THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PHD)

The role of the endocannabinoid system in regulating the biological processes of the skin

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DEBRECEN, 2014

Contents

List of Abbreviations

2-AG – 2-arachidonoylglycerol

2-AGE – 2-arachidonoylglycerol ether

AA – Arachidonic acid

AEA – N-arachidonoyl ethanolamine

AKT – Protein kinase B

BSA – Bovine serum albumin

CB1 – Cannabinoid receptor 1

CB2 – Cannabinoid receptor 2

CBD – Cannabidiol

CBG – Cannabigerol

CBGV – Cannabigivarin

CBN – Cannabinol

CCL8 – Chemokine (C-C motif) ligand 8

CCR7 – C-C chemokine receptor type 7

CK – Cytokeratin

CNS – Central nervous system

COX-2 – Cyclooxygenase-2

CPZ – Capsazepine

CXCL10 – C-X-C motif chemokine 10

Cyt-C – Cytochrome-C

DAGL – Diacylglycerol-lipase

DC – Dendritic cell

ECS – Endocannabinoid System

ERK – Extracellular-signal-regulated kinase

FAAH – Fatty acid amide hydrolase

FIL – Filaggrin

FLIPR – Fluorescent image plate reader

G6PD – Glucose-6-phophate-dehydrogenase

GPCR – G-protein coupled receptors

iDC – Immature dendritic cell

IFNγ – Interferon-γ

IL – Interleukin

INV – Involucrin

JNK – c-Jun N-terminal kinases

LOR – Loricrin

LOX – Lipooxigenase isoenzymes

LPI – Lysophosphatidyl inositol

MAGL – Monoacylglycerol lipase

MAPK – Mitogen-activated protein kinase

mDC – Mature dendritic cell

MMP – Matrix metalloproteinase

NAAA – N-acylethanolamine-hydrolyzing acid amidase

NADA – N-arachidonoyldopamine

NAGly – *N*-arachidonoyl glycine

NAPE-PLD – N-acyl phosphatidylethanolamine-specific phospholipase D

NFκB – Nuclear factor kappa-light-chain-enhancer of activated B cells

NRIP1 – Nuclear receptor interacting protein-1

OD – Optical density

PEA – Palmitoylethanolamide

PGE2 – Prostaglandin E2

PI-3K – Phosphatidylinositide 3-kinase

PKC – Protein kinase C

PLC – Phospholipase C

PPAR – Peroxisome proliferator-activated receptor

Q-PCR - Quantitative real-time PCR

RFU – Relative fluorescence units

RIPA – Radioimmunoprecipitation assay

RNAi – Small interfering RNA

RR – Ruthenium Red

TGFβ2 – Transforming growth factor-β 2

THC – (−)-trans-Δ9-tetrahydrocannabinol

THCV – Tetrahydrocannabivarin

TM – Transmembrane

TRIB3 – Tribbles homolog-3

TRP – Transient receptor potential

TRPA – Ankyrin subfamily of TRP channels

TRPC – Canonical subfamily of TRP channels

TRPM – Melastatin subfamily of TRP channels

TRPML – Mucolipin subfamily of TRP channels

TRPP – Polycystin subfamily of TRP channels

TRPV – Vanilloid subfamily of TRP channels

Introduction

The endocannabinoid system (ECS) was first described following the investigation of the main psychotropic component of the *Cannabis sativa* plant, (−)-trans-Δ9-tetrahydrocannabinol (THC). In the decades since these first results, the ECS has grown to encompass numerous cannabinoid ligands, the transmembrane receptors that bind and transduce the effects of said ligands and the enzymatic apparatus responsible for the synthesis and degradation of various endogenous cannabinoids. The discovery of numerous endocannabinoids ligands also broadened the receptor repertoire of the ECS, which currently encompasses not only G-protein coupled receptors but ionotropic receptors as well (mostly belonging to the transient receptor potential ion channel family). This "expansion" of the ECS is not only constrained to the molecular level; indeed, while the first cannabinoid receptors dubbed cannabinoid receptor 1 and 2 (CB1 and CB2) were found in the central nervous system and immune cells, respectively, it is now widely accepted that the ECS is expressed, and is functionally active, in almost all non-neuronal tissues as well.

One of the most exciting sites of this expansion is the human skin. Recent research has shown that the skin, far from being a purely passive physico-chemical barrier, also functions as an active neuro-immuno-endocrine organ. Our own workgroup has shown that various skin cells not only express multiple members of the ECS, but many of these cells also actively produce endocannabinoids. These locally produced cutaneous endocannabinoids influence diverse biological processes on numerous skin cells, such as epidermal keratinocytes, local cells of the innate and adaptive immune system as well as mini-organs such as the sebaceous gland or the hair follicle. The differential expression of cannabinoid receptors in various skin compartments, and the fact the level expression changes markedly depending on the differentiational status of the cells, suggest an important role of the ECS in regulating

fundamental cellular processes in the skin. Indeed, the ECS was found to play a profound role in the regulation of proliferation and differentiation, the production of interleukins and chemokines and the exocrine functions of these cells.

Although recent years have greatly expanded our knowledge on the expression and putative functions of the ECS on non-neuronal cells, and especially cells of the skin, our knowledge is far from complete. In the present work, as part of our extensive research effort on cutaneous functions, we aimed at examining the ECS on two cell types, namely human monocyte-derived dendritic cells and sweat gland cells.

