CANNABINOIDS AS NEUROPROTECTIVE AGENTS
A MECHANISTIC STUDY

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ABSTRACT

Glucose and oxygen supply to the brain is critical for its proper function and when it is restricted as during a stroke, neurons and glial cells quickly become necrotic leading to structural damage as well as functional impairment and even death. To date there are few effective therapies that inhibit the neurodegenerative process and improves the outcome for the affected individual. One possible target is the cannabinoid system. Cannabinoid receptor agonists reduce ischemic volume, endogenous cannabinoid levels are elevated during neurodegenerative insults and mice devoid of the central cannabinoid receptor are more seriously affected by experimental stroke than wild type mice. The cannabinoids are also ascribed anti-inflammatory properties and post ischemic inflammation has been proposed to contribute to the evolution of the ischemic damage. In this thesis mechanisms that can contribute to cannabinoid neuroprotection have been studied. In papers I and II the chick was used as a model species, since preparation of embryonic primary neuronal cultures from chick is relatively simple and time efficient compared to rodent primary cultures. Both adult and embryonic chick brain membranes contain functional CB₁ receptors and in the cultures they are coupled to inhibition of cAMP production. In embryonic primary cultures, neurons were not protected from glutamate toxicity by preincubation with CB receptor agonists suggesting that postsynaptic cannabinoid mediated neuroprotection is not effective in this system. The effect of cannabinoid agonists on neutrophil chemotaxis and transmigration was investigated in paper III. The CB₁/CB₂ agonist WIN 55,212-2 inhibited TNF-α-induced transmigration across ECV304 cell monolayers. The effect of WIN 55,212-2 on this process which was mediated by a reduction of IL-8 release from the ECV304 cells rather than a direct effect upon the migratory response to IL-8 was not possible to abolish with CB₁ or CB₂ agonists suggesting a mechanism distinct from the cannabinoid receptors is operative. In paper IV the photothrombotic ring stroke model was evaluated to determine if it is suitable in intervention studies targeting the cannabinoid system. Three major endpoints were of interest, ischemic volume, neutrophil infiltration and CB₁ receptor function. Consistent with previous studies the ischemic volume peaked at 48 hours after irradiation. Neutrophil infiltration was quantified using a myeloperoxidase activity assay. The assay revealed an increase in myeloperoxidase activity 48 hours after irradiation, albeit at a modest level. The function of the CB₁ receptor was assessed by radioligand binding and there was no change in either total binding or functional G-protein coupling following photothrombosis. Taken together these results indicate that it is feasible to undertake cannabinoid intervention studies in this model.
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<tr>
<td>Δ⁹-THC</td>
<td>Δ⁹-tetrahydrocannabinol</td>
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<tr>
<td>2-AG</td>
<td>2-arachidonylglycerol</td>
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<tr>
<td>AEA</td>
<td>anandamide, N-arachidonylethanolamine</td>
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<td>AM 251</td>
<td>CB₁ receptor antagonist/inverse agonist</td>
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<td>AM 630</td>
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<td>ANOVA</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>CP 55,940</td>
<td>CB₁ receptor agonist</td>
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<td>DIV</td>
<td>days <em>in vitro</em></td>
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<td>ECV304</td>
<td>endothelial cell line</td>
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<td>fatty acid amide hydrolase</td>
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<td>glial fibrillary acidic protein</td>
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<td>HU 210</td>
<td>CB₁ receptor agonist</td>
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<td>HUVEC</td>
<td>human umbilical cord vein endothelial cells</td>
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<td>IL-8</td>
<td>interleukin 8</td>
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<td>LDH</td>
<td>lactate dehydrogenase</td>
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<td>MAGL</td>
<td>monoacyl glycerol lipase</td>
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<td>N-acylethanolamines</td>
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<td>NAPE</td>
<td>N-acyl phosphatidyl ethanolamine</td>
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<td>NFKB</td>
<td>nuclear factor kappa B</td>
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<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
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<td>PEST</td>
<td>penicillin streptomycin</td>
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<td>SR141716A</td>
<td>CB₁ receptor antagonist/inverse agonist</td>
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<td>SR144528</td>
<td>CB₂ receptor antagonist/inverse agonist</td>
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<tr>
<td>TNF-α</td>
<td>tumour necrosis factor alpha</td>
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<td>WIN 55,212-2</td>
<td>CB₂/CB₁ receptor agonist</td>
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INTRODUCTION

The brains of higher vertebrates are highly complex organs which are under strict physiological control in order to ensure their proper function. One feature of the brain that makes it particularly susceptible to damage is its high and constant demand for oxygen and glucose. Although the brain makes up only 2% of the body mass, 20% of the blood oxygen is consumed there, and even short periods of oxygen deprivation can lead to irreversible brain damage. One common cause of oxygen deprivation in the brain is stroke, which can be of thrombotic or hemorrhagic nature. In either case the blood flow to the anatomical region perfused by the affected vessel is restricted and that region becomes ischemic (from Greek: *isch* –restriction and *hema* –blood).

Cerebral ischemia is a major cause of death or incapacitation in industrialised countries and in Sweden alone there are about 30 000 new cases each year. In stroke treatment, time is of the essence in order to restore perfusion of the ischemic region to reduce neurodegeneration, especially in the penumbra. The concept of the penumbra was introduced to define a region of reduced blood flow, below the threshold for electrical and membrane failure to occur (Astrup et al., 1981). The function of the penumbra is compromised but is structurally intact and can regain function if perfusion is restored (for review see Hossmann, 2006). The patophysiologial events during ischemia follow a certain timescale that have been well reviewed by Dirnagl et al., (1999). Two key events are excitotoxicity and inflammation (see Fig 1 for a schematic representation).

![ACUTE PHASE SUB ACUTE - CHRONIC PHASE](image)

**Fig 1.** Time frame of acute and sub acute damage during cerebral ischemia, the impact of the injury is represented by the vertical axis. Redrawn from Dirnagl et al., (1999).
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The acute phase, with a duration of 3-4 hours, has a major impact on the extent of neurodegeneration. During this phase, the cells of the brain are mainly affected by excitotoxicity that arises due to lack of oxygen and glucose, which cause the neurons to lose their membrane polarisation and release neurotransmitters, i.e. glutamate. In this respect, the acute phase should be the one where intervention would be most effective. However, the acute phase is of a short duration and an individual suffering a stroke is seldom under medical care and correctly diagnosed within this time span.

The most effective treatment up to date in intervening the acute phase is the use of thrombolytic agents such as recombinant tissue plasminogen activator (rtPA) in order to restore perfusion. The thrombolytic agents, however, are only of proven use during the first 3-6 hours and it is imperative that the patient is correctly diagnosed with having a thrombotic and not a hemorrhagic stroke, since in the latter case thrombolytic treatment would be deleterious (Frey, 2005).

Following the acute phase is the sub-acute phase that is prolonged up to days or weeks. During the sub-acute phase an inflammatory reaction starts as a consequence of the mainly necrotic cell death caused by excitotoxicity. The brain is normally protected from components of the peripheral immune system by the blood-brain barrier. However, during a stroke, the barrier becomes more permeable to blood components. In both human and other mammals, studies have shown that leukocytes cross the blood-brain barrier and migrate into the brain parenchyma early in response to ischemia (Garcia and Kamijyo, 1974). The neutrophils produce several highly reactive substances such as hypochlorous acid and oxygen radicals which can be toxic to surrounding cells. These substances are normally used to render pathogens harmless as a part of the innate immune response (Klebanoff, 2005). Early after an ischemic insult, leukocytes can be seen adhering to the walls of small vessels in the ischemic area. These leukocytes can subsequently transmigrate across the endothelial cells lining the vessels and into the brain parenchyma (for an extensive review see Barone and Feuerstein, 1999).

Neutrophils are the first leukocytes to migrate across the blood-brain barrier as a response to injury followed by macrophages and monocytes. In the brain parenchyma, neutrophils can be found for several days following a stroke. The neutrophils follow a certain pattern when transmigrating across an endothelial cell layer. The first step in transmigration is rolling where the neutrophil flow rate is reduced as a consequence of an increased expression of selectins on the endothelial cells. The second step is adhesion where the neutrophils link to specific adhesion molecules, such as intercellular cell adhesion molecule 1 (ICAM-1), on the endothelial cells. The third step is the actual transmigration and chemokinesis in the extracellular space towards the site of injury (for a detailed review see Ley, 1996). Transmigration and chemokinesis are largely
directed by cytokines that play a pivotal role in the whole process. As a consequence of cells dying or being damaged by ischemic processes, proinflammatory cytokines such as TNF-α and IL1β are released (Minami et al., 1992; Liu et al., 1994). These cytokines can in turn increase the expression of adhesion molecules and other chemotactic cytokines (chemokines) which result in neutrophil transendothelial migration.

Studies investigating the effect of reducing neutrophil migration have shown that when the recruitment of neutrophils into the brain is reduced, the lesion area and functional impairment is also reduced (Heinel et al., 1994; Zhang et al., 1994; Jiang et al., 1995), although there are also some negative results (Hayward et al., 1996). These observations pose a new therapeutic opportunity in stroke treatment since they offer a longer window of opportunity for interventions. The cannabinoid system has been reported to reduce excitotoxicity, neutrophil transmigration and produce hypothermia, effects that may have therapeutic value in the treatment of stroke.

The cannabinoid system

Background and discovery

Cannabis and cannabinoid ligands have a long history of use for the treatment of different ailments. In a publication from 1859, J.R. Reynolds described twenty-two cases where extracts from *Cannabis indica* were used (Reynolds, 1859). He concluded that *Cannabis indica* has a remedial action in as diverse maladies as “incipient insanity after yellow fever”, “intense cerebral congestion” and meningitis but is without effect on “temporary, recurrent religious melancholy”, sciatica and epilepsy. In 1964, Gaoni and Mechoulam reported the structure of Δ9-tetrahydrocannabinol (Δ9-THC; Fig 2), the main psychoactive component of preparations from the plant Cannabis sativa. The key to how the active constituent exerted its effects was somewhat elusive until 1988, when a G-protein-coupled receptor negatively coupled to adenylate cyclase and capable of binding Δ9-THC and the synthetic ligand CP 55,940 (structure see Fig 2) was described (Devane et al., 1988). This receptor, termed cannabinoid receptor 1 (CB1), was later cloned and found to be expressed mainly in the brain but also on peripheral neurons (Matsuda et al., 1990). In 1993, a second cannabinoid receptor was discovered which has a predominantly peripheral distribution, mainly in cells related to the immune system (Munro, 1993). There is evidence for additional cannabinoid receptors such as the “non-CB1, non-CB2 receptor” (Jarai et al., 1999) as well as an orphan G-protein-coupled receptor called GPR55 (for review see Baker et al., 2006) although these are not yet formally classified as cannabinoid receptors nor cloned (Howlett et al., 2002).
Compounds acting through the CB₁ receptor produces a multitude of effects, including the “tetrad”, namely hypothermia, antinociception, catalepsy and hypolocomotion (Compton et al., 1993). Upon ligand binding to the CB₁ receptor, several intracellular signalling mechanisms are activated. Cannabinoid receptor ligands have been reported to inhibit adenylyl cyclase (Howlett, 1984; Howlett and Fleming, 1984; Bidaut-Russell et al., 1990; Bonhaus et al., 1998; Breivogel and Childers, 2000), inhibit voltage gated Ca²⁺ channels of primarily N- and P/Q-type (Caulfield and Brown, 1992; Mackie and Hille, 1992; Felder et al., 1993), activate mitogen activated protein kinases (MAPKs) (Bouaboula et al., 1995; Sanchez et al., 1998; Rueda et al., 2000; Brandes et al., 2002) and activate inward rectifying potassium channels (Mackie et al., 1995; McAllister et al., 1999; Vasquez et al., 2003). The studies concerning the intracellular signalling downstream of the cannabinoid receptors that are referred to above have mainly used highly potent synthetic cannabinoid ligands. However, shortly after the cloning of the CB₁ receptor, an endogenous compound acting as an agonist at cannabinoid receptors, N-arachidinoylethanolamine (anandamide, AEA), was isolated from pig brain (Devane et al., 1992).

