The endocannabinoid system:
A translational study from Achilles tendinosis
to cyclooxygenase

Emmelie Björklund
“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein
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Original papers

The present thesis is based on the following papers, which are referred to in the text by their Roman numerals.


IV. **Emmelie Björklund**, Anders Blomqvist, Joel Hedlin, Emma Persson, Christopher J. Fowler. Involvement of fatty acid amide hydrolase and fatty acid binding protein 5 in the uptake of anandamide by cell lines with different levels of fatty acid amide hydrolase expression: a pharmacological study. *Submitted*

Abstract

The endogenous cannabinoids anandamide (arachidonoyl ethanolamide, AEA) and 2-arachidonoyl glycerol (2-AG) exert their effect by activating cannabinoid receptors (CB). These receptors mediate a broad range of physiological functions such as beneficial effects in pain and inflammation, although little is known about the expression of CB receptors in human pain conditions. AEA and 2-AG are short-lived molecules due to their rapid cellular accumulation and metabolism. The enzymes primarily responsible for their degradation are fatty acid amide hydrolase (FAAH) for AEA and monoacylglycerol lipase (MGL) for 2-AG. Inhibition of endocannabinoid metabolism is a potential approach for drug development, and there is a need for the identification of novel compounds with inhibitory effects upon FAAH and MGL.

In Paper I of this thesis, the expression of CB₁ receptors in human Achilles tendon was examined. We found expression of CB₁ receptors in tenocytes, blood vessel wall as well as in the perineurium of the nerve. A semi-quantitative analysis showed an increase of CB₁ receptors in painful human Achilles tendinosis.

In papers II and III, termination of AEA signalling was investigated via inhibition of FAAH. In Paper II, Flu-AM1, an analogue of flurbiprofen, was investigated. The compound inhibited both FAAH and the oxygenation of 2-AG by cyclooxygenase-2. In Paper III the antifungal compound ketoconazole was shown to inhibit the cellular uptake of AEA in HepG2, CaCo-2 and C6 cell lines in a manner consistent with inhibition of FAAH.

The role of FAAH in gating the cellular accumulation of AEA was investigated in Paper IV. FAAH has been shown to control the concentration gradient of AEA across the plasmamembrane in RBL2H3 cells, whereas no such effect is seen in other FAAH-expressing cell lines. To determine whether this effect is assay dependent or due to intrinsic differences between the cell lines, we assayed four cell lines with different levels of FAAH expression using the same methodology. We
found that the sensitivity of FAAH uptake inhibition was not dependent on the expression level of FAAH, suggesting that factors other than FAAH are important for uptake.

Paper V is focused on the inhibition of MGL. Prior to this study no selective inhibitors of the enzyme had been described. Thus, we screened a number of compounds for their inhibitory effect on MGL. Troglitazone was found to be an inhibitor of MGL, although its potency was dependent upon the enzyme assay used.
Populärvetenskaplig sammanfattning


Det är idag oklart hur dessa signaleringsmolekyler tas upp av cellen. Än så länge finns det inget identifierat transportprotein som står för förflyttningen över cellmembranet men det finns en rad hypoteser om hur endocannabinoider transporteras från utsidan av cellen till de metaboliserande enzymerna. De mest studerade enzymerna som bryter ner endocannabinoider är fettsyramidhydrolas (FAAH) och monoacylglycerollipas (MGL). Då man i en rad olika djurmodeller har påvisat en smärtlindrande effekt av cannabinoidsignalering ökar intresset för att farmakologiskt modifiera detta system och på så sätt få en ökad signalering. Användning av cannabinoider begränsas på grund av psykogena effekter men teoretisk skulle blockering av metabolismen av de kroppsegna cannabinoiderna leda till terapeutiskt effekt utan biverkan på centrala nervsystemet.

Den mesta forskningen som är gjord på endocannabinoidsystemet vid smärttilstånd är utförd i gnagare vilket gör att man inte helt känner till hur detta system är uttryckt hos människor. Inledningsvis studerade vi därför uttrycket av cannabinoidreceptorer i mänsklig frisk hälsa och jämförde detta mot uttrycket hos patienter med smärtande hälsor, så kallad Akilles tendinos. Vi fann att uttrycket skilde sig
mellan dessa grupper och det gav oss en grund till att systemet är förändrat även i mänskliga smärtstillstånd.

I de följande studierna använde vi oss av odlade celler och enzymextrakt för att undersöka olika substansers verkan på FAAH och MGL. Baserat på diskussionen ovan om att hämning av dessa enzymer potentiellt kan öka endocannabinoidsignaleringen och ge positiva terapeutiska effekter mot smärta var ett delsyfte med denna avhandling att finna substanser som har just denna hämmande effekt. Det finns FAAH-hämmande substanser som är under klinisk prövning men utfallet av dessa har varit svagt. Det finns därför ett behov av nya substanser som har denna FAAH-hämmande effekt. I studie II och III undersökte vi substanser som används eller har används kliniskt, alternativt substanser som har syntetiserats baserat på klinisk verksamma substanser och fann två substanser som var verksamma som FAAH-hämmare. Förutom studierna kring hämning av nedbrytning utreddede vi i studie IV FAAHs inverkan på upptaget av anandamide i olika celltyper. Processen kring cellens upptag av anandamide är omdiskuterat och resultaten skiljer sig mellan olika laboratorier och celltyper. Vi ville därför utreda huruvida detta beror på skillnad i metodologi eller hos de olika cellernas egenskaper. Vad gäller MGL så fanns det inga hämmande substanser som är selektiva mot det enzymet när vi startade arbetet med denna avhandling. I och med att behovet av sådana substanser är stort undersökte vi en rad substansers förmåga att påverka MGL och fann två lovande substanser som kan tjänstgöra som mall för framtida struktur-aktivitetssambands studier.

Sammanfattningsvis så visar resultaten från dessa studier att endocannabinoidssystemet är ändrat i smärtstillstånd hos människan. De visar även prov på substanser som potentiellt skulle kunna utgöra grund för nya FAAH- och MLG hämmande läkemedel.
**Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-AG</td>
<td>2-arachidonoylglycerol</td>
</tr>
<tr>
<td>2-OG</td>
<td>2-oleoylglycerol</td>
</tr>
<tr>
<td>ABHD6/12</td>
<td>ahydrolyase domain-containing protein 6 and 12</td>
</tr>
<tr>
<td>AEA</td>
<td>anandamide, arachidonoyl ethanolamide</td>
</tr>
<tr>
<td>AM404</td>
<td>N-(4-hydroxyphenyl)arachidonylamide (uptake inhibitor)</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>CB</td>
<td>cannabinoid</td>
</tr>
<tr>
<td>CB&lt;sub&gt;1&lt;/sub&gt;</td>
<td>cannabinoid receptor type 1</td>
</tr>
<tr>
<td>CB&lt;sub&gt;2&lt;/sub&gt;</td>
<td>cannabinoid receptor type 2</td>
</tr>
<tr>
<td>CB&lt;sub&gt;1&lt;/sub&gt;IR</td>
<td>CB receptor immunoreactivity</td>
</tr>
<tr>
<td>COX</td>
<td>cyclooxygenase</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DAGL</td>
<td>diacylglycerol lipase</td>
</tr>
<tr>
<td>DRG</td>
<td>dorsal root ganglion</td>
</tr>
<tr>
<td>FAAH</td>
<td>fatty acid amide hydrolase</td>
</tr>
<tr>
<td>FABP</td>
<td>fatty acid binding protein</td>
</tr>
<tr>
<td>FLAT</td>
<td>FAAH-like anandamide transporter</td>
</tr>
<tr>
<td>JZL184</td>
<td>4-nitrophenyl-4-(di-benzo[d][1,3]dioxol-5-yl(hydroxy)methyl)piperidine-1-carboxylate (MGL inhibitor)</td>
</tr>
<tr>
<td>LOX</td>
<td>lipooxygenase</td>
</tr>
<tr>
<td>MGL</td>
<td>monoacylglycerol lipase</td>
</tr>
<tr>
<td>NAE</td>
<td>N-acetylethanolamine</td>
</tr>
<tr>
<td>NAPE</td>
<td>N-acylphosphatidylethanolamine</td>
</tr>
<tr>
<td>NAPE-PLD</td>
<td>N-acylphosphatidylethanolamine phospholipase D</td>
</tr>
<tr>
<td>PEA</td>
<td>palmitoylethanolamide</td>
</tr>
<tr>
<td>PPAR</td>
<td>peroxisome proliferator-activated receptors</td>
</tr>
<tr>
<td>THC</td>
<td>Δ&lt;sup&gt;9&lt;/sup&gt;-tetrahydrocannabinol</td>
</tr>
<tr>
<td>TRPV1</td>
<td>transient receptor potential vanilloid type 1</td>
</tr>
<tr>
<td>URB597</td>
<td>cyclohexylcarbamic acid 39-carbamoylethylphenyl-3-yl ester (FAAH inhibitor)</td>
</tr>
</tbody>
</table>
Introduction

The endocannabinoid system

Extracts from the plant *Cannabis sativa* have been used for many centuries both for medicinal and for recreational purposes. The main psychoactive ingredient of cannabis is Δ⁹-tetrahydrocannabinol (THC). Initially, the term “cannabinoid” was used to indicate a structure with similarity to THC, however, the definition has evolved to include compounds that interact with cannabinoid receptors (see below). Most of the biological effects of THC and synthetic cannabinoids are mediated through specific G-protein-coupled receptors, named cannabinoid receptors. At present, there are two characterized cannabinoid receptors, CB₁ and CB₂, which were cloned in 1990 and 1993 (Matsuda *et al.*, 1990; Munro *et al.*, 1993) Signalling through cannabinoid receptors results in a broad repertoire of systemic responses such as the beneficial effects of analgesia and inflammation, appetite regulation, relief of spasticity in multiple sclerosis as well as decreased intestinal motility, and, of course, the psychoactive effects sought after by recreational users of cannabis (Howlett *et al.*, 2002).

The discovery of cannabinoid receptors led to the identification of endogenous ligands, called endocannabinoids. In 1992 the first endocannabinoid to be discovered was anandamide (*N*-arachidonylethanolamine, AEA) (Devane *et al.*, 1992). A few years later, 2-arachidonoylglycerol (2-AG) was identified as an agonist of cannabinoid receptors (Mechoulam *et al.*, 1995; Sugiura *et al.*, 1995). These are the most well-studied endocannabinoids although other compounds have been proposed to be endocannabinoids such as 2-arachidonoyl-glycerol ether (noladin ether) (Hanuš *et al.*, 2001) and the unsaturated fatty acid ethanolamides docosahexaenoyl ethanolamide (DHEA) and docosatetraenoyl ethanolamide (DEA) (Hanuš *et al.*, 1993).
Briefly, the endocannabinoid system comprises CB₁ and CB₂, the endogenous ligands and enzymes responsible for biosynthesis and degradation. These are considered in more detail below.

**Cannabinoid receptors**

CB₁ receptors were first identified in rat brain (Devane et al., 1988) and later cloned in both rat (Devane et al., 1988; Matsuda et al., 1990) and human (Gérard et al., 1991). CB₂ was discovered shortly after in the human promyelocytic leukemic cell line HL60 (Munro et al., 1993). The two receptors belong to the seven transmembrane domain family of G-protein-coupled receptors.

CB₁ receptors are abundantly expressed in the central nervous system (CNS). A high expression of CB₁ receptors is seen in, e.g. cerebral cortex, hippocampus, hypothalamus, basal ganglia and cerebellum (Glass et al., 1997; Herkenham et al., 1991). CB₁ receptors are also expressed in lower levels of the brain stem, spinal cord and in peripheral tissues such as the reproductive system, gastrointestinal tract, heart and vasculature (Bonz et al., 2003; Liu et al., 2000; Ruiz-Llorente et al., 2003; Wright et al., 2005). The distribution of CB₁ receptor expression correlates well with the known physiological effects of cannabinoids, e.g. modulation of cognition, memory, motor function and analgesia (Pertwee et al., 2010). Some of the physiological functions of peripheral CB₁ receptors are listed in Table 1.

Studies have shown that in the brain, CB₁ receptors are mainly located presynaptically (Katona et al., 1999). Endocannabinoids are generally considered as retrograde signals due to their synthesis and release into the synaptic cleft following elevated levels of intracellular calcium in postsynaptic neurons (Alger et al., 2011). They activate CB₁ receptors on the presynaptic membrane and suppress inhibitory or excitatory neurotransmitter release (see Fig. 1).
Table 1. Examples of physiological functions mediated by CB₁ receptors.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Agonist effect</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vasculature</td>
<td>induces hypotension</td>
<td>(Szekeres et al., 2012)</td>
</tr>
<tr>
<td>Prostate gland</td>
<td>inhibits contraction of the gland</td>
<td>(Ruiz-Llorente et al., 2003)</td>
</tr>
<tr>
<td>Testis</td>
<td>supresses hormone secretion</td>
<td>(Wenger et al., 2001)</td>
</tr>
<tr>
<td>Urinary tract</td>
<td>increases urine output</td>
<td>(Sofia et al., 1977)</td>
</tr>
<tr>
<td>Uterus</td>
<td>affects implantation of embryo</td>
<td>(Paria et al., 1998)</td>
</tr>
<tr>
<td>Intestinal tract</td>
<td>depresses gastrointestinal motility,</td>
<td>(Pertwee, 2001)</td>
</tr>
<tr>
<td></td>
<td>delays gastric emptying</td>
<td></td>
</tr>
</tbody>
</table>

CB₁ receptors undergo constitutive internalization and following activation of receptors at the plasma membrane, they are internalized via endocytosis. Receptors are trafficked through the recycling endosomal pathway back to the plasma membrane (Leterrier et al., 2004). It should be noted, however, that functional CB₁ receptors are also expressed in intracellular compartments (Brailoiu et al., 2011; Rozenfeld et al., 2008) Endosomes containing CB₁ receptors are believed to result from constitutive endocytosis.