Overview of literature

The endocannabinoid system (ECS) – a historical overview

The ECS was first discovered when research focused on the distinct natural compounds (called cannabinoids) responsible for the effects attributed to the *Cannabis sativa* plant (Figure 1.a) and its derivatives.

a b

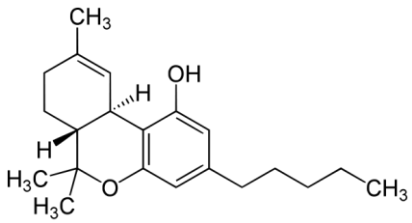


***Figure 1.*: (a)** The cannabis sativa plant and (**b**) its main psychoactive component (−)-trans-Δ9-tetrahydrocannabinol (from: http://commons.wikimedia.org/wiki/File:Cannabis_sativa_Koehler_drawing.jpg and http://en.wikipedia.org/wiki/File:Tetrahydrocannabinol.svg)

The first cannabinoid was identified before the turn of the 20th century (Wood, 1899), but it was the isolation of the main psychoactive component (−)-trans-Δ9-tetrahydrocannabinol (THC) (Figure 1.b), in 1964 by Mechaoulam's group, that truly sparked the study of cannabinoid chemistry. While the plant itself has been used in medicinal formulations for thousands of years, with definitive evidence of its application found in Chinese, Assyrian,

Persian and Egyptian historical finds, the discovery of endogenous substances that share both structural and functional characteristics with cannabinoids is much more recent. The first step in finding these endogenous molecules was the discovery that THC decreased prostanoid-stimulated cyclic AMP accumulation in membranes prepared from neuronally derived cells (Howlett 1984). The receptor in question was finally identified in 1988 from rat brain (Devane et al., 1988). Subsequent studies (Matsude et al., 1990, Gérard et al., 1991) managed to express the cDNA in immortalized cell lines, which lead to the determination of the amino acid sequence of the first cannabinoid receptor, i.e. CB1 (Figure 2.), from both rat and human tissues (the human protein shows 97.3% homology with the rat receptor). The next larger milestone was the discovery a second cannabinoid receptor (dubbed CB2) from HL-60 cells and identified in macrophages residing in the marginal zone of the spleen, and ultimately cloned from a mouse splenocyte cDNA library (Munro et al., 1993, Shire et al., 1996). The receptor shares a sequence homology of 82% with human CB1, but lacks 13 amino acids at the C-terminal (Figure 2.) (Griffin et al., 2000). Although these classical cannabinoid receptors were originally thought to be expressed in specific tissues (the central nervous system [CNS] for CB1 and non-neuronal tissues for CB2) it is now apparent that both receptors are widely distributed in many cell types (Herkenham et al., 1990; Gérard et al., 1991; Ishac et al., 1996; Liu et al., 2000; Onaivi et al., 2006; Teixeira-Clerc et al., 2006; Telek et al., 2007; Dobrosi et al., 2008; Xi et al., 2011).

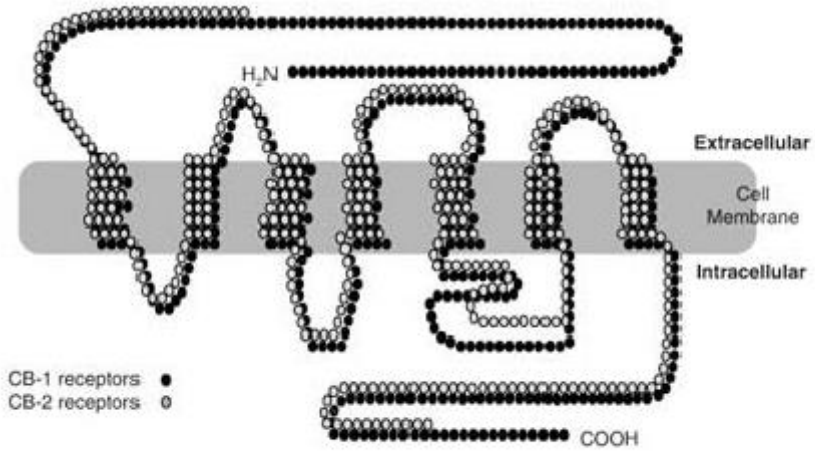
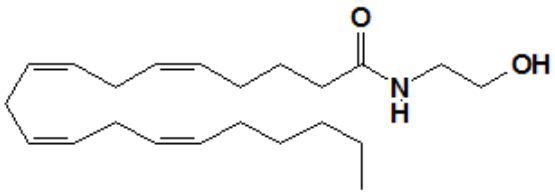


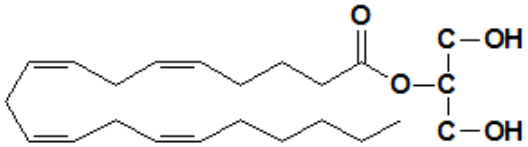
Figure 2.: Structure of CB1 and CB2 receptors (from: http://io9.com/)

Structure and metabolism of "classical" endocannabinoid ligands – AEA and 2-AG

The cloning of the first cannabinoid receptors triggered a search for endogenously occuring counterparts of plant-derived cannabinoids. The first two such endocannabinoids were isolated a mere three years apart; N-arachidonoyl ethanolamine (anandamide or AEA) from porcine brain (Devane et al., 1992) and 2-arachidonoylglycerol (2-AG) from canine gut and mouse brain (Mechoulam et al., 1995, Sugiura et al., 1995). Both are derivatives of arachidonic acid (AA), conjugated with ethanolamide (for AEA) or glycerol (for 2-AG) (Figure 3.).