Three years later, a second endogenous compound acting as an agonist at cannabinoid receptors, 2-arachidinoyl glycerol (2-AG), was described (Mechoulam et al., 1995; Sugiura et al., 1995). Both AEA and 2-AG bind and activate the two subtypes of cannabinoid receptor, although 2-AG acts with greater efficacy towards both subtypes (Gonsiorek et al., 2000; Savinainen et al., 2001). Even though AEA and 2-AG are potent and efficacious agonists towards the cannabinoid receptors there are other targets activated by these compounds. AEA is a powerful vasodilator by acting through the transient receptor potential vanilloid 1 (TRPV1) (Zygmunt et al., 1999) and is also a potent blocker of background K⁺-channel TASK-1 (Maingret et al., 2001). Although having striking similarities in structure and being active at cannabinoid receptors, 2-AG and AEA have rather dissimilar pathways for their synthesis and degradation.
Endocannabinoid synthesis and degradation

AEA belong to a group of fatty acid derivatives, the N-acyl ethanolamines. AEA is synthesised from membrane phospholipids by a two step process, first a fatty acyl chain is transferred in a Ca\(^{2+}\)-dependent manner from a phospholipid to a phosphatidyl ethanolamine, producing an N-acyl phosphatidyl ethanolamine (NAPE). The NAPE is subsequently hydrolysed by D-type phospholipase (NAPE-PLD), a phosphodiesterase, to yield AEA (Di Marzo et al., 1994; Okamoto et al., 2004). A slightly different pathway to produce AEA from its NAPE is by conversion via phospholipase A\(_2\) (PLA\(_2\)) and a lysophospholipase D (Sun et al., 2004). The synthesis of AEA is accompanied by the increased production of other N-acyl ethanolamines that however show very low or no affinity for cannabinoid receptors, although they possess biological activity (Fowler, 2003).

The synthesis of 2-AG is different from AEA both in the precursor molecules and in the enzymes involved. Pathways that have been characterised is hydrolysis of sn-arachidonoyl by selective diacylglycerol lipases (Sugiura et al., 1995; Lambert and Di Marzo, 1999) and conversion of lysophosphatidylinositol (LysoPI) by phospholipase C (Ueda et al., 1993; Lambert and Di Marzo, 1999). Whether or not these are the only pathways for AEA and 2-AG synthesis is as yet unclear. Recently though, the role of NAPE-PLD in NAE biosynthesis was tested in a NAPE\(^{-}\) mouse knock-out model where it was shown that the level of the polyunsaturated NAE anandamide was unaltered (Leung et al., 2006) and shows that additional pathways may be operative.

As with all signalling molecules, effective mechanisms are present for the metabolism of AEA and 2-AG. AEA and 2-AG are both substrates for the membrane bound enzyme fatty acid amide hydrolase (FAAH), first reported as a catabolic enzyme for AEA in 1993 (Deutsch and Chin, 1993). The importance of this enzyme, that was cloned in 1996 (Cravatt et al., 1996), in the hydrolysis of AEA is clearly shown in the FAAH\(^{-}\) knock out mice, where the AEA level is increased about 15-fold and tissue preparations from these animals are severely impaired in their ability to degrade AEA (Cravatt et al., 2001). FAAH has a wide substrate specificity with ability to hydrolyse additional N-acyl ethanolamines, such as palmitoylethanolamine (PEA) and oleoylethanolamine (OEA) (Di Marzo et al., 1998; Goparaju et al., 1998), as well as a variety of N-acyl amines such as the sleep-inducing lipid oleamide (Cravatt et al., 1996). The hydrolysis of AEA by FAAH produces arachidonic acid and ethanolamine (Deutsch and Chin, 1993), but other enzymes such as cyclooxygenase 2 (COX-2) and lipoxygenase (LOX) have also been reported to be able to degrade AEA (Ueda et al., 1995; Yu et al., 1997), although the physiological importance of these pathways is not fully elucidated.
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Even though 2-AG is a suitable substrate for FAAH, the enzyme responsible for the degradation of 2-AG in the brain is thought to be monoacyl glycerol lipase (MAGL) (Goparaju et al., 1998; Dinh et al., 2002). MAGL is a serine hydrolase and the resulting products from MAGL-catalysed hydrolysis of 2-AG is arachidonic acid and glycerol (Karlsson et al., 1997). MAGL is more selective in its action towards endocannabinoids since it hydrolyses 2-AG but not the N-acyl ethanolamines AEA and PEA (Dinh et al., 2002).

The identification of enzymes that are responsible for the degradation of endocannabinoids has led to the synthesis and characterisation of several compounds that inhibit endocannabinoid breakdown. The value of such compounds lies in an ability to potentiate the effect of released endocannabinoids and could be exploited to enhance the therapeutic potential of the endocannabinoids. A number of organosulfates, substrate analogues and carbamates have been identified as FAAH inhibitors. Phenylmethylsulfonyl fluoride (PMSF) belongs to the organosulfates, and is an irreversible FAAH inhibitor with efficiency in the low nanomolar range. However, the use of this compound as an FAAH inhibitor in vivo is hampered by its potential toxicity (Deutsch and Chin, 1993; Desarnaud et al., 1995; Quistad et al., 2002).

The substrate analogues include arachidonoyl trifluoromethylketone (ATMK) which is based on the anandamide structure and showing a similar or lower effective concentration than PMSF (Koutek et al., 1994). Among the carbamates one of the most potent FAAH inhibitors is found, URB 597, with an IC50 value in the low nanomolar region and with good specificity with respect to other “players” in the endocannabinoid system (Kathuria et al., 2003; Lichtman et al., 2004). Another useful compound that has been investigated is OL-135, that produces CB1-dependent analgesia in vivo (Lichtman et al., 2004), as does URB 597 (Jayamanne et al., 2006).

**Cannabinoid receptor ligands**

Researchers working today on the cannabinoid system have access to a wide arsenal of agonists, antagonists and inverse agonists. The agonists can be divided into four different groups depending on their origin. The “classical” cannabinoid group includes Δ9-THC and a number of dibenzopyran derivatives (Fig. 2). The second, sometimes referred to as the “nonclassical cannabinoid group” was developed by Pfizer in 1986. This group is made up of bicyclic and tricyclic analogs of Δ9-THC that lack a pyran ring. An important and model molecule of this group of compounds is CP 55,940, a thoroughly investigated substance that has been used in a great number of studies, especially in its tritiated form (Fig. 2). The third group consists of aminoalkylindoles, the model molecule of this group being WIN 55,212-2, a pravidoline derivative (Fig. 2). The aminoalkylindoles are structurally quite dissimilar to the first two. The
fourth and last group is the endocannabinoids with anandamide as the most prominent one (Fig. 2).

In common for the “nonclassical cannabinoids” and aminoalkylindoles is their high potency for binding to the cannabinoid receptors. For example, the $K_i$ values for CP 55,940 and WIN 55,212-2 binding to CB$_1$ receptors are approximately 0.5 – 15 and 2-40 nM, respectively, depending on the tissue investigated and whether the receptor is natively expressed or transfected (for an extensive review on this topic, see Pertwee, 1997). These $K_i$ values can be compared, for instance, to AEA and $\Delta^9$-THC with $K_i$-values of approximately 100-250 and 40-50 nM respectively (Felder et al., 1995; Rinaldi-Carmona et al., 1996; Showalter et al., 1996). Many of the agonists show more or less equal affinity to the cannabinoid receptor subtypes but there are some compounds that show selectivity for the CB$_1$ and CB$_2$ receptor, respectively. The anandamide analogues arachidonoyl 2’-chloroethylamide (ACEA) and ($R$)-(20-cyano-16,16-dimethyldocos-$cis$-5,8,11,14-tetraenoyl)-1'-hydroxy-2'-propylamine (O-1812) are two such compounds that are CB$_1$ selective with ~2000 fold and 1000-fold higher affinity for the CB$_1$ receptor, respectively (Hillard et al., 1999; Di Marzo et al., 2001). One example of the CB$_2$ selective compounds is JWH 133 (Huffman et al., 1999).
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A large step forward in determining if an effect of a cannabinoid agonist is really produced by binding to a cannabinoid receptor came in 1994 when SR141716A (rimonabant) was published as a CB₁ receptor selective antagonist (Rinaldi-Carmona et al., 1994). SR14176A is a diarylpyrazole derivative with a very high affinity for CB₁ receptors, see Fig 3, although originally described as an antagonist it has later been shown to act as an inverse agonist (Landsman et al., 1997; MacLennan et al., 1998; Pan et al., 1998). This compound has now been recognised for treatment of obesity (EMEA, 2006). Four years after the publication of SR141716A the same research group presented SR144528 as the first CB₂ receptor selective antagonist with over 700-fold higher affinity for the CB₂ receptor over the CB₁ receptor (Rinaldi-Carmona et al., 1998; see Fig 3). This compound has also subsequently been shown to act as an inverse agonist (Portier et al., 1999; Rhee and Kim, 2002).

![SR141716A](image1.png) SR141716A (Rimonabant) ![SR144528](image2.png) SR144528

Fig 3. Structure of the CB₁ and CB₂ antagonists SR141716A and SR144528, respectively.

The large number of agonists, antagonist and enzyme inhibitors that have been described, along with knock-out mice of CB₁, CB₂ and FAAH, since the discovery of the first cannabinoid receptor in 1988 have been very valuable tools to investigate the physiological role of the cannabinoid system.

Physiological and pathophysiological role of the cannabinoid system

The cannabinoid system is found in many species and seems to be evolutionary stable since the CB₁ receptor and FAAH, or homologues, are found in virtually all vertebrate species as well as in invertebrates such as leeches (for a review see McPartland, 2004). This evolutionary stability should imply that the cannabinoid system confers some advantage or advantages that make it prevail. By merely looking at the distribution of the receptors it would be tempting to outline a role for the cannabinoid system in CNS homeostasis, given the dense expression of CB₁ receptors in the brain. The high expression of CB₂ receptor on cells associated with the immune system.
would suggest a role for the cannabinoids in immune regulation or inflammation. In fact there are a multitude of biological effects affecting both normal physiologic and pathophysiologic events described for the cannabinoid system (for general reviews see Baker et al., 2003; Di Marzo et al., 2004; Howlett et al., 2004). I will focus on those concerning neurodegeneration and inflammation, since these are the most relevant for this thesis.