The CB₂ receptor was initially identified in a human promyelocytic leukemic cell line (Munro et al., 1993). CB₂ is often described as a peripheral receptor, mainly expressed in immune tissues, such as spleen, tonsils and immune cells (B-cells and natural killer cells) (Galiègue et al., 1995). When activated, CB₂ receptors can modulate immune cell migration and cytokine release (Pertwee et al., 2010). However, there is some evidence that CB₂ receptors have a limited distribution within the CNS and may be involved in memory consolidation (García-Gutiérrez et al., 2013).
Fig. 1. Activation of postsynaptic metabotropic glutamate receptors (mGluR) leads to release of intracellular Ca\(^{2+}\) and Ca\(^{2+}\) influx, triggering biosynthesis of endocannabinoids (see section below). The endocannabinoid (usually 2-AG) is released and presynaptic CB\(_1\) receptors are activated following retrograde diffusion leading to inhibition of Ca\(^{2+}\) channels. Decreased intracellular Ca\(^{2+}\) levels leads to reduced inhibitory or excitatory neurotransmitter release from the presynaptic terminal.

Upon stimulation both CB\(_1\) and CB\(_2\) receptors can inhibit adenylyl cyclase and activate mitogen-activated protein kinase by signalling through G\(_{i/o}\) proteins (see Fig. 1). CB\(_1\) receptors can also mediate increase of potassium current and inhibit calcium channel activity (Pertwee et al., 2010). Examples of known ligands to cannabinoid receptors are listed in Table 2.

In recent years functional studies have suggested that both endogenous and synthetic cannabinoids have effects independently of CB\(_1\) and CB\(_2\) receptors. In some cases, the target receptors are part of other receptor families (see below), whereas in others, putative additional cannabinoid receptors have been suggested. The most studied of the latter is the orphan G-protein receptor GPR55 (Ryberg et al., 2007). However there is conflicting evidence regarding the ligands with which this receptor interact.
and it is not yet clear whether or not GPR55 should be classified as a novel cannabinoid receptor (Pertwee et al., 2010).

Table 2. Commonly used agonists and antagonist and their $K_i$ values.

<table>
<thead>
<tr>
<th>CB Ligand</th>
<th>$K_i$ CB$_1$ (nM)</th>
<th>$K_i$ CB$_2$ (nM)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Agonists</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\Delta^9$-THC</td>
<td>25.1 (human)</td>
<td>35.2 (human)</td>
<td>(McPartland et al., 2007)</td>
</tr>
<tr>
<td>CP55,940</td>
<td>0.5 (rat)</td>
<td>2.8 (rat)</td>
<td>(Hillard et al., 1999)</td>
</tr>
<tr>
<td>HU-210</td>
<td>0.25 (human)</td>
<td>0.4 (human)</td>
<td>(McPartland et al., 2007)</td>
</tr>
<tr>
<td>AEA</td>
<td>239.2 (human)</td>
<td>439.5 (human)</td>
<td>(McPartland et al., 2007)</td>
</tr>
<tr>
<td>2-AG</td>
<td>3423.6 (human)</td>
<td>1193.8 (human)</td>
<td>(McPartland et al., 2007)</td>
</tr>
<tr>
<td>ACEA</td>
<td>1.4 (rat)</td>
<td>&gt;2000 (rat)</td>
<td>(Hillard et al., 1999)</td>
</tr>
<tr>
<td>WIN55,212-2</td>
<td>4.4 (rat)</td>
<td>Rat: 1.3 (rat)</td>
<td>(Hillard et al., 1999)</td>
</tr>
<tr>
<td>Noladin ether</td>
<td>21.2 (rat)</td>
<td>&gt;3000</td>
<td>(Hanuš et al., 2001)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(transfected COS cells)</td>
<td></td>
</tr>
<tr>
<td>Cannabidol</td>
<td>2210.5 (rat)</td>
<td>1000 (rat)</td>
<td>(McPartland et al., 2007)</td>
</tr>
<tr>
<td><strong>Antagonist</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>SR141716A</td>
<td>11.8 (mouse)</td>
<td>&gt;10 000 (mouse)</td>
<td>(Felder et al., 1995)</td>
</tr>
<tr>
<td>(Rimonabant ®)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

$K_i$ denotes the affinity of a ligand for a receptor. Measured using a radioligand competition binding assay, it refers to the concentration of the drug which would occupy 50% of the receptors if there was no radioligand present.
Non-cannabinoid receptors targeted by AEA

**Transient receptor potential vanilloid 1 (TRPV1)**

It is now generally accepted that the endogenous CB₁/CB₂ receptor agonist AEA and certain of its analogues are agonists for TRPV1 receptor (Howlett *et al.*, 2002; Zygmunt *et al.*, 1999). TRPV1 is part of a family of transient receptor potential channels. It is activated by a range of stimuli such as heat, low pH and compounds including capsaicin, the main pungent ingredient of chilli pepper (Caterina *et al.*, 1997; Tominaga *et al.*, 1998). The human receptor was cloned in 2000 and is most highly expressed in the dorsal root ganglion (DRG) (Hayes *et al.*, 2000). Although the vanilloid receptor is a molecular target for AEA the affinity towards this receptor is lower than that for CB₁ (Ross, 2003). However, inflammatory mediators can increase both the potency and efficacy of AEA (Singh Tahim *et al.*, 2005), suggesting a pro-nociceptive effect rather than an anti-nociceptive effect of AEA in pathological situations. The endocannabinoid 2-AG is a partial agonist at this receptor (Pertwee *et al.*, 2010), and sufficient 2-AG is synthesised in response to the appropriate receptor stimulation (see section on endocannabinoid synthesis below) to activate TRPV1 receptors expressed in HEK293 cells (Zygmunt *et al.*, 2013).

**Peroxisome proliferator-activated receptors**

Peroxisome proliferator activated receptors are ligand-activated transcription factors that belong to the nuclear receptor family with different fatty acids as classical agonists (Pertwee *et al.*, 2010). There are three isoforms of PPARs; α, β/δ and γ. PPAR-α is expressed in liver, kidney, muscle and fat (Desvergne *et al.*, 1999). PPAR-γ is highly expressed in intestine and adipose tissue (Braissant *et al.*, 1996). Studies have reported that AEA can activate PPAR-α and PPAR-γ (Bouaboula *et al.*, 2005; Sun *et al.*, 2006) while 2-AG activates PPAR-γ and PPAR-β/δ (Pertwee *et al.*, 2010; Rockwell *et al.*, 2006).

Other targets for AEA include some types of opioid, dopamine, and muscarinic acetylcholine receptors as well as a variety of ion channels (Hampson *et al.*, 1998; Kimura *et al.*, 1998; Lagalwar *et al.*, 1999; Pertwee *et al.*, 2010).
Synthesis of endocannabinoids

Under normal conditions the tissue concentrations of AEA are low. Thus, for example, in the mouse brain, a concentration of AEA of 13.6 ± 3.2 pmol/g, representing 1.3% of the total content of the N-acylethanolamines (the class of lipids to which AEA belongs) in the brain (Degn et al., 2007). In contrast, the concentration of 2-AG is much higher (12 ± 1 nmol/g) (Degn et al., 2007), although this represents the total concentration of this lipid, which is not only an endocannabinoid but also a metabolic intermediate. Interstitial levels of basal AEA and 2-AG in the nucleus accumbens are less divergent. In vivo microdialysis of this region in C57/BL6 mice gave AEA and 2-AG concentrations in the microdialysate of ~0.6 and ~4.5 nM, respectively. Selective blockade of the hydrolysis of 2-AG (discussed below) increased 2-AG levels in the microdialysate without affecting AEA levels (Long et al., 2009). AEA levels are not always low with respect to other N-acylethanolamines. In the periimplantation mouse uterus, for example, AEA is the most prominent of this class of lipids (Schmid et al., 1997).

Due to their highly lipophilic structure they are not stored in intracellular vesicles but are synthesized on demand (Freund et al., 2003). Examples of stimuli that trigger this response are listed in Table 3.

NAEs

AEA is a member of the N-acetylenolamine (NAE) family, a large group of bioactive lipids that also includes compounds such as N-oleylethanolamine (OEA) and N-palmitoylethanolamine (PEA). The latter two are not considered as endocannabinoids since they lack affinity towards cannabinoid receptors. However, they activate TRPV1 and PPARα and produce a range of biological effects such as appetite suppression and analgesia (Fu et al., 2003; Lo Verme et al., 2005; Movahed et al., 2005; Rodriguez de Fonseca et al., 2001).
Table 3. Examples of stimuli affecting endocannabinoid production.

<table>
<thead>
<tr>
<th>Stimulus triggering endocannabinoid synthesis</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>G-protein receptors coupled to phospholipase C</strong></td>
<td></td>
</tr>
<tr>
<td>e.g. Metabotropic glutamate receptors</td>
<td>(Maejima et al., 2001)</td>
</tr>
<tr>
<td>Subtype 1 (mGluR1) and 5 (mGluR5)</td>
<td>(Ohno-Shosaku et al., 2002)</td>
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<tr>
<td>Histamine H₁ receptors</td>
<td>(Zygmunt et al., 2013)</td>
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<td><strong>NMDA-receptors</strong></td>
<td>(Ohno-Shosaku et al., 2007)</td>
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<td><strong>Formalin</strong></td>
<td>(Walker et al., 1999)</td>
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<td><strong>Inflammation</strong></td>
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<td><strong>Nerve injury</strong></td>
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<td>Neuropathic pain</td>
<td>(Petrosino et al., 2007)</td>
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<td>(Mitrirattanakul et al., 2006)</td>
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<td>Closed head injury (increased 2-AG levels)</td>
<td>(Panikashvili et al., 2001)</td>
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<td>Concussive head trauma (increased AEA levels)</td>
<td>(Hansen et al., 2001) (Schäbitz et al., 2002)</td>
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<td>Stroke</td>
<td>(Naccarato et al., 2010) (Pisani et al., 2005)</td>
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<td>Parkinson's disease</td>
<td>(Eljaschewitsch et al., 2006)</td>
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<td>Multiple Sclerosis</td>
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Several different pathways have been suggested to contribute to the synthesis of NAEs from their corresponding N-acylphosphatidylethanolamine (NAPE) precursors, which exist as a minor component of membrane phospholipids. The most well studied pathway is a two-step enzymatic reaction involving NAPE-phospholipase D (NAPE-PLD). The first step is the transfer of a fatty acyl chain
from the sn-1 position of glycerophospholipids to phosphatidylethanolamine, catalyzed by Ca\(^{2+}\)-dependent N-acyltransferase (Ca-NAT) (Ueda et al., 2010). The second step comprises Ca\(^{2+}\)-sensitive NAPE-PLD which catalyses the hydrolysis of NAPE to produce the NAE (Okamoto et al., 2004). Even though NAEs are produced on demand, NAPE-PLD seems to be kept in a constitutively active form (Muccioli, 2010). A Ca\(^{2+}\)-independent NAT, named iNAT has been cloned (Jin et al., 2009). This enzyme is reported to be capable of forming NAPE. However, it is unclear whether or not iNAT contributes to the biosynthesis of NAPE and NAEs in vivo.

Two other pathways have been described. The first, which mainly has been characterized in macrophages and mouse brain, is the two-step process involving cleavage of NAPE by phospholipase C to yield phosphoanandamide. This is then dephosphorylated by phosphatases to yield AEA (Liu et al., 2006). In the second pathway, NAPE is first hydrolysed to lyso-NAPE by an enzyme with phospholipase 2 activity. NAE is then released from lyso-NAPE by a lysophospholipase D-like enzyme (Natarajan et al., 1983).