N-arachidonoyl ethanolamine (AEA)



2-arachidonoylglycerol (2-AG)

Figure 3.: Structure of classical endocannabinoids (from: commons.wikimedia.org)

Although both their general structure and functions are similar, the biochemical steps of their synthesis and degradation, as well as their receptor affinity differ widely. AEA is mainly thought to be generated via N-acyl phosphatidylethanolamine-specific phospholipase D (NAPE-PLD; Okamoto et al., 2004) while 2-AG is mainly synthesized by subsequent steps of

phospholipase C (PLC) and diacylglycerol-lipase (DAGL) α and β (Stella et al., 1997; Bisogno et al., 2003). The inactivation of both endocannabinoids is also dependent on separate degrading enzymes, namely fatty acid amide hydrolase (FAAH) and monoacylglycerol lipase (MAGL) for AEA and 2-AG, respectively (Vandevoorde and Lambert, 2007). Although both enzymes are regarded as the main route of inactivation for both endocannabinoids, AEA can also be hydrolyzed by N-acylethanolamine-hydrolyzing acid amidase (NAAA) (Ueda et al., 2010) and oxygenated by cyclooxygenase-2 (COX-2) (Yu et al., 1997), lipooxigenase isoenzymes (LOX) (Ueda et al., 1995) or by P-450 cytochrome (Bornheim et al., 1993). On the other hand, 2-AG can be hydrolyzed by a series of serine hydrolase α-β-hydrolase domain 6 or 12 (Marrs et al., 2010; Savinainen et al., 2012) (Figure 4.). Since both of these prototypic endocannabinoids bind to both CB1 and CB2 receptors (albeit with different affinities), early studies regarded them as mutually exchangeable in the modulation of synaptic signaling. More than two decades worth of observations have provided conclusive evidence that AEA and 2-AG are not similar in their functioning and that they have highly specific roles in synaptic remodeling regulation (Katona and Freund, 2012; for a comprehensive review also see Luchicci and Pistis, 2012).

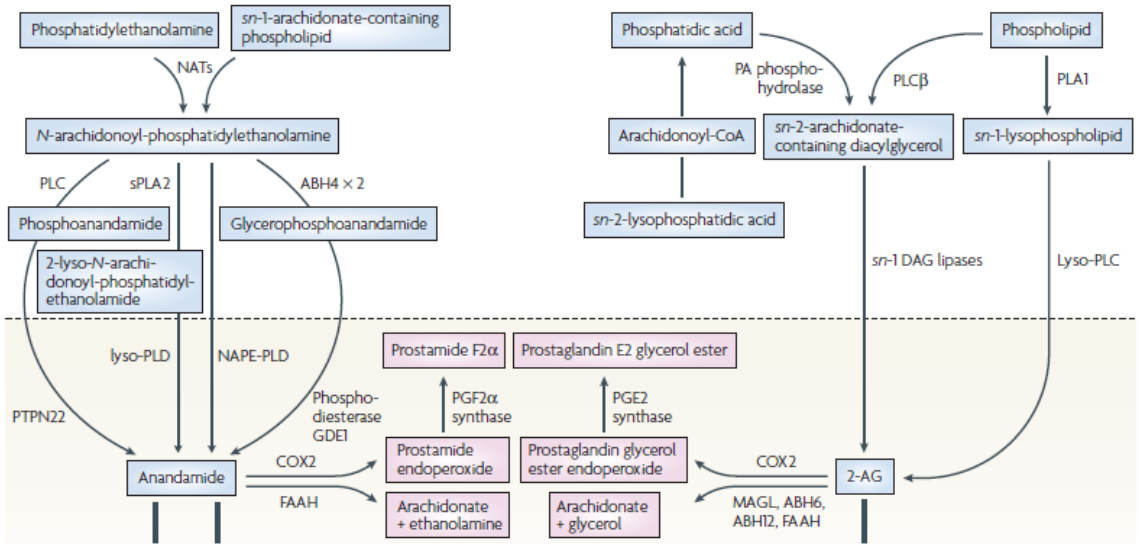
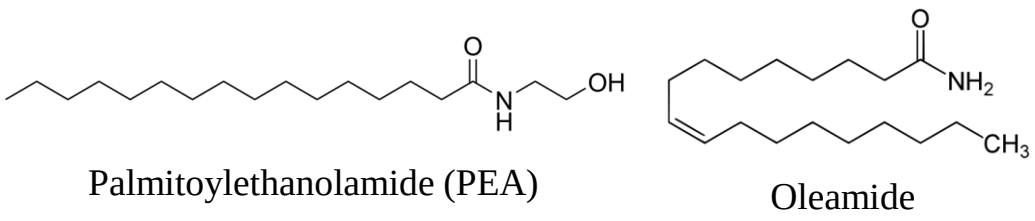


Figure 4.: Synthetic and degrading pathways of AEA and 2-AG (Di Marzo, 2008)

Novel endocannabinoid ligands

Although AEA and 2-AG are the two most extensively researched endocannabinoids, other endogenous ligands have been found to share some cannabimimetic actions. As such, the term "endocannabinoid" encompasses a steadily growing family of molecules which can in no way be considered complete. The following section aims to provide a brief overview of these ligands, more to highlight the complexity of the ECS than to provide a comprehensive reference.