**Cannabinoid neuroprotection in vivo**

There are several studies, *in vivo* and *in vitro*, to support a role for the cannabinoid system in neuroprotection. In a rat model of focal cerebral ischemia, pre-treatment with the CB$_1$/CB$_2$ agonist WIN 55,212-2 produced a smaller ischemic volume compared to vehicle-treated control animals (Nagayama et al., 1999). The neuroprotection afforded by WIN 55,212-2 was possible to largely abolish with SR141716A, and it was also found that the effect was stereoselective as evident from the lack of protection by the inactive enantiomer WIN 55,212-3, suggesting that the protection is CB$_1$ receptor mediated. In the same study a protective effect of WIN 55,212-2 *in vitro* was also described for cerebral cortical neurons subjected to hypoxia and glucose deprivation, but this effect was not abolished by cannabinoid receptor antagonists, nor was it stereoselective (Nagayama et al., 1999).

Other models have been used to investigate neuroprotective properties of cannabinoid ligands including intra-cerebral ouabain injection and closed head injury. In two studies using the Na$^+$/K$^+$/ATPase inhibitor ouabain, exogenous AEA reduced cytotoxic edema and neuronal damage and similar result was found when administering Δ$^9$-THC to the animals (van der Stelt et al., 2001a; van der Stelt et al., 2001b). The effect of AEA was blocked at a 7 day endpoint by co-administration of SR141716A, but not at earlier endpoints, whereas the effect of Δ$^9$-THC was possible to block at all endpoints with SR141716A. In a mouse trauma model, closed head injury (CHI), 2-AG was shown to reduce oedema, hippocampal cell death and improve clinical recovery when administered and the effect was dose-dependently inhibited by SR141716A (Panikashvili et al., 2001). Although the above studies show a role of the cannabinoid system, especially CB$_1$ receptor activation, in neuroprotection other studies have failed to determine such an effect unequivocally (Louw et al., 2000; Berger et al., 2004).

Further support for a neuroprotective role or at least involvement of the cannabinoid system during neurodegeneration comes from CB$_1$ knock-out animals. In 2002, a paper was published that reported increased infarct volume, reduced blood flow in the penumbra during reperfusion and increased NMDA neurotoxicity in CB$_1$$^-$$^-$ mice compared to their wild-type littermates (Parmentier-Batteur et al., 2002).
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Perhaps more speculative, but nonetheless intriguing, is the increased release of endocannabinoids following neurodegenerative insults. In a case study of an individual suffering a stroke, increased levels of NAE:s were detected (Schabitz et al., 2002) and in a rat permanent middle cerebral artery occlusion (MCAO) model there was a 30-fold increase in NAE:s in the ipsilateral hemisphere compared to the contralateral un-infarcted hemisphere (Berger et al., 2004). In the study by Panikashvili et al. (2002), it was observed that the amount of 2-AG in the brain increased following CHI. Following intra-cerebral NMDA injection in newborn rats, anandamide levels were upregulated 13-fold whereas 2-AG levels were unaltered (Hansen et al., 2001).

An attractive concept that would, at least in part, explain a neuroprotective effect of cannabinoids is if these substances act presynaptically to inhibit neurotransmitter release of glutamate. Gerdeman and coworkers, have convincingly shown in striatal slices that HU210 and WIN 55,212-2 reduce electrically evoked excitatory postsynaptic currents (EPSCs) in SR141716A sensitive manner and also that long term depression (LTD) is dependent on CB1 receptor activation (Gerdeman and Lovinger, 2001; Gerdeman et al., 2002). The same authors also presented data that supports a role for the endocannabinoid system in this process by showing that blockade of endocannabinoid uptake and postsynaptic loading with AEA facilitates LTD (Gerdeman et al., 2002). Similar results have been obtained in the nucleus accumbens and further strengthened the case for CB1 involvement by a lack of LTD by CB1 receptor agonists in CB1-/- mice (Robbe et al., 2002). The above studies derive a role for the endocannabinoids to act as retrograde messengers across the synaptic space. One well defined signal for endocannabinoid synthesis and release is elevated intracellular Ca2+, which is a consequence of e.g. NMDA receptor activation by glutamate (Di Marzo et al., 1994; Cadas et al., 1996). Hence, increased postsynaptic production and release of AEA and 2-AG following depolarisation and intracellular calcium elevation during e.g. ischemia, could act to inhibit further presynaptic glutamate release by inhibiting Ca2+ channels via the CB1 receptor. In conclusion to the in vivo studies described above, the cannabinoid system seems to act as a modulating system to maintain homeostasis in the CNS during both normal physiologic and pathophysiological processes.

Although several studies using interventions activating the CB1 receptor show receptor-mediated neuroprotection, there are some reports of the opposite where CB1 receptor antagonists rendered neuroprotection. In two reports employing transient and permanent MCAO, SR141716A exhibited similar effects, in that it decreased infarct size in the animals subjected to ischemia (Berger et al., 2004; Muthian et al., 2004). These reports are interesting contradictions to the previously discussed here and may be related to the ability of endocannabinoids to act as intrinsic retrograde signals of GABA release (Wilson and Nicoll, 2001). It is possible that depending upon the region of the brain which the predominant retrograde signalling regulates decide agonist or
inverse agonist effectiveness. An alternative explanation may be the involvement of other receptors than the cannabinoids. A recent publication also ascribe neuroprotective properties to SR141716A in gerbils, and the neuroprotection was possible to inhibit by using the TRPV1 antagonist capsazepine, suggesting a role for these receptors in neuroprotection elicited by SR141716A (Pegorini et al., 2006).

**Cannabinoid neuroprotection in vitro**

In addition to the *in vivo* studies that have been performed, several *in vitro* studies have been undertaken to define the underlying mechanisms of cannabinoid neuroprotection. In particular, a number of studies investigating neurotoxicity caused by excitotoxicity have been an area of interest. Reduction of Mg$^{2+}$ in the culture medium for primary rat hippocampal neurons cause an intense Ca$^{2+}$ spiking intracellularly that gives rise to excitotoxic damage. In using such a model, WIN 55,212-2 (100 nM) protected hippocampal neurons from toxicity and the protective effect was abolished by SR141716A (100 nM), consistent with a CB$_1$ receptor mediated effect (Shen and Thayer, 1998). In a model of kainate-induced excitotoxicity of cultured mouse spinal cord neurons, Δ$^9$-THC decreased kainate-induced cell death in a CB$_1$ receptor-mediated manner as demonstrated by the addition of SR 141716A (Abood et al., 2001).

A third study showing CB$_1$ receptor mediated neuroprotection by blocking protection with SR 141716A, also investigated the role of cAMP in this process. Rat primary cortical neuron cultures were subjected to glutamate for 15 min and the neurotoxic insult was measured 20 h later by measuring LDH-release into the culture medium. CP 55,940 up to 10 nM alone had no effect on LDH-release when added to the cultures during and after the glutamate exposure, but when a stable cAMP-analogue (db-cAMP) was added after glutamate, a significant reduction in LDH-release was found (Hampson and Grimaldi, 2001). This intriguing finding is perhaps a display of the fact that CB$_1$ receptors can couple to several intracellular signalling mechanisms, since negative coupling to adenylyl cyclase is not a logical way to explain the finding above, especially when it also was found that glutamate *per se* reduced intracellular cAMP. A more plausible explanation for the results obtained is an effect that is mediated by CB$_1$ receptor-mediated blockade of voltage sensitive Ca$^{2+}$-channels.

Other *in vitro* studies have suggested cannabinoids to produce neuroprotection but not by an obvious receptor-mediated mechanism. In a study by Sinor et al. (2000), cortical cerebral cultures were exposed to hypoxic conditions and the effect of the endocannabinoids AEA and 2-AG, and a non-hydrolysable anadamide analogue was assessed. All three substances increased cell viability following hypoxia compared to control but the increase in cell survival could not be attenuated by CB$_1$ or CB$_2$ antagonists. Nagayama and co-workers obtained similar results, hypoxia induced cell
death could be inhibited by WIN 55,212-2 and anandamide, whereas Δ⁹-THC and the inactive enantiomer WIN 55,212-3 were ineffective. The involvement of CB₁ receptors in the protection was further ruled out by the finding that the effect of WIN 55,212-2 could not be inhibited by CB₁ or CB₂ antagonists (Nagayama et al., 1999).

A publication in 1998 reported that the plant-derived cannabinoids Δ⁹-THC and cannabidiol, afforded protection of rat primary cortical neurons towards neurotoxic levels of glutamate, NMDA and kainate (Hampson et al., 1998). Cannabidiol and Δ⁹-THC had similar potencies in this process, although cannabidiol does not interact with cannabinoid receptors, and it was concluded that the neuroprotective effect seen was due to the potent antioxidant effect of these substances.

Cannabinoids and inflammation

Immune cells are active in inflammatory reactions by release of fatty acids such as arachidonic acid, release of pro-inflammatory cytokines and chemokines as well as migration towards sites of inflammation and infection. As previously mentioned the cannabinoid receptors, primarily CB₂, are expressed on immune cells and these cells can also synthesise and release endocannabinoids as a response to inflammatory stimuli. Given this background, it is not surprising that modulation of the cannabinoid system have in a large number of publications been shown to be active in the regulation of inflammatory processes (for review see Klein, 2005). The properties of immune and inflammatory modulation by cannabinoids that are of most relevance to this thesis, cytokine and chemokine release, neuroinflammation and cellular migration, are discussed in more detail below.

Multiple sclerosis (MS) is a disease that demonstrates a link between loss of autoimmune control and neurodegeneration and has been subject of intense research on cannabinoid intervention (Jackson et al., 2005). In search of novel therapies for MS, mouse models using infections with Theiler's murine encephalomyelitis virus (TMEV) and experimental allergic encephalomyelitis (EAE) have shown beneficial effects of synthetic as well as plant-derived cannabinoids on the symptoms of MS (Baker et al., 2000; Croxford and Miller, 2003; Pryce et al., 2003). Leukocytes, predominantly macrophages, infiltrating the perivascular space and parenchyma are essential in the progress of the disease (for review see Noseworthy et al., 2000). The CB₁/CB₂ receptor agonist WIN 55,212-2 has been reported to attenuate leukocyte rolling and adhesion to cerebral microvessels in EAE mice. In the same experiments the neurologic damage was also attenuated and the effect was possible to block with the CB₂ antagonist SR144528 but not SR141716A, suggesting that the effect was CB₂-mediated (Ni et al., 2004).
In different inflammatory models, cannabinoid agonists have proved to be able to restrict neutrophil migration in vivo. In a mouse myocardial ischemia-reperfusion model, WIN 55,212-2 attenuated neutrophil migration as well as the levels of the proinflammatory cytokines IL-1β and IL-8 (Di Filippo et al., 2004). In this study the CB2 receptor antagonist AM630 inhibited the effect of WIN 55,212-2, suggesting a role for the CB2 receptor in the process. A different inflammatory model, injection of 12-O-tetradecanoylphorbol-13-acetate (TPA), has been used to induce neutrophil infiltration in mouse ear (Oka et al., 2006). WIN 55,212-2 could inhibit TPA-induced neutrophil infiltration and in addition 2-AG-induced HL-60 cell migration. However, inhibition of neutrophil migration by cannabinoids is not noted in all assays. In vitro, fMLP-induced neutrophil migration across HUVEC monolayers was not affected by Δ9-THC, a result consistent with a lack of CB1 receptor expression in the neutrophils (Deusch et al., 2003).