2-arachidonoylglycerol

2-AG, similar to AEA, is produced in a stimulus-dependent fashion. Increased intracellular Ca\(^{2+}\) triggers synthesis but the synthetic pathways differs (Bisogno et al., 1997b; Kondo et al., 1998). 2-AG can be synthesized in a two-step reaction. Diacylglycerol (DAG) is generated from phosphatidylinositol (PI), a minor component of membrane phospholipids, by phospholipase C (PLC). DAG is then hydrolysed by a diacylglycerol lipase (DAGL) to yield 2-AG. Inhibition of these enzymes results in decreased 2-AG levels. In DAGL-alpha knockout mice the levels of 2-AG are reduced by up to 80% in brain and spinal cord and about 60% in liver. Depletion of the other subtype of DAGL, DAGL-beta, yields a 50% reduction of 2-AG levels in brain but no difference in spinal cord (Gao et al., 2010). These lowered concentrations of 2-AG result in a loss of retrograde endocannabinoid signalling in the hippocampus of mice (Gao et al., 2010). The key intermediate DAG can also be
produced from phosphatidic acid by a phosphatidic acid hydrolase. This represents an alternative pathway to DAG production (Muccioli, 2010).

A second pathway for 2-AG production involves 2-arachidonoyl-lyso phosphatidylinositol (lyso-PI) as intermediate. Phosphatidylinositol-prefering phospholipase A₄ produces the lyso-PI intermediate from PI. Secondly, a lysophosphatidylinositol-selective phospholipase C generates 2-AG in a Ca²⁺-independent manner in rat brain (Ueda et al., 1993). The relevance of this pathway to generate 2-AG compared to the PLC-DAGL cascade is less clear. In addition, DAG and 2-AG are intermediates in several pathways, one being arachidonic acid release. It is likely, therefore, that not all the pathways leading to 2-AG are actually involved in physiological endocannabinoid signalling (Muccioli, 2010). Additionally, there are studies suggesting that all 2-AG is not necessarily produced on demand but that there is a pool that is pre-synthesised and stored until needed (Min et al., 2010; Zhang et al., 2011). However, this requires further study, and new reports support the idea that on demand 2-AG biosynthesis is required for retrograde endocannabinoid signalling (Hashimotodani et al., 2013).

**The cellular processing of endocannabinoids**

Termination of endocannabinoid signalling occurs through an uptake mechanism followed primarily by enzymatic hydrolysis. The mechanism(s) responsible for cellular uptake has not yet been clarified. There are studies of endocannabinoid uptake showing a time- and temperature dependency, saturability but also an ATP- and sodium independency (Chicca et al., 2012; Di Marzo et al., 1994; Hillard et al., 1997). In addition, the fact that AEA analogues such as AM404 inhibit the uptake process indicates the presence of a membrane transporter (Beltramo et al., 1997). All these characteristics are consistent with a facilitated transport mechanism. Nonetheless, at the time of writing of this thesis, a plasma membrane transporter protein has still not been cloned.
If AEA uptake is driven by passive transport, e.g. in absence of a transporter protein, the uptake will cease once the extracellular and intracellular levels of AEA reach equilibrium. However, this equilibrium can be affected both by sequestration of the intracellular AEA, such has been described in lipid droplets (Kaczocha et al., 2010; Oddi et al., 2008), and/or by intracellular metabolism of endocannabinoids (the degradation of endocannabinoids are described in more detail in the section below). Fatty acid amide hydrolase (FAAH) is the key enzyme responsible for the hydrolysis of AEA (Cravatt et al., 1996; Deutsch et al., 1993). At present, there are clearly disagreements in the literature concerning the importance of FAAH in controlling the cellular uptake of AEA. In FAAH-containing neuroblastoma (N18), glioma (C6) and rat basophilic leukaemia cells, the net uptake of AEA is decreased in the presence of FAAH inhibitors (Day et al., 2001; Deutsch et al., 2001). However, compounds such as AM404, which are structurally similar to AEA, decrease AEA uptake in cells lacking FAAH (Fegley et al., 2004; Ligresti et al., 2004; Ortega-Gutiérrez et al., 2004). One explanation for these differences is that they are due to cellular differences, but it might also be a matter of methodological artefacts. The cellular accumulation of 2-AG is not dependent upon its subsequent metabolism in RBL2H3, AT-1 and PC3 cells, but may gate the uptake in Neuro-2a cells (Fowler et al., 2008).

Due to their highly lipophilic nature, endocannabinoids require intracellular transporters to carry them throughout the cytoplasm of the cell to their catabolic enzymes and/or intracellular targets (the AEA-binding site of the TRPV1 receptor, for example, is located on the intracellular face of the receptor) once they have crossed the plasma membrane. Several intracellular AEA carrier proteins have been proposed including fatty acid binding protein (FABP), heat shock protein 70, albumin and FAAH-like anandamide transporter (FLAT) (Bojessen et al., 2003; Fu et al., 2012; Kaczocha et al., 2009; Oddi et al., 2009), although such a role of FLAT has been questioned (Leung et al., 2013). In an effort to reduce the cellular uptake of AEA, blockade of intracellular carrier proteins is in theory an alternative to FAAH inhibitors. Recently, Berger et al. identified SB-FI-26 as a novel inhibitor of FABP5. SB-FI-26 reduces FABP-mediated AEA uptake in HeLa cells and produces
antinociceptive and anti-inflammatory effects in mice (Berger et al., 2012). Recently, ARN272 was found to block AEA binding to FLAT selectively. Systemic administration of this compound in mice caused a dose-dependent reduction of formalin-induced pain behaviour. In addition, it produced anti-inflammatory and anti-hyperalgesic effects when injected intraplantarally (Fu et al., 2012).

**Degradation of AEA**

N-Acylethanolamines are hydrolysed to free fatty acids and ethanolamines. The key enzyme in this process is the intracellular enzyme fatty acid amide hydrolase (FAAH) (Cravatt et al., 1996; Deutsch et al., 1993). FAAH is a 63kDa membrane-bound serine hyrolyse enzyme distributed widely throughout the body. Examples of tissues expressing FAAH is brain, liver, testis, uterus and spleen (Bobrov et al., 2000; Cravatt et al., 1996; Deutsch et al., 1993; Maccarrone et al., 2000; Watanabe et al., 1998). In the brain, the distribution of FAAH and CB1 receptors are complementary, CB1 is principally located presynaptically in contrast to the FAAH, which is postsynaptic (Egertová et al., 1998). FAAH has a wide substrate specificity and is capable of metabolising not only AEA but also other fatty acid amides such as PEA and oleamide as well as 2-AG (Bisogno et al., 1997a; Cravatt et al., 1996; Goparaju et al., 1999; Lang et al., 1999; Maccarrone et al., 1998; Tiger et al., 2000).

In 2006, an isoenzyme of FAAH, referred to as FAAH-2, with ~20% sequence identity at amino acid level was found to be expressed in humans but not rodents (Wei et al., 2006).

Early studies of FAAH demonstrated that it is inhibited by non-selective compounds such as phenylmethylsulfonyl fluoride (Deutsch et al., 1993) and compounds structurally related to the substrates of FAAH such as arachidoloyl trifluoromethyl ketones (Boger et al., 1999; Koutek et al., 1994). The carbamate-type inhibitor URB597 is frequently used as a selective FAAH inhibitor (Kathuria et al., 2003). In rodents, URB597 elevates the AEA levels, induces antidepressant-like effects, reduces blood pressure, analgesia and reduces inflammation (Adamczyk et al., 2008; Bátkai et al., 2004; Fegley et al., 2005; Holt et al., 2005; Jayamanne et al., 2006; Kathuria et al., 2003). There are FAAH inhibitors in Phase I and Phase II clinical
trials. PF-04457845, which possesses antinociceptive effect in rodents (Ahn et al., 2011), has recently been investigated in a randomized, placebo-controlled clinical trial of patients with osteoarthritis of the knee. Despite increasing levels of four NAEs in the plasma at the doses used, it did not have an analgesic effect (Huggins et al., 2012). One possible explanation is at least in part to cyclooxygenase-2 (COX-2) metabolism of AEA, i.e. that the increased AEA resulting from the FAAH inhibition is rerouted along the COX-2 metabolic pathway (see section below).

**Other enzymes responsible for endocannabinoid inactivation**

Apart from FAAH, AEA can also be metabolised by several other enzymes (see Fig. 2). COX-2, which is expressed during inflammation, converts AEA to several prostaglandin ethanolamides (PG-EAs) (Kozak et al., 2002a; Yu et al., 1997). The major metabolites from LOX- oxidation are hydroxylated derivatives of AEA. The lipooxygenase (LOX) derivate 12-hydroxyanandamide is capable of binding to the CB₁ receptor with twice the affinity that of AEA (Hampson et al., 1995). Cytochrome P450 oxygenases metabolises arachidonic acid to form epoxyicosatetraenoic acids (EETs) and hydroxyeicosatetraenoic acid (HETEs) but also to metabolise AEA in both mouse and human (Bornheim et al., 1995; Snider et al., 2007). One of the many P450-derived metabolites of AEA, 5,6-EET-EA has been show to bind and functionally activate recombinant human CB₂ receptor (Snider et al., 2009).

![Fig. 2. The main degrading enzymes and products of AEA.](image)

Anandamide

- FAAH
- COX-2
- LOX
- P450

EA, Ethanolamine; AA, arachi-donic acid; PG-EA, prostaglandin ethanolamide; HPETE, hydroper-oxyeicosatetraenoic acid; HETE, hydroxyeicosatetraenoic acid; EET, epoxyeicosatrienoic acid
Degradation of 2-AG

The main hydrolysing enzyme converting 2-AG to glycerol and arachidonic acid is monoacylglycerol lipase (MGL). This enzyme is a 33kDa protein originally cloned and purified in adipose tissue (Karlsson et al., 1997). Later in 2002, northern blot and in situ hybridization analyses revealed that MGL mRNA is heterogeneously expressed in the rat brain, with highest levels in regions where CB1 receptors are also present (hippocampus, cortex, anterior thalamus and cerebellum) (Dinh et al., 2002). Like CB1 receptors, it is predominantly localized to presynaptic axon terminals (Gulyas et al., 2004). Its primary mechanism is inactivation of 2-AG (Dinh et al., 2002) and functional proteomic approaches have been used to explore different 2-AG hydrolases in mouse brain membrane homogenates. Under the conditions used, approximately 85% of brain 2-AG is hydrolysed by MGL, the remaining 15% is mostly catalysed by the serine hydrolases ABHD6 and ABHD12 (Blankman et al., 2007). MGL has been described to be both cytosolic- and membrane bound (Sakurada et al., 1981) with a pH optimum of ~8 (Tornqvist et al., 1976). MGL is expressed in a number of tissues including adipose tissue, adrenal gland, ovary, heart, brain, spleen, lung, liver, skeletal muscle, kidney and testis (Karlsson et al., 1997).

At the time when this thesis work was started, selective inhibitors of MGL were not available. Early studies suggested that MGL activity is sensitive to general serine hydrolase inhibitors such as phenylmethanesulfonyl fluoride (PMSF), arachidonoyl trifluoromethylketone, and hexadecysulfonylfluoride (Ghafouri et al., 2004; Saario et al., 2004), compounds also inhibiting FAAH. It has also been implied that MGL is inhibited by non-specific sulfhydryl agents, including p-chloromercuribenzoic acid and N- ethylmaleimide (Sakurada et al., 1981; Tornqvist et al., 1976). The carbamate compound URB602 was initially reported as the first selective inhibitor of MGL (Hohmann et al., 2005). However its selectivity has been a matter of debate since it has been shown to be equally potent against FAAH in vitro (Vandevoorde et al., 2007). In 2009, JZL184, a novel highly potent selective MGL inhibitor was reported (Long et al., 2009). When administrated to mice, JZL184 raised brain 2-AG levels by eight-fold without altering AEA levels. In agreement with previous
studies (Blankman et al., 2007), brain membranes maintained a residual of ~15% 2-AG hydrolysis activity even at the highest concentrations of JZL184 tested (Long et al., 2009). JZL184 showed to exhibit analgesic properties in different pain assays including the acetic acid writhing test of visceral pain, tail-immersion test of acute thermal pain sensation and the formalin test of noxious chemical pain. The CB1 antagonist Rimonabant blocked these effects. However, in contrast to FAAH inhibitors JZL184 induced hypothermia and hypomotility but not catalepsy (Long et al., 2009).

**Other enzymes responsible for 2-AG metabolism**

As with AEA, 2-AG can also be metabolised by FAAH, COX-2, LOX and CYP (Goparaju et al., 1998; Guindon et al., 2008; Hu et al., 2008; Kozak et al., 2002a; Kozak et al., 2002b; Kozak et al., 2002c). As mentioned previously, Blankman et al. confirmed that MGL is the key enzyme (~85% of hydrolysis) responsible for metabolism of 2-AG in mouse brain whereas ABHD6 and 12 is responsible for about 4% and 9% respectively. It is suggested that based on their distinct cellular and/or subcellular localization, MGL, ABHD6 and 12 regulate distinct pools of 2-AG in the brain. While MGL is a soluble enzyme that associates with membranes, ABHD6 and ABHD12 are integral membrane enzymes (ABHD6 facing the cytoplasm and ABHD12 the extracellular compartments of the cell) (Blankman et al., 2007).

