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"A. AVOGADRO"

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ENDOCANNABINOIDS AND MURINE MEGAKARYOCYTIC DIFFERENTIATION

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Coordinatore del corso:
Prof. Umberto Dianzani

Supervisore:
Prof. Fabiola Sinigaglia

Dottoranda
Dott.ssa Alessandra Bagarotti

CONTENTS

1. INTRODUCTION

Cannabis is a genus of flowering plants that includes three species: *Cannabis sativa, Cannabis indica, and Cannabis ruderalis*. These three taxa are indigenous to Central and South Asia. *Cannabis* has long been used for medical purposes, for fibre, for seed and seed oils, and as a recreational drug.

Figure 1. Cannabis sativa, scientific drawing (1900)

Cannabis is an annual, dioecious, flowering herb. The leaves are digitate or palmately compound, with serrate leaflets. The first pair of leaves usually have a single leaflet, the number gradually increasing up to a maximum of about thirteen leaflets per leaf.

Cannabis, also known as marijuana, refers to any number of preparations of the Cannabis plant.

For many centuries, marijuana has been used both recreationally, as a result of its psychoactivity, and medicinally. The considerable therapeutic potential of the drug has been documented ever since the fourth century BC (Mechoulam et al., 1986; Zias et al., 1993). Under Emperor Chen Nung's rule, the Chinese used marijuana for the treatment of constipation, malaria, rheumatic pains, female disorders, and absentmindedness. Use of marijuana spread west from early Chinese culture to India and finally to Eastern Europe, where anecdotal information about its health benefits were finally put to scientific scrutiny early in the nineteenth century.

Recently, its use as an appetite stimulant has been indicated in patients with cachexia or wasting disease observed, for example, in AIDS victims (Beal et al., 1995; Grinspoon et al., 1995). The recent Food and Drug Administration (FDA) approval of an oral preparation of synthetic Δ9-tetrahydrocannabinol (Δ9-THC) (the primary psychoactive compound of cannabis) (Figure 2), and the recent approval of bills in California and Arizona allowing the medicinal use of smoked marijuana have renewed interest in the therapeutic potential of cannabinoids. The modern development of cannabinoids as therapeutic agents has been hampered largely because of their abuse potential and difficulties in separating the psychotropic effects from possible therapeutic effects.

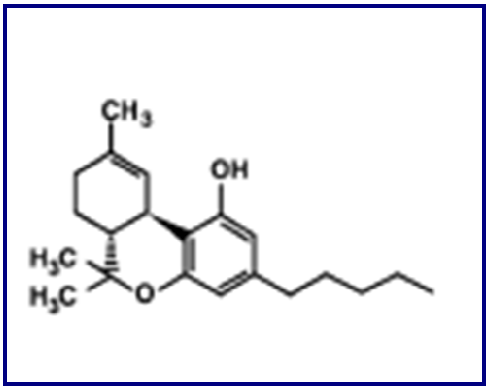

Figure 2. Δ9-tetrahydrocannabinol (Δ9-THC)

Still in the 1970s, it was believed that THC produced its effects by perturbing neuronal cell membranes due to its lipid-soluble, hydrophobic nature. However, the structural and steric selectivity of the actions of THC and its synthetic analogs suggested the involvement of specific receptors. This was subsequently demonstrated in radioligand-binding studies

(Devane et al., 1988). In the early 1990s, two distinct G protein-coupled cannabinoid receptors were identified by molecular cloning: the brain-type cannabinoid receptor later termed CB1, which is highly expressed in the brain but is also present in peripheral tissues, and CB2 receptors, whose expression is mainly limited to the cells of the immune and haematopoietic systems (see below). The existence in mammalian cells of specific membrane receptors for plant-derived substances triggered a search for an endogenous ligand. In 1992, this search lead to the identification of arachidonoyl ethanolamide, named anandamide (AEA), a lipid that binds to cannabinoid receptors and mimics the biological effects of THC (Devane et al., 1992). A second endogenous cannabinoid, 2-arachidonoylglycerol (2-AG), was isolated three years later, from brain tissue (Sugiura et al., 1995) and gut (Mechoulam et al., 1995). Subsequently several other related lipids with endocannabinoid properties were identified, but these have been less characterized than AEA or 2-AG (Hanus et al., 2001; Porter et al., 2002).

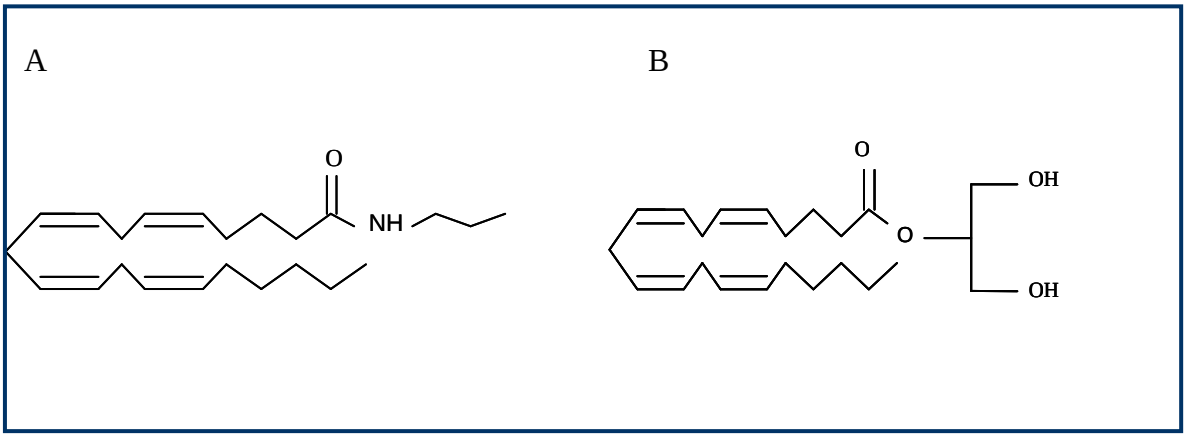

Figure 3. Chemical structures of anandamide (A) and 2-arachidonoylglycerol (B)

The endocannabinoid system (ECS) refers to: the cannabinoid receptors, the endogenous arachidonate-based lipids (collectively termed "endocannabinoids") and the enzymes that sinthetize and degrade the endocannabinoids.

1.1 Endocannabinoids: an overview

There are several novel structural analogs of AEA: Porter and collegues has demonstrated that an ester-linked isomer of AEA is present in the rat brain and human hippocampus and named this compound ‘virodhamine’(Porter et al., 2002). Virodhamine acts as an antagonist or a partial agonist towards the CB1 receptor and acts as a full agonist towards the CB2 receptor. On the other hand, Huang et al. isolated N-arachidonoyldopamine from the bovine brain. N-arachidonoyldopamine binds to both the cannabinoid receptors and the vanilloid receptor and induces analgesia upon systemic administration and hyperalgesia when intradermally injected (Huang et al., 2002). It is not still clear if these AEA analogs play specific physiological roles as intercellular signalling molecules in living animals. Hanus (Hanus et al., 2001) reported that 2-AG ether (noladin ether), an ether-linked analog of 2-AG, is also present in the pig brain (0.6 nmol/g tissue).

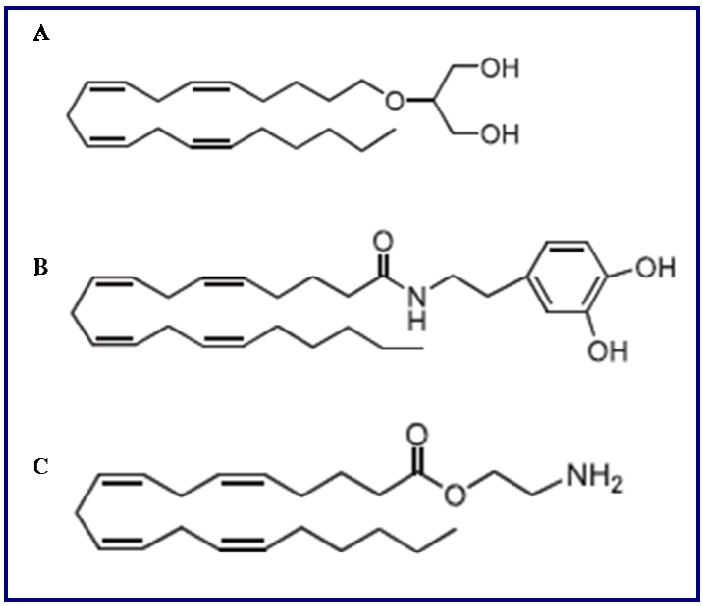

Figure 4. Chemical structures of noladin ether (A), N-arachidonoyldopamine (B) and virodhamine (C).

1.2 CB1 and CB2 receptors

The biological effects of endogenous, plant-derived and synthetic cannabinoids are mediated through specific G protein-coupled cannabinoid (CB) receptors. There is an extensive literature on CB1 and CB2 receptors, I will summarize here only some features.

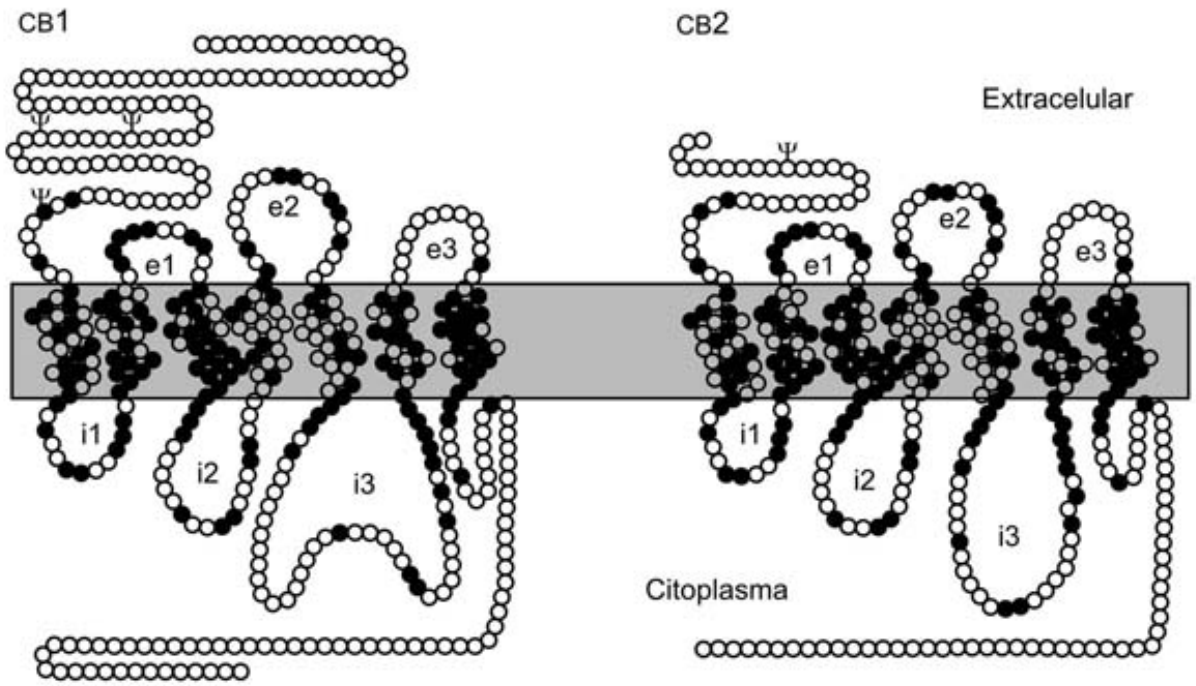

Figure 5. A detailed diagram of the Cannabinoid receptor 1 and Cannabinoid receptor 2 structures. As it is typical of G protein-coupled receptors, the cannabinoid receptors contain seven transmembrane spanning domains. The protein sequences of CB1 and CB2 receptors are about 44% similar. (Sourge: http://www.upf.edu).

Both receptors belong to the rhodopsin-like family of G-protein-coupled receptors superfamily. They share a common seven transmembrane-spanning α-helical serpentine structure. The α-helical segments are linked by alternating intracellular and extracellular peptide loops to the N-terminal region, with potential glycosylation sites, located on the extracellular side and C-terminal region, with phosphorylation residues, located on the intracellular side.

Cannabinoids agonist ligands interact with the receptor within the pore formed within the transmembrane helical cluster (Shire et al., 1996 - a). The three cytosolic loops and a putative fourth loop formed by palmitoylation at the juxtamembrane C-terminal region contribute to the activation of G proteins.

The CB1 functional identity was revealed by the perfect overlap between the brain distribution of its mRNA and the specific binding sites for a radiolabeled cannabinoid (Herkenham et al., 1990). The human homolog of the CB1 was identified shortly thereafter (Gerard et al., 1991), and a second cannabinoid receptor was cloned from rat spleen and named CB2 (Munro et al., 1993). A splice variant of the mRNA encoding the CB1 receptor has also been identified in human and rat tissues (Shire et al., 1996 - b), but the existence of the protein product of this mRNA has not yet been demonstrated. CB1 receptors are expressed predominantly in the central nervous system (CNS) with particularly high levels in cerebellum, hippocampus and basal ganglia. In fact, of all known neurotransmitter and hormone receptors, the CB1 receptor is by far the most abundant in the mammalian brain.

CB1 receptors are also expressed, albeit at much lower levels, in the peripheral nervous system as well as on the cells of the immune system, in the heart, vascular tissues, and the testis (Herkenham et al., 1990; Gerard et al., 1991; Ishac et al., 1996; Pertwee, 1997; Gebremedhin et al., 1999; Liu et al., 2000; Bonz et al., 2003). In tissues naturally expressing CB1 receptors and in transfected cell lines, both CB1 (Losonczy et al., 2004) and CB2 receptors (Bouaboula et al., 1999) have been shown to have a high level of ligand-independent activation (i.e. constitutive activity). It was estimated that in the population of wild-type CB1 receptors only 30% exist in the activated state, while 70% are inactive (Kearn et al., 1999).

The expression of the CB2 receptor is more restricted, limited primarily to immune and haematopoietic cells (Munro et al., 1993): it is almost exclusively present in the immune system such as the spleen, tonsil and lymph nodes, although the expression of the CB2 receptor in the brainstem has recently been noted (Van Sickle et al., 2005). The CB2 receptor is abundantly expressed in several types of leukocytes, such as B lymphocytes, natural killer cells and macrophages/monocytes, and is assumed to participate in the regulation of immune responses and/or inflammatory.

The human CB2 receptor shows 68% amino acid homology with the CB1 receptor in the transmembrane domains and a 44% overall homology (Munro et al., 1993).

Both CB1 and CB2 receptors are coupled through Gi and Go proteins to inhibit adenylyl cyclase and regulate calcium and potassium channels (Mackie et al., 1992; Mackie et al., 1995). Moreover, various intracellular kinases, including the mitogen-activated protein kinases, extracellular signal regulated kinases type 1 and 2, JUN N-terminal kinase, focal adhesion kinase, and protein kinase B/Akt, are also activated by CB1 receptors (Bouaboula et al., 1995, Bouaboula et al.,1997; Derkinderen et al., 1996, Derkinderen et al., 2001, Derkinderen et al., 2003; Gomez del Pulgar et al., 2000; Rueda et al., 2000).

1.3 Non-CB1/non-CB2 cannabinoid receptors

The first indication that cannabinoid receptors other than CB1 or CB2 may exist derived from studies of the mesenteric vasodilator effect of cannabinoids. In the rat isolated perfused mesenteric arterial bed preparation, AEA, and R-methanandamide (metAEA) elicit long-lasting vasodilation, whereas synthetic cannabinoids potent at both CB1 and CB2 receptors or THC do not have a dilator effect (Wagner et al., 1999). The most extensevly studied non-CB1/CB2 site occurs in the vasculature, but also in the central nervous system and on immune cells (Brown, 2007).