The most abundant cells in the central nervous system, astrocytes, play an important role in regulating the homeostasis of the brain. When activated, the astrocytes respond by release of inflammatory mediators such as cytokines and chemokines, as well as nitric oxide (NO). Interleukin 1β (IL-1β) is produced in a number of neuropathologic conditions and stimulates release of TNF-α and CCL5/RANKL from human astrocytes (Aloisi et al., 1992). WIN 55,212-2 reduced the IL-1β induced release of the proinflammatory cytokine TNF-α and chemokine CCL5 from human foetal astrocytes in a dose-dependent manner suggesting an anti-inflammatory action of this substance (Sheng et al., 2005). Consistent with this report is the finding that WIN 55,212-2 decreased IL-1 induced IL-8 release in 1321N1 astrocytoma cells in a manner that was not inhibited by CB1 or CB2 antagonists, nor by pertussis toxin, indicating a mechanism that is not G-protein receptor mediated (Curran et al., 2005). CP 55,940 has also been shown to be able to alter cytokine production as it decreased TNF-α mRNA production, albeit at high concentrations, in rat microglial cells activated with lipopolysaccharide LPS (Puffenbarger et al., 2000).

Collectively, the above mentioned studies on modulation of cytokine and chemokine release with high affinity cannabinoid receptor agonists, do not give a clear picture of how the modulation is brought about. In the study by Sheng et al. (2005) the inhibitory effect of WIN 55,212-2 could be partially blocked by both SR141716A and SR144528, but in the study by Curran et al (2005) neither of those substances could block the effect of WIN 55,212-2. The latter group suggested that the effect of WIN 55,212-2 is mediated by inhibition of activation of NFκB. Other investigators have suggested a similar mechanism to be operative in inhibition of TNF-α induced IL-8 release in HT-29 epithelial cells (Mormina et al., 2006), but in this case as a downstream effect of CB2 receptor activation as shown previously by the same group (Ihenetu et al., 2003).
AIMS

The general aims of this thesis are to investigate mechanisms of neuroprotection by activation of the cannabinoid system. Although I am not the first to explore this specific field, existing data in the area are far from consensus as to how neuroprotection is achieved by manipulating the cannabinoid system. In the above introduction the emphasis, with respect to therapeutic potential of the cannabinoid system, is put on neuroprotection and regulation of inflammation and the innate immune system. A key objective of the present thesis was to develop and use simple model systems in vitro to assess cannabinoid function in excitotoxicity and inflammatory processes, and then to investigate them in an in vivo model. The specific aims of the publications and manuscript included in this thesis were as follows:

Paper I: To investigate if avian species, i.e. chick, express cannabinoid receptors and if they are functionally coupled to G-proteins by means of a radioligand binding assay.

Paper II: To elucidate, in vitro, if primary chick neurons in culture are protected from cytotoxicity due to excess glutamate by pretreatment with cannabinoid receptor agonists.

Paper III: To establish if cannabinoid receptor agonists can alter TNF-α induced neutrophil transmigration in vitro across an endothelial barrier. Moreover, the ability of cannabinoid receptor agonists to act directly on neutrophils to alter IL-8-induced chemokinesis was also investigated.

Paper IV: To explore if the photothrombotic ring stroke model is a suitable model system to use for study of cannabinoid intervention of neurodegenerative insults with respect to secondary neuroinflammatory damage.
METHODOLOGICAL CONSIDERATIONS

In this section are only the most important methods used in the papers (I-IV) discussed. All the studies in this thesis were reviewed and accepted by the local ethical review boards.

Radioligand binding (Papers I and IV)

The method of radioligand binding measuring inhibition of \[^{[3]}H\]SR141716A was the same as used previously by Thomas et al. (1998). For species comparison, adult Sprague-Dawley rat brains (minus cerebellum) and brains from 35 week old chickens were homogenized in 20 mM HEPES buffer pH 7.0 containing 1 mM MgCl\(_2\). The homogenates were centrifuged at 36000 \(\times\) g for 20 min at 4 °C after which the pellets were resuspended in homogenization buffer and the centrifugation repeated. After resuspension, the homogenates were incubated at 37 °C for 15 minutes to degrade endogenous ligands. The homogenates were centrifuged once more and thereafter resuspended in homogenization buffer and stored at -80°C until used for assay.

For the radioligand binding assay, flat bottomed 96-well microtiter plates were used with a final volume of 250 µl in each well. To all wells, membrane preparation, test compounds and \[^{[3]}H\]SR141716A (final concentration 2 nM) was added and the samples were incubated at 30 °C for 60 min. All test compounds, except the membrane preparation, were diluted to their appropriate concentration in assay buffer (50 mM Tris HCl, pH 7.4 containing 1 mM EDTA, 3 mM MgCl\(_2\), and 0.5% BSA (w/v)). By means of vacuum filtration the labelled membranes were rinsed in ice cold 50 mM Tris HCl, pH 7.4 containing 0.1% BSA (w/v) and harvested onto Whatman GF/C filters pre-soaked in 0.3% polyethylenimine. The filters were transferred to vials containing scintillation fluid (Ultima Gold™) and analyzed in a Beckman LS1801 liquid scintillation counter with quench correction.

Comments on radioligand binding

Radioligand binding is an efficient method to use in the study of ligand-receptor interactions. It is a high throughput method and straightforward to use for receptors in cell membranes. Both these properties are advantageous, since a greater number of compound concentrations can be tested over a limited time period. One drawback in this radioligand binding method is that it only measures affinity of different compounds to the receptor added and not the efficacy of them. In order to discriminate between agonist and antagonist binding, stable GTP-analogues (GppNHp and GTP\(\gamma\)S) were included in the assay. Stable GTP-analogues clamp the receptor in a conformation that is less favoured by agonists since the G-protein cannot reassemble when the stable GTP cannot be hydrolyzed.
Antagonists, with equal affinity for either receptor conformation, are not affected by this process and make it possible to discriminate agonists and antagonists in this experimental setting. The inclusion of stable GTP-analogues also makes it possible to investigate if there is a functional coupling between the receptor and its G-protein since when a stable analogue is present a rightward shift of the dose-response curve for an agonist is observed.

Another method to use if one wants to investigate a functional coupling between receptor and G-protein is the use of radiolabeled GTP such as $^{35}$S$\text{GTP}_\gamma$S which measures the amount of radiolabeled GTP that associates with the G-protein upon receptor activation in presence of excess GDP (Selley et al., 1996). In presence of an agonist the amount of $^{35}$S$\text{GTP}_\gamma$S recruited to the $\alpha$-subunit of the G-protein is increased. By this method it is also possible to quantitatively measure the G-protein activation upon receptor binding. This method has been used, for example, to measure the efficacy of several cannabinoid receptor agonists in rat cerebellar membrane preparations (Griffin et al., 1998).

For both $^{35}$S$\text{GTP}_\gamma$S binding and inhibition of $[^3\text{H}]\text{SR141716A}$ binding assays the specificity of the ligands are of importance. For example, it has been shown that both anandamide and WIN 55,212-2 can dose-dependently increase $^{35}$S$\text{GTP}_\gamma$S binding in CB$^-$ mice brain membranes, albeit with lower efficacy compared to wild type, in a manner that is not inhibited by SR141716A (Breivogel et al., 2001). These results imply a binding site that is distinct from CB$^+$ in the brain, that could be a novel CB receptor, also distinct from CB$^-$.

Culture of primary chick telencephali neurons (Paper II)

Fertilized White leghorn eggs (Gallus gallus) were incubated for eight days at 37.8 °C in a humidified egg incubator. At embryonic day eight the eggs were cracked open and the telencephali carefully dissected out in cold HEPES-buffered HBSS (Hank’s balanced salt solution) without Ca$^{2+}$ and Mg$^{2+}$. The tissue was carefully triturred in HEPES-buffered HBSS without Ca$^{2+}$ and Mg$^{2+}$ and thereafter 2 volumes of Dulbeccos modified eagles medium (DMEM) with 20% foetal bovine serum (FBS) and 1% penicillin streptomycin (PEST) were added. Non-dissociated tissue was allowed to settle for two minutes after which the supernatant was transferred to a new tube and centrifuged at 200 x g for one minute. The resulting pellet was resuspended and the cell suspension was passed through a cell strainer with 70 µm pore size and diluted with DMEM containing 20% FBS and 1% PEST to the appropriate cell density.

The cells were seeded onto poly-D-lysine-coated 24 well plates at a density of 3 x 10$^5$ cells/well with a multichannel pipette. The cells were cultured at 37 °C and on the first day in vitro (DIV) the medium was changed to DMEM with 5% FBS and 1% PEST in which it was cultured until
three DIV when the medium was changed to Neurobasal medium supplemented with 2% B-27, 0.25% glutamine and 1% PEST. At six DIV the cell viability tests were performed on the cell cultures.

Comments on primary cultures

This chick neuronal cell culture model has several advantages compared to immortalised cell lines or mammalian primary cultures. Cells in primary culture have a higher degree of biological accuracy and are more likely to reflect the in situ properties than an immortalised cell line. The primary cultures of embryonic chick telencephali are easy to produce compared to their rodent counterparts since the removal of embryos from pregnant dams is avoided. The chick neuron cultures are also very low in non-neural cells contaminating the culture and the culture time to obtain a culture to use for experiments is shorter than for mammalian counterparts. This, however, can be a disadvantage since native removal mechanisms of glutamate are lost. In six days an in vitro culture sensitive to glutamate is achieved. The same would take approximately 18-20 days in a rodent model.

Primary embryonal chick neurons are possible to culture without serum, which is advantageous since the contents of serum are not well defined and are likely to contain endocannabinoids such as anandamide. However, some drawbacks of using chick embryonic neurons instead of rodent neurons are present. The culture conditions for this type of primary neurons are not as well defined as for the traditionally used rodent neurons in conjunction with a certain shortage of anti-chicken antibodies that would be advantageous for the characterisation of these cell cultures.

Glutamate toxicity measurements (Paper II)

The effect of glutamate on cell death was measured by lactate dehydrogenase (LDH) release into the tissue culture medium. Briefly, glutamate dissolved in culture medium was added to the cell cultures and the cells were incubated for one hour with glutamate. Afterwards the medium was changed and the cells were left for 24 hours. After the incubation, aliquots of cell free culture medium was taken for LDH analysis. To measure maximal LDH release cell cultures were treated with 1% Triton X100 in order to lyse the cells. LDH was measured with a LDH cytotoxicity detection kit (Roche Molecular Biochemicals, Mannheim, Germany) in a Thermomax spectrophotometer. LDH activity is determined in a two step enzymatic test. In the first step, NAD⁺ is reduced to NADH/H⁺ by the LDH-catalyzed conversion of lactate to pyruvate. In the second step, the catalyst (diaphorase) transfers H/H⁺ from NADH/H⁺ to the tetrazolium salt INT, which is reduced to formazan which in turn is detectable at 480 nm. The amount of
absorbance is proportional to the amount of LDH that is released from damaged cells and hence a measure of cell damage.

*Comments on glutamate toxicity measurements*

The use of LDH release as a measure of cell death is a reliable and easy to use method. One drawback in measuring cell death by LDH is that cells that undergo apoptosis do not release LDH to the same extent as necrotic cells and if there are a substantial number of apoptotic cells in the population of dying cells the total cell death might be underestimated. Apart from the LDH activity assay, there are numerous different ways to measure cell death, or indeed cell viability, by spectrophotometric or fluorescent methods. These methods include MTT reduction assay measuring mitochondrial function, assays to measure nucleic acid content and calcein AM to label live cells. All these methods are now routinely used in the lab (see e.g. De Lago et al., 2006).