The physiological and pathophysiological roles of ABHD6 and 12 have not been well examined but mutations of the ABHD12 gene cause the human neurodegenerative disease PHARC (polyneuropathy, hearing loss, ataxia, retinitis pigmentosa, and cataract) (Fiskerstrand et al., 2010). Recently ABHD6 has also been shown to control 2-AG accumulation in Neuro2A cells, which lack MGL (Hsu et al., 2012).

**The importance of the COX-2 pathway in endocannabinoid signalling**

As mentioned above, COX-2 converts AEA to several prostaglandin ethanolamides (PG-EAs). This pathway, first demonstrated *in vitro* by (Yu et al., 1997) is of
importance in vivo, particularly under inflammatory conditions (Duggan et al., 2011; Gatta et al., 2012), or in the absence of FAAH following a priming dose of AEA (Weber et al., 2004). Indirect evidence for the importance of COX-2 as an endocannabinoid metabolic enzyme has been obtained in studies of AEA uptake by the mouse brain in vivo: inhibition of COX-2 resulted in higher AEA uptake and stability (Glaser et al., 2010). AEA and 2-AG levels are also higher in the brains of COX-2 knockout mice compared to wild type animals (Hermanson et al., 2013). Inhibitors of COX-2 has also been shown to potentiate retrograde signalling in hippocampal pyramidal cells, e.g. activate presynaptic CB₁ receptors and transiently reduce GABAergic transmission, a process termed depolarization induced suppression of inhibition (Kim et al., 2004). Recently, an indomethacin analogue, LM-4131, has been identified as a substrate-selective COX-2 inhibitor, inhibiting the oxygenation of AEA and 2-AG, but not arachidonic acid, by this enzyme form. LM-4131 increased AEA levels without affecting central or peripheral prostaglandins. It also had effect on 2-AG levels, albeit more modest. Furthermore, LM-4131 treatment did not further increase AEA and 2-AG levels in COX-2 knockout mice over and above the effects produced by the genetic deletion per se. In line with its substrate selectivity, LM-4131 did not affect levels of arachidonic acid or prostaglandin levels in the brain, in contrast to indomethacin, which increased arachidonic acid levels and decreased prostaglandin levels as expected for a general inhibition of COX-2. Other N-acylethanolamines such as palmitoylethanolamine, were not affected. The authors also found that LM-4131 decreased anxiety-like behaviours in a Rimonabant-sensitive manner, suggesting that the augmentation of endocannabinoid signalling by the compound in vivo is sufficient to produce behavioural effects (Hermanson et al., 2013).

Endocannabinoids and pain

According to the International Association for the Study of Pain (IASP), pain is defined as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage. IASP have further classified pain in to different categories. Nociceptive pain is described as pain that
arises from actual or threatened damage to non-neural tissue and is due to the activation of nociceptors, whereas neuropathic pain is caused by a lesion or disease of the somatosensory nervous system. Pain resulting from tissue inflammation is referred to as inflammatory pain. Inflammation gives rise to peripheral sensitization, which is defined as increased responsiveness of nociceptive neurons to their normal input, and/or recruitment of a response to normally sub-threshold inputs. Pain that persists more than 3 months is defined as chronic pain (www.iasp-pain.org). Chronic pain is common and affects quality of life negatively (Breivik et al., 2006).

Studies using experimental animals show that the anti-nociceptive effects of cannabinoids are not only centrally mediated, but that spinal and peripheral cannabinoid receptors are involved (Guindon et al., 2009). Support for supraspinal sites for the analgesic action of cannabinoids was found in studies in which synthetic CB receptor agonists were injected into different brain regions, including the periaqueductal grey (PAG) (Lichtman et al., 1996; Martin et al., 1995), thalamus (Martin et al., 1996) and amygdala (Marsicano et al., 2002; Martin et al., 1999) among others, in rat. These studies suggest that cannabinoids might act in the midbrain to produce antinociception under physiological conditions. In 1999, Walker et al. found that electric stimulation of the dorsolateral PAG produced antinociception in the tail-flick test and increased levels of endogenous AEA in this area as measured by microdialysis. This analgesic effect was blocked by Rimonabant (Walker et al., 1999). Intraplantar administration of formalin was also shown to increase levels of endogenous AEA in the dorsolateral PAG (Walker et al., 1999). Endocannabinoid levels are altered in specific brain regions following nerve injury. For example, injury of the sciatic nerve increases the levels of AEA and 2-AG in the PAG as well as in the spinal cord (Petrosino et al., 2007).

A number of animal studies have demonstrated that cannabinoids act at the spinal level to modulate pain. Intrathecal administration of cannabinoids produces antinociception and suppresses nociceptive neuronal activity (Hohmann et al., 1998; Smith et al., 1992; Welch et al., 1995; Yaksh, 1981). Additionally, the CB2-selective agonist AM1241 suppresses C-fibre-evoked responses of dorsal horn neurons in rats
in the presence of inflammation (Nackley et al., 2004). Also, CB1 receptors are observed to be up-regulated in the spinal cord following nerve injury (Lim et al., 2003; Sagar et al., 2005). Non-opioid, stress-induced analgesia increases 2-AG but not AEA levels in the lumbar spinal cord. Spinal administration of inhibitors of endocannabinoid hydrolysis (URB597 for FAAH) and (URB602 for MGL) enhanced stress-induced analgesia through a CB1 mediated mechanism (Suplita et al., 2006). Spinal cord levels of AEA and 2-AG are increased following cisplatin-induced peripheral neuropathy. Also, inhibition of FAAH (URB937, URB597) and MGL (JZL184) suppresses cisplatin-evoked mechanical and cold allodynia (Guindon et al., 2013).

Evidence for peripheral involvement of cannabinoids in the modulation of pain has been presented in numerous animal pain models. Peripheral, but not systemic administration of AEA inhibits oedema, capsaicin-evoked plasma extravasation into the hind paw and carrageenan-induced thermal induction of hyperalgesia. Peripheral administration also reduces hyperalgesia after its development via interaction with CB1 receptors, as revealed by using the CB1 antagonist Rimonabant (Richardson et al., 1998). In the formalin-evoked pain model AEA prevents pain when injected locally (intraplantar), an effect which was blocked by Rimonabant. A further experiment showed that 94% of the injected AEA remained associated with the injected paw. This indicates that AEA inhibits nociception after formalin injection by activating peripheral CB1 receptors located on sensory neurons involved in pain transmission (Calignano et al., 1998). In inflamed paw following carrageenan-induced inflammation a decrease in FAAH activity is seen compared to the non-inflamed mice. Also intraperitoneal injections of the FAAH inhibitor URB597 reduce oedema formation (Holt et al., 2005). Intraplantar administration of AEA and ibuprofen have antinociceptive effects in rat paw formalin test. The compounds showed synergistic effects that were completely antagonised by the CB1 antagonist AM251 (Guindon et al., 2006).

The peripheral contribution of endocannabinoid-mediated analgesia has been investigated further by generation of transgenic mice lacking CB1 receptors in
nociceptors, preserving expression in the spinal neurons, brain and all other organs. These genetically modified mice shows that specific loss of CB₁ in nociceptors leads to reduced response to noxious heat, reduced response thresholds to mechanical stimuli and greater responses to intraplantar injections of capsaicin and formalin (Agarwal et al., 2007). Additionally, low doses of a peripherally applied synthetic cannabinoid reduced inflammatory and neuropathic pain, an effect that was nearly completely lost on nociceptor-specific deletion of CB₁ receptors (Agarwal et al., 2007). Following the induction of neuropathy, e.g. by spinal nerve ligation, both AEA and 2-AG are increased only in the ipsilateral DRG (Mitrirattanakul et al., 2006). FAAH has been found in the rat DRG, spinal cord and peripheral nerve tissue (Lever et al., 2009). An increase of FAAH in the ipsilateral DRG occurred after spinal nerve lesion but not after chronic inflammation of the rat hind paw 2 d after injection of complete Freund's adjuvant (Lever et al., 2009). This reveals the location of FAAH in neural tissue involved in peripheral nociception and provides targets for manipulation of the endocannabinoid system for the treatment of pain.

As mentioned earlier, AEA can also activate the TRPV1 receptor (Zygmunt et al., 1999). N-arachidonoyl-5-hydroxytryptamine (AA-5-HT), a compound with a “dual” ability to inhibit the fatty acid amide hydrolase (FAAH) and to antagonize the TRPV1 receptors shows strong analgesic activity after systemic administration in acute or chronic pain models in rodents (Maione et al., 2007). Intra-periaquedetal grey (PAG) administration of the compound significantly increased basal levels of 2-AG and OEA (which activates TRPV1 receptors) but not those of AEA. Injection of AA-5-HT also produced anti-nociceptive effects in the formalin model of pain, an effect erased by co-injection by either AM251 (a CB₁ antagonist) or I-RTX (a TRPV1 antagonist) (de Novellis et al., 2008). In 2013, Zygmunt et al. demonstrated that 2-AG and 1-AG activate the TRPV1 receptor. Additionally, the MGL inhibitor JZL184 produced a TRPV1-dependent anti-nociceptive effect in the first phase of the mouse formalin test (Zygmunet et al., 2013).
Inhibition of endocannabinoid hydrolysis as a therapeutic target for the treatment of pain

Although AEA binds to CB\textsubscript{1} receptors and has been implicated in the suppression of pain, its rapid degradation by FAAH is a challenge in investigating the physiological functions of this endocannabinoid. There is evidence that FAAH knockout mice have a 15-fold increase of AEA levels in brain and display reduced pain sensation that is reversed by Rimonabant (Cravatt et al., 2001). In a mouse collagen-induced arthritis (CIA) model, FAAH knockout mice displayed decreased severity of CIA and associated hyperalgesia (Kinsey et al., 2011). However, as mentioned in a previous section, the irreversible FAAH inhibitor PF-004457845 has been investigated in a randomized, placebo-controlled clinical trial of patients with osteoarthritis of the knee. Although elevation of AEA plasma levels were seen, this inhibitor did not produce significant analgesia in the patient population investigated (Huggins et al., 2012). One explanation for this might be the choice of outcome measures (Rice et al., 2008). Another explanation is that AEA uses metabolic pathways other than FAAH in the presence of an FAAH inhibitor. AEA and 2-AG are also substrates of COX-2 (see above) and FAAH inhibitors have been shown to give synergistic analgesic interactions together with non-steroidal anti-inflammatory drugs in experimental animals (Naidu et al., 2009; Sasso et al., 2012). Further, the FAAH inhibition reduced the gastrointestinal disturbances produced by the non-steroidal anti-inflammatory drugs (Naidu et al., 2009; Sasso et al., 2012).

Most of the research examining the role of endocannabinoid catabolic enzymes in nociception has focused on FAAH, largely due to the lack of a selective MGL inhibitor. At the start of the present thesis, the only compound available was URB602 (Hohmann et al., 2005), the selectivity of which had been questioned (Vandevoorde et al., 2007). The development of JZL184 (Long et al., 2009) opened up for experiments to evaluate the role of 2-AG in pain perception. JZL184 when administered acutely increases 2-AG brain levels, without altering AEA brain levels (Long et al., 2009). Systemic administration of JZL184 has been demonstrated to reduce nociceptive responses in several different animal models including tail withdrawal, formalin, acetic acid stretching tests and chronic constriction injury.
model of neuropathic pain in mice (Kinsey et al., 2009; Long et al., 2009). Intraplantar injection of JZL184 produces antinociception in the formalin test and in the capsaicin model of nociception (Guindon et al., 2011; Spradley et al., 2010). Additionally, JZL184 significantly inhibits inflammatory pain in the carrageenan assay and more specifically, JZL184 attenuated the development of paw oedema and mechanical allodynia. The compound also reversed oedema and allodynia when administered after carrageenan (Ghosh et al., 2013).

There are drugs used in the clinic, such as Sativex®, that affect the cannabinoid system. However, compounds with one primary mechanism of action can have additional biological targets, including components of the endocannabinoid system. One interesting approach in the design of novel inhibitors of endocannabinoid metabolism is the use of clinically known compounds as a template. This has an advantage that clinical data is available, at least for the starting compound. An example of this is ibuprofen, which has a primary action to inhibit COX, but which has been demonstrated to also inhibit FAAH (Fowler et al., 1997). Moreover, studies have shown that paracetamol (acetaminoprofen) can be metabolised in the brain into the anandamide uptake inhibitor AM404 in a FAAH-dependent manner (Högestätt et al., 2005). Ibuprofen and paracetamol analogues with low to sub-micromolar potency have been identified (De Wael et al., 2010; Holt et al., 2007; Onnis et al., 2010; Patel et al., 2013) and one of these, the N-(3-methylpyridin-2-yl)amide derivative of ibuprofen, combines an FAAH inhibitory effect with a substrate-selective inhibition of COX-2 (Fowler et al., 2013).