In addition to the well-characterized receptors, pharmacological studies have revealed the existence of other endocannabinoid targets among which the vanilloid receptor (Zygmunt et al., 1999) and at least two non-CB1 non-CB2 receptors. It has been recently demonstrated that, for instance, 2-AG induced full platelet activation and aggregation with a non-CB 1/CB2 receptor-mediated mechanism (Baldassarri et al., 2008).

TRPV-1

The transient receptor potential cation channel, subfamily V, member 1 (TRPV1), also known as the capsaicin receptor, is a protein that, in humans, is encoded by the TRPV1 gene. TRPV1 is a member of the Transient Receptor Potential Vanilloid (TRPV) group: a family of transient receptor potential channels that are selective for calcium and magnesium over sodium ions. Like other TRP channels, TRPV1 has a carboxyl terminus containing a TRP domain close to the sixth transmembrane domains and a long amino terminus containing three ankyrin-repeat domains. Oligomer formation analysis using perfluorooctanoic acid polyacrylamide gel revealed that TRPV1 forms multimers with a homotetramer as the predominant form (figure 6).

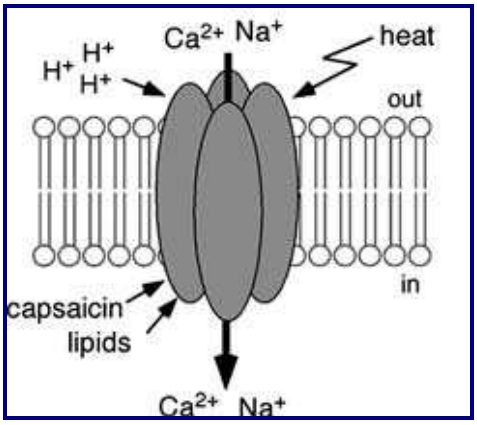

Figure 6. Proposed tetrameric structure of transient receptor potential vanilloid 1 (TRPV-1) in the plasma membrane. (Tominanga et al., 2005)

Like other members of the TRP superfamily, TRPV channels can be activated through seemingly disparate mechanisms. The best-known activators of TRPV1 are heat greater than 43°C and capsaicin, the pungent compound in hot chili peppers. The activation of TRPV1 leads to painful, burning sensation. Its endogenous activators include: low pH (acidic conditions), AEA but not 2-AG (Starowicz et al., 2007) and N-arachidonoyl dopamine. TRPV1 receptors are found mainly in the nociceptive neurons of the peripheral nervous system, but they have also been described in many other tissues, including the central nervous system. TRPV1 is involved in the transmission and modulation of pain (nociception), as well as the integration of diverse painful stimuli (Cui et al. 2006, Huang et al., 2002).

The capsaicin binding domain on TRPV1 is intracellular (Jung et al., 1999) and selective AEA membrane transporter (AMT) inhibitors block AEA activity at TRPV1 (De Petrocellis et al., 2001), suggest that AEA activates this receptor by acting from the cytosolic side of the cell (De Petrocellis et al., 2001; Jordt et al., 2002) and that the AMT plays a permissive role in the interaction of AEA with TRPV1. Moreover, activity of AEA at recombinant TRPV1 receptors is significantly enhanced when protein kinase (PK) C or A are stimulated (De Petrocellis et al., 2001; Premkumar et al., 2000; Vellani et al., 2001). It is possible that, for example during inflammation or cell damage, when PKC is activated, pH is decreased and palmitoylethanolamide is biosynthesized by cells (Bisogno et al., 1997; Hansen et al., 1995), AEA becomes more active at vanilloid receptors than at CB1 or CB2 receptors, thus possibly producing different effects on pain perception, inflammation and cell survival depending on whether it only activates or also immediately desensitizes TRPV1.

G protein-coupled receptor

The cloning of the human G protein-coupled receptor 55 (GPR55) was originally reported in 1999, when it was described as a classical intronless GPCR that maps to chromosome 2 and consists of 319 amino acids. High levels of human GPR55 mRNA transcripts have been found in brain regions implicated in the control of memory, learning and motor functions, such as the dorsal striatum, caudate nucleus and putamen, and in several peripheral tissues, including the ileum, testis, spleen, tonsil, breast and omental adipose tissue (Brown, 2007; Sawzdargo et al., 1999), as well as some endothelial cell lines (Waldeck-Weiermair et al., 2008). It is generally agreed that certain cannabinoid ligands interact with GPR55 with high affinity, and that the relative affinities of different cannabinoid ligands are distinct from the relative affinities of the same ligands established for either CB1 or CB2 receptors (Godlewski et al., 2009): Δ9-THC (Ryberg et al, 2007) and the endocannabinoids AEA, 2-AG and noladin ether bind GPR55 in the low nanomolar range.

AEA also bind G protein-coupled receptor 119 (GPR119). It is expressed predominantly in the pancreas and gastrointestinal tract in rodents and humans, and in rodents brain (Overton et al., 2006). Activation of the receptor has been shown to cause a reduction in food intake and body weight gain in rats (Overton et al., 2006). GPR119 has also been shown to regulate incretin and insuline hormone secretion (Ning et al., 2008; Swaminath, 2008; Lan et al., 2009). As a result, new drugs acting on the receptor have been suggested as novel treatments for obesity and diabets (Overton et al., 2006; Swaminath, 2008). Cells expressing GPR119 at high levels display a constitutive increase in intracellular cAMP, which implies coupling of GPR119 to Gs. (Chu et al., 2007).

Peroxisome proliferator-activated receptors

In addition to G-protein-coupled receptors (GPCR), another potential candidate for CB1/CB2-independent effects of cannabinoids is the PPAR (peroxisome-proliferator-activated receptor) family of nuclear receptor transcription factors. PPARs are members of a nuclear hormone receptor superfamily of ligand-activated transcription factors.

The natural ligands for PPARs include fatty acids and eicosanoid derivatives. The study of cannabinoids effects on PPARs started from the investigation of N-acyl ethanolamine (OEA), a naturally occurring lipid derivative structurally related to AEA, which shares the anorectic property of other cannabinoids.

Recently, THC was found to activate one member of the PPAR family, PPARγ, in a concentration-dependent manner in transactivation assays in human embryonic kidney (HEK-293) cells (O'Sullivan et al., 2005). It also stimulated adipocyte differentiation in 3T3L1 cells, a well-accepted property of PPARγ ligands. Finallt it has also been demonstrated that THC can cause vasorelaxation through activation of PPARγ. Recently, AEA also has been found to directly activate PPARγ (Bouaboula et al. 2005) and PPARα (Sun et al., 2006). AEA can bind PPARγ ligand binding domain directly and induce transcriptional activation of PPARγ in different cell types. AEA can stimulate 3T3L1 adipocyte differentiation and induce the expression of adipocyte differentiation markers (Bouaboula et al. 2005). Cannabinoids effects on the PPARβ subtype have not been comprehensively studied yet, probably due to shortage of apparent pharmacological significance.

AMT: anandamide membrane transporter

As previously decribed, the endogenous cannabinoid/vanilloid agonists AEA (and N-arachidonoyl-dopamine (NADA)), bind to CB1 and CB2 and TRPV1. Since the proposed binding site for TRPV1 agonists is on an intracellular domain, many scientists have proposed that the cellular uptake of AEA (Beltramo et al., 1997; Di Marzo et al., 1994) and NADA (Bisogno et al., 2000; Huang et al., 2002) could be mediated by the putative anandamide membrane transporter (AMT) in order to gain access to this intracellular binding site. Although the AMT has yet to be molecularly identified, multiple lines of evidence suggest its existence. The cellular uptake of AEA is a saturable process that is time- and temperature-dependent (Beltramo et al., 1997). Moreover, AEA uptake is inhibited by multiple proposed AMT inhibitors and there are strict molecular determinants for the pharmacological inhibition of uptake (Hillard et al., 2000; Hillard et al., 2003; Reggio et al., 2000) and uptake itself (Piomelli et al., 1999). AEA uptake has been demonstrated in many cell lines as well as in CNS neurons (Beltramo et al., 1997; Di Marzo et al., 1994; Hajos et al., 2004; Ortega-Gutierrez et al., 2004) and astrocytes (Beltramo et al., 1997). Additionally, AMT inhibitors augment extracellular AEA concentrations in vivo (Giuffrida et al., 2000) and increase the

behavioral effects of exogenously administered AEA (de Lago et al., 2004). Hence, the primary role of the AMT in the CNS has been proposed as concentrating AEA within neurons and glia that contain the AEA-degrading enzyme fatty acid amide hydrolase (FAAH) (Giuffrida et al., 2001; Hillard et al., 2003; Piomelli, 2003). FAAH also appears to be involved in concentrating AEA within cells (Day et al., 2001; Glaset et al., 2003; Ortega-Gutierrez et al., 2004), and so the relative contribution of FAAH and AMT to AEA uptake remains controversial (Hillard et al. 2003). However, the persistence of AEA accumulation in FAAH knockout mice indicates that another mechanism, different from FAAH, and pharmacologically blocked by AMT inhibitors, plays an essential role in this process (Fegley et al., 2004; Ortega-Gutierrez et al., 2004).

1.4 Endocannabinoids: biosynthesis and metabolism

The endocannabinoids are produced by neurons ‘‘on demand’’, act near the site of their synthesis and, as it is typical for neuromodulators, they are effectively metabolized to ensure rapid signal inactivation (Devane et al. 1992, Mechoulam et al., 1995; Sugiura et al., 1995).

AEA biosynthesis and metabolism

AEA is a member of the N-acylethanolamine (NAE) family, a large group of bioactive lipids that also includes non-endocannabinoid compounds. Initially, NAEs were thought to be produced by a phospholipase D and hydrolyzed by an amidase called ‘fatty acid amide hydrolase’ (FAAH). This rather simple picture has now evolved into a more complex array of biochemical pathways, with newly discovered enzymes. N-acylphosphatidylethanolamines (NAPEs) are considered the general precursors for NAEs; the molecular characterization of the enzyme responsible for the activity-dependent transfer of the sn-1 acyl chain from 1,2-diacylglycerophospholipids (e.g. phosphatidylcholine) or 1-acyl lysophospholipids to phosphatidylethanolamine (an N-acyl transferase, NAT) is still unclear (Di Marzo et al., 1994; Astarita et al., 2008). An N-acyl transferase able to synthesize NAPEs was recently cloned but differs from NAT – in its relative insensitivity to Ca2+ activation, for example – and, therefore, was named ‘iNAT’(Jin et al., 2009).

The second step in the canonical transacylation–phosphodiesterase pathway leading to NAEs is NAPE hydrolysis by a Ca2+-sensitive NAPE-selective phospholipase D (NAPE-PLD) (Wang et al., 2008 -a). Its molecular cloning revealed a phosphodiesterase of the metallo-blactamase family (Okamoto et al., 2004), and the characterization of the purified enzyme

confirmed that it is kept constitutively active in its membrane-associated form by membrane components such as phosphatidylethanolamine (Okamoto et al., 2004; Petersen et al., 2009). It is noteworthy that even though NAEs are produced on demand, NAPE-PLD seems to be kept in a constitutively active form. Although the transacylation–phosphodiesterase pathway is considered the major route for NAE production, generation of NAPE-PLD knockout mice clearly showed the presence of additional metabolic pathways responsible for NAE, especially AEA, synthesis. These mice have wild-type brain levels of AEA and moderately reduced N-palmitoylethanolamine and N-oleoylethanolamine levels, contrasting with the strong decrease in long-chain saturated NAE levels (Leung et al., 2006). Thus, with NAPEs generally accepted as NAE precursors, at least two pathways distinct from NAPE-PLD are now being characterized (figure 7). One pathway has glycerophospho-N-acylethanolamine lipids (GP-NAEs) as key intermediates. The serine hydrolase ABHD4 (α/β-hydrolase 4, Abh4) was put forward as a B-type lipase producing GP-NAEs by double O-deacylation of NAPEs. Accordingly, cells overexpressing ABHD4 have increased levels of both NAPE-lipase and lyso-NAPE-lipase activities, with ABHD4 showing a marked preference for lyso-NAPE substrates over other lysophospholipids (Simon et al., 2006). GP-NAEs' role as intermediates in NAE synthesis was confirmed by their detection in mouse brain and by the characterization of glycerophosphodiesterase 1 (GDE1) as a metal-dependent GP-NAE phosphodiesterase (Simon et al., 2008). Another pathway, to date mainly characterized in macrophagelike RAW264.7 cells, uses phospho-N-arachidonoylethanolamine (pAEA) as a key intermediate in the synthesis of AEA (figure 7). In the presence of lipopolysaccharide (LPS), these cells have increased AEA levels but decreased NAPE-PLD expression. In the same conditions, expression of the phosphatase protein tyrosine phosphatase PTPN22 is increased and pAEA detected. Upon PTPN22 silencing in LPS-activated RAW264.7 cells, AEA levels are decreased, confirming the role of PTPN22 in LPS-induced enhancement of AEA levels. Thus, the suggested pathway involves the production of pAEA from Narachidonoylphosphatidylethanolamine by a PLC, which, in turn, is hydrolyzed into AEA by a phosphatase, with PTPN22 and SHIP1 being the prime candidates for this activity (Liu et al., 2006; Liu et al., 2008).

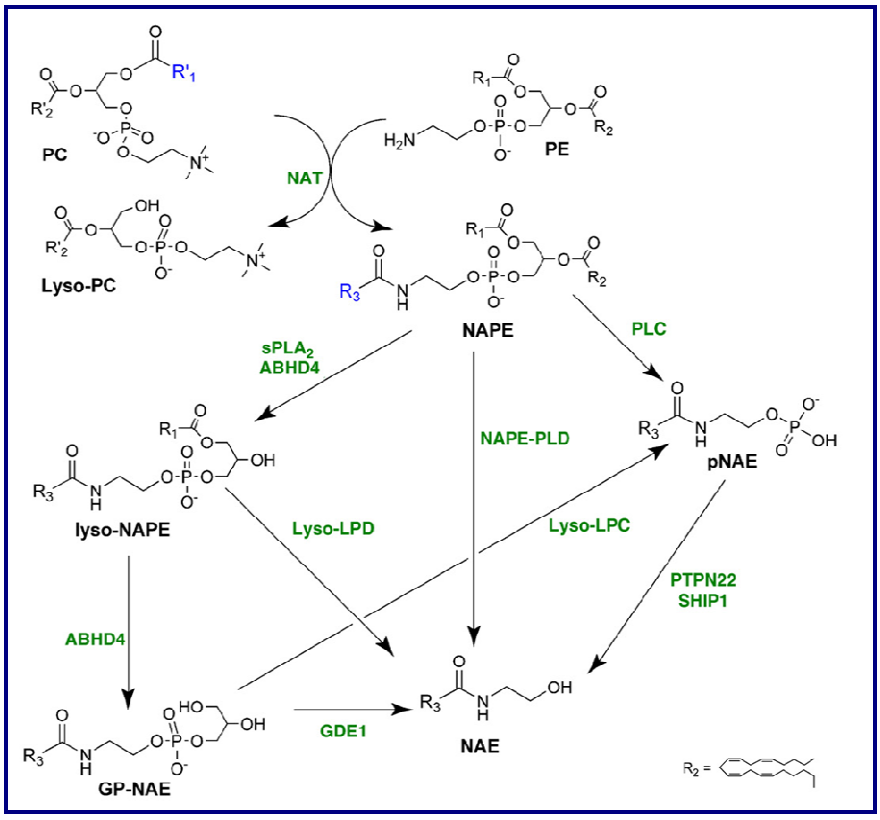

Figure 7. All the described NAE synthetic pathways have NAPEs, produced from membrane phospholipids by NAT, as key intermediate. Note that the pNAE pathway was described for AEA only (modified by Muccioli et al., 2010)

Although differing in the nature of their acyl moiety, NAEs are characterized by a common ethanolamide moiety and, thus, are hydrolyzed by a common group of amidases (figure 8). Fatty acid amide hydrolase (FAAH), cloned in 1996 and now extensively characterized, has received a great deal of attention because its pharmacological or genetic invalidation results in strongly enhanced NAE levels, both in the CNS and in the periphery (Ahn et al., 2008). FAAH (fatty acid amide hydrolase) is a membrane-bound enzyme member of the amidase signature family of enzymes characterized by a Ser-Ser-Lys catalytic triad. FAAH has an alkaline optimal pH and preferentially hydrolyzes AEA over other NAEs, such as N-oleoylethanolamine or N-palmitoylethanolamine. Although this is suggestive of a FAAH primary role in controlling NAE levels, two additional amidases hydrolyzing AEA and related compounds have been described. A second amidase signature enzyme, FAAH-2, was found in human, but not rodent, tissues (Wei et al., 2006). Although it shares the Ser-Ser-Lys catalytic triad with FAAH, the two enzymes have only limited sequence homology (20%). They also differ in their subcellular localization: FAAH-2 is localized in cytosolic lipid droplets and not in the endoplasmic reticulum as is FAAH (Kaczocha et al., 2010). Although FAAH-2 is less efficacious than FAAH a hydrolyzing NAEs (Wei et al., 2006; Kaczocha et al., 2010), its high

expression in peripheral tissues – including liver, where AEA is known to have crucial metabolic roles – suggests that FAAH-2 might have a rescue role in hydrolyzing NAEs upon FAAH inactivation. A third NAE-hydrolyzing enzyme, N-acylethanolamine-hydrolyzing acid amidase (NAAA), is highly expressed in immune cells, specifically in macrophages, and localized into the lysosomes, where it is activated by autoproteolytic cleavage. Accordingly, and in contrast to FAAH, NAAA is most active at acidic pH (Wang et al., 2008 -b). Given that the preferred substrate of NAAA is N-palmitoylethanolamine (the levels of which are increased during inflammation) and that NAAA is highly expressed in macrophages, NAAA is to be considered an interesting target in tackling inflammatory states. The presence of at least three NAE hydrolases, with only partially overlapping tissue expression, raises the question of their respective roles in regulating NAE levels. Whereas it is generally accepted that FAAH is the principal contributor to AEA hydrolysis in the CNS, the role of FAAH-2 and NAAA is still an open question.