Two fatty acid amides that have been shown to have such cannabimemitic actions are palmitoylethanolamide (PEA) and oleamide (Lambert and Di Marzo, 1999) (Figure 5.).

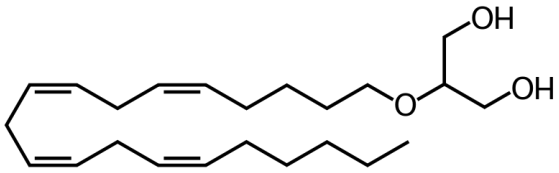


Palmitoylethanolamide (PEA) Oleamide

Figure 5.: Structure of palmitoylethanolamide and oleamide (from: commons.wikimedia.org)

PEA has been shown to have anti-allergic/anti-inflammatory effects on both animals and humans. It was found to inhibit peripheral inflammation (Mazzari et al., 1996; Berdyshev et al., 1998) and mast-cell degranulation (Aloe et al., 1993) as well as to exert neuroprotective (Lambert et al., 2001) and anti-nociceptive effects in rats and mice (Jaggar et al., 1998). These actions were accompanied by changes in nitric oxide production (Ross et al., 2000), neurotrophil influx (Farquhar-Smith and Rice, 2003) and expression of pro-inflammatory proteins such as inducible nitric-oxide synthase and COX-2 (Costa et al., 2002). The nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α) was identified as the molecular target responsible for the anti-inflammatory properties of PEA (Lo Verme et al., 2005). PEA and anandamide are synthesized from different precursors through the action of the same enzyme, NAPE-PLD, and are hydrolyzed by the same amidase enzymes (Okamoto et al., 2004). Interestingly, although the effects of PEA were found to mimic those of phyto- and

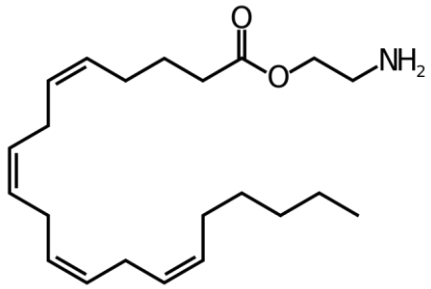
endocannabinoids PEA has minimal affinity for either CB1 or CB2 (Lambert and Di Marzo, 1999). Although the effects of PEA are not relegated through classical cannabinoid receptors, they have been described to have an "entourage effect", whereby the application of PEA potentiates the effect of anandamide (Ho et al., 2008). A similar mechanism was first suggested to explain the effects of oleamide (Lambert and Di Marzo, 1999), whereas later reports seem to point to direct actions on the CB1 receptor. The classification of oleamide as a cannabinoid ligand is still disputed however, since relatively high (micromolar) concentrations are required to activate CB1 (for a review see: Hiley and Hoi, 2007). This entourage effect was described not only for AEA but 2-AG as well, the effect of which could be potentiated by 2-linoleoylglycerol and 2-palmitoylglycerol (Ben-Shabat et al., 1998).



Noladin ether (2-AGE)

Figure 6.: Structure of noladin ether (from: commons.wikimedia.org)

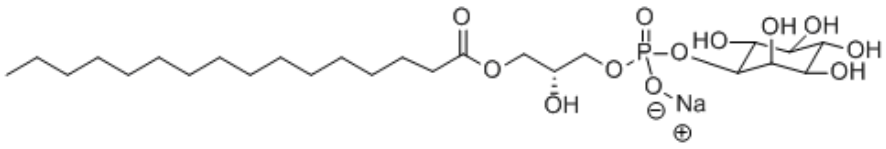
2-arachidonoylglycerol ether (also known as noladin ether; 2-AGE) (Figure 6.) is an ether-linked analogue of 2-AG. It was first isolated from porcine brain, and was shown to bind to both classical cannabinoid receptors (although with much lower affinity to CB2; Hanus et al., 2001). Its concentration in the brain is too low for it to act on cannabinoid receptors (Oka et al., 2003); hence, the exact function of the molecule is still in question. It has also been shown to activate PPAR-α (Sun et al., 2007), another target of certain cannabinoids.