**Endocannabinoid system in human pain states**

Current data from experimental animals, summarised above, suggest that the endocannabinoid system is dysfunctional in pain states. As mentioned earlier, there is evidence for this in animal pain models but at the time when this thesis was started little was, and still is, known about the situation in human pain. In 2008 it was reported that CB1 receptor expression in pancreatic nerves was negatively correlated to pain symptoms of patients with pancreatic cancer (Michalski et al., 2008). One year later, in 2009, it was stated that patients with complex regional pain
syndrome show higher AEA plasma concentrations compared to age- and sex-matched controls (Kaufmann et al., 2009). Additionally, a study suggests that increased CB₁ receptor immunoreactive nerve fibres may be related to bladder pain in painful bladder syndrome (Mukerji et al., 2010). A negative correlation of CB₂ mRNA in human spinal cord is seen in patients with joint chondropathy (Burston et al., 2013).

**Chronic pain in the human Achilles tendon**
The Achilles tendon is the strongest tendon in the body (O’Brien, 1992). When there is chronic pain, swelling and impaired function in the Achilles tendon, the condition is referred to as tendinopathy (Khan et al., 1999). If patients with tendon pain, swelling and impaired function also demonstrate structural tissue changes, the condition is termed tendinosis (Alfredson et al., 2005). Biopsies of symptomatic tendons show changes in the appearance of the tenocytes, such as rounded nuclei and a less spindle-shaped appearance, hypercellularity, neovascularization, degeneration and disordered arrangement of collagen fibres (Khan et al., 1999; Åström et al., 1995). There are no signs of classic inflammation in chronic tendinosis, i.e. presence of inflammatory cells and elevated prostaglandin levels (Alfredson et al., 1999; Khan et al., 1999). The aetiology of Achilles tendinopathy is still unclear although several molecular candidates have been identified and proposed as mediators of the pain in Achilles tendinosis (Riley, 2008). Given the ubiquity of the endocannabinoid system and its role in pain, a dysregulation of the endocannabinoid system might also occur in Achilles tendinosis.
Aims of the thesis

It is known that the endocannabinoid system plays an important role in the control of pain and that activation of the cannabinoid receptors shows clinical utility in a variety of pain states. However, there are many gaps in the knowledge about the situation in human pain syndromes. Thus, nothing is known about the potential involvement of CB₁ receptors in Achilles tendinosis. There is also a need for a better understanding of how the endocannabinoid system can be modulated pharmacologically in order to strengthen existing signalling patterns. One approach is to investigate compounds that are already in clinical use, to determine whether they inhibit endocannabinoid metabolism as an additional effect. Such an approach can then form the basis of structure-activity studies designed to optimise the endocannabinoid component of the drug in question. An example of this is ibuprofen, which in addition to their effects upon COX, inhibits FAAH (Fowler et al., 1997). The N-(3-methylpyridin-2-yl)amide analogue of ibuprofen is 2-3 orders of magnitude more potent as an inhibitor of FAAH but retains the COX inhibitory properties of the parent compound (Holt et al., 2007). The aims of the thesis are as follows:

Paper I: To evaluate if CB₁ receptors are expressed in Achilles tendons, and whether this expression is anomalous in Achilles tendinosis.

Paper II: To determine whether the N-(3-methylpyridin-2-yl)amide analogue of flurbiprofen has greater potency than the corresponding ibuprofen analogue as an FAAH inhibitor, and whether it shows a substrate-selective inhibition of COX-2.

Paper III: To determine whether, and how, the antifungal agent ketoconazole inhibits the cellular uptake of AEA at pharmacologically relevant concentrations.

Paper IV: To determine whether the expression level of FAAH is an absolute determinant of the sensitivity of AEA uptake to FAAH inhibition.

Paper V: To determine whether compounds inhibiting the activity of MGL can be identified from a screen of a library of drugs and biologically active compounds.
Methodological considerations

During the work of this thesis, several methods have been used. The details of each method are given in the original papers and are summarized in this section.

Subjects (Paper I)

The subjects participating in the study were from a group of patients suffering from Achilles tendinosis or healthy controls. In total, samples from 24 individuals; 11 males and 13 females (Table 4) were analysed. All participants were healthy, free from medication, non-smokers and chose to be included in the research program on voluntary basis.

Achilles tendinosis patients

The Achilles tendinosis group consisted of 17 patients suffering from chronic painful mid-portion Achilles tendinosis verified by ultrasonography and clinical examination.

Inclusion criteria for this group were:

- Pain in the Achilles tendon for more than 3 months
- Clinical symptoms: tender thickening in the Achilles tendon mid-portion and ultrasound verified tendinosis changes corresponding to the region with clinical findings

Exclusion criteria were:

- Diseases or injuries causing radiating pain in the lower limb
- Smokers
- Acute or chronic inflammatory diseases

There were 9 females (aged 47, 52, 53, 55, 59, 61, 61, 68 and 75 years) and 8 males (ages 28, 28, 29, 36, 53, 58, 60 and 70 years) in this group.
**Controls**

This group included 7 individuals (4 females, aged 21, 47, 47 and 47 years; 3 males ages 39, 39 and 46 years) with no history of pain symptoms from their Achilles tendons. Ultrasonography showed normal tendons.

Inclusion criteria for this group were:

- No diseases or injuries affecting the lower extremities
- Non-smokers
- No ongoing or previous pain in the Achilles tendon
- Normal findings on ultrasonography
- Good health and free from medication

Table 4. Overview of subjects for the study. M/F: Male/Female, Age: Mean age

<table>
<thead>
<tr>
<th>Subjects</th>
<th>24</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/F</td>
<td>11/13</td>
</tr>
<tr>
<td>Age (range)</td>
<td>48 (21-70)</td>
</tr>
<tr>
<td>Tendinosis</td>
<td>17</td>
</tr>
<tr>
<td>M/F</td>
<td>8/9</td>
</tr>
<tr>
<td>Age (range)</td>
<td>51 (28-70)</td>
</tr>
<tr>
<td>Controls</td>
<td>7</td>
</tr>
<tr>
<td>M/F</td>
<td>3 / 4</td>
</tr>
<tr>
<td>Age (range)</td>
<td>41 (21-47)</td>
</tr>
</tbody>
</table>
Ethics (Paper I)

The Committee of Ethics at the Faculty of Medicine, Umeå University and the Regional Ethical Review Board in Umeå approved the study. All participants read an explanatory statement and received a verbal summary of the project before they gave verbal consent to participate in the research. All procedures followed the principles of the Declaration of Helsinki.

Sampling, fixation and sectioning (Paper I)

All biopsies were taken during surgical treatment and under strict sterile condition. In the tendinosis group, tendon tissue (macroscopically abnormal) was taken through a longitudinal incision lateral to the tendon mid-portion from the ventral part of the Achilles tendon. The samples were taken from different depths of the tendon and were approximately 2 mm wide and 1–5 mm long. Biopsies from the control group (same size as from tendinosis patients) were carefully taken from the dorsal part of the tendon using a longitudinal plain incision under local anaesthesia. The dorsal part of the tendon was chosen for ethical and practical reasons.

The samples were fixed overnight at 4 °C in a solution of 4 % formaldehyde in 0.1 M phosphate buffer, pH 7.0 and were thoroughly washed in Tyrode’s solution containing 10% sucrose. The samples were then mounted on thin cardboard in OCT embedding medium and frozen at -80 °C until sectioning.

The samples were cut in a series of sections (7 µm thick) using a cryostat. The sections were mounted on slides, pre-coated with Crome Alum Gelatin solution, dried and thereafter used for immunohistochemistry. Reference tissues (human colonic and rat dorsal root ganglion tissue), which had been fixed and handled in the same way as the tendon samples were examined in parallel.
**Immunofluorescence and control staining (Paper I)**

Briefly, sections were initially treated with acid potassium permanganate in order to enhance the visualization of specific immunofluorescence reaction sites (Hansson et al., 1995) prior to incubation for 20 min in a 1% solution of Triton X-100. After rinsing three times in PBS the sections were incubated in 5% normal swine serum supplemented with 0.1% bovine serum albumin (BSA) for 15 min in a humid environment in room temperature. The samples were thereafter incubated with the primary antibody for 60 min at 37 °C in a humid environment. Prior to a 30 min incubation at 37 °C with the secondary antibody the samples were again incubated with normal swine serum. This antibody corresponded to tetramethylrhodamine isothiocyanate (TRITC)-conjugated swine antirabbit IgG.

All sections were evaluated for the intensity of CB₁ receptor immunoreactivity in the normal and Achilles tendinosis tendons by two independent investigators (this author and Prof. Sture Forsgren). Tenocytes were scored for immunoreactive intensity (0–3 where 0 is absent and 3 is high), and the average value was taken.

To confirm the validity of the method, parallel staining on reference tissue (human colon and dorsal root ganglia) were performed. For control purposes, the primary CB₁ antibody was pre-absorbed overnight at 4 °C with its immunogenic peptide (20–100 mg/ml; ab50542; Abcam, Cambridge, UK) prior to incubation on the sections. As an additional control, the primary antibody was substituted with PBS/BSA. The PGP9.5 (protein gene product 9.5) (see Table 5) antibody for staining nerve fibres is a well-used antibody in the laboratory and in numerous studies by others (Andersson et al., 2007; Bjur et al., 2005).

**Hematoxylin-eosin staining**

To outline tissue morphology, hematoxylin-eosin staining of parallel sections to those processed for immunohistochemistry was performed. Hematoxylin gives a blue colour to basophilic structures such as cell nuclei and ribosomes whereas eosin
colours eosinophilic structures such as intra- and extracellular proteins in different shades of red.

**Antibodies (Paper I)**

Polyclonal antibodies were used as primary antibodies in immunochemical staining (Table 5)

Table 5. Properties of primary antibodies used

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody code</th>
<th>Source</th>
<th>Raised in</th>
<th>Raised against (antigen)</th>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>CB₁</td>
<td>ab23703</td>
<td>Abcam, Cambridge, UK</td>
<td>Rabbit</td>
<td>C-terminal amino acid 461–472 of human CB₁</td>
<td>1:20-1:100</td>
</tr>
<tr>
<td>PGP9.5</td>
<td>7863-0504</td>
<td>Biogenesis, Poole, UK</td>
<td>Rabbit</td>
<td>Native brain PGP9.5</td>
<td>1:100</td>
</tr>
</tbody>
</table>

*Comments*

The use of antibodies is among one of the most critical techniques performed in laboratory research; for discussion see (Rhodes et al., 2006). The specificity and reliability of the antibody is of upmost importance in order to study the expression of proteins. The commercially available antibodies for the CB₁ receptor have been questioned regarding their specificity (Grimsey et al., 2008). However, the antibody used in this thesis was not investigated in that study, and its specificity has been supported by the lack of staining in forebrain tissue from CB₁ knock-out mice (Chung et al., 2009; Gustafsson et al., 2011).
Assay for FAAH and MGL activities (Paper II, III, IV, V)

The assays used for the activity of either FAAH or MGL are based on the measurement of [3H]ethanolamine formed from hydrolysis of [3H]AEA and [3H]glycerol formed from [3H]2-OG hydrolysis, respectively (Dinh et al., 2002; Omeir et al., 1995). The original assay described by Omeir et al., 1995 used chloroform:methanol extraction for separation of the water-soluble molecules (products) from lipophilic molecules (substrates). In the present form, the hydrophilic products ([3H]ethanolamine and [3H]glycerol) were separated from the lipophilic substrates ([3H]AEA and [3H]2-OG) by addition of charcoal:HCl (Boldrup et al., 2004).

For FAAH assays, the homogenates used were prepared from adult Sprague-Dawley or Wistar rats. Frozen brains (minus cerebellum) were thawed, homogenized and thereafter centrifuged at 4 °C. The pellets were resuspended in buffer followed by recentrifugation and a second resuspension in buffer. After incubation at 37 °C, in order to hydrolyse all endogenous FAAH substrates, the pellets were again centrifuged, resuspended in 50 mM Tris-HCl buffer (pH 7.4) and frozen at -80 °C in aliquots until used.

For cell homogenates, cells were grown in culturing flasks. Cells were washed two times with ice-cold PBS prior to collection using a rubber policeman. Cells were centrifuged, resuspended in 10 mM Tris-HCl buffer and stored at -80 °C until use. The protein concentration was determined using the Bradford assay with BSA as standard (Bradford, 1976).

For MGL assays, homogenates of adult Sprague-Dawley or Wistar rats cerebella were prepared in 50 mM sodium phosphate buffer (pH 8.0) containing 0.32 M sucrose and centrifuged for one hour at 4 °C. The supernatants (cytosolic fraction) were saved and the pellets (membrane fraction) were resuspended in 50 mM sodium phosphate buffer (pH 8.0). The protein concentration of all homogenates was
determined by the method of Lowry or Bradford using bovine serum albumin as standard (Bradford, 1976; Lowry et al., 1951).

For both the FAAH and the MGL assay, enzyme (homogenates or cytosolic fraction) were pretreated with test compound prior to addition of $[^3H]$AEA (labelled in the ethanolamine part) or $[^3H]2$-OG (labelled in the glycerol part). The samples were incubated for 10 min at 37 °C and the reaction was then stopped by addition of active charcoal:HCl, vortex mixing of the tubes and placing them on ice. The tubes were then centrifuged and aliquots of the aqueous phase (containing the hydrophilic $[^3H]$EA and $[^3H]$glycerol products) were transferred into scintillation vials and counted with scintillation spectroscopy with quench correction. The lipophilic AEA molecule remains bound to the charcoal. Blanks contained assay buffer instead of homogenate solution.