Besides the hydrolytic enzymes described, endocannabinoids can be enzymatically transformed by other enzymes (cyclooxygenase 2 is one example) into pharmacologically active chemical entities (e.g. prostamides) (Rouzer et al., 2008) (figure 8). Although they are interesting because they represent pathways to additional bioactive lipids, to date only the hydrolytic enzymes are considered to be responsible for terminating endocannabinoid signaling.

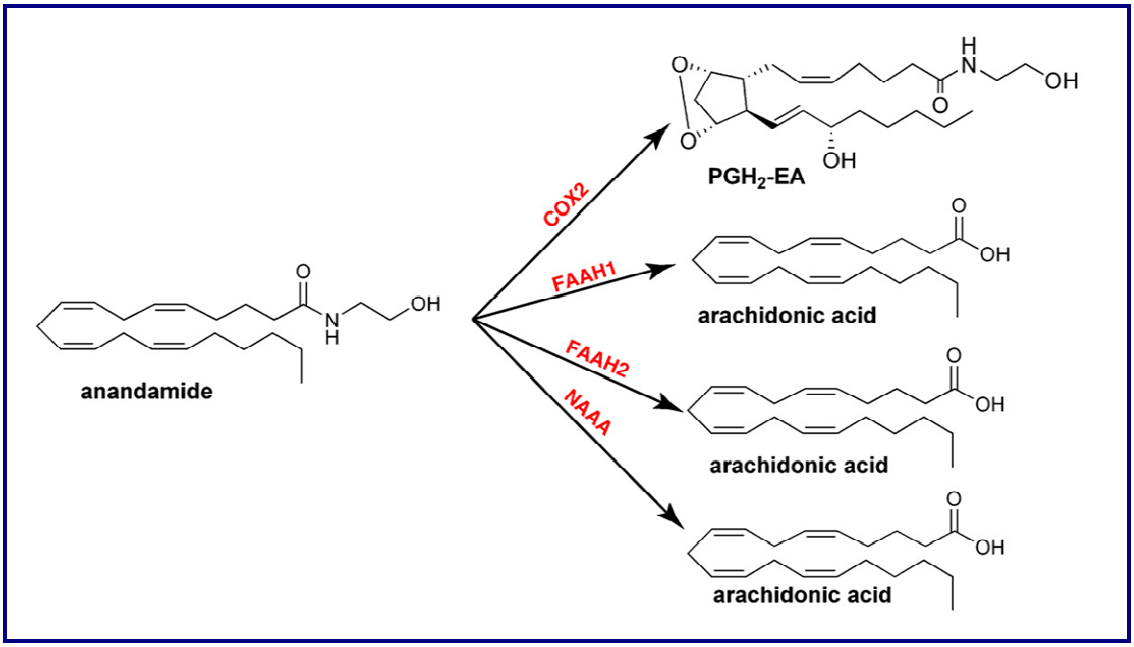

Figure 8. Schematic representation of the endocannabinoid inactivating pathways. AEA signaling is inactivated by FAAH1, FAAH2 or NAAA mediated hydrolysis into arachidonic acid or by COX2 oxidation into PGH2-EA.

Abbreviations: COX2, cyclooxygenase2; FAAH, fatty acid amide hydrolase; NAAA, N-acylethanolamine-hydrolyzing acid amidase; PGH2-EA, PGH2-ethanolamide. (modified by Muccioli et al., 2010).

2-AG biosynthesis

It is well known that 2-AG is produced in a stimulus-dependent way in both the CNS and peripheral cells (Bisogno et a., 1997; Kondo et al., 1998). Stimuli can be cell depolarization, inducing a strong Ca2+ influx; activation of Gq/11-coupled-receptors, such as metabotropic glutamate receptors; or a combination of both mechanisms leading to increased 2-AG production. This acylglycerol can be synthesized in two steps via generation of 1-acyl-2-arachidonoylglycerol (diacylglycerol, or DAG) from phosphatidylinositol by PLC activity and subsequent hydrolysis of DAG by a diacylglycerol lipase (figure 9) (Stella et a., 1997; Kondo et al., 1998).

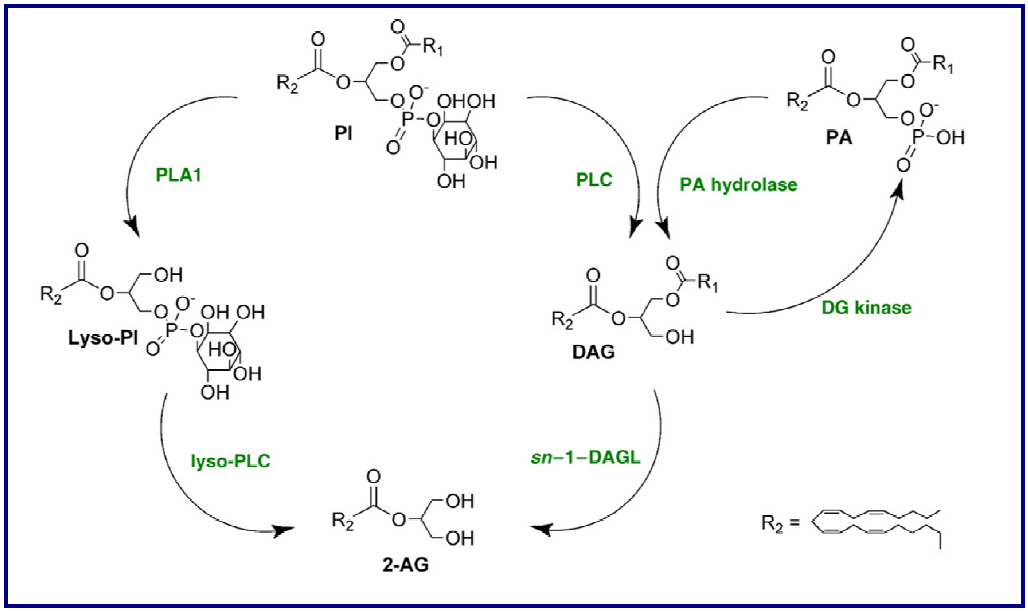

Figure 9. Starting from membrane phospholipids, 2-AG is produced via a lyso-PI intermediate or a DAG intermediate. DAG can also be obtained from PA. (modified by Muccioli et al., 2010).

It is thought that this PLCβ–DAGL pathway is involved in 2-AG retrograde signaling after activation of $G_{q/11}$-type-coupled-receptors. Indeed, the phenomenon is absent in brain preparations from PLCβ knockout mice or after inhibition of these enzymes (Hashimotodani et al., 2005; Edwards et al., 2006). The molecular cloning of sn1-DAGLα and sn1-DAGLβ was a crucial step in further characterizing 2-AG biosynthesis (Bisogno et al., 2003). Cellular activity of these enzymes correlates with 2-AG production and, conversely, their inhibition

results in decreased endocannabinoid levels. The key intermediate DAG can also be produced from phosphatidic acid by a phosphatidic acid hydrolase, which thus represents an alternative pathway to PLC-driven DAG production (Bisogno et al., 1999). A second pathway leading to 2-AG features a 2-arachidonoyl-lysophosphatidylinositol (lyso-PI) intermediate (figure 9). It involves the sequential actions of a phosphatidylinositol-preferring phospholipase A1, producing the lyso-PI intermediate, and of a lysophosphatidylinositol-selective phospholipase C (lyso-PLC) producing 2-AG (Ueda et al., 1993). Compared with the PLC–DAGL pathway, however, the actual relevance of this 2-AG-producing cascade in generating 2-AG as an endocannabinoid is less clear. It is important to keep in mind that most of these molecules, including DAG and 2-AG, are intermediates in several pathways, the most notable of which is arachidonic acid release. It is likely, therefore, that not all the pathways leading to 2-AG are actually involved in physiological cannabinoid signaling. As mentioned above, 2-AG production is stimulated by cell depolarization or $G_{q/11}$-coupled-receptor activation. It seems, however, that differing pathways are responsible for 2-AG synthesis, depending on the stimuli. Indeed, 2-AG-mediated retrograde suppression of synaptic transmission after $G_{q/11}$-coupled-receptor activation is absent in PLCβ knockout mouse neurons, whereas 2-AG-mediated depolarization-induced suppression of inhibition (DSI) or excitation (DSE) is still present in both PLCβ knockout and PLCδ knockout mouse neurons (Hashimotodani et al., 2005; Hashimotodani et al., 2008). However, stimuli-induced 2-AG production in mouse forebrain is affected by $G_{\alpha q}/G_{\alpha 11}$ protein invalidation, whereas basal production is unaffected by the mutation (Wettschureck et al., 2006). Although additional studies are needed to further dissect the pathways activated by a given stimuli, these differences already suggest that, using specific inhibitors, one could finely tune 2-AG signaling.

It is now firmly established that the serine hydrolase monoacylglycerol lipase (MAGL) is the main contributor to brain 2-AG hydrolysis (Dinh et al., 2004; Saario et al., 2005; Blankman et al., 2007). MAGL has long been considered the pivotal enzyme controlling the duration of 2-AG-mediated retrograde signalling (Szabo et al., 2006; Makara et al., 2005). However, this was only confirmed recently using a potent and selective MAGL inhibitor (JZL184) (Pan et al., 2009; Straiker et al., 2009). Similarly, novel evidence demonstrated that 2-AG increased levels upon MAGL inhibition induce CB1-receptor-dependent behavioral effects (Long et al., 2009 a; Long et al., 2009 b). Beyond the CNS, MAGL inhibition resulted in substantially increased monoacylglycerol levels in several peripheral tissues, including liver and adipose tissue (Long et al., 2009 c). Thus, MAGL also regulates the levels of monoacylglycerols, such as palmitoylglycerol and oleoylglycerol, which have not been implicated in endocannabinoid

signaling. Besides regulating 2-AG brain levels, MAGL activity affects brain arachidonic acid levels. Using organophosphorous inhibitors, Nomura and coworkers showed that MAGL inhibition results in decreased fatty acid concentrations. Because these inhibitors do not inhibit cPLA2, their effect on arachidonic acid tissue content was suggested to be mediated by MAGL (Nomura et al., 2008 a; Nomura et al., 2008 b). MAGL involvement was later confirmed using the selective inhibitor JZL184 (Long et al., 2009 a; Long et al., 2009 c). Nevertheless, the physiological relevance of MAGL in controlling arachidonic acid metabolism – and, thus, a wealth of downstream mediators – remains to be established. One characteristic feature of MAGL structure is the presence of a lid made of two loops surrounding the α helix. This helix has a high content of apolar and lipophilic residues pointing outside the enzyme, suggesting that it enables MAGL to anchor itself to cell membranes. This hydrophobic helix, therefore, could explain why MAGL is found in both cytoplasmic and membrane fractions. A residual 2-AG hydrolysis upon MAGL inhibition or immunodepletion, as well as 2-AG hydrolysis in cells not expressing MAGL, suggested the existence of additional enzymes controlling the endocannabinoid levels in vivo (Dinh et al., 2004; Saario et al, 2005; Maione et al., 2006). FAAH could be one of these enzymes because it efficiently hydrolyzes 2-AG in vitro, and URB597 (a relatively selective inhibitor of FAAH) was shown in some studies to increase 2-AG tissue levels (Maione et al., 2006; Jhaveri et al., 2006). Note, however, that in numerous studies, FAAH inhibition (e.g. using URB597 (Patel et al., 2005 -a) or PF-3845 (Ahn et al., 2009)) or genetic invalidation (Patel et al., 2005 -a) had no effect on 2-AG levels. This suggests either a role for FAAH in specific conditions and/or tissues only or a lack of specificity for some of the inhibitors used in these studies. Using a functional proteomic approach, two additional enzymes, ABHD6 and ABHD12, were identified as 2-AG hydrolases (Blankman et al., 2007). These novel 2-AG α/β hydrolases make up for most of the non-MAGL-dependent 2-AG hydrolysis found in mouse brain. Because MAGL, ABHD6 and ABHD12 display different subcellular – and probably cellular – localizations, it is believed that they might control independent pools of 2-AG and, thus, signaling events. Because arachidonic acid is also a bioactive lipid at the crossroads of several signaling pathways, the presence of three 2-AG-hydrolyzing enzymes might also be relevant for endocannabinoid-unrelated processes.