**Cyclic AMP measurement (Paper II)**

To confirm the existence of functional CB₁ receptors in primary cultures of embryonic chick neurons the levels of intracellular cyclic AMP (cAMP) was measured. The cells were cultured in poly-D-lysine coated 24-well plates and at six DIV the medium was exchanged and fresh medium containing 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) was added in which the cultures were preincubated for 20 min at 37 °C.

After the preincubation, the medium was replaced with fresh medium containing 0.5 mM IBMX, 5 µM forskolin and varying concentrations of CP 55,940. After a 15 minute incubation at 37 °C, the medium was aspirated and replaced with lysis buffer from a cAMP enzyme immunoassay (EIA) kit (Amersham Biosciences, Little Chalfont, UK). The cell cultures were lysed for 15 minutes at room temperature on a microplate shaker. Aliquots from the culture wells were taken and stored at -80 °C until analysed according to the manufacturers instructions with the cAMP EIA kit.

*Comments on cAMP measurement*

Determining the existence of CB₁ receptors by measuring cAMP levels is possible since the CB₁ receptor is negatively coupled to adenylyl cyclase and the activation of the receptor results in a decreased production of cAMP. Since a decrease in cAMP levels was expected we activated adenylyl cyclase with forskolin to increase the levels of cAMP, which makes it easier to measure a decrease and hence obtain a functional measure of receptor activation. cAMP is in most cases a
reliable indicator of the existence of functional CB receptors. In some cases however, the response to CB receptor activation is not relayed via cAMP as a second messenger but rather due to direct effects on Ca\(^{2+}\) and inward rectifying K\(^+\) (GIRK) channels (Mackie and Hille, 1992; Henry and Chavkin, 1995). In these instances measurements of cAMP levels might not give information on receptor function even though the ligand-receptor interaction in fact produces an effect.

**Endothelial cell model (Paper III)**

In order to model the wall of a blood vessel we cultured ECV304 cells in medium 199 (M199) supplemented with 10% foetal bovine serum (FBS), 1% penicillin streptomycin (PEST) and 2 mM glutamine. The cells were initially cultured in T-75 culture flasks and subsequently seeded in Transwell™ inserts with a 3 μM pore size in 12-well microtiter plates. The cells were cultured for 9-13 days with medium change every day before the experiments were performed. For follow up on the quality of the monolayers of cells cultured on the Transwell™ inserts the transendothelial electrical resistance (TEER) was measured every second day and before the start of the experiments.

ECV304 was originally reported as a cell line derived from spontaneously transformed human umbilical vein endothelial cells (HUVEC) and has been characterised and used in co-culture models of the blood-brain barrier (Hurst et al., 1998). These authors show that this system provides several properties that mimics the normal environment of endothelial cells of the blood-brain barrier. The identity of ECV304 as an endothelial cell line has later been disputed, on the basis that it shares the same genomic fingerprint as the urinary bladder carcinoma cell line T24/83 (Brown et al., 2000). However it has subsequently been shown that these two cell lines have separate morphologies and phenotypes (Kiesling et al., 1999; Tan et al., 2001).

**Comments on endothelial cell models**

There are a number of considerations to take into account when deciding what type of cells to use in order to model the endothelial cells of a vessel for transmigration studies. First, the cells should form a monolayer with tight junctions *in vitro* as is seen for endothelial cells *in vivo*. Second, if investigating the transmigration of human leukocytes the endothelial cells should be of human origin in order to avoid immunological reactions due to species differences. Third, and optimally, the endothelial cells should be in a cell environment similar to that seen *in vivo*, i.e. there should be other cells types present in the culture or include the use of conditioned medium.

In the case of cerebral vessels the endothelial cells should be co-cultured with glial cells as is found in the blood-brain barrier. At present, the study of endothelial cells *in vitro* is limited to the use of
primary cell cultures isolated from e.g. human umbilical cord veins or cerebral microvessels from rat or ox. This is in some circumstances an obstacle in the studies since primary cultures are only possible to keep in culture for a limited number of passages. Primary cultures display a larger degree of variability than corresponding cell lines, since they are prepared from different donors. The alternative, cell lines such as ECV304, are easy to keep in prolonged culture and are more reproducible.

Neutrophil chemotaxis and transmigration (Paper III)

For chemotaxis and transmigration experiments, venous blood was collected from healthy donors into EDTA-treated tubes. The tubes were centrifuged for 20 min at 400 x g to produce a leukocyte rich “buffy coat”. The buffy coat was collected and for the chemotaxis experiments the neutrophils were purified by magnetic activated cell sorting (MACS) using a CD15+ antibody coupled to magnetic beads according to the manufacturers instructions (Miltenyi Biotech, Bergisch Gladbach, Germany). After the magnetic separation the neutrophils were resuspended in RPMI 1640 medium supplemented with 5% FBS. The neutrophils were labelled with 5 µM Calcein AM (Molecular Probes, Eugene, USA) and preincubated with the drugs investigated before being applied to ChemoTX 96-well (Neuroprobe, Gaithersburg, USA) plates for the chemotaxis experiments. In the bottom well, 1 nM IL-8 in RPMI 1640 with 10% FBS was applied. On top of the wells, a filter with 3 µm pore size was placed on to which the neutrophil suspension was applied. The neutrophils were allowed to migrate for one hour in a humidified atmosphere at 37 °C and 5% CO₂.

After the migration period the amount of migrated neutrophils was analysed by measuring the fluorescence intensity of Calcein AM in the bottom wells at 485 nm excitation and 520 nm emission wavelength, respectively, on a Fluostar Galaxy spectrofluorometer (BMG Labtech, Offenburg, Germany). For the neutrophil transmigration experiments the whole blood was pipetted onto a Polymorphprep gradient and centrifuged at 500 x g for 20 min to yield a fraction of granulocytes and pellet the remaining erythrocytes. The granulocyte fraction was recovered and resuspended in medium 199 with 10% FBS, 1% PEST and 2 mM glutamine and labelled with Calcein AM. The granulocytes were then preincubated with the drugs for 45 min and subsequently added to confluent ECV304 cells cultured on 3 µm pore size Transwell membranes. Before the addition of the neutrophils the ECV304 cells had been stimulated with 20 ng/ml TNF-α. The neutrophils were allowed to migrate for two hours in a cell culture incubator before the number of migrated neutrophils were analysed by measuring the fluorescence intensity of Calcein AM in the bottom wells as described above.
Comments on neutrophil chemotaxis and transmigration

There are a number of substances that stimulate neutrophil migration including cytokines and bacterially derived peptides. Several previous studies have used fMLP as chemotactic factor with good results but in this study it is of less relevance since fMLP is derived from bacteria and the main objective here is to study inflammatory reactions that results from cell damage rather than bacterial infection. IL-8 was chosen as chemotactic factor in this study since it is the most potent neutrophil chemoattractant known, and with a selective effect upon neutrophils over other types of leukocytes. One drawback of both the ChemoTX™ and Transwell™ system used here for the chemotaxis and transmigration assays is that it is a static system as opposed to the in vivo situation in the microvessels where there is a constant flow.

A second drawback is the effects on processes like rolling, tethering and firm adhesion are not assessed, which are crucial parts in the process of recruiting leukocytes to inflammatory sites. Systems measuring this require specially designed flow systems and powerful microscopes to make good quality measures. However, in the flow systems, the endpoint of transmigration is not readily measured.

In vivo stroke model (Paper IV)

To investigate neutrophil transmigration across the blood brain barrier in vivo, we used a photothrombotic ring stroke (PRS) model as described by (Hu et al., 2001). Anaesthesia of male Wistar rats was induced by 3.5% halothane in a mix of 70% nitrous oxide and 30% oxygen delivered by a Fluotec 5 evaporator into the induction chamber. When the animal was properly anaesthetised it was moved to the operating table and the anaesthesia was maintained with 2% halothane delivered through a face mask. The body temperature was kept constant at 37°C by a rectal thermistor probe connected to a heating pad. The right femoral artery and vein were exposed by blunt dissection and catheterised with PE 50 polyethylene tubing for blood pressure and heart rate monitoring and delivery of drugs respectively. Through a midline incision along the neck, the sternohyoid muscle was separated with a retractor to expose the trachea and the animal was intubated with a PE 250 polyethylen tube.

After the intubation, the anaesthesia was maintained with approximately 1% halothane delivered through a small rodent ventilator and the rat was transferred to a stereotaxic frame. The skull bone was exposed by blunt dissection. A ring-formed 514.5 nm argon laser (5 mm diameter, 46 mW effect) was projected on to the skull bone symmetrically between lambda and bregma over the right hemisphere. The laser was turned on for 2 min with simultaneous infusion of 17 mg/kg erythrosine B through the femoral vein catheter for the first 30 seconds to produce transient
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ischemia in the area enclosed by the laser ring. After the laser irradiation the rat was left in the
sterotaxic frame for 45 minutes with the head temperature kept at 38 °C by a needle probe in the
temporal muscle connected to a heating lamp.

After the operation, all incisions were sutured and the rats were transferred to individual cages for
1-7 days. After the different time periods of recovery (reperfusion) the animals were killed by first
being anaesthetised with 3.5% halothane and then the right atrium and the apex of the left
ventricle was opened with small scissors. A PE250 polyethylene tube was introduced to the
ascending aorta and the animal was perfused with 200 ml isotonic saline at a pressure of 100 mg
Hg. After the perfusion the skull bone was opened and the brain was taken out and frozen at -80
°C until analysed. 10 µm frozen sections were stained with haematoxylin and eosin and the lesion
volume was calculated by using the Morfometri® software.

Comments on stroke models

The most widely used model for transient focal cerebral ischemia is the middle cerebral artery
occlusion (MCAO) model. In this model the middle cerebral artery is exposed and the artery is
occluded for the time desired by either a metal clip or a nylon filament that is introduced a
specific distance. This feature has relevance in stroke models since the middle cerebral artery often
is occluded when a human is suffering a thrombotic stroke. A drawback in the MCAO model is
that the resulting lesions are dispersed in the hemisphere and the locations are dependent upon
which segment of the MCA is occluded and rendered ischemic.

The photothrombotic ring stroke produces, in contrast to the MCAO model, a photochemical
reaction that damages the vessel wall and results in an occlusion of the vessel in a fashion similar
to what is observed in vivo and rendering the area inside the ring ischemic. Correct concentration
of the photosensitive chemical erythrosine B and correct tuning and positioning of the laser ring
produces an ischemic area that is spontaneously reperfused with time. This method also gives the
advantage of knowing where the injury will be located since a predefined area is targeted. One
drawback with the PRS model is that it only produces a cortical injury whilst occlusion of the
MCA also gives hippocampal injuries.
Western blotting (Paper IV)

Western blotting was used qualitatively to assess the expression of proteins associated with inflammation in the photothrombotic ring stroke model. Briefly, brain sections from rats subjected to PRS and different reperfusion times or from sham-operated control animals were used. The sections were homogenised with a glass douncer on ice in ice-cold homogenisation buffer (20mM TRIS, 274mM NaCl, 2% Triton X-100, 2% deoxycholate, pH 7.4) mixed 1:1 with protease inhibitor cocktail (Sigma, St Louis, USA). After centrifugation at 13000 x g at 4 °C the supernatants were recovered and stored at -80 °C until analysed. Protein content in the supernatants were analysed according to the method described by Harrington (1990). The protein samples were electrophoresed on SDS-PAGE. The gels were blotted onto PVDF membranes and incubated at 4 °C over night with the primary antibodies.