For experiments using intact cells, $[^3H]$AEA and $[^3H]2$-AG hydrolysis were measured as described by Paylor et al., 2006. However a slightly modified protocol was used. Briefly, cells were seeded in a 24-well plate and grown over night. Wells were washed with warm assay buffer (Krebs-Ringer HEPES (KRH) buffer, pH 7.4), preincubated with test compound and then incubated with $[^3H]$AEA (labelled in the ethanolamine part) or $[^3H]2$-AG (labelled in the glycerol part). To stop the reaction, plates were put on ice and a mixture of active charcoal:HCl was added. The plates were left at room temperature for approximately 30 min. prior to centrifugation. Aliquots of the upper aqueous phase (containing the hydrophilic $[^3H]$EA and $[^3H]$glycerol products) were transferred to scintillation vials and assayed for tritium content by liquid scintillation spectroscopy with quench correction. The lipophilic AEA molecule remains bound to the charcoal.

The spectrophotometric assay for MGL used in this thesis was developed by Muccioli et al., 2008. Briefly, human recombinant MGL (either clear lysates or enzyme further purified through a nickel column) in 10 mM Tris–HCl, 1 mM EDTA, pH 7.4 and selected test compounds were added to a 96-well microtiter plate. The reaction was started by rapid addition of the substrate 4-nitrophenyl
acetate (NPA). Blanks contained buffer alone. The absorbance was measured at 405 nm after 0 min (to rule out effects of the compounds per se on the absorbance) and at least two 20 min intervals thereafter using a Thermomax Microplate Reader.

Comments

A number of different assays for FAAH, including chromatographic separation of substrate, spectrophotometric assays with p-nitroanilide substrates, coupled assays and simple organic separation of radiolabelled product from radiolabelled substrates, have been described (Boldrup et al., 2004). The principle of the assay was originally described by Omeir et al., 1995 and Dinh et al., 2002, although these authors used a chloroform/methanol extraction procedure. The use of charcoal to separate the metabolites of AEA (Boldrup et al., 2004) avoids exposure to potentially hazardous organic solvents.

One advantage of this assay is that it is simple and easy to perform. However, the observed activity of FAAH is highly pH-dependent, and the same can be true for its inhibition (Holt et al., 2001; Paylor et al., 2006). Throughout the experiments of this thesis, an assay pH of 7.4 has been used in order to measure activity under physiological conditions. However, in Paper III a pH of 9 was used to be as close to the pH optimum for FAAH as possible.

As discussed in the introduction, FAAH and MGL are not the only enzymes involved in the degradation of AEA and MGL. Oxidative enzymes might also contribute to activity, at least in theory. However, oxidation of AEA by COX or LOX generates lipophilic products that will not be extracted in the water phase measured in our assays (Patrignani et al., 2005; Ueda et al., 1995). Evidence of FAAH as the major contributing enzyme for the metabolism of AEA have been shown in studies where mice lacking FAAH possess a 50-100 fold reduced rate of AEA hydrolysis (Cravatt et al., 2001). Furthermore, the FAAH isoenzyme FAAH-2, with a pH optimum of 8.0, is not as effective as FAAH-1 in the hydrolysis of AEA. In addition, it is not expressed in rodents (Wei et al., 2006). As mentioned earlier,
brain 2-AG can be metabolised not only by MGL but also by ABHD6 and 12. In Paper V we found that, using rat cerebellar cytosolic fractions and the charcoal assay to separate the products, the selective MGL inhibitor JZL184 inhibited the hydrolysis of 2-OG by ~90%, suggesting that the involvement of other enzymes is minor, consistent with the literature (Blankman et al., 2007).

The number of available MGL inhibitors is limited, mostly due to the lack of rapid and accurate pharmacological assays for the enzyme. The spectrophotometric assay using 4-nitrophenylacetate (4-NPA) as a substrate simplifies the screening for novel inhibitors of the enzyme. Since 4-NPA is a substrate for many esterases, human recombinant MGL was used instead of cytosol fraction of brain homogenates. Throughout the experiments reported in this thesis, the 20 min. time point was used to ensure that initial activities were measured but at the same time allow for sufficient product formation. Given that the MGL used is a recombinant his-tagged enzyme, the assumption is made that the recombinant enzyme behaves in an identical manner to the natural enzyme.

**Cell culture (Paper II, III, IV)**

RBL2H3 rat basophilic leukaemia cells (ATCC® CRL-2256™), C6 rat glioma cells (ATCC® CCL-107™), SH-SY5Y human neuroblastoma cells (ATCC® CRL-2266™), P19 mouse teratocarcinoma (ATCC® CRL-1825™), R3327 AT-1 rat prostate cancer cells (kind gift from Prof. Anders Bergh, Umeå University), HepG2 human liver hepatocellular carcinoma cells (ATCC® HB-8065™), CaCo-2 human epithelial colorectal adenocarcinoma cells (ATCC® HTB-37™) and PC-3 human prostate epithelial cells were used (ATCC® CRL-1435™). All cells were grown in 75 cm² culturing flasks at 37 °C with 5% CO₂ in humidified atmospheric pressure.

**Comments**

RBL2H3 cells were the first peripheral cell line investigated in detail for AEA uptake (Bisogno et al., 1997a; Rakhshan et al., 2000). C6 cells are commonly used
in neurobiological research and are also widely used when investigating the cellular uptake of AEA. The well-characterized accumulation of AEA in C6 (and RBL2H3) cells makes it easier to compare findings between laboratories (Deutsch et al., 2001; Ligresti et al., 2004). All cells chosen for this thesis are well characterized regarding the endocannabinoid system, and have been reported to express endocannabinoid components such as FAAH. In contrast, PC-3 cells do not express this enzyme (Endsley et al., 2008; Ligresti et al., 2003; Pasquariello et al., 2009; Thors et al., 2007; Wu et al., 2010) which makes them useful as a negative control for the FAAH-involvement in AEA uptake.

**Uptake assay of [3H]AEA, [3H]2-AG and [3H]PEA (Paper II, III, IV)**

The assay for [3H]AEA, [3H]2-AG and [3H]PEA accumulation for adherent cells was originally described by (Rakhshan et al., 2000) and later modified by (Sandberg et al., 2005). Cells were plated in 24-well plates and incubated over night. The cells were washed with warm assay buffer (Krebs-Ringer HEPES (KRH) buffer, pH 7.4) prior to preincubation with selected compounds or vehicle. To start the reaction, [3H]AEA, [3H]2-AG (both labelled in the arachidonate part of the molecule) or [3H]PEA (labelled in the palmitoyl part of the molecule) were added to the wells. Placing the plates on ice stopped the reaction. The wells were then washed three times with ice-cold assay buffer containing 1% bovine serum albumin (BSA). Finally, 0.2 M NaOH was added and the plates were incubated at 75 °C to solubilise cellular material. Aliquots were transferred to scintillation vials and the tritium content was measured by liquid scintillation spectroscopy with quench correction.

**Comments**

As discussed in the introduction, the mechanism of uptake of AEA is a matter of debate. Also, the specific conditions used for AEA uptake assays have not been standardized and may be a confounding issue for the analysis of the data.
Throughout the experiments of this thesis we have used BSA to stabilize AEA in the buffer. BSA is known to bind to AEA and thereby prevent its binding to plastics (Bojesen et al., 2003). However, the use of BSA in assay media is discussed widely among researchers (Glaser et al., 2005). It was reported that addition of BSA to the transport media greatly reduced AEA uptake (Di Marzo et al., 1994), although this is presumably because it decreases the amount of free AEA available for uptake, rather than directly affecting the uptake process itself. For discussion see Thors et al., 2006. On the other hand, BSA decreases non-specific binding of AEA to plastic wells, which is an advantage (Karlsson et al., 2004). In all the assays the BSA concentration never exceeded 0.1%.

**Binding to CB₁ receptors (Paper II)**

The protocol of Thomas et al., 1998 was used with minor modifications. The binding of [³H]CP55,940, a CB₁ receptor agonist (Hillard et al., 1999) were undertaken in flat-bottomed 96-well plates using rat brain (without the cerebellum) homogenates. The homogenates were prepared as described above. Non-specific binding was determined in the presence of 10 µM CP55,940. Samples were incubated for 90 min. at 37 °C and the bound radioactivity was separated from unbound by vacuum filtration through pre-wetted FilterMAT filters using a cell harvester. The tritium retained by the filters was measured by liquid scintillation spectroscopy with quench correction.

**RNA extraction, cDNA synthesis and PCR (Paper IV)**

Total RNA was extracted using the miRNeasy Kit (Qiagen, Hilden, Germany, Cat. No. 217004) following the manufacturer's instructions. Briefly, cells were washed and lysed in the recommended volume of QIAzol Lysis Reagent. Rat prefrontal cortex was lysed in the same buffer using a homogenisator and RNA was extracted as described above. The RNA concentration was determined by measuring the absorbance at 260 nm using a NanoDrop spectrophotometer.
cDNA was synthesized using the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems, Foster City, CA, USA). RNA was diluted with RT buffer (keeps the enzyme stable), RT random primers (bind to mRNA in order for reverse transcription to occur), dNTP mix (for cDNA synthesis), multiscrbe transcriptase (RNA dependent-DNA polymerase, MuLV), RNAse inhibitor together with nuclease-free water. The thermal cycler conditions used were an initial step of 10 min. at 25 °C followed by 120 min. at 37 °C, 85 s at 85 °C and finally cooled to 4 °C.

For detection of rat and mouse faah and fabp5 mRNA, samples of equal cDNA concentrations were amplified in a polymerase chain reactions (PCR) using a Taq PCR Core kit (Qiagen, Hilden, Germany, Cat. No. 201223). The primers used were: forward rat faah forward 5’-GTGTGCTGACGAGTGGACC-3’, reverse 5’-GGGCCTGGACAGCTGAGTCT-3’, rat fabp5 forward 5’- CGACCGTGTGTTTC TTGCACC-3’, reverse 5’-TGCCATTGTTCATGACGCAC-3’, mouse faah forward 5’-GTGGTGCTAAACCCCATGCTGG-3’, reverse 5’-TCCACCTCCCCGCATGAA CGCAGACA-3’, mouse fabp5 forward 5’-GACGGTCTGCACCTTCCAAG-3’, reverse 5’-CAGGATGACGAGGAAAGCCC-3’. Samples were amplified with the following conditions; an initial denaturation step at 94 °C for 2 min., followed by 35 cycles with denaturing at 94 °C for 40 s, annealing at 60 °C for 40 s and elongation at 72 °C for 60 s, followed by a final elongation step at 72 °C for 3 min. The PCR products were separated electrophoretically on a 1.5 % agarose gel. The expected sizes of the fragments were: rat FAAH 382 bp, rat FABP5 192 bp, mouse FAAH 302 bp, and mouse FABP5 176 bp.

Other methodologies

Other methodologies in the papers include: synthesis of racemic Flu-AM1, Flu-AM2, Nap-AM1 and the in vivo interaction of Flu-AM1 with rat brain FAAH, performed at the University of Cagliari, Italy and the Centre for Addiction and Mental Health, Toronto, Toronto, Canada, respectively; sampling and fixation of human and animal material for Paper I, undertaken at the Section for Anatomy, Department of Integrative Medical Biology and at the Sports Medicine Unit,
Department of Surgical and Perioperative Sciences, Umeå University, Sweden; assays of COX inhibition in Paper II, performed by Ph. D. Mariateresa Cipriano at our laboratory. For details of these methodologies, the reader is referred to the individual papers.

Statistics

With the exception of analyses conducted using the R computer programme (paper IV), all statistical calculations were undertaken using the computer software GraphPad Prism 5 or 6 for the Macintosh (GraphPad Software Inc., San Diego, CA, USA). Statistical significance was set to \( p<0.05 \).

**Mann-Whitney U test**

The non-parametric test is used for comparison of two independent groups. This test was applied in Paper I.

**One-way and two-way ANOVA**

The one-way ANOVA (analysis of variance) test compares whether the mean of three or more samples differ significantly, and the two-way ANOVA tests the influence of different independent variables upon a dependent variable. Upon significance, Dunnett's multiple comparison test was undertaken to compare every mean with a control mean or Šidák’s multiple comparison test to compare selected means with each other. These tests were used in papers II, III and IV.