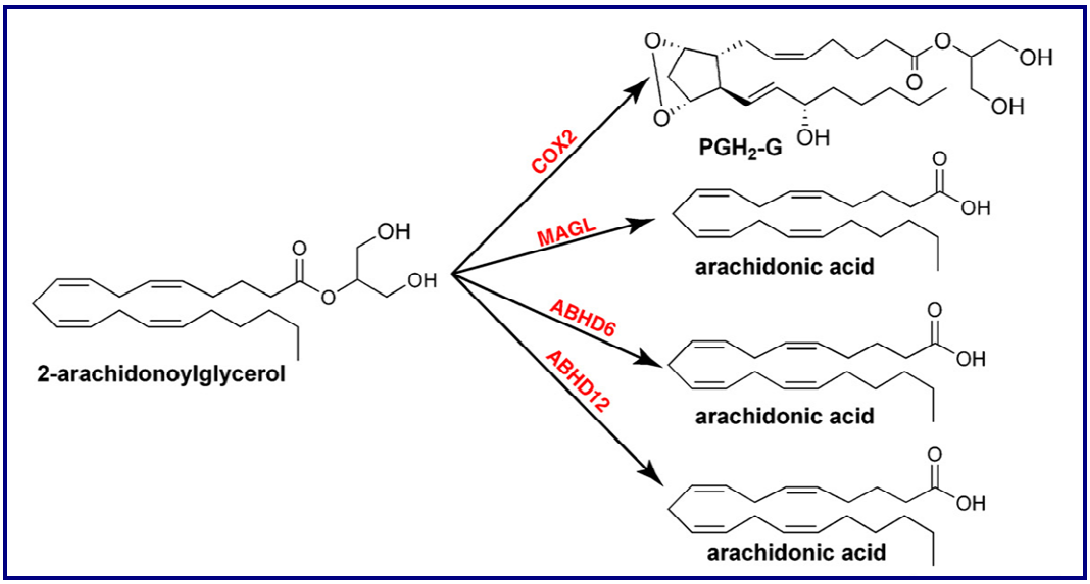

Figure 10. 2-Arachidonoylglycerol signaling is terminated by MAGL, ABHD6 or ABHD12-mediated hydrolysis or by COX2 oxidation into PGH2-G. Note that arachidonic acid, PGH2-EA and PGH2-G can be further transformed into other bioactive lipids, such as prostaglandins and endocannabinoid-derived prostaglandins, respectively. Abbreviations: ABHD6 and 12, α/β-hydrolase 6 and 12; COX2, cyclooxygenase2; MAGL, monoacylglycerol lipase; PGH2-ethanolamide; PGH2-G (modified by Muccioli et al., 2010)

1.5 The endocannabinoid system: functions

Clinical and experimental studies have demonstrated that endogenous cannabinoids and the concurrent activation of their receptors result in a plethora of effects both in central and peripheral tissues. The ECS is implicated in the hormonal regulation of food intake, cardiovascular, gastrointestinal, immune, behavioral, antiproliferative and mammalian reproduction functions.

Endocannabinoid-mediated control of food-intake and of energy metabolism

It has been known for centuries that smoking cannabis is a potent stimulator of appetite, and because of this property it is often prescribed for medicinal purposes to stimulate eating. Studies involving rodents confirmed the role of the EC system and the CB1 receptor in appetite regulation: mice lacking the CB1 receptor reduce their food intake, even after fasting, and lose weight compared to wild type mice, and when wild type mice receive a dose of the CB1 antagonist Rimonabant, food intake is also decreased (Di Marzo et al, 2001). These results imply that the CB1 receptor is responsible for the changes observed in food intake. In fact, antagonism of the CB1 receptor was found to have significant appetite reducing and weight reduction effects. Actually, we know that endocannabinoids through

CB1Rs control homeostatic regulation of energy imbalance by stimulating the central, hypothalamic, orexigenic system and enhance food consumption by mediating motivational processes of the nucleus accumbens (Di Marzo et al., 2004; Howlett et al., 2004; Bellocchio et al., 2006; Pacher et al., 2006; Cota, 2007; Stern et al., 2007; Soria-Gómez et al., 2007; Woelkart, et al., 2008).

The ECS presence has also been verified in both human and rodent white adipocytes and adipose tissue (Pacher et al., 2006; Roche et al., 2006; Despres, 2007; Gonthier et al., 2007; Matias et al., 2007; Valassi et al., 2008). So far, it has been demonstrated that the ECS is involved in free fatty acids synthesis and therefore in cholesterol metabolism, in adipokines biosynthesis as well as in their signalling pathways where applicable, in glucose metabolism, insulin sensitivity and inflammation processes. The ECS takes part in adipogenesis and fat accumulation. On the other hand, endocannabinoids might also negatively affect fatty acid oxidation. (Roche et al., 2006; Gonthier et al., 2007; Matias et al., 2008).

Cardiovascular functions

Both endogenous and synthetic cannabinoids exert noticeable cardiovascular effects. CB1Rs have been detected in the human, rat, and mouse myocardium mediating negative inotropy, which leads to vasodilation and eventually to a hypotensive effect of AEA in anaesthetized rodents (Sarzani, 2008). A vasorelaxant effect of endocannabinoids and their synthetic analogues *in vitro* has been reported and associated with CB1 and TRPV1 receptor- and NO-mediated or NO-independent mechanisms (Randall, 2007; Sarzani, 2008) During shock states, platelets and macrophages exert elevated levels of endocannabinoids and their injection into normal rodents resulted in CB1 mediated hypotension. CB1R blockade prevented or reversed the hypotension associated with shock states; however, mortality was increased in the setting of cardiogenic shock, suggesting endocannabinoid-mediated vasodilation may be protective by improving tissue oxygenation and countering vasoconstriciton mediated by increased sympathetic tone (Grassi et al., 2008). Experimental data suggest that the ECS is mainly inactive under normal hemodynamic conditions and gets activated when the body finds itself in a stressful situation. The ECS is involved in increasing heart rate, vasodilation and bronchodilation (Calignano et al., 2000; Wagner et al., 2001).

Gastrointestinal functions

Cannabis has been widely used as an appetizer stimulant and in order to decrease emesis and diarrhoea. It has been shown thata AEA and some CB1 agonists, but not CB2-selective agonists, inhibit gastrointestinal motility in rodents *in vivo* and in isolated ileum and colon from both experimental animals and humans (Massa, 2006). Endogenous substrates of FAAH also inhibit intestinal motility in wild-type but not in FAAH knockout mice. The mechanism through which CB1Rs mediate enteric contractility and peristalsis reduction remains unclear (Caraceni et a., 2008). The ECS has also been implicated in the regulation of gastric acid and intestinal secretions.

Immune and behavioural functions

Although the available data are limited, there is a number of preclinical and clinical data assigning the ECS with several functions in the immune and behavioural functions. Lutz (Lutz et al., 2002) showed antinociceptivity (reduction in painful stimuli) effects mediated by ECS. Data reported in literature also demonstrated that cannabinoids can inhibit movement control and short term memory. Martin and co-workers (Martin et al., 2002) have shown an involvement of CB1 in emotional behaviour; their findings showing that CB1 knockout mice presented an increase in the aggressive response and, in the light/dark box, an anxiogenic-like response. Furthermore, in CB1 knockout mice an higher sensitivity to exhibit depressive-like responses in the chronic unpredictable mild stress procedure was observed (suggesting an increased susceptibility to develop an anhedonic state in these animals). Finally, CB1 knockout mice showed a significant increase in the conditioned responses that suggests an improvement of learning and memory processes. In CNS of mammals, there is strong evidence emerging that the CB1 and its ligands comprise a neuromodulatory system functionally interacting with other neurotransmitter systems. Furthermore, the presynaptic localization of CB1, together with the results obtained from electrophysiological experiments, strengthen the notion that, in cerebellum and hippocampus and possibly in other regions of the central nervous system, endocannabinoids act as retrograde messengers to suppress neurotransmitter release at the presynaptic site. Data reported in literature show that ECS, through action on hypothalamus-hypophysis-adrenal axis, also regulates anxiety (Navarro et al., 1997) and modulates immune and inflammatory response (De petrocelli et al., 2000). It is also involved in neuro-protection against trauma and hypoxia conditions (Panikashvili et al., 2001).

Since 1970, when the first studies on marijuana smoking effects on immune cells were reported, the effects of cannabinoids on immune function have been extensively studied. It is actually well known that CB1 is expressed in T lymphocytes, and may be involved in cannabinoid-induced T-helper cell biasing. CB1 is up-regulated in T-cells by stimuli such as cannabinoids themselves, and this effect is mediated by IL-4 (Borner et al., 2008).

Strong evidence demonstrates that CB2 is mainly expressed on immune cells (especially those macrophage-derived: microglia, osteoclasts) and neurons (Galiegue et al.,1995; Ofek et al., 2006).

CB2 mRNA has been also found at lower levels in human B cells, natural killer cells, monocytes, polymorphonuclear neutrophils and T cells (Galiegue et al., 1995). The main immune consequences of CB2 activation include changes in cytokine release from immune cells and migration of immune cells inside or outside the CNS (Cabral et al., 2005). In the immune organs, CB2 expression has been verified in thymus and spleen. The presence of CB2 also in dendritic cells, potent antigen-presenting cells, suggests a role for cannabinoids in modulating antigen presentation (Matias et al., 2002). New pathways for studies on ECS and neuroprotection and neuroinflammation have been opened thanks to the discovery of CB2 receptors in the brain.

Microglia (a major cell type responsible for chronic neurodegenerative and neuroinflammatory processes) are able to produce endocannabinoids (2-AG and AEA) and they express both CB1 and CB2 (Carrier et al., 2004; Carrier et al., 2005). In particular, CB2 expression is higher in microglial activation states, like ‘primed’ and ‘responsive’ microglia (Carlisle et al., 2002; Cabral et al., 2008). During these states, cannabinoids exert a stronger influence on activated microglia functions. Thus, it has been suggested that a CB2-dependent ‘‘time-window’’ for functional modulation of microglial actions exists, and that synthetic and endogenous cannabinoid analogues have different modulator effects at this level (Walter et al., 2003; Cabral et al., 2008).

Generally, in the immune cells, CB stimulation is implicated in the regulation of DNA binding of different nuclear factors (Massi et al., 2006). These effects are mainly achieved via down-regulation of cAMP formation and signal transduction involving adenylate cyclase (AC) (Koh et al., 1995). Rapid and transient bursts in AC activity are in fact associated with previous lymphocyte activation by mitogens; also cytokine transcription in macrophages is regulated via cAMP signaling cascade (Kaminski et al., 1994). cAMP analogues variably inhibit or stimulate immune responses in a concentration-dependent manner, and can antagonize the effect of cannabinoids on T lymphocyte-dependent production of antibodies

(Koh et al., 1995). CB stimulation seems to antagonize the regulatory role of cAMP pathway in the early events in immune cell activation, but these effects are probably more complex since natural cannabinoids (unlike synthetic cannabinoids) act as inverse AC agonists or antagonists in some circumstances (Bayewitch et al., 1996; Massi et al., 2006).

Cannabinoids exert different effects on cellular immunity:

- T cells: cannabinoids can affect T cells number and proliferation, but may also have important effects on T helper 1- and 2-specific cytokines and TGF-β secretion (Croxford and Yamamura, 2005).
- Natural killer cells (NK): various animal studies showed that both proliferation and cellular cytolytic activity can be influenced by cannabinoid treatment, and that these effects can be mediated by CB1 and CB2 (Massi et al., 2006). However, in humans, NK cell functions do not seem to be significantly affected by cannabinoids.
- Macrophages: macrophages exert important roles in both innate and adaptive immunity and express both cannabinoid receptors, but predominantly CB2 (Sinha et al.,1998). Cannabinoid ligands can interfere predominantly by inhibition with macrophage migration (CB2 mediated) (Raborn et al., 2008), antigen presentation to T cells and phagocytic capacity (Sacerdote et al., 2005). They can also influence the release of inflammatory mediators (Berdyshev et al.,2001; Cabral et al.,1995). On the other hand, macrophages can synthesize AEA and 2AG.
- Neutrophils: CB can be expressed by neutrophils (Galiegue et al.,1995); endocannabinoids, phytocannabinoids and related ligands are considered potent inhibitors of human neutrophilic migration (Deusch et al., 2003).
- Mast cells (MC): despite controversy on CB expression and cannabinoid effects on MC (Croxford and Yamamura, 2005), it is accepted that CB2 can be expressed by MC (De Filippis et al., 2008). Cannabinoid ligands may act in order to control mast cell activation and degranulation early during the inflammatory response (De Filippis et al., 2008).

Finally, it must be noted that cannabinoid compounds may affect B cells number, proliferation, migration, Ig production or isotype switching (Croxford and Yamamura, 2005).

Bone metabolism

The correlation of the ECS with the regulation of skeletal and bone remodelling has been derived upon several preclinical observations (Zhao, 2008; Bab, 2008; Tam, 2008). For instance, in the case of bone formation and bone mass, the central production of 2-AG, is subject to negative regulation by leptin, while traumatic head injury stimulates both bone formation and central 2-AG production. On the other hand, bone metabolism is subject to

biochemical pathways involving signaling by the hypothalamic receptors of leptin and neuropeptide Y, which are regulated by the ECS. Several preclinical data confirm the expression of CB receptors in bones. The presence of endocannabinoids has also been substantiated in the skeleton (Bab et al., 2009). AEA and 2-AG levels in bones are equally high to their hypothalamic levels, while there is evidence of their synthesis by osteoblastic cells in culture (Bab et al., 2008).

Antiproliferative functions

THC was recognized as a potential anti-cancer almost 30 years ago, while cannabinoids have been known to afford palliative effects in cancer patients including appetite stimulation, chemotherapy-related nausea inhibition (Nabilone, a synthetic derivative of THC, has been launched under this indication), pain and insomnia relief as well as mood elevation (Guzman, 2003; Hall et al., 2005; Pertweee, 2005; Pacher et al., 2006). Plentiful studies have associated cannabinoids with cancer growth inhibition in many cancer types but in most cases in a cell type specific mechanism.

Cannabinoids are known to interact with tumor cells cycles resulting in growth arrest, apoptosis inhibition, angiogenic activity and reduced tumor cell migration. The mechanisms for these activities are not yet clear since cannabinoid receptors have been found to interfere with various intracellular signaling pathways (Flygare, 2008). It must be noted that endocannabinoids levels vary according to cell type and malignancy. For example, AEA concentration in normal colon tissue triples upon malignant transformation. Using a xenograft model of thyroid cancer, endocannabinoid degradation inhibitors have been found to increase AEA and 2-AG levels in tissue and reduced tumor growth (Sarfaraz, 2008). The interaction with CB1/CB2-and VR1 receptors is another mechanism through which AEA, 2-AG, and some endocannabinoid transport inhibitors induce apoptotic cell death and inhibit cell proliferation in glioma, oligodendroglioma, astrocytoma, neuroblastoma, pheochromocytoma, colon carcinoma, uterine cervix carcinoma, leukemia, and lymphoid tumors (Pushkarev, 2008).

Reproductive functions

There are several data indicating that the ECS is involved in reproductive functions in both male and female animals and humans. Blastocysts, spermatozoa, uterus and testis synthesize endocannabinoids and mainly AEA, which is present throughout the whole pregnacy period.

Sexual behavior and gonadal hormone function are strongly influenced by ECS (Gorzalka et al, 2010).

1.6 The endocannabinoid system and cellular differentiation

As previously decribed, since its discovery, the endocannabinoid system (ECS) has been shown to be implicated in several fundamental physiological functions as well as in many pathological conditions. The ECS is modulated during cell proliferation, differentiation and apoptosis through alterations of the expression levels of cannabinoids receptors and of the enzymes involved in the biosynthesis and degradation of the two main endocannabinoids (AEA and 2-AG). It is known that endocannabinoids regulate differentiation in many cellular models. For example, cannabinoids promote astroglial and glioma stem-like cell differentiation (Aguado et al., 2006); they regulate bone mass (Bab et al., 2008) and they are involved in neural progenitor proliferation (Aguado et al., 2006; Aguado et al., 2007). In addition AEA, through DNA metilation, inhibits human keratinocytic differentiation (Paradisi et al, 2008; Maccarrone et al., 2003) and CB2 seems to play an important role in neutrophilic differentiation block (Jordà et al., 2002).

It is also known that endocannabinoids have a function in the regulation of proliferation and differentiation of haematopoietic cell lines. Valk et al. reported that AEA, *in vitro*, acted via cannabinoid CB2 receptors to synergize with colony-stimulating factors, interleukin-3 and erythropoietin to stimulate haemotopoiesis (Valk et al., 1997). Moreover, it has been recently reported that murine embryonic stem cells (mES) express both CB1 and CB2 receptors and both receptors are functional. Addition of exogenous selective cannabinoid agonists augmented the embryoid body formation derived from mES cells, indicating that cannabinoid ligands induced the haematopoietic differentiation of mES cells through cannabinoid receptors in both mES cells and EB-derived mES cells (Jiang et al., 2007). Thus, these results strongly suggest that cannabinoid receptors are involved in the maintenance of mES cells and that the endocannabinoid system is essential in stem cell survival and stem cell haematopoietic differentiation. Despite this data, however, little is so far known concerning haematopoietic cell differentiation and endocannabinoids.