Following washing the membranes were incubated for one hour at room temperature with the corresponding HRP-linked secondary antibody. After the last washing steps the membrane was covered with ECL reagent and the membrane was developed on Hyperfilm (Amersham Biosciences, Little Chalfont, UK).

Comments on Western blotting

Although a reliable and well documented qualitative method of protein detection, Western blotting has some caveats that should be noted. Samples should be handled with greatest care in order not to be subjected to temperatures above 4 °C during preparation which would induce degradation of the proteins and result in multiple bands detected after the electrophoresis separation.

One should also take the time to carefully titrate the antibodies to determine suitable dilutions of both the primary and secondary antibodies to yield a distinct detection of the protein of interest with a minimum of background staining. When comparing samples run in separate lanes on the polyacrylamide gels or on different gels, the need to include an antibody detecting a ubiquitous protein such as actin is pivotal, in order to confirm that the same amount of protein is loaded onto the gel and hence draw conclusion as if to a protein is up- or down-regulated. Optimally, only a single band is detected after blotting and developing, when the above criteria are fulfilled.

Myeloperoxidase assay (Paper IV)

To quantify the infiltration of neutrophils into the brain parenchyma myeloperoxidase (MPO) activity was taken as a measure of the amount of neutrophils. The protocol of (Barone et al.,
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1991) was essentially followed with minor modifications as described below. Sections from rat brains were homogenised with a glass homogeniser in cold 5 mM in phosphate buffer and centrifuged at 30 000 x g after which the pellets were extracted resuspended in 50 mM phosphate buffer containing 0.5% hexadecyltriammonium bromide (HTAB). The samples then underwent three freeze-thaw cycles with 10 sec sonication after which the samples were centrifuged at 15500 x g for 15 min. Instead of using the carcinogenic substance o-dianisidine hydrochloride as a substrate for myeloperoxidase we used the TMB substrate (Sigma, St Louis, USA) and analysed the supernatants in a Thermomax spectrophotometer (Molecular Devices, Sunnyvale, USA).

**Comments on myeloperoxidase assay**

When aiming to estimate the amount of neutrophil influx to the brain parenchyma, one obvious way to quantify this process is to stain sections of brain tissue from the region of interest with a standard histological stain such as haematoxylin and eosin and count the number of neutrophils dictated by their morphology. This, however, requires a skilled histologist to recognise neutrophils by their morphology and also demands good quality tissue sections. Such a study should also be carried out by an operator blinded to the belonging to treatment groups of the animals. A similar approach is to use an antibody that recognises neutrophils with high specificity which gives a more binary result in the sense that negative and positive cell are easier to differentiate and gives less interpretation to the person analysing the stained tissue. However, both these approaches are quite labour intensive and there is a possibility for bias in the determination of positive and negative cells.

When using a myeloperoxidase enzymatic activity assay to asses the amount of neutrophil influx, the end point is a optical density value, i.e unbiased. In order to obtain good quality measurements that give the resolution necessary to detect changes between treatment groups, a number of precautions must be taken. First, the animals must be thoroughly perfused to wash away blood components from the vasculature that would otherwise contribute to a high background level of MPO activity. Second, the smallest anatomical area of interest possible to use for the assy should be obtained. The importance of dissecting out different regions was shown by Barone et al (1991) when simple separation of ipsilateral and contralateral hemispheres showed no statistical difference in MPO activity after different treatments but such a difference could be seen on the cortical level.
RESULTS AND DISCUSSION

Radioligand binding (Paper I)

Excitotoxicity is an important neurotoxic process during cerebral ischemia. The study of protective mechanisms against excitotoxicity requires robust and well characterised *in vitro* systems. Neuronal cultures of rodent origin are the most common types of primary cultures used for such assays. However, in avian species little is known about the cannabinoid system. As a first step to investigate if chicken is an appropriate species to conduct research on the cannabinoid system, radioligand binding measuring inhibition of [$^3$H]SR141716A binding by cannabinoid agonist, was undertaken in adult chicken brain. The initial pharmacological characterisation had been done by co-author Andersson while my contribution was with respect to the experiments including the stable GTP-analogues in the assay.

The initial experiments revealed that CP 55,940 could dose-dependently inhibit [$^3$H]SR141716A binding in membrane preparations of chick brain with a $p_{50}$ of 7.77 (IC$_{50}$ = 17 nM). Similar results were obtained for the same experiment done in rat brain membranes, albeit with a higher total binding and a slightly higher $p_{50}$ value of 7.41 (IC$_{50}$ = 38 nM). The result obtained in the rat is consistent with data for inhibition of [$^{125}$I]AM251 by CP 55,940 with an IC$_{50}$ value of 4 nM (Gatley et al., 1997).

Having established that binding of [$^3$H]SR141716A does indeed occur in chicken brain membranes the GTP-analogues GTP$_{\gamma}$S and GppNHp were added to the membranes before binding experiments were conducted. The inclusion of these stable analogues in the assay produced a rightward shift of the dose-response curve for inhibition of [$^3$H]SR141716A binding by CP 55,940 in chick brain membranes. When performing non-linear regression for the experiments including GTP$_{\gamma}$S and GppNHp, with constraints for the bottom values set to the corresponding binding values at the highest CP 55,940 concentration, the EC$_{50}$ values were 310 and 250 nM respectively. These values, however, are highly dependent upon the constraints used in the curve fitting process, and should thus be considered in this context. Nonetheless, the rightward shift in the dose-response curves in this study is in line with previous reports obtained for WIN 55,212 and CP 55,940 with stable GTP-analogues in rat cerebellar membranes (Griffin et al., 1998; Houston and Howlett, 1998).

The results obtained in our study reveal that in the chick brain homogenates, specific receptor binding of the antagonist SR 141716A occur, and that the receptors are functionally coupled to G-proteins as shown by the rightward shift of the dose-response curve.
Glutamate toxicity in cultured embryonic chick telencephali cultures (Paper II)

In paper I, it was concluded that adult chick brain membranes display cannabinoid receptors that retain a functional coupling to G-proteins. This finding raises the possibility that primary embryonic chick telencephali cultures can be used as a model system to study toxic effects of excess glutamate and if this process can be influenced by cannabinoid receptor ligands. The culture model was set up previously in the laboratory and has been characterised here with respect to NMDA-receptor expression and glutamate sensitivity (Jacobsson et al., 1998; Jacobsson and Fowler, 1999). With respect to cannabinoid receptors, it was not known at the start of the study, whether chick embryonic telencephali cultures display functional cannabinoid receptors. To address this issue, an intracellular cAMP assay was employed to determine the effect of CP 55,940 on forskolin induced cAMP levels. The experiments demonstrated that CP 55,940 could potently and efficaciously reduce forskolin-induced intracellular cAMP level in primary chick neurons. The maximal decrease was to 41% of forskolin induced control with 300 nM CP 55,940 (i.e. a 59% inhibition) and already at a concentration of 3 nM a substantial decrease to 60% of forskolin induced control (40% inhibition) was seen. The effect of CP 55,940 was abolished by preincubation with the CB$_1$ receptor antagonist/inverse agonist AM251. AM 251 per se had no effect on the forskolin induced cAMP levels. These results show that primary embryonal chick telencephali cell in culture express functional CB$_1$ receptors and that they in this respect are suitable as a model system to study cannabinoid receptor mediated neuroprotection. The level of cAMP reduction achieved in this culture system is similar or larger compared with other cell systems, summarised in table 1. Furthermore, the concentration of agonist producing an effect is much lower here, compared to the threshold levels in table 1.

Table 1. Effect of cannabinoid agonists on cAMP reduction in tissues and cell preparations.

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Stimulus</th>
<th>Compound</th>
<th>Maximal% inhibition</th>
<th>Inhibition by SR141716A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rat cerebellar membranes</td>
<td>Basal</td>
<td>10 µM WIN 55,212-2</td>
<td>40</td>
<td>N.D</td>
</tr>
<tr>
<td>Cultured rat cortical neurons</td>
<td>1 µM forskolin</td>
<td>1 µM CP 55,940</td>
<td>46</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table continued overpage
Table 1 (continued)

<table>
<thead>
<tr>
<th>Tissue/cell type</th>
<th>Stimulus</th>
<th>Compound</th>
<th>Maximal% inhibition</th>
<th>Inhibition by SR141716A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cultured mouse cortical neurons&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1 µM isoproterenol</td>
<td>10 µM WIN 55,212-2</td>
<td>60</td>
<td>Yes</td>
</tr>
<tr>
<td>Cultured mouse striatal&lt;sup&gt;c&lt;/sup&gt; astrocytes</td>
<td>1 µM isoproterenol</td>
<td>10 µM WIN 55,212-2</td>
<td>60</td>
<td>No</td>
</tr>
<tr>
<td>Rat cerebellar membranes&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Basal</td>
<td>10 µM CP 55,940</td>
<td>40</td>
<td>N.D</td>
</tr>
<tr>
<td>Rat cerebellar membranes&lt;sup&gt;d&lt;/sup&gt;</td>
<td>Basal</td>
<td>10 µM AEA</td>
<td>33</td>
<td>N.D</td>
</tr>
<tr>
<td>Rat cerebellar membranes&lt;sup&gt;e&lt;/sup&gt;</td>
<td>Basal</td>
<td>3 µM WIN 55,212-2</td>
<td>34</td>
<td>Yes</td>
</tr>
<tr>
<td>CHO-hCB&lt;sub&gt;f&lt;/sub&gt;</td>
<td>50 nM forskolin</td>
<td>1 µM HU 210</td>
<td>75&lt;sup&gt;f&lt;/sup&gt;</td>
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<tr>
<td>T-cells&lt;sup&gt;g&lt;/sup&gt;</td>
<td>50 µM forskolin</td>
<td>22.5 µM (15 µM)&lt;sup&gt;h&lt;/sup&gt; Δ&lt;sup&gt;2&lt;/sup&gt;-THC</td>
<td>-45</td>
<td>N.D</td>
</tr>
<tr>
<td>Rat cerebellar membranes&lt;sup&gt;h&lt;/sup&gt;</td>
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<td>Yes</td>
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<td>Rat striatum (in vivo)&lt;sup&gt;i&lt;/sup&gt;</td>
<td>30 µM forskolin</td>
<td>1 mM WIN 55,212-2</td>
<td>-46</td>
<td>Yes</td>
</tr>
<tr>
<td>N18TG2 neuroblastoma&lt;sup&gt;j&lt;/sup&gt;</td>
<td>1 µM prostacyclin</td>
<td>1 µM levonantradol</td>
<td>56</td>
<td>N.D</td>
</tr>
<tr>
<td>Rat cortical brain slices&lt;sup&gt;k&lt;/sup&gt;</td>
<td>10 µM isoproterenol</td>
<td>10 µM levonantradol</td>
<td>25</td>
<td>N.D</td>
</tr>
</tbody>
</table>

<sup>a</sup>(Pacheco et al., 1993), <sup>b</sup>(Jung et al., 1997), <sup>c</sup>(Sagan et al., 1999), <sup>d</sup>(Childers et al., 1994), <sup>e</sup>(Hirst et al., 1996), <sup>f</sup>(Glass and Felder, 1997), <sup>g</sup>(Schatz et al., 1997), <sup>h</sup>(Breivogel and Childers, 2000), <sup>i</sup>(Wade et al., 2004), <sup>j</sup>(Howlett, 1984), <sup>k</sup>(Bidaut-Russell et al., 1990). * indicates threshold concentrations.
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To test if endocannabinoids and synthetic cannabinoids could prevent glutamate induced cytotoxicity, embryonic chick telencephalic cultures at 6 days *in vitro* were exposed to either 100 µM or 1 mM L-glutamate for one hour, and after medium change the LDH activity was measured 24 hours later. The same experiments were also performed in the presence of db-cAMP during, or both during and after the glutamate exposure. Exposure of the cells to glutamate produced a large increase in LDH release into the tissue culture supernatant. Pretreatment of the cell cultures with CP 55,940 at concentrations from 3-30 nM (*i.e.* concentrations known to activate the CB₁ receptor and reduce forskolin-induced cAMP elevation), however, did not change the amount of LDH released into the medium during the following 24 hours, regardless if db-cAMP was present or not.