**\( p_{I50} \) and \( IC_{50} \)**

\( IC_{50} \) is defined as the molar concentration of a substance needed to produce 50 % of maximum possible inhibition. \( p_{I50} \) is the negative logarithm of the \( IC_{50} \) value. The \( p_{I50} \) and \( IC_{50} \) were calculated using the built-in equation log(inhibitor) vs. response from the data expressed as % of control. The top was set to 100 and the bottom to either 0 or allowed to float. For comparison between the curves Akaike’s Informative Criteria was used. These calculations were used in papers II, III and V.
Results

CB<sub>1</sub> receptor immunoreactivity in normal Achilles tendon and Achilles tendinosis (Paper 1)

CB<sub>1</sub> receptor immunoreactivity (CB<sub>1</sub>IR) was seen in normal Achilles tendon as well as in Achilles tendinosis (Fig. 3). In the normal Achilles tendon, tendon cells (tenocytes) had the characteristic appearance of being elongated, and CB<sub>1</sub>IR was seen along the length of the cells (Fig. 3A). In the case for Achilles tendinosis, CB<sub>1</sub>IR appeared in a granular pattern, which was seen especially in the abnormally-formed tenocytes, e.g. rounded/swollen tenocytes and in tenocytes with a wavy appearance, which is characteristic for Achilles tendinosis (Fig. 3B).

CB<sub>1</sub>IR in the form of fine pointed reactions was also seen in the walls of small blood vessels (confirmed by haematoxylin-eosin staining). CB<sub>1</sub>IR was also seen in the perineurium of nerve fascicles (demarcated with PGP9.5 immunoreactivity) but not within the interior of nerve fascicles. CB<sub>1</sub>IR in blood vessels and nerve structures was seen especially in the tendinosis samples (Fig. 3C, D).

As a positive control, CB<sub>1</sub>IR was also investigated in human colon, where distinct reactivity was seen in both cells the mucosal, submucosal and the epithelial layers and in dorsal root ganglia where CB<sub>1</sub>IR was seen as granular, intracellular reactions (Paper I, Fig. 1c, e) No immunostaining was seen when the antibody was preabsorbed with the corresponding peptide (Paper I, Fig. 1b, d, f and Fig. 2b).

Comparison of tenocyte CB<sub>1</sub>IR in normal Achilles tendon with Achilles tendinosis

A semi-quantitative analysis was performed based on the scoring of the CB<sub>1</sub>IR intensity in tenocytes from patients either having pain-free Achilles tendon or from patients suffering from Achilles tendinosis. Analysis of the data indicated that CB<sub>1</sub> receptor expression was significantly upregulated in Achilles tendinosis (p<0.05) (Fig 4). The tendon from the control with the highest CB<sub>1</sub>IR showed characteristic tendinosis pathology, although the individual was asymptomatic at the time of the
biopsy. This makes the categorisation of this sample as a control questionable, and when it was excluded from the data the significance level increased to p<0.005.

**Fig. 3. Immunofluorescence for CB1 in normal tendon (A) and Achilles tendinosis (B-D).** Panels show sections processed for CB1 immunostaining. Immunoreactivity (white arrows) is seen in normal tendon tissue (A), Achilles tendinosis tendon (B), small blood vessels (C) and a nerve fascicle (D). Original magnification x40 (A), x63 (B, C, D).

**Fig. 4. CB1 IR scores for biopsies from control patients and patients with Achilles tendinosis.** Boxplot of CB1 IR scores from control patients (normal pain-free Achilles tendon, n=7) vs. Achilles tendinosis patients (n=17). *p<=0.05, two-tailed Mann-Whitney U test. Values are median of the individual scoring made by the two investigators. Whiskers represents min. to max. values.
Inhibition of FAAH and COX by Flu-AM1 and Nap-AM1 (Paper II)

Analogues of flurbiprofen (Flu-AM1) and naproxen (Nap-AM1) were synthesised by our collaborators at the University of Cagliari, Italy (for details regarding the synthesis and the structure of the compounds, see Paper II, Fig. 1). These were tested for their inhibitory capacity towards FAAH using rat brain homogenates and 0.5 µM \[^{3}H\]AEA as a substrate. A summary of the data is shown in Fig. 5. Flurbiprofen inhibited rat brain FAAH with an IC\(_{50}\) value of 29 µM whereas its AM1 analogue was about 60-fold more potent (IC\(_{50}\) of 0.44 µM). The analogue of naproxen, Nap-AM1, had a similar potency towards FAAH with an IC\(_{50}\) value of 0.74 µM. Naproxen, however was a weak inhibitor of FAAH, producing less then 50% inhibition at the highest concentration tested (100 µM). The mode of inhibition by Flu-AM1 towards FAAH describes Flu-AM1 as mixed-type reversible inhibitor of rat brain FAAH.

![Fig. 5. Summary of the inhibitory potencies of flurbiprofen, Flu-AM1 and Nap-AM1 towards FAAH and COX. Data are from Table 1 of Paper II. Note that the values for COX-2 are approximate.](image)

Flu-AM1 was found to inhibit FAAH in intact RBL2H3 cells using the \[^{3}H\]AEA uptake assay. The potency of Flu-AM1 in intact cells was similar to that seen towards rat brain FAAH. Hence, a 50% inhibition of the AEA uptake was seen for 1µM Flu-AM1. Flu-AM1 did not interact with CB\(_{1}\) receptors (Paper II). Two doses of Flu-AM1 (2 and 20 mg/kg i.p.) were tested for their ability to prevent the binding of \[^{18}F\]DOPP, an irreversible FAAH inhibitor, to the enzyme. However, neither
dose affected the binding profile of this imaging agent measured ex vivo (Fig. 5 of Paper II).

From the COX assay performed by Ph. D. Mariateresa Cipriano, it was found that Flu-AM1 is a potent inhibitor of the COX-1 catalysed cyclooxygenation of arachidonic acid. COX-2 was less affected by Flu-AM1 when arachidonic acid was used as a substrate. However, when using 2-AG as a substrate, COX-2 catalysed oxygenation was greatly reduced. A similar pattern was seen for flurbiprofen. Nap-AM1, on the other hand, was a poor inhibitor of both COX isoforms (Fig. 5).

Inhibition of cellular uptake of AEA by ketoconazole (Paper III)

The antifungal agent ketoconazole was found to inhibit the cellular uptake of AEA in HepG2 and CaCo-2 cells with IC$_{50}$ values of 17 µM and 18 µM respectively (Fig. 6). The compound also reduced the retention of AEA by the wells, i.e. the binding of AEA to plastic. However, experiments using different incubation times revealed that ketoconazole significantly reduced the rate of cellular uptake of AEA by HepG2 cells, while the retention to the wells alone was low over time.

Inhibitory effect of ketoconazole upon FAAH activity

UBR597 produced a large reduction of AEA accumulation in the uptake assay with both HepG2 and CaCo-2 cells, demonstrating the importance of FAAH in preserving the gradient across the plasma membrane for these cells. Further investigation of the inhibitory effect of FAAH by ketoconazole was undertaken where the inhibition produced by ketoconazole was not additive to that produced by URB597 alone (Fig. 7). This indicates that these compounds act on the same pathway. This was further validated using PC-3 cells, which lack FAAH. In this cell line ketoconazole produced a modest inhibition of AEA uptake (Fig. 7). Experiments using HepG2 and CaCo-2 lysates revealed an inhibitory effect of ketoconazole upon the hydrolysis of AEA.
Fig. 6. The inhibitory effect upon the uptake of [³H]AEA by ketoconazole in CaCo-2 cells, HepG2 cells, PC-3 cells and wells alone. Cells (or wells) were preincubated with ketoconazole for 10 min followed by addition of [³H]AEA (100 nM) and an additional incubation time for 4 min. Shown are means ± s.e.m., n=3-9.

Fig. 7. The effect of ketoconazole upon [³H]AEA uptake in HepG2 cells in combination with URB597. Cells were preincubated with ketoconazole in combination with URB597 followed by incubation with [³H]AEA. Shown are means ± s.e.m., n=3.
Expression of FAAH mRNA in AT-1, RBL2H3, C6 and P19 cells (Paper IV)

The role of FAAH in gating AEA uptake was further explored in Paper IV. *faah* mRNA expression in three rat cell lines, one mouse cell line together with rat brain, as a positive control, was investigated using RT-PCR. Specific primers were designed to amplify a PCR product corresponding to the +4 to +385 region of the coding sequence of the rat *faah* gene. The PCR analysis displayed the presence of a band in agreement with the expected fragment size of 382 bp for rat *faah* and 302 for mouse *faah*. Equal amount of cDNA was added to the reaction and the relative *faah* expression levels were found to be RBL2H3 > C6 > AT-1 > P19 cells (Fig. 8). There was no evidence of a band with the predicted molecular weight of FLAT (178 bp), a finding consistent with the study of Leung et al. (2013). FLAT resembles the *faah* gene except that it lacks a 204 bp segment encoding amino acid residues 9-76 (Fu et al., 2012).

Fig. 8. RT-PCR analysis of mRNA expression of *faah*. Expression of *faah* mRNA was determined in rat brain lysate (lane 2), and in AT-1 (lane 3), C6 (lane 4) RBL2H3 (lane 5) and P19 (lane 6) cells using RT-PCR. Lane 1 and 7 are a 100 bp marker and the arrows show the expected size of rat FAAH (382 bp) and mouse FAAH (302 bp) products. Total RNA was isolated and reverse transcribed into cDNA. Samples (n=4) were pooled. Note that the photograph has been inverted to show the bands more clearly.
Activity of FAAH and accumulation of $[^{3}H]$AEA in RBL2H3, C6, AT-1 and P19 cells (Paper IV)

The FAAH activity in intact cells (RBL2H3, C6, AT-1 and P19) was measured by following the accumulation of tritiated product after incubation of the cells with 100 nM $[^{3}H]$AEA. C6 showed the highest rate of hydrolysis with an activity of 3.3±0.66 pmol/well/10 min. RBL2H3, AT-1 and P19 hydrolysed the substrate with activities of 2.1±0.16, 0.31±0.09 and 0.7±0.09 pmol/well/10 min.

The cellular accumulation of $[^{3}H]$AEA was measured in all four cell lines (C6, RBL2H3, AT-1 and P19) in the absence and presence of URB597. A significant decrease in uptake was seen for all cell lines tested. Despite differences in the rates of uptake in the absence of URB597, a rather similar residual uptake in all cells was seen in its presence (Paper IV, fig 1 and 2).

We also compared Compound 33, known to be an inhibitor of FAAH (Onnis et al., 2010), for its ability to inhibit the hydrolysis of AEA in intact AT-1, C6 and RBL2H3 cells in relation to its capability of affecting uptake of AEA in the same cells. We found a clear relationship between % inhibition of AEA metabolism and uptake in all of the three cell lines. However, the slopes of the regression lines were different. This indicates that a given degree of FAAH inhibition may not lead to the same degree of inhibition of AEA uptake for different cells despite similarities in FAAH expression (Paper IV, Fig 3 and 4).

Expression and activity of FABP5 in RBL2H3, C6, AT-1 and P19 cells (Paper IV)

AEA is shuttled to FAAH for hydrolysis by transporting proteins. The differences seen between the cells might be due to different expression levels of such carriers. Therefore, we examined the expression and function of FABP5, an intracellular carrier protein of AEA (Kaczocha et al., 2009), using the FABP5 inhibitor SB-FI-26 (Berger et al., 2012). The PCR revealed a band, which correlates well with the
expected size of the fragment of 192 bp for rat FABP5 mRNA and 179 bp for mouse FABP5 mRNA (Paper IV, Fig 5a). The cells show similar expression levels of FABP5. Further, in all cells tested 50 µM SB-F1-26 significantly decreased the accumulation of AEA (Paper IV, Fig 5b-f). Thus, the differences between the cells cannot be ascribed to differences in expression of FABP5, although other carriers may still be involved.

**Screening of selected compounds for inhibition of MGL using the spectrophotometric NPA assay (Paper V)**

Initial experiments were undertaken by co-authors Norén and Nilsson to characterise the assay with respect to enzyme concentrations and incubation times used, pH optima, sensitivity to inhibition by 2-AG and 2-OG, and maximum tolerable solvent concentrations (Norén & Nilsson, undergraduate thesis project, 15 hp, Autumn term 2008). The screening included 96 compounds known to interact with the CB system or have other actions upon targets in the brain, NSAIDs or naturally occurring compounds. Selected compounds and their hMGL activity are listed in Paper V, Table 1. In the initial screen, compounds were tested at two different concentrations (3 and 10 µM). A compound producing >60% inhibition was classified as a “hit”. Further characterisation of six of the “hits” was performed by dose-response curves over the range of 0.1-10 µM (Paper V, Fig. 1a). The pI \textsubscript{50} values (IC \textsubscript{50} values in brackets) were for troglitazone, CP55,940, N-arachidonoyl dopamine and AM404 5.95±0.04 (1.1 µM), 5.31±0.07 (4.9 µM), 6.11±0.08 (0.78 µM) and 5.53±0.09 (3.1 µM) respectively. There was a large variability in the observed inhibition at the highest concentration for WIN55,212-2 and JWH015 yielding difficulties to determine their IC \textsubscript{50} values.