1.8 Megakaryocytopoiesis

Megakaryopoiesis is the process by which mature megakaryocyte (MKs) are derived from pluripotent stem cells (HSCs). The major function of the MK is to produce platelets (thrombopoiesis), which are critical for the haemostasis in the peripheral blood vasculature. During its lifespan, a mature MK can produce up to 10^4 platelets (Long, 1998). Each day the human adult produces 1 x 10^{11} platelets and this number can increase tenfold on demand (Branehog et al., 1975).

Megakaryocytopoiesis involves the commitment of haematopoietic stem cells, and the proliferation, maturation and terminal differentiation of the megakaryocytic progenitors.

Megakaryocyte development

Megakaryocytes (MKs) give rise to circulating platelets (thrombocytes) through commitment of the multipotent stem cell to the MK lineage, proliferation of the progenitors and terminal differentiation of MKs. This process is characterised by DNA endoreduplication, cytoplasmic maturation and expansion, and release of cytoplasmic fragments as circulating platelets. Within the bone marrow (BM), MKs are derived from haematopoietic stem cells (HSCs), which evolve from the multipotential haemangioblast. The haemangioblast gives rise to all blood and blood vessel precursor cells. The HSC gives rise to the early common myeloid progenitor which can be cloned as the multi-lineage (granulocyte, erythrocyte, MK and monocyte) colony-forming unit (CFU-GEMM). Erythroid and MK lineages arise from a common MK-erythroid progenitor (MEP) derived from the early common mieloid progenitor (Fig 11). Common mieloid progenitor differentiation is orchestrated by molecular signals controlled by regulatory transcription factors. Two major transcription factor involved in common mieloid progenitor differentiation are GATA-1, which drives differentiation of MEP and PU.1, which regulates granulocyte-monocyte precursors (Nutt et al., 2005). In response to environmental factors, cytokines and chemokines, the bipotential MEP can develop into the highly proliferative, early MK burst-forming unit (BFU-MK), or the more mature smaller CFU-MK, which both express the CD34 antigen (Briddell et al., 1989). Alternatively, MEP can progress to early and late erythroid progenitors, the BFU-E and CFU-E (Schulze et al.,

2004 -a). The proliferating diploid MK progenitors (megakaryoblasts) lose their capacity to divide, but retain their ability for DNA replication (endoreduplication) and cytoplasmic maturation.

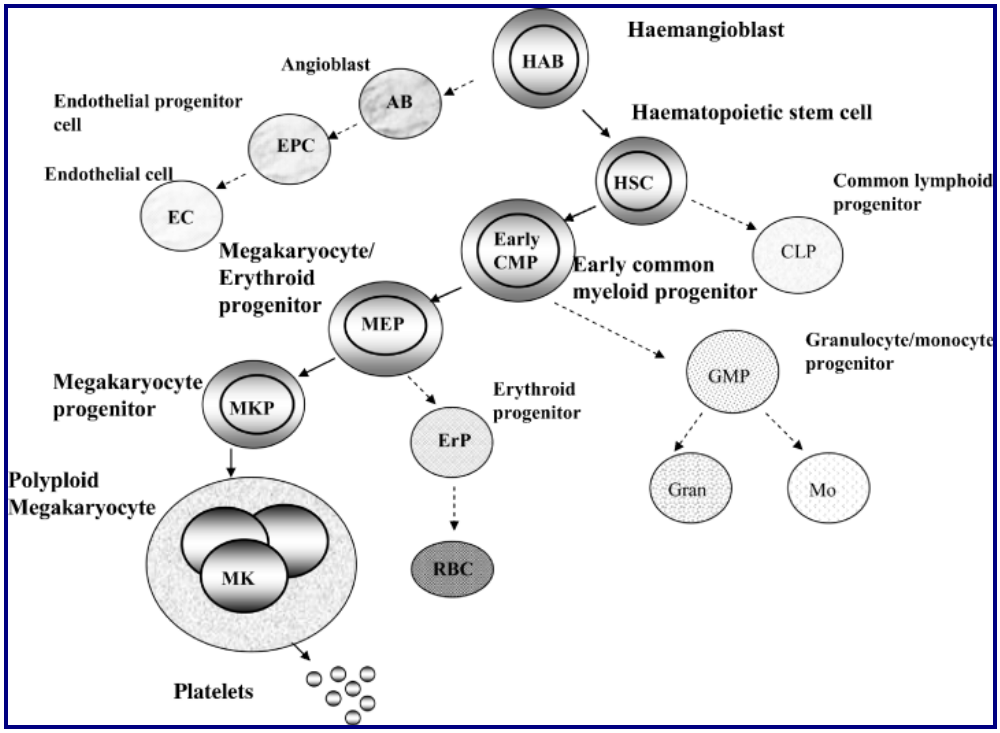

Figure 11. The megakaryocytopoietic developmental pathway. The figure illustrates the development of megakaryocytes from the haemangioblast (HAB), which gives rise to both vascular and haematopoietic stem cells (HSC). In response to physiological demand, the HSC can produce early progenitor cells of all the haematopoietic lineages, including the common myeloid progenitor (CMP) and the common lymphoid progenitor (CLP). The megakaryocyte progenitor (MKP) is derived from the common megakaryocyte-erythroid progenitor (MEP). The MEP maintains many characteristics of the HSC, which includes the expression of CD34, c-Mpl thrombopoietin receptor, erythropoietin receptor; CD41, glycoprotein IIb/IIIa or αIIβ3-integrin receptor, and is regulated by GATA-1. Pathways leading to platelet production are indicated by solid arrows, other pathways are indicated by dashed arrows. RBC, red blood cell; Gran, granulocyte; Mo, monocyte. (modified by Deutsch et al., 2006)

Megakaryocyte maturation

The hallmarks of MK maturation are endoreduplication (polyploidisation) and expansion of cytoplasmic mass. Mature MKs give rise to circulating platelets by the acquisition of the cytoplasmic structural and functional characteristics necessary for platelet action (Patel et al.,

2005 -b; Richardson et al., 2005), reaching cell sizes <50–100 microns in diameter, with ploidy ranges up to 128 N (Tomer et al., 1987, Tomer et al.,1988). Early megakaryoblasts have the highest nuclear/cytoplasmic ratio. These immature cells contain elevated RNA levels, prominent ribosomes and rough endoplasmic reticulum, express platelet peroxidase, contain α-granules and dense bodies, and primitive demarcation membrane. As the MK matures, the polyploid nucleus becomes horseshoe-shaped, the cytoplasm expands, and platelet organelles and the demarcation membrane system are amplified (Breton-Gorius et al., 1976). The robust cytoplasmic mass forms proplatelet projections which give rise to de novo circulating platelets (Italiano et al., 1999; Italiano et al. 2003, Shivdasani et al., 2003).

While the large polyploid MKs are easily identified, the small MK progenitor and immature MK are difficult to discern. Their identification is facilitated by using antibodies to major platelet membrane glycoproteins (GPs) including the integrin αIIbβ3 (CD41a or GPIIb/IIIa complex), CD41b (GPIIb),

CD61 (GPIIIa), CD42a (GPIX), CD42b (GPIb) and CD51 (αV), as well as against the platelet α-granule proteins, von Willebrand factor (VWF), platelet factor 4 (PF4), β-thromboglobulin (β-TG), fibrinogen, coagulation factor VIII, and factor V (Tomer, 2004).

The nuclear endomitotic cell cycle consists of a DNA replication S-phase, an M-phase with multiple pole spindles, aborted anaphase B, aborted cytokinesis and a Gap-phase that enables re-entry into the next S-phase. Cyclin D3 is overexpressed in the G1-phase of maturing cells and is a key inducer of MK polyploidisation (Ravid et al., 2002). Cyclin E may also be important as cyclin E null mice have defective endomitosis of MK and trophoblasts (Geng et al., 2003). Aurora-B/AIM-1, the fundamental regulator of mitosis, has normal localisation and expression during prophase and early anaphase but is absent or mislocalised at late anaphase MK (Geddis et al., 2004; Zhang et al., 2004). Chromosomes segregation is asymmetrical with normal metaphase/anaphase checkpoints (Roy et al., 2001). Platelet release occurs when the MK cytoplasm is transformed into proplatelets, followed by the release of 2000–5000 new platelets/cell.

1.9 Regulation of megakaryocytopoiesis

The processes of megakaryocytopoiesis and platelet production occur within a complex BM microenvironment where chemokines, cytokines as well as adhesive interactions play a major role (Avecilla et al, 2004). Mechanisms regulating megakaryocytopoiesis operate at the levels of proliferation, differentiation and platelet release (Gewirtz et al., 1995; Kaushansky, 2003).

Thrombopoietin

Thrombopoietin, also known as c-Mpl ligand, is the primary physiological growth factor for the MK lineage, which also plays a central role in the survival and proliferation of HSC (Kaushansky, 2006). TPO stimulates MKs to increase in cell size and ploidy, and to form proplatelet processes that then fragment into single platelets (Kaushansky, 2005). Because MK volume and ploidy attain predictable maximum values simultaneously, MK ploidy is an accurate measure of the Mpl-ligand stimulation of megakaryocytopoiesis (Tomer et al., 1996; Harker et al., 1997). TPO can also act in synergy with other haematopoietic cytokines and has been utilised effectively to expand human HSC and MK-progenitor cells in vitro (Pick et al., 2002; Bruno et al., 2003; Ivanovic et al., 2006). In platelets, TPO enhances α-granule secretion and aggregation that is induced by thrombin in a phosphoinositide-3 kinase (PI3K)-dependent fashion (Kojima et al., 2001). This powerful cytokine also affects mature platelets, reducing the level of ADP, collagen, or thrombin necessary for aggregation (Oda et al., 1996; Pasquet et al, 2000), and stimulates platelet adhesion (Van Os et al, 2003).

TPO shares high homology with erythropoietin (EPO) in its N-terminal half, reflecting a close evolutionary relationship between their receptor signalling pathways. TPO binds to its receptor on MKs and selectively initiates proliferation, maturation and cytoplasmic delivery of platelets into the circulation (Kaushansky, 1997, Kaushansky, 2003). TPO is produced constitutively by the liver and its circulating levels are regulated by the extent of binding to c-Mpl receptors on circulating platelets and marrow MKs, resulting in the elimination of TPO-c-Mpl complexes (Kaushansky, 1997; Scheding et al., 2002). Blood and marrow levels of

TPO are usually inversely related to BM MK mass and platelet counts (Kaushansky, 2005). The plasma level of TPO in normal state is 95 ± 6 pg/L.

The TPO receptor (c-Mpl) is predominantly expressed in haematopoietic tissues, haemangioblasts, MKs at all stages of differentiation and platelets. TPO avidly binds and activates the c-Mpl receptors on MK and platelets, which undergo conformational changes to initiate signal transduction. The Janus kinase (JAK) family kinases constitutively bind to the membrane-proximal cytoplasmic domains of c-Mpl and initiate signalling. The active JAK kinase also phosphorylates tyrosine residues within the receptor itself, as well as downstream signal transducers and activators of transcription (STATs), PI3K, and the mitogen-activated protein kinases (MAPKs). This cascade drives cell survival and proliferation, requires PI3K activation and activates SHP1 and SHIP1 phosphatases and suppressors of cytokine signalling (SOCSs) to limit cell signalling (Solberg et al., 2005).

Additional growth factors

While TPO is the main physiological regulator of megakaryocytopoiesis, it is not exclusive in this activity: other pleiotropic haematopoietic growth factors that stimulate MK growth alone or in combination with TPO include granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, IL-6, IL-11, stem cell factor, FLT ligand, fibroblast growth factor (FGF) and EPO (Kaushansky et al., 2002; Tomer et al., 1987, Tomer et al., 1988; Broudy et al., 1995; Deutsch et al., 1995; Kaushansky et al., 1995; Bruno et al., 2003).

A novel MK growth stimulating peptide has recently been described, which drives robust proliferation of CD34+ haematopoietic progenitor cells and MK in vitro and MK progenitors in transgenic mice via protein kinase C (PKC) signalling pathways. This unique peptide is derived from the cleavable C-terminus of the stress associated form of acetylcholinesterase (AChE), a molecule known to be involved in the regulation of megakaryocytopoiesis (Long et al., 1982; Lev-Lehman et al, 1997). This readthrough variant (AChE-R) is physiologically functional during stress thrombopoiesis and recurs in preclinical development as a new thrombopoietic factor (Grisaru et al., 2001; Deutsch et al., 2002; Pick et al, 2005).

Negative regulators of megakaryocytopoiesis

Several factors are known to inhibit MK development, including transforming growth factor-β1 (Kuter et al., 1992), PF4 and IL-4 (Han et al., 1991; Zauli et al., 1995). Src kinase inhibitors have recently been shown to negatively regulate MK proliferation by inducing MK differentiation and functional platelet-like fragments formation in vitro (Gandhi et al., 2005; Lannutti et al., 2005). This important discovery may enable new therapeutic modalities for treating thrombocytosis.

Cellular interactions and chemokines

Megakaryocytopoiesis and thrombocytopoiesis require cytokines, chemokines and cellular interactions between HSC and marrow stromal cells (Avecilla et al., 2004). Stromal-derived factor-1 (SDF-1), of the CXC family, is the key chemokine involved in the retention of haematopoietic precursor cells in the BM. SDF-1 supports megakaryocytopoiesis and homing of HSCs to the BM during foetal development (Wang et al., 1998).

The SDF-1 receptor, CXCR4, is expressed along the entire MK differentiation pathway from early progenitors to platelets (Wang et al., 1998). Platelet production is enhanced during transendothelial migration of CXCR4+ MK in response to SDF-1 (Lane et al., 2000).

Nuclear transcription factors

Megakaryocyte development and thrombocytopoiesis are controlled by the concerted action of transcription factors, which form complexes that co-ordinately regulate the chromatin organisation to specifically activate the genes of MK lineage precursors and/or concurrently repress gene expression that supports other cell types. Many MK-specific genes are co-regulated by GATA and friend of GATA (FOG) together with acute myeloid leukaemia/runt-related transcription factor 1 (AML/RUNX1) and ETS proteins.

The zinc-finger protein GATA-1 is the principle transcription factor directing MK development by forming complexes with other transcription factors (FOG, ETS and RUNX - 1) (Tsang et al., 1997). One of the initial events during MK/E lineage restriction is downregulation of PU.1, the main transcription factor responsible for myeloid cell

differentiation (Nutt et al., 2005) and upregulation of GATA-1. Reciprocal antagonism of PU.1 and GATA factors balances lineage commitment decisions (Rekhtman et al., 2003; Schulze et al, 2004). While the loss of GATA-1 leads to differentiation arrest and apoptosis of erythroid progenitors and accumulation of immature MKs (Gurbuxani et al., 2004), forced GATA-1 expression reprogrammes the common lymphoid and myeloid progenitors to the Meg/E lineage (Iwasaki et al., 2003). GATA-1 possesses a robust MK-specific genetic programme, regulating all stages of MK development (Kaushansky, 2005).

Nuclear factor erythroid 2 (NF-E2) is a heterodimeric leucine zipper transcription factor that comprises an MK-erythroid specific 45-kDa subunit and a non-lineage specific p18 Maf family subunit which controls terminal MK maturation, proplatelet formation and platelet release (Lecine et al, 1998; Schulze et al., 2004 -b), by regulating a panoply of MK genes which are crucial elements in the process of platelet production.