These results are in contrast to previously published results (Hampson and Grimaldi, 2001), where CP 55,940 protected primary cortical neuron cultures from glutamate-induced excitotoxicity in a fashion that was possible to block with SR141716A, when db-cAMP was included in the assay but not in its absence. The use of db-cAMP in the assay was motivated by the fact that following cerebral ischemia, the level of cAMP is elevated in brain, plasma and cerebrospinal fluid (CSF) during post-ischemic reperfusion (Mrsulja et al., 1984; Blomqvist et al., 1985; Suyama et al., 1995) and administration of cAMP analogues or PDE inhibitors decreases morbidity (Toung et al., 1996; Block et al., 1997).

In a second series of experiments the ability of AEA and a mix of the *N*-acylethanolamines AEA, palmitoylethanolamide (PEA), stearoylethanolamide (SEA) and oleoylethanolamide (OEA) to inhibit glutamate induced excitotoxicity of the chick neuronal cell cultures was tested. These substances were tested based on the notion that, *in vivo*, the brain levels of AEA, PEA, SEA and OEA are markedly during neurodegeneration and intracerebral NMDA injection of rat pups (Hansen et al., 2001) and the rationale for including PEA, SEA and OEA, although they are not active at cannabinoid receptors to any substantial degree (Lambert et al., 1999), is that they might modulate a putative neuroprotective effect of AEA by competing as substrates for fatty acid amide hydrolase (FAAH) and hence increasing the effect of AEA by reducing its hydrolysis (Lambert and Di Marzo, 1999). However, these substances did not cause any reduction in the amount of LDH release compared to their vehicle controls.

To explain the lack of effect by CP 55,940 and the *N*-acylethanolamines in the chick neuronal cell cultures, as opposed to the findings by Hampson and Grimaldi (2001), is not totally straightforward. A species difference between the chick and the rat with respect to CB₁ receptor sensitivity seems unlikely based on the obtained data in paper I. Factors such as that the chick neuronal cultures are held in serum-free condition could influence the outcome of the cannabinoid treatment as well as the lack of glial cells in the chick neuron cultures. Glial cells are
important in the reuptake of glutamate released from neurons and the loss of this function could increase the sensitivity of neurons to glutamate (for review see, Bambrick et al., 2004).

Neutrophil chemotaxis (Paper III)

Anti-inflammatory effects of cannabinoids are well documented, especially in vivo, but in this study the interest was to investigate if neutrophil migration is affected by cannabinoid treatment given the role of neutrophils following ischemia and the neuroprotective effects seen when administering cannabinoids in neurodegenerative models in vivo, (see introduction). IL-8 was chosen as chemotactic factor because that most previous studies have use fMLP, a bacterial-derived substance, while our interest was more focussed on post-ischemic inflammation which does not necessarily involve infectious agents. IL-8 (1 nM) in the lower chamber of the chemotaxis plate produced a significant increase in neutrophil chemotaxis compared to random chemotaxis. Thapsigargin, used as a positive control (Elferink and de Koster, 2000), at concentrations of 1 or 10 µM decreased the IL-8 induced chemotaxis to levels comparable to the basal rate of random migration. Preincubation of the neutrophils with the synthetic cannabinoids HU 210, CP 55,940 and WIN 55,212 did not change the chemotactic response towards IL-8, similarly AEA was without any significant effect. However, an AEA-induced decrease in fMLP-induced neutrophil migration has been reported, albeit in a non-CB1-, non-CB2-dependent fashion (McHugh and Ross, 2004). This would suggest that the observed effect of AEA is to interfere with the response to fMLP downstream of the fMLP receptor in a way that does not occur downstream of the IL-8 receptor. Both IL-8 and fMLP receptors are G-protein coupled and share many intracellular signalling pathways upon receptor activation (Fujita et al., 2005). However, several intracellular mechanisms act in concert to induce migration and it is possible that AEA can act differently depending on the chemotactic stimulus.

More recently, McHugh (2005) have proposed that AEA may act as a precursor and its metabolites are responsible for the inhibition of fMLP-induced neutrophil chemotaxis (McHugh, 2005). Although CB2 receptors are considered as “immune cell receptors”, the expression in neutrophils is not entirely clear. In 1995, it was reported in an often cited publication, that CB2 receptors are detected in neutrophils among other subsets of leukocytes (Galiegue et al., 1995). However, these results were obtained using RT-PCR which is a very sensitive method of detection, raising the possibility that it could be a false positive result originating from incomplete removal of other types of leukocytes than neutrophils in the preparation of the material from whole blood. Alternatively, neutrophils might carry CB2 mRNA without expressing the receptor protein. Consistent with this notion, Deusch et al. (Deusch et al., 2003) reported that the CB2 protein could be detected by Western blotting in the lymphocyte fraction, but not in the neutrophil fraction from the same blood samples.
Chemotaxis, the directional movement in response to a chemical gradient, is a fairly simple biological process. Chemotaxis is, however, only the final step in the more complicated process when leukocytes move from blood vessels, across a tight layer of endothelial and epithelial cells, into surrounding tissues. In the experiments presented in this section, confluent monolayers of ECV304 cells were preincubated with TNF-α, after which calcein AM-labelled neutrophils were allowed to migrate across the monolayers. This assay was used, in a much simplified way, to model in vitro the transmigration of neutrophils from cerebral blood vessels, across the blood-brain barrier, into the brain parenchyma. PD 98059, a MEK inhibitor acting downstream of the TNF-α receptor, dose dependently decreased the amount of migrated neutrophils to the lower chamber in the assay system used, as expected (Stein et al., 2003). Similarly, the non-steroidal anti-inflammatory drug (NSAID) ibuprofen, could also significantly decrease TNF-α migration, a response previously reported for TNF-α induced migration across HUVEC (Hofbauer et al., 1998). Four substances active at cannabinoid receptors, HU 210, CP 55,940, WIN 55,212-2 and AEA were tested for their ability to inhibit TNF-α induced neutrophil transmigration. Out of these four, only WIN 55,212-2 produced a significant decrease in neutrophil migration.

To investigate further the mechanism of action by WIN 55,212-2, the ECV304 cells and the neutrophils were preincubated with CB1 and CB2 receptor antagonists AM 251 and AM 630, respectively (for structures of the compounds see Fig 3). Neither of the CB receptor antagonists could reverse the effect seen for WIN 55,212-2, suggesting that the effect of WIN 55,212-2 is not CB1 or CB2 receptor mediated. Non-receptor mediated effects of WIN 55,212-2 has been reported several times before, especially in the micromolar range that gave a significant effect in this study, in several different model systems and tissues (Martinez-Orgado et al., 2003; Nieri et al., 2003; Price et al., 2004; Steffens and Feuerstein, 2004).

The explanation for how WIN 55,212-2 decreases the TNF-α induced transmigration of neutrophils is not entirely clear but it has been reported that preincubation of HT 29 cells with TNF-α induces IL-8 release and that this can be inhibited by WIN 55,212-2 by a non-CB receptor dependent mechanism (Ihenetu et al., 2003; Kim et al., 2005). To investigate if a similar mechanism, reduced IL-8 release from the ECV304 cells, is responsible for the reduced transmigration caused by WIN 55,212-2, the IL-8 release produced by TNF-α incubation of ECV304 cells was assessed. TNF-α induced a robust increase compared to cells not subjected to TNF-α and the increase was inhibited by preincubation with WIN 55,212. Addition of AM251 and AM630 did not produce a significantly different effect compared to that seen for WIN
55,212 alone. Once again the results suggest that the effect of WIN 55,212-2 is not mediated by any to date characterised cannabinoid receptor. Regardless if the decrease in transmigration is CB receptor-dependent or not, a tempting conclusion is that the effect is manifested via the ECV304 cells rather than directly on the neutrophils. This would explain the lack of effect from WIN 55,212-2 on the chemotaxis towards IL-8 alone. The key question though, how WIN 55,212-2 interferes with the TNF-α induced IL-8 release, is not answered by the experiments presented here. In a recent study, however, it was shown that preincubation with WIN 55,212-2 before TNF-α treatment of HT 29 cells decreased the release of IL-8 and that this was accompanied by inhibition of IκB degradation and subsequent NFκB binding (Mormina et al., 2006). These authors further showed by the use of actinomycin D and cycloheximide that the TNF-α induced IL-8 release requires de novo synthesis as opposed to mere release from intracellular stores. An alternative way for WIN 55,212-2 to interfere with TNF-α induced IL-8 release is by interacting in the sphingomyelin-ceramide signalling as outlined in Fig 4.

Ceramide has been shown to be involved in intracellular signalling following treatment of different cell types with cannabinoid receptor ligands, since the intracellular ceramide levels increase upon cannabinoid treatment, albeit in a manner not blocked by CB receptor antagonists, causing phosphorylation of ERK1/2 which subsequently can lead to NFκB activation (Galve-Roperh et al., 2000; Gomez del Pulgar et al., 2002; Blazquez et al., 2004). The spingomyelin-ceramide signalling pathway could perhaps represent a point of intersection for TNF-α signalling and WIN 55,212-2. Upon TNF-α binding to one of its cognate receptors, ceramide levels are
increased and subsequent enzymatic metabolism of ceramide by ceramidase produces sphingosine (Xia et al., 1998). Sphingosine is in turn metabolised to sphingosine phosphate (S1P) by sphingosine kinase. S1P have been shown to be a key molecule in TNF-α-induced up regulation of VCAM-1 and E-selectin in HUVEC, both proteins being imperative for leukocyte transmigration *in vivo* (Xia et al., 1998). It is a possibility that WIN 55,212-2 interact in the signalling cascade from TNF-α receptor binding to S1P formation and thereby reduces TNF-α-induced neutrophil transmigration. This is admittedly a speculation, but nonetheless a testable one.

**Photothrombotic lesion and myeloperoxidase activity (Paper IV)**

A transient and reproducible ischemic lesion can produced through a non-invasive method using erythrosine B activated with a circular laser beam. This robust method to inflict a neurodegenerative insult has been thoroughly investigated previously by other investigators with respect to ischemic volume, cerebral blood flow, inflammatory markers, neurogenesis and apoptosis (Wester et al., 1995; Gu et al., 1999; Hu et al., 1999; Gu et al., 2000; Hu et al., 2004). In this study, however, it was examined if neutrophil infiltration to the ischemic area could be detected by a myeloperoxidase assay. The photothrombotic procedure produced an ischemic volume that expanded significantly from 0-48 hours and gradually decreased during the course of the next five days (see Fig 5). The maximum ischemic volume was detected at 48 hours after photothrombosis and is consistent with previous reports. The interest in this study was in particular to define a time frame for neutrophil infiltration following photothrombosis and explore if this measure would be possible to use as an endpoint if intervening with compounds of the cannabinoid system. Using an assay for myeloperoxidase activity as a measure of neutrophil content, an increase in myeloperoxidase activity that had a statistical significance could be detected at 48 hours after the irradiation. At the other time points measured, the MPO activity was not altered compared to control or sham operated rats. Although there is a statistically significant increase in MPO activity at 48 hours, it is at a level barely above the detection limit of the assay, (see Fig 5).