Virodhamine

Figure 7.: Structure of virodhamine (from: commons.wikimedia.org)

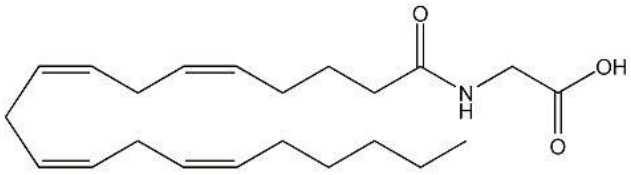
The ester of arachidonic acid and ethanolamine, virodhamine (O-arachidonoylethanolamine) (Figure 7.) is proven to be a full agonist on CB2. On CB1 it has been described to be both a partial agonist and an antagonist, depending on the concentrations applied (Porter et al., 2002). There is an interesting relationship between virodhamine and AEA, since virodhamine is rapidly converted to AEA because of its chemical instability in the brain (Porter et al., 2002); on the other hand, AEA can also be converted to virodhamine by spontaneous or enzymatic chemical rearrangement (Markey et al., 2000). It might also share pathways of uptake and intracellular hydrolysis with AEA, due to its ability to block AEA transport (Porter et al., 2002). Although it is unclear how it is produced, degraded or stored, multiple receptors have been shown to be activated by virodhamine; namely PPARα, GPR55 and transient receptor potential vanilloid 1 (TRPV1; Sun et al., 2006; Gkoumassi et al., 2009; Sharir et al., 2012, see below). N-arachidonoyldopamine (NADA) has also been reported to activate similar receptors, namely CB1, TRPV1 and PPARγ (Bisogno et al., 2000; Huang et al., 2002; O'Sullivan 2007) (all detailed below).



Lysophosphatidyl inositol (LPI)

Figure 8.: Structure of lysophosphatidyl inositol (from: commons.wikimedia.org)

As the short overview above shows, many "novel" endocannabinoids do not only activate classical cannabinoid receptors but numerous others as well, thereby effectively expanding the receptor-arsenal of the ECS. Conversely, the addition of newer receptors that belong to the ECS led to the discovery of novel endogenous ligands as well. GPR55 is one of the most conclusively "de-orphanized" G-protein coupled receptors (GPCR) newly classified as a cannabinoid receptor. It can be activated not only by 2-AG as described previously, but by lysophosphatidyl inositol (LPI; Figure 8.) in both murine (Lauckner et al., 2008) and human cells (Oka et al., 2007; Kapur et al., 2009; Whyte et al., 2009). Recent evidence has also led to another similar compound, 2-arachidonyl n-glycero-3-phosphoinositol, which activates GPR55 more potently then LPI in HEK293 cells; this might suggest that this molecule is the true intrinsic natural ligand for GPR55 (Oka et al., 2009).



N-arachidonoyl glycine

Figure 9.: Structure of N-arachidonoyl glycine (from: commons.wikimedia.org)

Research focused on phytocannabinoids (cannabinoid ligands derived from the *Cannabis sativa* plant) has also led to the discovery of novel endocannabinoid ligands, as well as the "de-orphanization" of GPCRs; namely Δ^9-THC was shown to activate GPR18, another novel endocannabinoid receptor (see characterization below). The endogenous ligand of GPR18 was identified as *N*-arachidonoyl glycine (NAGly; Figure 9.) (Bradshaw et al., 2009; McHugh et al., 2010, 2012), an oxygenated metabolite of AEA. It was first described as a full agonist of GPR18 at nanomolar concentrations by Kohno et al., 2006, who used stably transfected cell lines to establish the connection. Further research has shown that NAGly has mainly anti-

inflammatory effects, by decreasing the viability of pro-inflammatory macrophages (Burstein et al., 2011; Takenouchi et al., 2012).

Endocannabinoid	Receptors affected	References
N-arachidonoyl ethanolamine	Agonist on: • CB1 and CB2 • TRPV1 • PPARα and γ	Katona and Freund, 2012; Luchicci and Pistis, 2012
2-arachidonoylglycerol	Agonist on: • CB1 and CB2 • PPARγ	Katona and Freund, 2012; Luchicci and Pistis, 2012
Palmitoylethanolamide	Agonist on PPARα Entourage effect on CB1 and CB2	Aloe et al., 1993; Mazzari et al., 1996; Berdyshev et al., 1998; Jaggar et al., 1998; Lo Verme et al., 2005; Ho et al., 2008
Oleamide	Entourage effect on CB1 and CB2	Lambert and Di Marzo, 1999
2-arachidonoylglycerol ether	Agonist on: • CB1 and CB2 • PPARα • GPR55	Hanus et al., 2001; Oka et al., 2003; Sun et al., 2007
Virodhamine	Agonist on: • CB2 • PPARα • GPR55 • TRPV1 Antagonist/partial agonist on: • CB1	Bisogno et al., 2000; Markey et al., 2000; Huang et al., 2002; Porter et al., 2002; Sun et al., 2006; O'Sullivan 2007; Gkoumassi et al., 2009; Sharir et al., 2012;
Lysophosphatidyl inositol	Agonist on: • GPR55	Oka et al., 2007; Kapur et al., 2009; Whyte et al., 2009
***N*-arachidonoyl glycine**	Agonist on: • GPR18	Bradshaw et al., 2009; McHugh et al., 2010, 2012

Table 1.: Major endocannabinoid ligands

„Classical" cannabinoid receptors

The activation of the most widely investigated CB1 induces analgesia, attenuation of nausea/vomiting in cancer chemotherapy, appetite stimulation in wasting syndromes (which effect is exploited by the drug Sativex, a mixture of THC and cannabidiol [CBD]) and decreases intestinal motility as beneficial effects; however, the untoward side effects that can accompany

these therapeutic responses include alterations in cognition/memory, dysphoria/euphoria and sedation (reviewed in: Abood and Martin 1992; Hollister 1986). A similar tetrad of responses has also been described in rodents (hypothermia, analgesia, hypoactivity and catalepsy). Nerve-muscle tissue preparations respond to CB1 activation with an inhibition of contraction, most probably due to the inhibition of neurotransmitter release (Pertwee et al., 1992; Roth 1978). Indeed, it is now generally accepted that the main function of CB1 receptors located at central or peripheral nerve terminals is to mediate the inhibition of on-going release of certain excitatory and inhibitory neurotransmitters (Pertwee et al., 1992; Pertwee and Ross, 2002; Szabó and Schliker, 2005).