The proto-oncogene c-myb, which functions together with p300 as an early mandatory haematopoietic transcription factor, is also a powerful negative regulator of thrombopoiesis in mice (Metcalf et al., 2005). Impairment of its association with the transcriptional coactivator p300 increased the numbers of HSCs, MKs and platelets (Sandberg et al., 2005). Mpl−/− mice harbouring mutations in the DNA-binding domain (called plt3) or within the leucine zipper domain (called plt4) of the c-Myb gene, exhibit TPO independent excessive thrombopoiesis (Carpinelli et al., 2004)

1.10 Platelet production and release

During MK maturation, internal membrane systems, granules and organelles are assembled in bulk. Platelet production by cytoplasmic fragmentation requires highly structured intricate changes in the MK cytoskeleton and concomitant assembly of anucleate platelets. High ploidy MKs form an extensive internal demarcation membrane, which is continuous with the plasma membrane and serves primarily as a membrane reservoir for the formation of the precursors of cytoplasmic extensions called proplatelets. The open canalicular system, a channelled system for granule release and a dense tubular network, is formed prior to the initiation of proplatelet assembly (Patel et al., 2005). Some platelet proteins, such as VWF and fibrinogen receptors, are synthesised and directed to the MK surface membrane while others proteins are conveyed into secretory granules. Individual organelles migrate from the cell body to the proplatelet ends, with approximately 30% of organelles/granules in motion at any given time

(Richardson et al., 2005). Fibrinogen is taken up by MK from the plasma through endocytosis and/or pinocytosis and selectively transferred to platelet-specific granules.

There are two models of thrombopoiesis. One model proposes that, within the MK cytoplasm, there are preformed territories with internal membranes demarcating prepackaged platelets that are released upon fragmentation of the cytoplasm. This theory is based on electron microscopy analysis of the internal membranes of MKs (Zucker-Franklin et al., 1984; Mori et al., 1993).

The other model of platelet biogenesis proposes that platelet assembly and budding off the tips of proplatelets, which operate like assembly lines for platelet production at the end of each proplatelet (Patel et al., 2005 -b). Patel describes the assembly of platelets from megakaryocytes as “an elaborate dance that converts the cytoplasm into 100- to 500-μm-long branched proplatelets, on which the individual platelets develop”. The proplatelet and platelet formation process generally commences from a single site on the megakaryocyte where 1 or more broad pseudopodia form. Over a period of 4–10 hours, the pseudopodial processes continue to elongate and become tapered into proplatelets with an average diameter of 2–4 μm. Proplatelets are randomly decorated with multiple bulges or swellings, each similar in size to a platelet, which gives them the appearance of beads connected by thin cytoplasmic strings (Figure 12). The generation of additional proplatelets continues at or near the original site of proplatelet formation and spreads in a wavelike fashion throughout the remainder of the cell until the megakaryocyte cytoplasm is entirely transformed into an extensive and complex network of interconnected proplatelets (Italiano et al., 1999; Andrews et al., 1993). The multilobed nucleus of the megakaryocyte cell body is compressed into a central mass with little cytoplasm and is eventually extruded and degraded. Platelet-sized swellings also develop at the proplatelet ends and are the primary sites of platelet assembly and release, as opposed to the swellings along the length of the proplatelet shaft (Figure 12). The precise events involved in platelet release from proplatelet ends have not been identified.

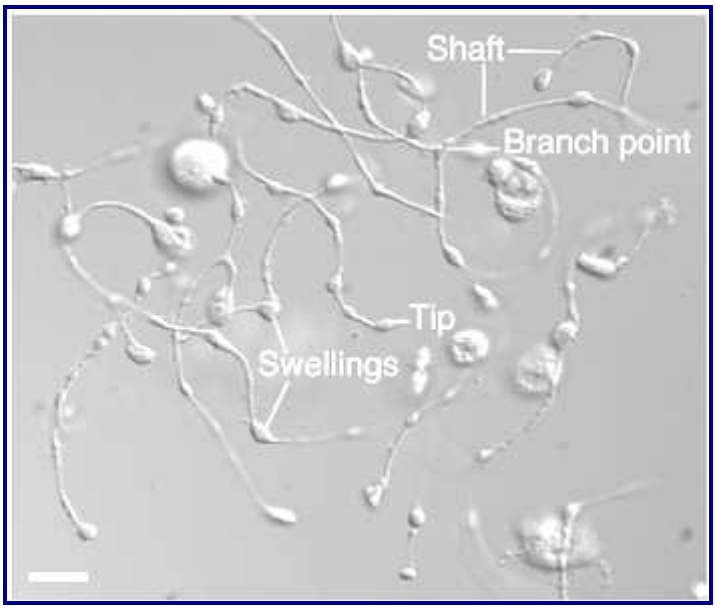

Figure 12. Anatomy of a proplatelet. Differential interference contrast image of proplatelets on a mouse megakaryocyte in vitro. Some of the hallmark features of proplatelets, including the tip, swellings, shafts, and a branch point, are indicated. Scale bar, 5 mm. (modified by Patel et al., 2005)

2. AIM OF THE STUDY

Haematopoietic stem cells express cannabinoid receptors CB1 and CB2, and these receptors have been involved in the mechanisms that regulate proliferation and/or differentiation in myeliod and lymphoid cells (Valk et al., 1997; Jiang et al., 2007; Randall, 2007). Moreover, it has been reported that platelets, primary cells derived from the myeloid precursors, express the cannabinoid receptors CB1, CB2 (Catani et al., 2010) and TRPV1 (Authi, 2007).
However, very little is known about the receptoral pattern of haematopoietic cells of the megakaryocyte/erythroid lineage, precursors of platelets and red blood cells; nothing is actually known about the role of endocannabinoids in megakaryo- and thrombopoiesis. This background prompted me to investigate the presence of a complete and functional AEA-related endocannabinoid system in our cellular model of megakaryocytopoiesis and its impact on this process.

In this project mononuclear cells from murine bone marrow have been employed to study:

(1) the expression pattern of the main endocannabinoid receptors (CB1, CB2 and TRPV1) in haematopoietic stem cells.

(2) how the pattern of expression is modified during megakaryocyte differentiation.

(3) the role of endocannabinoid action in megakaryocytic differentiation.

3. MATERIALS AND METHODS

3.1 Bone marrow cell isolation

Bone marrow -cells were obtained according to the protocol of Shiraga and collegues (Shiraga et al., 1999). Briefly: cells were harvested from tibiae and femora of 8–12-wk-old BALB/c female mice by flushing the marrow with sterile catch buffer (PBS pH 7.4 containing 0.38 % trisodium citrate, 2% BSA and 0,1 μg/mL DNase), separated by lyering the cell suspension onto Ficoll Paque Premium (1.084 g/mL) and centrifugated for 30-min, 400 g, at room temperature. Mononuclear cells at the interface were collected and re-spinned for 10 min. Cells were then counted and cultured in a differentiation medium (IMDM opportunely supplemented with: 100 mg/mL penicillin/streptomycin, 200 mg/mL transferrin, 0.5% BSA, 50 mM 2-ME, 4 mg/mL LDL, 10 mg/mL insulin, 10 mg/L nucleotides, 10 ng/mL IL-6, 10 ng/mL IL-11, and 10 ng/mL TPO) at a starting density of $1x10^6$ cells/mL.
Cells at different stages of differentiation have been collected as follows: cells at day 0 of differentiation were let adhere to plastic for 2 h and non-adherent cells were subsequently centrifuged -for 10 min at 400 g. Cells cultured in differentation medium for 3 days were spinned for 10 min at 300 g. Cells cultured for 5 days were instead separated over a discontinuous (3%-2%-1%) BSA gradient.

3.2 Electrophoresis and Immunoblotting analysis

Pellets obtained as previously described were lysated with RIPA 1X (75 mM NaCl, 50 mM TRIS, 1% Nonidet P40, 0,1% SDS and 0,5% Sodium Deoxycholate) supplemented with protease inhibitor mixture. Samples were then dissociated with 0,25 volumes of SDS-SAMPLE buffer (50% glycerol; 10% SDS; 312 mM TRIS-HCl; 25% beta Mercaptoethanol; 0.3% Bromophenol-blue) and subsequently heated at 95° C for 5 minutes. Proteins were separated by SDS-PAGE on 10% polyacrylamide gel, transferred to PVDF membranes and probed with specific primary antibodies. Immunoreactive bands were detected by an ECL reaction and visualized by means of VersaDoc Imaging System - Quantity One software (BIORAD).

3.3 RNA extraction

Cells at different stages of differentiation have been collected as previously described. Total RNA was extracted with 1 mL of Tri-Reagent (Ambion), incubated for 5 min at room temperature, and mixed with 0.2 mL of chloroform. Organic and aqueous phases were partitioned by centrifugation (12.000 g, 15 min, 4° C); the aqueous (upper) layer obtained was then transferred to a 1.5-mL tube. RNA from this phase was precipitated with isopropanol (0.5 mL), washed with 75% ethanol (1 mL), dried, and finally resuspended in sterile distilled water. Effective genomic DNA elimination and subsequent reverse transcription (750 ng RNA/sample) have been performed using QuantiTect Reverse Transcription Kit (Quiagen), according to the manufacturer's instructions.

3.4 Reverse transcriptase - Polymerase chain reaction

cDNA obtained was amplified through GoTaq Green Master Mix 2X (Promega), containing GoTaq DNA Polymerase, Reaction Buffer pH 8.5, 400 μM dATP, 400 μM dGTP, 400 μM dCTP, 400 μM dTTP and 3 mM MgCl2. Amplification of GAPDH cDNA was performed as control.

The sequences of the primers used are:

CB1 :
primer forward 5'- GTG CCG AGG GAG CTT CTG - 3'
primer reverse 5'- TTG GAT GCC ATG TCT CCT TT- 3'
amplicon: 204 bp

CB2:
primer forward 5'-CTG CCT GAT AGG CTG GAA GA- 3'
primer reverse 5'- TCA GAA AGA ATC CGG GTC TG- 3'
amplicon: 244 bp

GAPDH (controllo positivo):
primer forward 5'- CTG GCC AAG GTC ATC CAT GA - 3'
primer reverse 5'- AGG GGC CAT CCA CAG TCT T - 3'
amplicon: 78 bp

CB1 and CB2 receptor PCR amplification was peformed in PTC-100 Peltier Thermal Cycler (Celbio) under the following conditions: 94° C for 2 min; thirty-five rounds at 94° C for 30 sec, 55° C for 30 sec, and 72° C for 1 min. Finally, after a final extension step at 72° C for 5 min, PCR products (20 μL of each sample) were separated on 1,5% agarose gels.

3.5 REAL-TIME Polymerase chain reaction

TaqMan chemistry was used to perform Real-time RT-PCR. The assays used for the detection of CB1, CB2 and VR1 expression were Mm00432621_s1, Mm00438286_m1 and Mm01246301_m1 respectively (Applied Biosystem). The reactions were carried in a 7000 System Software (Applied Biosystem). Levels of amplified cDNA were normalized to the level of GAPDH housekeeping control detected by mouse GAPDH (20X) probe dye FAM-MGB 4352932-0808024 assay (Applied Biosystem). Data were analyzed normalizing GAPDH expression to cells at day 0 of differentiation.

3.6 Megakaryocyte differentiation evaluation

Haematopoietic stem cells were plated in 6-wells dishes at the starting density of $1x10^6$ cells/mL in differentiation medium. Cells were properly treated with 10 μM R-1 Methanandamide (a stable chiral analogue of AEA) alone or in combination with 1 μM Am251 (CB1 antagonist). An equivalent amount of ethanol (vehicle) was added to control cells. In order to investigate the role of endocannabinoids in megakaryocyte differentiation, cells at day 0, 3 and 5 of differentiation were collected as previously described and expression of the surface protein CD41, nuclear ploidy, and cell dimension have been used as differentiation markers.

Morphological analysis

Cells collected at different days of culture were cytocentrifuged onto glass slides, stained with DIFF QUICK Staining Set (Medion Diagnostics) and then identified by morphological analysis. Briefly: $1,5x10^4$ cells were resuspended in 100 μL PBS supplemented with 1% BSA, and cytospinned 400 rpm, 5 minutes (Cytocentrifuge Shandon 3). Spots obtained were then stained with DIFF QUICK Staining Set according to the manufacturer's instructions.

Megakaryocytes were examined under the microscope (Leica EC3) and cellular images were acquired with digital camera.

Cellular dimensions, which in our system represent a differentiation marker, have been so analized: mononuclear haematopoietic stem cells were plated in 6-wells dishes at the starting density of $1x10^6$ cells/mL in differentiation medium and adequately stimulated. After 3 and 5 days of differentiation, megakaryocytes were examined under microscope (Axiovert 40 CFL, Zeiss, 20x objective), and images were acquired and analyzed using Image-Pro Plus Software (Media Cybernetics).

Flow cytometric analysis

Cells obtained as previously described were incubated for 30 min with 10 mg/mL FITC-conjugated antibody or the corresponding rat IgGs (Becton Dickinson). Cells were then washed with PBS, re-pelleted and resuspended in PBS containing 1 μg/mL propidium iodide. Samples were finally analyzed by FACScan (Becton Dickinson) by means of the CellQuest (Becton Dickinson) program for fluorescence intensity analysis.

DNA staining

Cells obtained as previously described were gently resuspended in PBS containing 70% Et-OH. Samples have been then re-pelleted, washed with PBS alone and finally analyzed by cytofluorimetric technique after 30-min DNA staining with 50 mg/mL propidium iodide and 0.2 mg/mL RNase A. Samples were acquired using FACScan flow cytometer and analized using CellQuest software.

4. RESULTS

4.1 Megakaryocytic differentiation: validation of the method

The first goal of my project was to confirm that mononuclear cells harvest from murine tibiae and femora of 8–12-wk-old BALB/c female mice and cultured in a differentiation medium, really differentiated in megakaryocytes. Since the hallmarks of MK maturation are expansion of cytoplasmic mass and endoreduplication (polyploidization), to validate our experimental protocol I have performed preliminary experiments that demonstrate the effective increase of cellular size and ploidy during the differentiative process.

Mononuclear cells obtained from murine tibiae and femora were collected, counted and finally plated in 6-wells dishes at the starting density of $1x10^6$ cells/mL, in differentiation medium.

Cells at day 0, 3 and 5 of differentiation were collected and cell size has been evaluated under microscope. Figure 13 is representative of the other images obtained. As expected, there is a significant and progressive increase in cellular dimension: at day 0 there is an homogeneous population of small cells; with the passing of the time, is possible to note an augmentation in cellular size. Expecially at day 5 of differentiation there is a significantly large population of differentiated cells.

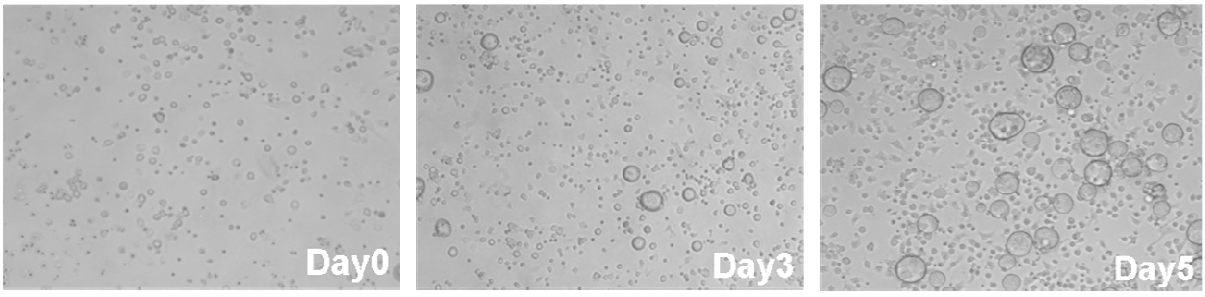

Figure 13. Progressive cellular size increase at different stages of differentiation. Megakaryocytes are readily recognized by their large size. Images are representative of three independent experiments performed.