The fact that the variation of myeloperoxidase activity in the samples is at a very low level would hamper the value of this endpoint to investigate if cannabinoid ligands can decrease neutrophil infiltration following neurodegenerative insults. This obstacle could probably be overcome by using larger tissue samples in the preparation but it would make determination of the ischemic volume in the same animal more difficult. Previous studies measuring MPO activity following transient ischemia have found increases at both earlier and later time points than 48 hours as was found in this study (Barone et al., 1991; Matsuo et al., 1994; Yamasaki et al., 1995; Barone and Feuerstein, 1999).
Fig 5. Ischemic volume and myeloperoxidase activity in rat brain tissue at different time points following photothrombosis. Ischemic volume is presented as % of hemisphere volume and the activity is expressed in units per gram tissue (wet weight). *P and †P < 0.05 vs sham operated control with for ischemic volume and myeloperoxidase activity, respectively, with Dunnett’s multiple comparison test following significant one-way ANOVA.

These studies have used MCA occlusion to achieve transient ischemia that affects a larger region involving caudate putamen as well as the cortical layers, which can be one explanation to the substantially higher MPO levels detected (Beray-Berthat et al., 2003). A distinct advantage in using the photothrombotic model instead of the traditional MCAO model is the reproducibility of the lesion and that a pre-defined region is affected. To avoid invasive methods and to take advantage of knowing where the lesion will be situated, magnetic resonance imaging (MRI) makes a method possible to employ in the study of lesion growth and leukocyte infiltration (Schroeter et al., 2001). The drawback of using this method in a research setting is in part image resolution and, the expense of the equipment required.

Radioligand binding properties following ischemia-reperfusion (Paper IV)

The key to a successful intervention is that the target, i.e. cannabinoid receptor 1, of the intervention is intact. In view of this we wanted to investigate if antagonist binding to CB₁ receptors was altered after photothrombotic stroke in adult rats. In a preliminary experiment,
consecutive slices from control brains were analysed to define if there are differences between adjacent areas in the binding of the radioligand but this was not the case (see Fig 6).

Fig 6. Inhibition of \(^3\)H\SR141716A binding by CP 55,940 in four 1 mm consecutive slices prepared from a single rat brain.

In tissue samples collected at 24, 48, 72, 120 and 168 hours after irradiation, the binding of \(^3\)H\SR141716A in the presence of increasing concentrations of CP 55,940 was analysed. Upon comparison between the different time points, total binding and decreased binding with CP 55,940 present was unaltered. Furthermore the expected rightward shift of the dose-response curves when stable GTP-anallogues were included in the assay was also unaltered suggesting that the coupling between the receptor and the G-protein remains intact after photothrombosis.
CONCLUSIONS

This thesis work was undertaken to explore different aspects and mechanisms of neuroprotection involving the cannabinoid system. Different methods and species and methods have been employed and yielded finding that can be concluded as follows.

- The adult chick brain expresses CB₁ receptors that are functionally coupled to G-proteins and the functional coupling is persistent after oxidative stress.
- Primary cultures of embryonic chick telencephali express functional CB₁ receptors coupled to adenylyl cyclase but are not protected from glutamate toxicity by CB₁ agonists.
- The CB₁/CB₂ receptor agonist WIN 55,212-2 can inhibit TNF-α induced neutrophil transmigration across endothelia ECV304 cells. Underlying this effect is most likely decreased release of IL-8 or other cytokines from the endothelial cells by a mechanism distinct from the CB₁ and CB₂ receptor.
- The photothrombotic ring stroke model produced a robust lesion and a modest influx of neutrophils to the brain tissue as assessed by measurement of MPO activity. The CB₁ receptor remain intact following the photothrombotic stroke, indicating that it can be a valid target for intervention studies in this model.
GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

As mentioned in the introduction of this thesis, the cannabinoid system influences a vast number of biological functions through a number of different mechanisms. The findings in paper I and II, that both the adult and embryonal chick brain express functional CB1 receptors are interesting per se, but also provides simple model systems to use in cannabinoid research. The lack of protective effect of the cannabinoid agonists on their own in the primary culture system used in paper II, rules out these cultures as model system for mechanistic studies of neuroprotection. This culture system, however, could be expanded to address questions such as the involvement of glial cells in neuroprotection and the influence of cannabinoids. Such questions could be investigated in co-culture systems of neurons and glial cells and would hopefully contribute with interesting data.

The relevance of coculture systems could be further expanded in blood-brain barrier models such as the one used in paper III. Glial cells are undoubtedly involved in the regulation of immune cell entry to the brain from the blood stream since they are an important part of the function of the blood-brain barrier. Studies of neutrophil transmigration across a coculture of endothelial and glial cells would add additional relevance to the situation in vivo as well as data on the importance of glial cells in this process. Furthermore the effect of cannabinoid preincubation on, for example, astrocytes subjected to in vitro ischemia and their release of pro- and anti-inflammatory cytokines would give interesting clues to the involvement of the cannabinoid system in neuroinflammation. The subject of cannabinoid involvement in neuroinflammation is not only important in multiple sclerosis as previously shown (Baker et al., 2000; Croxford and Miller, 2003; Ni et al., 2004) but also potentially in cerebral ischemia. The finding in paper III that WIN 55,212-2 could inhibit TNF-α induced neutrophil transmigration by a mechanism separate from the cannabinoid receptors is interesting and should warrant further investigation. Recent publications on this subject have suggested that WIN 55,212-2 could exert an effect through the NFκB pathway which is central in the regulation of the release of several cytokines (Curran et al., 2005; Mormina et al., 2006).

The ability of WIN 55,212-2 to affect inflammatory processes as well as being neuroprotective (Nagayama et al., 1999) makes it an obvious candidate to test for intervention in the photothrombotic ring stroke model used in paper IV. In this respect it might be possible to compare this compound with other cannabinoids that do not affect neutrophil transmigrations to gauge the importance of this effect in neuroprotection. Such studies will hopefully contribute to a better understanding of the role of the cannabinoid system in the body.
Hjärnan som utgör cirka två procent av den totala kroppsvikten förbrukar ca 20 procent av den totala mängden tillgängligt syre och glukos. När blodflödet i hjärnan hämmas på grund av att det bildats en propp eller att en blödning uppstått så uppstår brist på både syre och glukos i den region som är försörjd av de påverkade blodkärlen. Denna process som kallas ischemi (från grekiskans ischemia - flöde och hemostasis - blod) ger snabbt upphov till skador på nerv- och glia-celler i hjärnan. Ischemi i hjärnan, kallas i dagligt tal stroke, är en sjukdom som drabbar cirka 30 000 individer i Sverige varje år. De som överlever lider ofta av efterföljande handicapp som nedsatt fysisk funktion eller afasi. 85% av alla strokefall orsakas av blodproppar och den mest effektiva behandling som finns att tillgå idag bygger på att man ger läkemedel som löser de blodproppar bildats. För att behandlingen skall vara effektiv krävs det att den drabbade individernas hjärntapade blodflöde återställs inom loppet av cirka 3 timmar. Utöver detta finns det få modifierats så att de inte uttrycker CB1-receptorer åtvisat en större hjärnskada av en experimentell stroke än normala möss. Dessutom har studier visat att olika typer av hjärnskadade djur blöder till skillnad mot hela kroppen.
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preparationer av kycklinghjärna innehåller funktionella cannabinoidreceptorer. Syftet med detta var att bedöma om denna art kan vara lämplig att använda som modell att studera mekanismerna bakom cannabinoidinducerad neuroprotektion. Utifrån detta arbete kunde det konstateras att i vuxen kycklinghjärna finns cannabinoidreceptorer, samt att de är funktionellt kopplade till de proteiner som förmedlar effekten av molekyler som aktiverar receptorerna.

I det andra arbetet undersökt dels om funktionella cannabinoidreceptorer uttryckts i odlingar av nervceller från embryonala kycklinghjärnor, samt om agonister till CB₁-receptorn kan verka skyddande när nervcellsdölingarna utsätts för höga koncentrationer av aminosyran glutamat. Syftet med dessa studier var att i ett renodlat cellodlingssystem efterlikna vissa av processerna som uppstår i samband med en stroke, nämligen att stora mängder glutamat frisätts vilket är skadligt för nervcellerna. Därutöver saknades kunskap om nervceller från embryonala kycklingar uttrycker funktionella CB₁-receptorer, vilket är en förutsättning för att molekylen som aktiverar receptorn skall kunna ha någon effekt i detta modellsystem. Resultaten visade att det finns fungerande CB₁-receptorer i nervcellsdölingarna men att aktivering av dem inte skyddar mot glutamatinducerad celldöd.

I det tredje arbetet har effekten av agonister till cannabinoidreceptorer på ett inflammatoriskt förlöp undersökt. Bakgrunden till att denna studie genomfördes är att vid en stroke så uppstår först en akut fas där nervceller dör på grund av excitotoxicitet (överstimulering av receptorer med t. ex glutamat), för att sedan följas av en inflammatorisk fas då vita blodkroppar kan förflytta sig från blodomloppet in i hjärnvävnaden. Denna infiltration av vita blodkroppar, främst neutrofiler, tros kunna bidra till hjärnskadan som fortsätter att utvecklas även efter den akuta fasen. Blodhjärnbarnären, som utgörs av celler som bildar blodkärl, nervceller och stödjeceller i hjärnan (gliaceller), studerades om cannabinoider kan påverka neutrofilers förmåga att migrera över ett lager endotelceller. Modellen baseras på att neutrofiler renas fram från blodprover och sedan tillsätts till en odling av endotelceller, vilka stimulerats med en substans som främjar inflammation och migrering av neutrofiler. I detta system mättes mängden neutrofiler som passerade över lagret av endotelceller med eller utan cannabinoider närvarande. Av resultaten framgick det att en av de testade substanserna, WIN 55,212-2 som aktiverar både CB₁- och CB₂-receptorer, kunde hämma neutrofilernas förmåga att passera över endotelcellslagret. Ytterligare försök visade att detta inte berodde på en direkt effekt av WIN 55,212-2 på neutrofilerna utan snarare på en effekt som påverkar frisättningen av interleukin 8 från endotelcellerna, en substans som stimulerar neutrofilers förflyttning.

I det fjärde och sista arbetet så undersöktes en experimentell modell för stroke i rätta för att se om den skulle kunna passa för att studera mekanismer bakom cannabinoidinducerad neuroprotektion i levande djur. Kortfattat innebär modellen att skallbenet på en sövd rätta friläggs och belyses med

Sammanfattningsvis har studierna som ingår i denna avhandling bidragit med breddad kunskap om cannabinoidsystemets utbredning och funktion i kyckling, en tidigare dåligt undersökt art i detta avseende. Utöver detta har också funktioner som ingår i inflammatoriska processer efter stroke visats påverkas av cannabinoidsystemet samt att det är möjligt att undersöka sådana förlopp i en djurmodell. Framtida forskning inom fältet får utvisa huruvida påverkan av cannabinoidsystemet är en framkomlig väg för att förbättra utfallet för personer som drabbas av stroke.
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