Although the expression of CB2 receptors is more difficult to track than that of CB1 due to their lower immunogenicity (Thomas, 2009; Atwood and Macke, 2010), the expression of CB2 has been conclusively proven in numerous tissues; namely in pulmonary endothelial cells (Zoratti et al., 2003), osteocytes, osteoblasts and osteoclasts (Ofek et al., 2006), gastrointestinal cells (Storr et al., 2002; Hillsley et al., 2007; Duncan et al., 2008), sperm cells (Grimaldi et al., 2009), adipocytes (Roche et al., 2006), cirrhotic (but not normal) liver cells (Julien et al., 2005) and keratinocytes (Ibrahim et al., 2005).

While both CB1 and CB2 receptors signal through $G_{i/o}$ proteins, which results in the inhibition of adenylyl-cyclase and modulation of ion channel functions (McAllister et al., 1999), CB1 has also been shown to signal through G_s proteins (Glass and Felder, 1997; Chen et al., 2010). In addition, cannabinoid receptors also regulate various intracellular kinase cascades, such as the mitogen-activated protein kinase (MAPK) pathway, c-Jun N-terminal kinases (JNKs), and protein kinase B (AKT), in a G-protein-independent manner (reviewed in Di Marzo, 2009).

Recent research into the signaling pathways activated by cannabinoids have brought to light several more factors that increase the complexity of these secondary messenger pathways;

namely, CB1 receptors have been shown to form multimers (both homo- and heteromers, Mukhopadhyay et al., 2000) whereas cannabinoid ligands show biased agonism on CB1 receptors (Hudson et al, 2010). While the exact functional relevance of CB1 homomers has not been elucidated to date, several CB1-X heteromers have been studied more extensively. There is definite data supporting CB1-D2 dopamine, CB1-opioid and CB1-orexin-1 receptor heteromers (reviewed in Pertwee et al., 2010). The exact functional consequence of these heteromers is still not known, but current studies suggest that CB1-D2 heteromers function *in vivo* to convert G-protein preference from G_i to G_s. Furthermore, CB1-μ-opoid receptor association was suggested to be a factor in receptor internalization and also modulate the efficacy of MAPK activation by agonists of both receptors, while the CB1-orexin-1 pairing increases the MAPK sensitivity to orexin-1 by 100-fold in a CB1-dependent manner.

Novel endocannabinoid receptors

The expansion of the ECS, as described previously, has led to the inclusion of new metabotropic and ionotropic as well as intranuclear receptors. Although both AEA and 2-AG were discovered via their interactions with classical cannabinoid receptors, both have been shown to modulate activities of other receptors as well. As such, the number of receptors associated with the ECS has also increased.

The list of novel metabotropic receptors includes previously orphan receptors such as GPR18, GPR55 and GPR119. Although many new receptors were found based on functional findings, GPR18 was first identified from various broad expression studies of GPCRs (Gantz et al., 1997); it was shown that GPR18 was expressed in numerous tissues, notably the spleen, thymus, peripheral blood leukocytes, small intestine, appendix and lymph nodes, which already suggested a possible role in immune regulation. The most significant expression was identified in several cell types of the testis, with the highest levels in the most terminally differentiated

cells (Gantz et al., 1997). A subsequent paper that examined the GPCR repertoires of humans and mice via Q-PCR tissue profiling partly supported these results, and reported four different levels for GPR18: (i) no expression in the amygdala, frontal cortex, hippocampus, liver, and muscle; (ii) low expression in the cortex, thalamus, adrenal tissue, colon, intestine, kidney, prostate, skin, spleen, stomach, and uterus; (iii) moderate expression in the lung, ovary, testis, thymus, and striatum; and (iv) strong expression in the hypothalamus, thyroid, peripheral blood leukocytes, cerebellum, and brain stem (Vassilatis et al., 2003). Although the distribution of the receptor has been described as seen above, its exact function has not been elucidated conclusively. Recent results (McHugh et al., 2010 and 2012) have put forth the hypothesis that GPR18 is actually the abnormal CBD receptor, which has previously been implicated in the modulation of microglial, endothelial and glioma cell migration, and a selection of cardiovascular responses (Járai et al., 1999; Franklin and Stella 2003; Offertáler et al., 2003; Walter et al., 2003; Mo et al., 2004; Begg et al., 2005; Vaccani et al., 2005; Mackie and Stella 2006).