During megakaryocytic differentiation, megakaryocytic precursors switch from a mitotic to an endomitotic process characterized by DNA duplication without cytokinesis. This process leads to the formation of large polyploid cells with polylobulated nuclei. To evaluate if nuclei of differentiated cells really were poliploid, cells at day 5 of differentiation have been separated over a discontinuous (3%-2%-1%) BSA gradient. Biggest cells, that sediment more quickly, have been collected and then spun down on microscope slides by a cytocentrifuge.
Cell morphology has been then evaluated using a commercial histological staining (DIFF QUICK Staining Set). Morphologic analysis of megakaryocyte cultures confirmed the presence of polilobated nucleus. Figure 14 shows the tipical morphological features of cells at day 5 of differentiation: as easily observable, there is a poli-lobated nucleus (white arrow), with high DNA content.

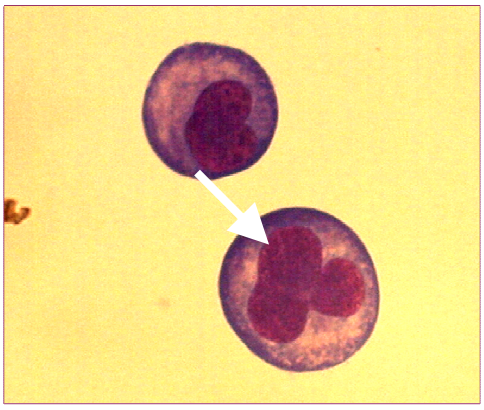

Figure 14. Cell morphology of cells at day 5 of differentiation. Iterative rounds of endomitosis (hallmark of MK development) lead MKs to become large polyploid cells. Morphologic analysis performed shown a progressive augmentation of poliplody also in our experimental model: cells at day 5 of differentiation (figure) exhibited a poli-lobated nucleus (white arrow). The image is representative of three independent experiments performed.

The process of cellular differentiation is always associated with the acquisition of cellular specific markers. Since integrin beta-3, also named CD61, is a cluster of differentiation found on thrombocytes and its expression is reported to gradually increase during megakaryocytopoiesis, I have finally performed Western Blotting analysis to evaluate its expression in our experimental model. Samples of proteins obtained from MKs at different stages of differentiation have been collected at day 0 and 3 of differentiation with a simpli centrifuge. Cells cultured for 5 days were instead separated over a discontinuous (3%-2%-1%) BSA gradient and biggest cells, that sediment more quickly, have been collected and used. Data obtained showed, as expected, a significant and progressive increase in integrin beta-3 expression.

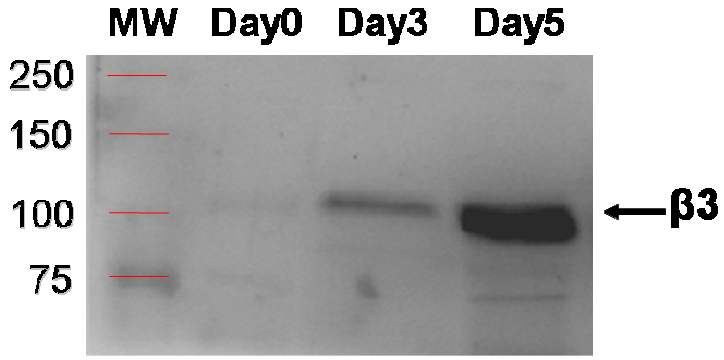

Figure 15. CD61 expression: Western Blotting analysis. Samples of MKs at different stages of differentiation were lysated and dissociated. Proteins were separated by SDS-PAGE and detected with specific antibodies. The panel reported in the figure is representative of three independent experiments performed.

4.2 Expression pattern of the endocannabinod receptors

Before to investigate the potential effects of cannabinoids on megakaryocytopoiesis, I have performed Wester Blotting analysis to evaluate whether these cells express classical cannabinoid receptors (CB1, CB2 and TRPV-1). Samples of MKs at different stages of differentiation were lysated and dissociated. Proteins have been separated by SDS-PAGE and finally detected with specific antibodies. Data obtained (figure 16) showed a progressive and significant increase in CB1 expression from day 0 to day 5 of differentiation (upper panel); by contrast CB2 expression (central panel) seems to be unmodulated. TRPV1 (lower panel) is detectable only in the more differentiated cells.

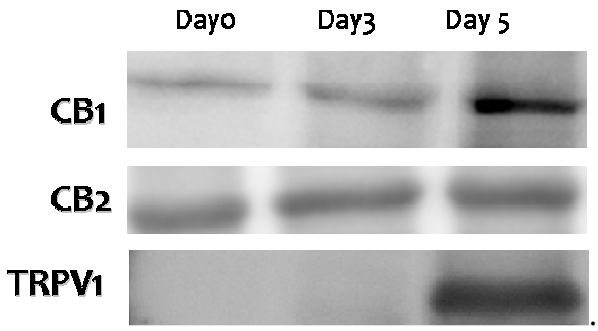

Figure 16. Expression pattern of the main cannabinoid receptors: immunoblotting analysis. Mononuclear cells obtained from murine tibiae and femora were collected, counted and finally plated at the starting density of $1x10^6$ cells/mL, in differentiation medium. Cells were collected at Day 0 and 3 of differentiation by a centrifuge; cells used at Day 5 of differentiation were instead obtained by sedimentation on a BSA gradient to collect only fully differentiated megakaryocyte. CB1, CB2 and TRPV-1 expressions were detected with specific commercial antibodies. The panels reported in the figure are representative of three independent experiments performed.

To confirm data obtained, I have evaluated cannabinoid receptors expression also using polymerase chain reaction experiments: RNA from MKs at different stages of differentiation was used to perform RT-PCR analysis. The results obtained, shown in figure 17, essentially support data previously obtained: there is a significant increase in CB1 expression from day 0 to day 5 of differentiation and unmodulated expression of CB2.

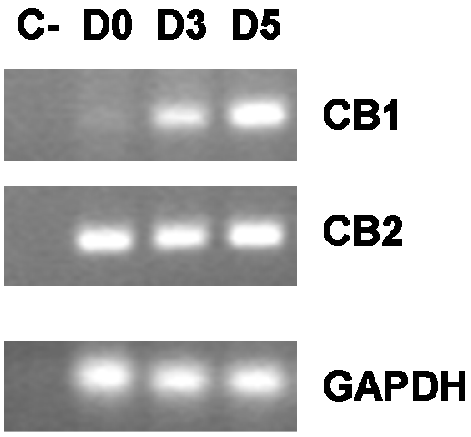

Figure 17. Expression pattern of cannabinoid receptor: RT-PCR analysis. Cannabinoid CB1 and CB2 amplicons (of expected sizes of 204 bp and 244 bp, respectively) obtained after the amplification of cDNA prepared from RNA of megakaryocyte at different stages of differentiation (to note: RNA from cells at Day 5 of differentiation has been obtained using only fully mature cells presents in the sample and isolated through a discontinuous BSA gradient. D0= Day 0; D3= Day 3; D5= Day 5. C- = negative control. Amplification of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) cDNA, was performed as positive control. Results are representative of 4 different experiments.

In order to evaluate cannabinoid receptors expression in a quantitative manner, I have performed Real Time RT-PCR experiments. Data obtained are reported in figure 18 and essentialy confirm results obtained in Wester Blotting and PCR analysis: there is a progressive and statistically significant increase in CB1 mRNA levels from day 0 to day 3 and day 5 of differentiation. As for previous experiments, RNA from cells at Day 5 of differentiation has been obtained using only fully mature cells of the sample. CB2 receptors showed unmodulated transcriptional levels in all the stages of differentiation evaluated and TRPV-1 mRNA levels gradually increase.

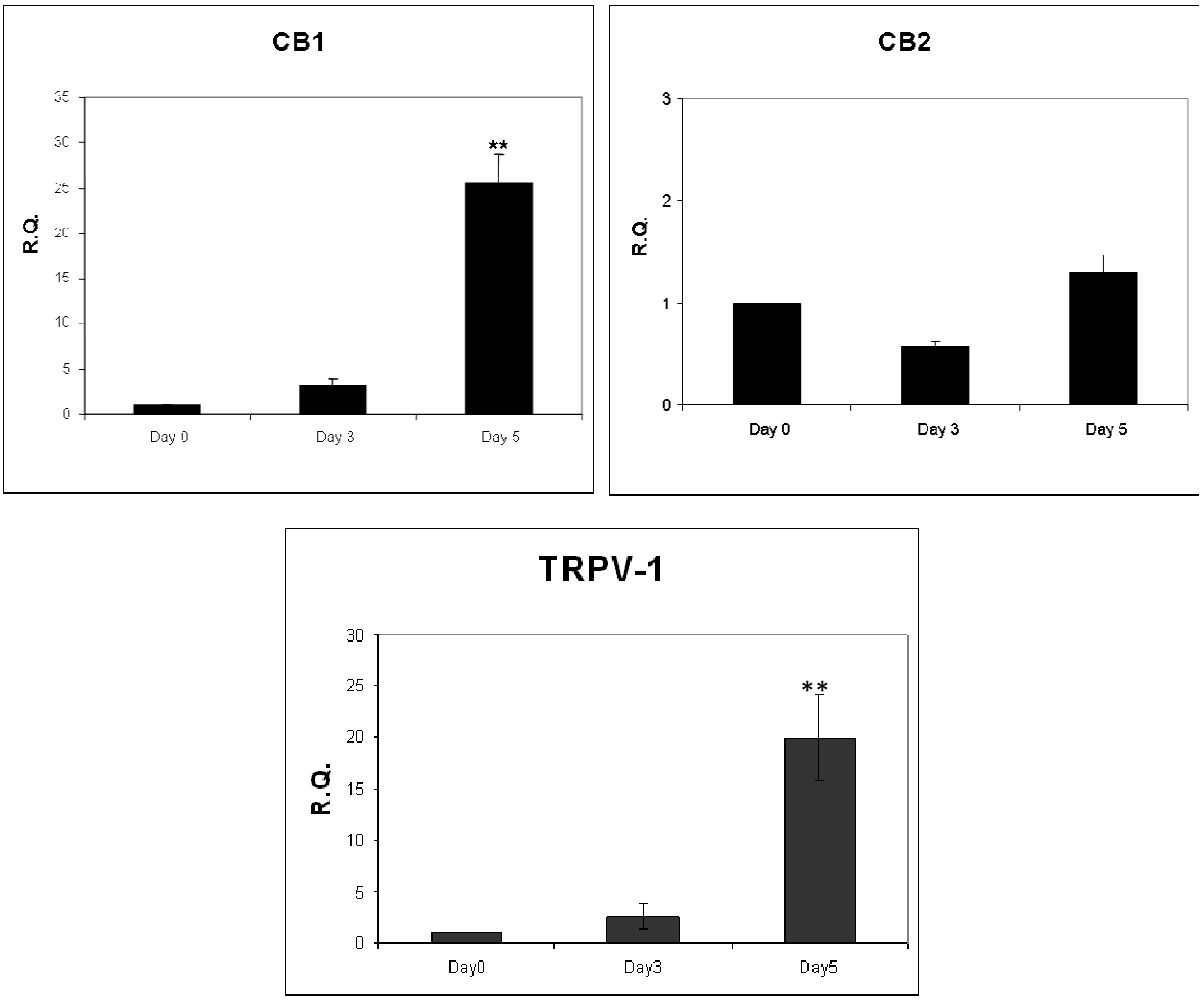

Figure 18. Expression pattern of cannabinoid receptor: Real-Time RT-PCR analysis. Gene expression analysis were performed using the comparative ΔΔCt method; non-adherent cells at day 0 of culture were used as calibrator and expression in these cells was arbitrarily set to 1; endogenous control used: GAPDH (glyceraldehyde-3-phosphate dehydrogenase). R.Q. = relative quantification. Each sample was analyzed in triplicate and results of triplicate analysis of each sample were consistent. Bar graphs represent the means ± SEM of three independent experiments. Statistical analysis: ANOVA **p<0.01.

4.3 Megakaryocytic differentiation: the role of endocannabinoids.

The main goal of my project was to clarify the role of endocannabinoids in megakayocytic differentiation. In order to do so, megakaryocytic differentiation has been induced in the presence or absence of endocannabinoids in the culture media. Since data reported in literature showed that AEA (more than 2-AG) is involved in cellular differentiation, and because of its biological function is previously mediated by CB1 (receptor with a strongly modulated expression in our system), I have performed my experiments using the stable chiral analogue of AEA: methanandamide (MetAEA) alone (10 μM), or in combination with 1 μM Am251 (CB1 antagonist).

The surface proteins CD41, the nuclear ploidy, and the cell dimension have been used to evaluate differentiative process.

4.4 MetAEA increases the number of fully mature megakaryocyte

Cellular dimension, which in our system represents a differentiation marker, has been initially evaluated in cells treated or not (at day 0) with 10 μM MetAEA. After 3 and 5 days of differentiation, megakaryocytes size was examined under the microscope and cells were classified according to cell size criteria: fully mature cells (radius > 10 μm) have been properly selected and counted. Data obtained are reported in the graph bar in figure 19: after 3 and 5 days of culture, MetAEA increases the number of fully mature cells (fold increase: 2.035 ± 0.073 and 1.207 ± 0.382 respectively). The effect was statistically significant only at day 3 of differentiation.

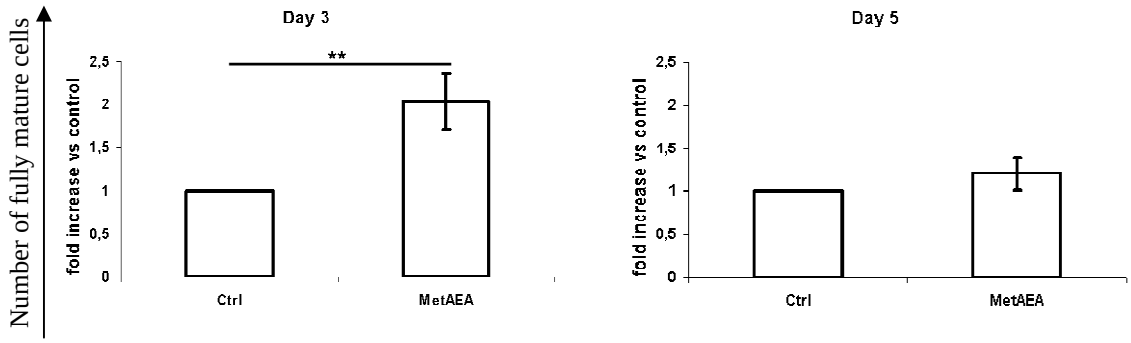

Figure 19. MetAEA increases the number of fully mature megakaryocytes. Murine bone marrow cells were induced to differentiate in the presence or absence of 10 μM Methanandamide (MetAEA). At day 3 and 5 of differentiation cells were analyzed and fully mature cells (radius > 10 μm) were counted. Treatment with MetAEA significally increased the number of fully mature cells. Values were normalized vs control (arbitrarily set to 1). Bar graphs represent the mean ± SD of 3 independent experiments. Statistical analysis: t-test **p<0.01.

Since preliminary experiments performed suggested that metAEA can affect megakaryocytic differentiation, cellular dimensions have been evaluated by means of citofluorimetric studies: cells at day 0, 3 and 5 of differentiation were sorted according to size in a dot plot SSC vs FSC (figure 20). The selected area (—) represents the chosen gate for subsequent analysis and contains cells with an increased size and internal complexity (a differentiation marker in our system).

