be possible using TRPV1-deficient mice.

The ability of cannabinoid receptor agonists to suppress peritoneal inflammation and to inhibit inflammation-induced gastrointestinal hypermotility when administered intracerebroventricularly has recently been shown (45, 50), indicating that cannabinoids may also act in the CNS to exert their anti-inflammatory activities. As we used systemic treatments and mutant mice bearing gene deletions in all cells of the body, we cannot exclude a central component of the anti-inflammatory effects of the endogenous cannabinoid system during DNBS-induced colitis. However, the early alterations in electrophysiological properties of inflamed *CB1*$^{-/-}$ colons and the increased levels of CB1 mRNA in myenteric plexuses of DNBS-treated colons seem to point to an important, if not predominant, function of the endogenous cannabinoid system at peripheral sites. Use of central administration of drugs or, conversely, of cannabinoid agonists and antagonists unable to cross the blood-brain barrier, or the generation of conditional CB1 mutant mice with tissue-specific deletion of the gene, will help to clarify this important issue.

In conclusion, this study shows that the endogenous cannabinoid system is physiologically involved in the protection against excessive inflammation in the colon, both by dampening smooth muscular irritation caused by inflammation and by controlling cellular pathways leading to inflammatory responses. These results strongly suggest that modulation of the physiological activity of the endogenous cannabinoid system during colonic inflammation might be a promising therapeutic tool for the treatment of several diseases characterized by inflammation of the gastrointestinal tract.

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