**Troglitazone and N-arachidonoyl dopamine as inhibitors of MGL**

The PPAR\textgamma ligand troglitazone was compared to other PPAR\textgamma ligands toward their ability to inhibit hMGL. The potency order was troglitazone > ciglitazone > rosiglitazone > 15-deoxy-\Delta^{12,14}-prostaglandin J\textsubscript{2} ≈ CAY10415 > CAY10514 (Paper V, Fig. 1b).
The inhibition of hMGL with troglitazone showed no time-dependency indicating that the inhibition is reversible (Fig. 9a). However, additional experiment indicates that this reversibility is not rapid. Saturation curves for NPA incubated with 0 and 0.75 µM troglitazone gave $K_m$ values of 0.17 and 0.70 mM respectively (Fig. 9b), assuming a competitive mode of action.

Both troglitazone and the endogenous TRPV1 ligand $N$-arachidonoyl dopamine were investigated in two assay systems in order to determine if the inhibition of MGL was assay, substrate and/or species dependent. $N$-arachidonoyl dopamine inhibited the hydrolysis by the hMGL of NPA and 2-OG with $IC_{50}$ values in the low µM range (0.78 µM and 2.2 µM respectively). However, the compound was less potent as an inhibitor of the hydrolysis of 2-OG by rat cytosol ($IC_{50}$ value of 20 µM) (Fig. 10a). For troglitazone there was a large inter-assay variability. The compound inhibited purified hMGL with a threefold lower potency then seen for the lysate in the NPA assay. Yet, in the radiochemical assay troglitazone was a weak inhibitor of both rat cytosol and hMGL with 2-OG as substrate (Fig. 10b).

Different BSA concentrations in the two assays were found to affect the observed inhibitory properties of troglitazone. In the NPA assay, the inhibition of hMGL produced by both troglitazone and $N$-arachidonoyl dopamine were greater in the absence of BSA. Also, when assayed radiochemically, $N$-arachidonoyl dopamine inhibited hMGL lysate better without BSA in the buffer compared to the presence of BSA. Troglitazone was also a better inhibitor of hMGL lysate in the radiochemically assay in the absence of BSA, although its potency was still lower then that seen in the NPA assay.
Fig. 9. Mode of inhibition by troglitazone upon hydrolysis of 4-NPA by hMGL. In panel A, lysates were preincubated with troglitazone at room temperature prior to addition of 4-NPA (0.25mM). In panel B, no preincubation was used prior to addition of 4-NPA (0.08-0.64 mM). Shown are means ± s.e.m., n=3 except for the data in Panel B with 0.32 mM NPA, where n=2. To illustrate the competitive nature of the inhibition, a double reciprocal plot of the mean data with 0 and 0.75 mM troglitazone is inserted in Panel B.

Fig. 10. The potencies of N-arachidonoyl dopamine (A) and troglitazone (B) towards MGL possessed in different assays. No preincubation phase were used prior to addition of substrate in any of the assay system used. Shown are mean ± s.e.m., n=3-6.
Discussion

Overall comments

The studies included in this thesis provides fruitful novel information about the endocannabinoid system in a human pain state, e.g. Achilles tendinosis as well as a better understanding on how to pharmacologically modify this system. The results demonstrate that CB₁ receptors can be observed immunohistochemically in Achilles tenocytes and that their immunochemical staining intensity in increased in tendinosis. This is consistent with the suggestion of a dysregulated endocannabinoid system in a painful human condition. As a consequence of this, an enhanced knowledge on how to modulate this system in a pharmacological manner would be beneficial. The findings presented here delineate new openings for the development of novel inhibitors of endocannabinoid metabolism.

Achilles tendinosis

Prior to Paper I, as discussed in the introduction, little was known about the endocannabinoid system in human pain. Likewise, the Achilles tendon is the strongest tendon in the body but the reasons behind the pain experienced in Achilles tendinosis remains unclear. Based on this gap in our knowledge, we investigated the expression of CB₁ receptors in Achilles tendon tissue using biopsy samples from patients suffering from Achilles tendinosis and from individuals having asymptomatic pain free tendons. The result of the semi-quantitative analysis, performed by two independent blinded researchers, implied an up-regulation of CB₁ receptor expression in Achilles tendinosis. We found CB₁IR along the length of tenocytes in normal tendons. For tenocytes having a characteristic tendinosis morphology, i.e. rounded/swollen tenocytes and tenocytes with a wavy appearance, the CB₁IR had a granular intracellular pattern. The presence of intracellular reactions can also be seen in the literature for other cells in the body (Bridges et al., 2003). In 2001 Brailoiu et al., 2011 showed that AEA can activate intracellular CB₁
receptors and thereby provided evidence that these intracellular receptors are functional.

Recently, Dean et al., 2013 have critically reviewed the reliability of published articles in the tendinosis research field. They conducted a systematic review of the scientific literature where inclusion criteria and methods of analysis were specified in advance. The search, selection of studies and data analysis was performed by two individuals. The methodological quality of the selected papers was assessed using a 10-point scale where a point was awarded for each of the following criteria: inclusion/exclusion criteria clearly described; study population clearly described; study population representative of those in clinical practice; control group clearly described; sampling method clearly described; live study subjects; live control subjects; quantitative method or semi-quantitative method using minimum of two independent observers; reliability and/or validity of methods described; and study limitations addressed (Dean et al., 2013). Our study attained a high score (9/10), although it was felt that the study limitations should have been further discussed. Hence, these are addressed below.

Our control group consisted of healthy asymptomatic patients (as opposed to cadaveric controls or patients with Achilles tendinosis rupture). Although this is a strength - the tendon rupture patients might not yet show symptoms of tendinosis albeit the tendon tissue is somehow impaired which leads to the rupture - the use of healthy controls is a study limitation in terms of the size of the control group. Further, for ethical reasons, the tendon samples taken from the controls were from the dorsal part of the tendon. Another limitation of the study is that CB1 immunoreactivity alone does not tell us whether or not CB1 receptors are functional or active. One way of investigating this would be the study of CB1-mediated receptor signalling in primary tenocyte cultures. In collaboration with the research group of Ph. D. Patrick Danielsson (Department of Integrative Medical Biology, Section of Anatomy, Umeå University), we have tried to evaluate this possibility, but we were unable to obtain robust data in the time frame available. Additionally, although immunoreactivity gives information about protein expression, it does not
provide data concerning the underlying regulation accounting for observed
differences, e.g. regulation at the level of mRNA expression or post-translationally.
Moreover, small or local changes in endocannabinoid signalling would be difficult
to detect. Although the latter question is beyond the scope of our paper, it would be
of clear interest to investigate local endocannabinoid levels using, for example the
microdialysis technique. An additional approach, which we initiated, was to evaluate
whether the expression of the endocannabinoid metabolising enzyme FAAH was
altered in Achilles tendinosis. However, we were not able to find a robust antibody
with which to assess immunoreactivity.

Inhibitors of endocannabinoid metabolism and uptake

Termination of endocannabinoid signalling is known to occur through cellular
uptake followed by hydrolysis by metabolising enzymes. Although the catabolic
process is well defined, the mechanism(s) of cellular uptake has not yet been
clarified. A number of inhibitors of AEA uptake and AEA metabolism have been
developed, most of them with reported effect both in vitro and in vivo. Inhibitors of
FAAH are in phase I and II clinical trials, but so far no compounds have
successfully reached the market. When this thesis was started, no selective MGL
inhibitors had been disclosed. The irreversible inhibitor of MGL, JZL184, was
reported in 2009 but there are issues of tolerance with this compound (Schlosburg et
al., 2010). Our goal was to identify novel compounds, preferably inhibiting
endocannabinoid metabolism in a reversible manner. Our approach was to
investigate compounds that are already in clinical use and elucidate whether
inhibition of endocannabinoid metabolism is an additional effect either per se, or a
result of chemical modification of the compound in question.

A previous article from our lab revealed the inhibitory effect of ibuprofen on FAAH,
in addition to COX (Fowler et al., 1997). Furthermore, its N-(3-methylpyridin-2-yl)amide analogue proved to be 2-3 orders of magnitude more potent as an FAAH
inhibitor but with preserved effect on COX (Holt et al., 2007). Mice treated with
FAAH inhibitors together with compounds inhibiting COX had a lower incidence of
gastric haemorrhages (Naidu et al., 2009). Based on these results, in Paper II we synthesised the analogue of flurbiprofen, Flu-AM1, in order to investigate its ability to inhibit FAAH and COX. We found that Flu-AM1 inhibited FAAH with preserved effect upon COX. By using RBL2H3 cells, in which FAAH has been shown to gate AEA uptake (Deutsch et al., 2001), we found that Flu-AM1 had the ability to cross membranes and inhibit FAAH but it did not affect brain FAAH. This raises the question as to whether or not the concentrations tested were too low, or the compound has a limited capacity to cross the blood-brain barrier. The answer to this was beyond the scope of this project. Nonetheless, identification of novel dual-action compounds is clearly an area worth pursuing, given the synergistic effects of FAAH inhibitors and NSAIDs in preclinical pain models (Naidu et al., 2009).

In line with the concept of finding novel actions of established compounds, in Paper III we investigated whether or not the antifungal compound ketoconazole affects AEA uptake. Ketoconazole has previously been shown to interact with CYP3A4 (Sai et al., 2000) and lipoxygenase (Beetens et al., 1986), two enzymes that can metabolise AEA. Ketoconazole inhibited AEA uptake in two FAAH-containing cell lines, namely HepG2 and CaCo-2, in a manner consistent with inhibition of FAAH. This is supported by the fact that ketoconazole had no effect upon the accumulation of AEA in PC-3 cells, which have low expression of FAAH. In addition we found that the inhibitory effects of ketoconazole and the FAAH inhibitor URB597 upon AEA uptake were not additive suggesting that the two compounds act on the same pathway.

As pointed out above, due to the lack of selective MGL inhibitors, more research about the activity of the enzyme and potential inhibitors is needed. As already discussed regarding ketoconazole, our basic idea was to investigate a range of compounds that are either currently, or have been previously, in clinical use and/or that are known to have some kind of activity towards the endocannabinoid system. We used the assay of Muccioli et al., 2008, which allows the screening of a larger number of compounds than usually possible in our radiochemical assay. The “hits” from this preliminary screen were then further evaluated. We found that the PPARγ
ligand troglitazone (Willson et al., 1996) inhibited MGL in the low micromolar range but also that there was a large inter-assay difference. Troglitazone was withdrawn from the market due to hepatotoxicity and related ligands such as rosiglitazone are associated with cardiovascular events, but it may be possible to use troglitazone as a starting point for novel MGL inhibitors devoid of these issues.

Taken together, by screening compounds that are, or previously have been, in clinical use for their FAAH and MGL inhibitory properties gives us good templates with which to design novel FAAH and MGL inhibitors. By synthesising analogues of a promising compound, it may be possible to optimise effects upon the endocannabinoid system while retaining (or not, as appropriate) its effects on other systems.

As mentioned in the introduction, the uptake process of AEA in to cells is a matter of debate. FAAH activity has been shown to gate AEA uptake in RBL2H3 cells but in other FAAH-expressing cells, the effect is not seen (Deutsch et al., 2001). This raises the question as to whether or not the degree of FAAH-sensitivity is the result of methodological differences in different laboratories, or if it reflects the properties of the cells themselves. To answer this we investigated the effect of FAAH inhibition upon AEA accumulation in four cell lines. We found that RBL2H3 and C6 cells, two cell lines having roughly similar levels of faah gene expression, had different sensitivities to a given level of FAAH inhibition. This implies that the reported differences in FAAH sensitivities of AEA uptake in the literature are not due to methodological differences alone but to the properties of the cells themselves. One suggestion might be that different cells may take up and accumulate AEA for different purposes (Hillard et al., 2005), e.g. as a requirement for cell signalling or as a source for the production of arachidonic acid, and in consequence utilise different transport mechanisms. The presence of intracellular AEA transporting proteins might also contribute to the differences in FAAH sensitivity between the cells, although variations in FABP5 expression can be ruled out.
Future perspectives

Future directions of this work could be:

• Examine whether or not the CB₁ receptors in Achilles tendinosis are functionally active, for example by investigating the signalling of the CB₁ receptors in fresh tissue from tendinosis patients.

• Determine the strength of local endocannabinoid signalling in the normal and pathological Achilles tendon. Ideally, this could be undertaken by measuring local endocannabinoid levels, but a more indirect measure would be the determination of expression levels of the endocannabinoid synthetic and degradative enzymes, or by investigating the activation of downstream targets of CB₁.

• *In vitro* and *in vivo* experiments with analogues of ketoconazole, troglitazone and Flu-AM1 optimised for inhibition of FAAH, MGL and FAAH/COX-2, respectively.
Conclusions

CB₁ receptors are expressed in tenocytes of Achilles tendon. In painful Achilles tendinosis, expression is up-regulated compared to normal, healthy tendons.

The analogue of flurbiprofen, Flu-AM1, shows promising potency as a dual-action compound effecting both FAAH and COX.

Ketoconazole, an antifungal compound with inhibitory effect on enzymes in the CYP family, can also inhibit the cellular uptake of AEA primarily due to effects upon FAAH.

Cells with broadly similar levels of FAAH-expression show different sensitivities to FAAH inhibition upon AEA uptake.

Troglitazone inhibits MGL in a manner dependent upon the enzyme assay used.
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References


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