GPR55 was discovered *in silico* and cloned in 1999 (Sawzdargo et al., 1999). Multiple cannabinoids bind with high affinity to GPR55 – including synthetic CB1 and CB2 agonists HU210 and JWH015, endocannabinoids 2-AG and LPI, CB1 antagonists AM251 and rimonabant as well as phytocannabinoids such as CBD and THC; yet, the receptor shows a lack of significant alignment of amino acid residues with classical cannabinoid receptors (Sawzdargo et al., 1999; Ryberg et al., 2007; Lauckner et al., 2008). GPR55 expression has since been proven to be nearly ubiquitous outside of the CNS, with expression reported in the spleen, lung, adrenal gland, urinary bladder, uterus, adipose tissue, gastrointestinal tract, liver, bone and kidney (Ryberg et al., 2007; Pertwee, 2007; Whyte et al., 2009). In the CNS, GPR55 expression is limited to certain areas of the brain such as the hippocampus, hypothalamus, cerebellum and frontal cortex; however, the levels of GPR55 are certainly lower than that of

CB1 (Herkenham et al., 1991). Since the pharmacology of the receptor is complex, as mentioned above, numerous cannabinoid ligands (including endo-, phyto- and synthetic cannabinoids) bind to GPR55; yet, the true endogenous ligand has not been conclusively identified. The most likely candidates are (i) 2-AG, which is considered by some to be the true endogenous ligand of classical cannabinoid receptors (Sugiura and Waku, 2000) since it was shown to activate the GPR55 receptor at nanomolar concentrations (Ryberg et al., 2007); (ii) 2-arachidonoyl-containing LPI, which possesses significantly higher potency and maximal efficacy as a GPR55 ligand, so it may represent the true natural ligand of this receptor (Gasperi et al., 2013). In spite of the ambiguity of the pharmacology of the receptor, numerous (patho)physiological processes have been linked to GPR55. These include putative roles in the cardiovascular system (since knockout mice showed hypertension), in nociception (where it is pro-nociceptive in neuropathic and inflammatory conditions), in inflammation (partly through CB2 modulation), in the regulation of energy metabolism (by increasing adipogenic gene expression in visceral adipose tissue and cooperation with CB1 receptors) and in bone metabolism (by regulating the formation and activity of osteoclasts). GPR55 is also implicated the regulation of cellular proliferation and cancer formation, since its expression is augmented in different neuronal and peripheral cells, with mRNA and protein levels appeared to correlated with tumor aggressiveness (Ford et al., 2010; Oka et al., 2010; Andradas et al., 2011; Piñeiro et al., 2011). GPR55 deficient mice are more resistant to papilloma and carcinoma formation (Pérez-Gómez et al., 2013), although GPR55 was also shown to induce specific apoptotic pathways (Huang et al., 2011).

GPR119 was assigned to the receptor cluster encompassing the cannabinoid receptors through a bioinformatic search of the human genome database (Fredriksson et al., 2003; Oh et al., 2006). Subsequently, its expression was proven in β-cells of the pancreatic islets of Langerhans and in proglucagon positive cells of the small intestine (Chu et al., 2007; Semple

et al., 2008; Reimann et al., 2008; Lauffer et al., 2009). In line with these data, GPR119 was found to take part in metabolic homeostasis and in the control of energy balance via the regulation of incretin-dependent insulin release and the secretion of glucagon-like peptide 1 from enteroendocrine cells (Soga et al., 2005; Chu et al., 2007, 2008; Semple et al., 2008; Lan et al., 2009; Lauffer et al., 2009; Hughes, 2009; Shah 2009; Flock et al., 2011; Oh and Lagakos 2011).

Although endocannabinoid ligands have classically and most extensively been described to activate metabotropic receptors, the promiscuity of the ECS also encompasses ionotropic receptors; namely the thermosensitive transient receptor potential (TRP) channels. TRP channels represent a superfamily of (mostly) non-selective cation channels further classified into – as of the current writing – six subfamilies: canonical (TRPC), vanilloid (TRPV), melastatin (TRPM), polycystin (TRPP), mucolipin (TRPML) and ankyrin (TRPA). All TRP channels share certain structural characteristics (they are all 6 transmembrane (TM) domain plasma membrane proteins with intracellular C- and N-termini and a pore region between TM5 and TM6 α helices). Most are involved in the transduction of polymodal stimuli such as temperature, mechanical and osmotic changes, electrical charge, light, hypotonic swelling and chemical stimuli such as xenobiotic substances (first described, but not limited to olfactive, taste and chemesthetic stimuli) and endogenous lipids (Moran et al., 2011; Nelson et al., 2011). Certain TRP channels may also be activated by various temperature changes; namely TRPA1 (<17°C), TRPM8 (<25-28°C), TRPV4 (>27-35°C), TRPV3 (>32-39°C), TRPV1 (>43°C) and TRPV2 (>52°C) (see Figure 10).