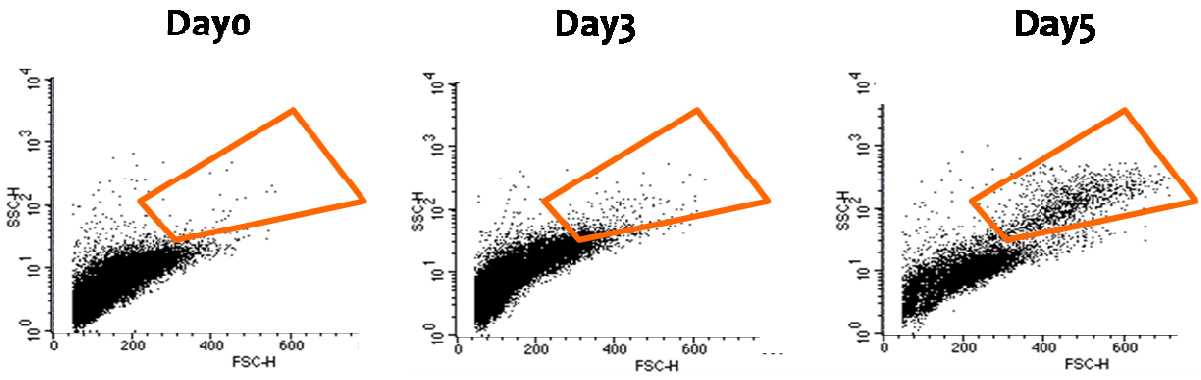

Figure 20. SCATTER PLOT of cells at day 0, 3 and 5 of differentiation. The panels reported in the figure are representative of all the independent experiments performed.

In order to have quantitative data and confirm results previously observed (that is that tratement with metAEA increases the number of fully mature cells), cellular dimensions have been evaluated by means of citofluorimetric studies. Murine bone marrow cells were induced to differentiate in the presence or the absence of 10 µM Methanandamide (MetAEA) alone or in combination with 1 µM Am251. At day 3 and 5 of differentiation cells were analyzed by flow cytometry and sorted according to size. Fully mature cells (gated in — , see figure 20) were counted. Results are reported in figure 21: treatment with MetAEA significantly increases the number of fully mature cells both at day 3 and 5 of differentiation.

In order to verify if also in our system metAEA exerts its biological function mainly via CB1 (as reported in many scientific articles), I have performed my experiments using MetAEA in combination with 1 µM Am251 (CB1 antagonist). In all the samples so treated (MetAEA and Am251) the number of fully mature cells was not significant different with respect to control. In the light of these findings, it can be speculated that, also in our system, MetAEA action is CB1 receptor mediated.

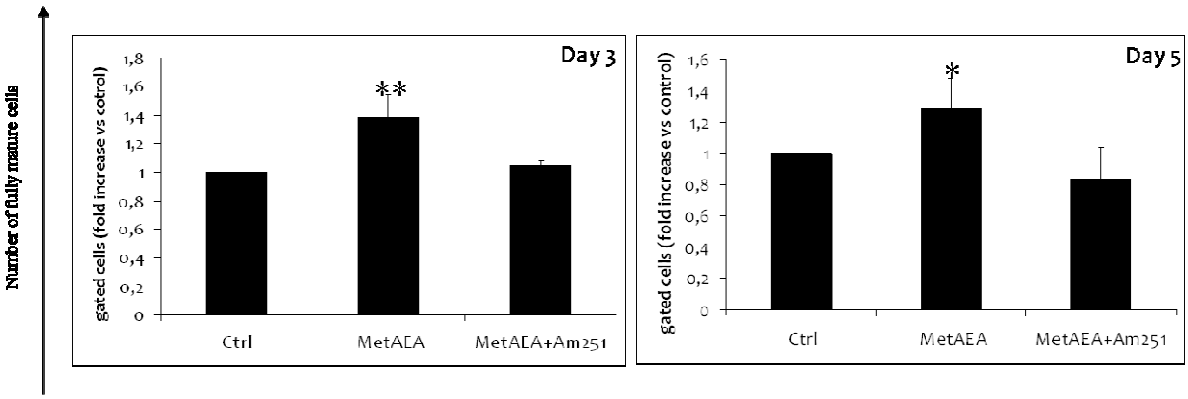

Figure 21. The citoflurimetric analysis performed showed that treatment with MetAEA significantly increased the number of fully mature cells via CB1. Murine bone marrow cells were treated at day 0 of differentiation with 10 µM MetAEA alone or with 1 µM Am251 and left in differentiation medium. At day 3 and 5 of differentiation cells were stained with 10 mg/ml FITC anti-CD41 antibody and FACS-sorted according to size in a graph SSC vs FSC. CD41+ cells gated in the orange area (see figure 20) were selected and analyzed. Values have been opportunely normalized to the number of cells gated in the control (arbitrarily set to 1). Bar graphs represent the mean ± SD of 3 independent experiments. Statistical analysis: t-test *p<0.05; **p<0.01.

4.5 Methanandamide does not affect CD41 expression during megakaryocytopoiesis

Data previously showed, suggested that MetAEA, maily via CB1, is able to modulate megakaryocytic differentiation. In order to better understand the cellular processes involved, I have studied if other differentiation markers were modulated by metAEA. Since the cellular differentiation is always associated with the acquisition of cellular specific markers, in order to investigate the role of metAEA during megakaryocytopoiesis, I have evaluated modulation of a platelet lineage specific marker (CD41) expression. Cells properly treated at day 0 (vehicle control or 10 μM MetAEA alone or in combination with 1 μM Am251) have been collected as previously described, incubated with specific antibodies, analyzed by flow cytometry and sorted according to size. CD41 expression of fully mature cells (gated in — , see figure 20) were analyzed. Our data (figure 22) showed that treatment with metAEA alone seems lightly increase CD41 expression only at day 3 of differentiation but this difference is not statistically significant. In samples treated with MetAEA and Am251, CD41 expression was not visibily significant different with respect to control.

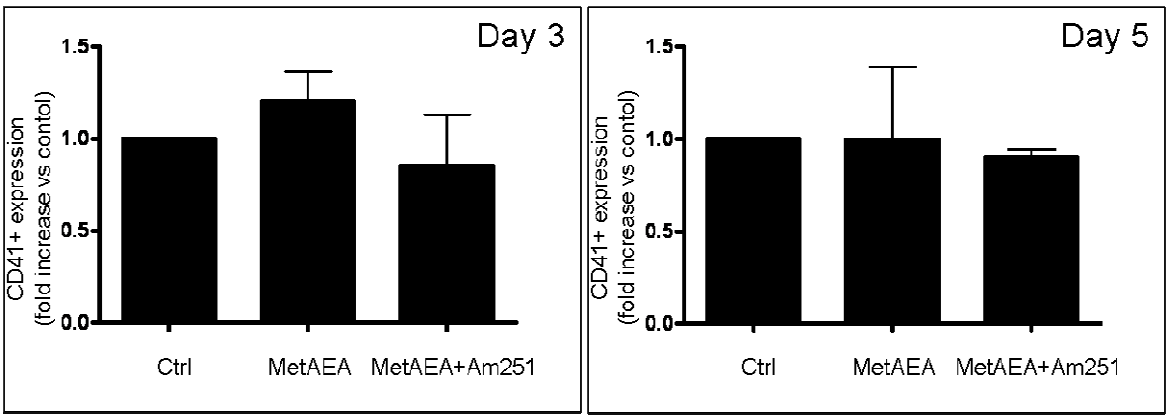

Figure 22. The citoflurimetric analysis performed showed that treatment with MetAEA did not significantly affect CD41 expression at the different stages of differentiation evaluated. Values have been opportunely normalized to mean fluorescence of the control (arbitrarily set to 1). Bar graphs represent the mean ± SD of 3 independent experiments. Statistical analysis: t-test *p<0.05; **p<0.01.

4.6 Methanandamide does not increase cellular ploidy during megakaryocytopoiesis

Since the hallmarks of MK maturation are expansion of cytoplasmic mass and endoreduplication (polyploidization), I have evaluated if treatment with MetAEA could increase cellular ploidy during megakaryocytopoiesis. Murine bone marrow cells were treated at day 0 of differentiation with 10 μM MetAEA (—), 10 μM MetAEA and 1 μM Am251 (—) or vehicle control (—).Cells have been collected as previously described and, to evaluate DNA content, cells were stained with propidium iodide. Cytofluorimetric assays were performed to understand if metAEA modulates nuclear ploidy during megacariocitopoiesis. As expected (figure 23), during megakaryocytic differentiation there is a significant increase of cellular ploidy (number of peaks) but treatment with MetAEA (both alone or in combination with Am251) did not modify cellular ploidy profiles with respect to control (traces of the different samples overlap perfectly).

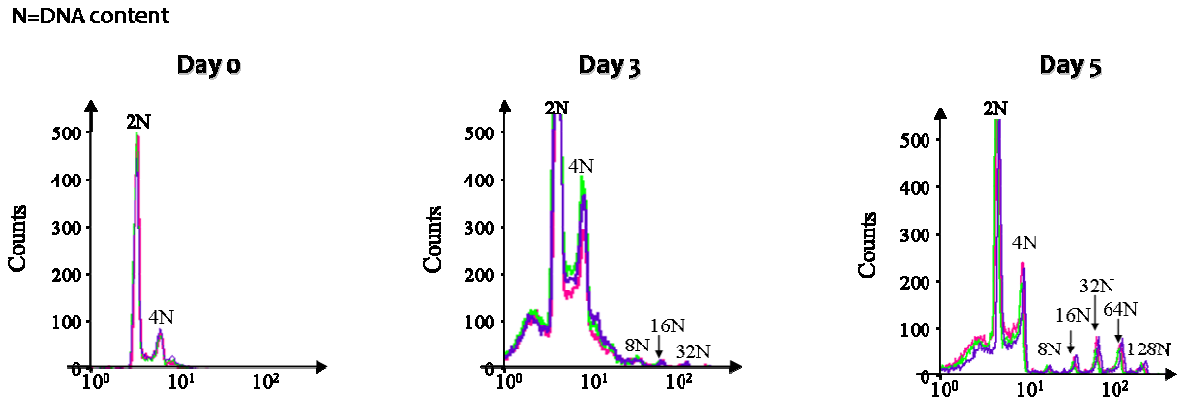

Figure 23. Cellular ploidy profiles at day 0, 3 and 5 of differentiation. The citoflurimetric analysis performed showed that during megakaryocytic differentiation there is a significant increase of cellular ploidy (number of peaks). Treatment with MetAEA (both alone or in combination with Am251) did not modify cellular ploidy profiles with respect to control. Profiles here reported are representative of all the independent experiments performed.

5. DISCUSSION

Since its identification, AEA has been shown to exert many physiological activities. Althought first described in the nervous system, it is becoming increasingly clear that AEA's effects can be either central, in the brain, or peripheral, in other parts of the body. Endocannabinoids exert many functions and, in the case of AEA, the control of cell fate seems to be the core of its biological activity. It is in fact well documented that AEA plays an important role in the modulation of cellular differentiation processes.

As previously said, the megakaryocyte is a bone marrow cell responsible for the production of blood thrombocytes (platelets), which are necessary for normal blood clotting. Megakaryocytes normally account for 1 out of 10.000 bone marrow cells but can increase nearly 10-fold in certain diseases. The availability of stem cells is essential for this process and, in many cases, it provides new approaches for the treatment of human diseases. Congenital amegakaryocytic thrombocytopenia (CAMT), for instance, is a disorder characterized by thrombocytopenia and absence or decline in the number of megakaryocytic precursors in the bone marrow. It is caused by mutations in the thrombopoietin receptor gene, c-mpl, involved in the proliferation and differentiation of megakaryocytes and platelets. Many efforts are actually done to develop a gene therapy for this disorder, but the elucidation of the regulatory mechanisms responsible for stem cell differentiation is crucial for therapy, too.

Since the biological action of AEA is mainly CB1 mediated and G-coupled protein receptor (GPCR) members (such as CB1) have been related to many functions including cell proliferation, maturation, survival, apoptosis and migration, I have evaluated the role of MetAEA on megakaryocytic differentiation.

The first goal of my project has been to confirm that mononuclear cells obtained from murine tibiae and femora really differentiated in megakaryocytes. Since the hallmarks of MK maturation are the expansion of cytoplasmic mass and endoreduplication (polyploidization), to validate our experimental protocol I have performed different experiments. All the data obtained validate the method: during the differentiation process there is a significant increase in cellular size (figure 13), polyploidization (figure 14) and expression of cellular specific markers (figure 15).

The second step of my work was to characterize the expression pattern of the main endocannabinoid receptors (CB1, CB2 and TRPV-1) during *ex vivo* differentiation of murine haematopoietic stem cells. The data obtained by means of several techniques clearly demonstrate that there is a progressive and significant increase of CB1 expression from day 0

to day 5 of differentiation whilst CB2 expression seems to be unmodulated. TRPV1 expression was instead detectable only in the last differentiative stage evaluated (day 5). This first observation led me to suppose that especially CB1 could play a regulatory role in haematopoietic differentiation and, to test this hypothesis, cells were adequately treated with MetAEA alone or in combination with AM251 (the CB1 cannabinoid antagonist). In order to investigate the role of endocannabinoids in megakaryocyte differentiation, expression of the surface protein CD41, nuclear ploidy and cell dimension have been used as differentiation markers. Both manual count (figure 19) and cytofluorimetric analysis (figure 21) showed that MetAEA increases the number of fully mature cells in a statistically significant way. This effect was particulary evident at day 3 of differentiation and lower in the last differentiative stage evaluated. This event could be due to MetAEA consume as time goes by or simply because since day 3 the system is "saturated". Moreover, I have observed that in samples treated with MetAEA and Am251, the number of fully mature cells was not significantly different in respect to control. This data strongly suggest that MetAEA action is CB1 receptor mediated.

To conclude, the present work shed some light on the effects of endocannabinoids on *ex vivo* differentiation of murine haematopoietic stem cells. In particular, here I provide the first evidence that MetAEA, via CB1, increases the numer of fully mature cells without affecting expression of the surface protein CD41 or nuclear ploidy. Further studies are necessary to better understand the molecular pathways activated downstream of CB1 receptors during this process. One possibility is that, as for keratinocytic differentiation (Paradisi et al., 2008), AEA, via CB1, induces DNA methylation and consequent modulation in differentiating gene expression. Derocq (Deroq et al., 2000) has reported that the activation of CB2 in the promyelocytic cells HL-60 induced an up-regulation of genes involved in cytokine synthesis, regulation of transcription and cell differentiation. Many features of the transcriptional events downstram CB2 activation appeared to be related to a cell differentiation program, suggesting that CB receptors could play a role in the initialization of cell maturation. It is also possible that, as suggested by Valk (Valk et al., 1997), AEA acts as a synergistic growth stimulator for haematopoietic cells, in synergy with other interleukins (for instance IL-3, IL-11 or TPO, which are present in the differentiation medium). Recently, Catani and co-workers (Catani et al., 2009) have elegantly demonstrated that AEA extends platelets survival through CB1-dependent Akt signalling; it is possible that the same event also occurs in our cellular system. Finally, it is well known that one of the most important pathways downstream CB activation involves mitogen-activated proteinkinases (MAPKs). Since the results obtained by Conde

(Conde et al., 2010) indicate that a precise orchestration of signals, including ERK1/2 and p38 MAPKs as well as PI3K pathway, is necessary for acquisition of features of mature megakaryocyte, these pathways could be investigated in future, too.

6. PhD PAPERS

1. Baldassarri S, Bertoni A, **Bagarotti A**, Sarasso C, Zanfa M, Catani MV, Avigliano L, Maccarrone M, Torti M, Sinigaglia F. (2008) The endocannabinoid 2-arachidonoylglycerol activates human platelets through non-CB1/CB2 receptors. *J Thromb Haemost.* 10:1772-9.

2. Di Vito C, Bergante S, Balduini A, Rastoldo A, **Bagarotti A**, Surico N, Bertoni A, Sinigaglia F. (2010) The oestrogen receptor GPER is expressed in human haematopoietic stem cells but not in mature megakaryocytes. *Br J Haematol.* 1:150-2.

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