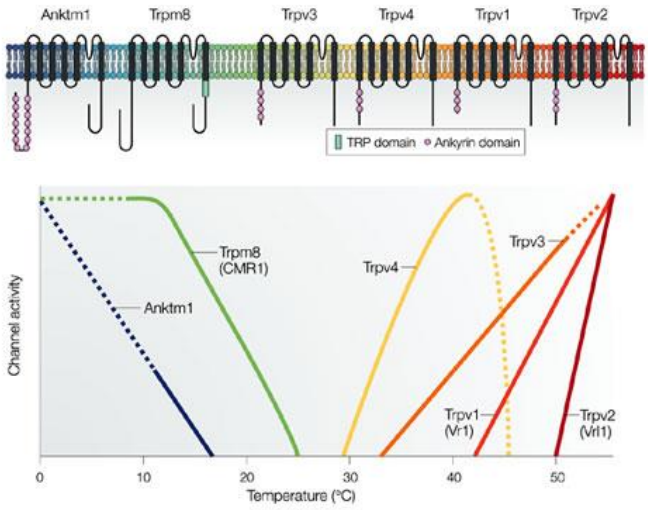


Figure 10.: Thermosensitive TRP channels (Belmonte and Viana , 2008)

Most of these receptors (with the notable exception of TRPV3) were first described in neurons where they have been shown to take part in the regulation of sensory functions (especially TRPA1 and TRPV1; the latter of which has been shown to act as an integrator of painful stimuli) (reviewed in: Moran et al., 2011). Outside of their neural functions, there is accumulating evidence about the roles of thermo-TRPs in physiological and pathological conditions of non-neuronal tissues; indeed, TRP channels are involved in cell homeostasis and growth control, regulation of cell fate and survival, immune and inflammatory mechanisms, as well as endocrine and exocrine secretory processes (Nilius and Owsianik, 2010; Boesmans et al., 2011; Denda and Tsutsumi, 2011; Moran et al., 2011; Fernandes et al., 2012).

The link between TRP channels and the ECS was first suggested based on functional and anatomical similarities between TRPV1 and proteins of the ECS (Di Marzo et al., 1998; Melck et al., 1999). Indeed, the first endogenous ligand of cannabinoid receptors, AEA (Devane et al., 1992) has the distinction of being the first endogenous ligand of TRPV1 as well (Zygmunt et al., 1999). Further research following this discovery has deepened the understanding of TRP-ECS crosstalk to the point that thermosensitive TRP channels are rightly considered as ionotropic cannabinoid receptors. Although AEA was first shown to activate TRPV1 channels at relatively high concentrations, and the efficacy and potency of AEA was found to be less

than that of capsaicin (the natural agonist of TRPV1) in most experiments (for a review see Ross, 2003), more recent findings support the hypothesis that AEA is a relevant physiological ligand on TRPV1 channel. For example, Garami et al. showed that the hypolocomotor and hypothermic effects of this compound which are present in CB1 receptor null mice (Di Marzo et al., 2000) are absent in TRPV1 null mice (Garami et al., 2011). AEA was also shown to cause nasal pain (Alenmyr et al., 2012) and to increase the response to heat of the carotid artery sinus nerve afferents by acting on TRPV1 (Roy et al., 2012). Intra-plantar injection of AEA in rats excite cutaneous C-nociceptor activity and produce nocifensive behaviour via TRPV1 activation (Potenzieri et al., 2009). AEA was also shown to have effect on central TRPV1 channels, where the concomitant pharmacological blockade of CB1 with AEA injected into the prefrontal cortex unmasked the contribution of TRPV1 to anxiety- and panic-like behaviors (Rubino et al., 2008; Casarotto et al., 2012). Further evidence supporting the physiological relevance of TRPV1-AEA interactions came from experiments utilizing URB597, an inhibitor of FAAH, which effectively raises the endogenous levels of AEA, and was found to lead to TRPV1-mediated effects (Maione et al., 2006; Morgese et al., 2007; Rubino et al., 2008).

The second most studied endocannabinoid, 2-AG has been shown to have very little functional activity on TRPV1 channels, using pharmacological methods (Zygmunt et al., 1999; De Petrocellis et al., 2000). However, certain effects such as the stimulation of Ca^{2+} influx in microvascular endothelial cells and the anti-proliferative action on C6 glioma cells (Jacobsson et al., 2001; Golech et al., 2004) were attenuated by capsazepine (CPZ), a TRPV1 antagonist. Whether this effect can be attributed to the lack of selectivity of CPZ, or to the conversion of 2-AG to diacylglycerols which might activate TRPV1 (Woo et al., 2008) has not been elucidated as of this writing.

NADA is also capable of activating both CB1 and TRPV1; however, its efficacy and affinity for TRPV1 is higher than that of AEA (Bisogno et al., 2000; Huang et al., 2002). As

such, it is possible to observe NADA-dependent and TRPV1-mediated stimulatory effects on nociceptors or central neurons without the need for concomitant CB1 receptor blockade (Sagar et al., 2004; Huang and Walker 2006; Marinelli et al., 2007). NADA was also shown to activate non-neuronal TRPV1 channels, since it caused the cell death of blood mononuclear cells (especially in donors with high TRPV1 expression; i.e. end-stage kidney disease patients; Saunders et al., 2009).

Although other previously mentioned endocannabinoids have no appreciable activity on TRP channels, the "entourage effect" described for PEA and oleamide in relation to CB1 is also present in relation to TRPV1 and AEA interactions in heterologous systems (De Petrocellis et al., 2001, 2004; Smart et al., 2002), *in vitro* (Ho et al., 2008), and *in vivo* (García Mdel et al., 2009).

Phytocannabinoids have also been shown to activate thermo-TRP channels. CBD was the first such exogenous ligand to be proven to activate TRPV1 with affinity and efficacy similar to that of capsaicin, but with significantly lower potency on recombinant TRPV1 in HEK-293 cells (Bisogno et al., 2001). Since then numerous phytocannabinoinds have been shown to act on TRPV1; namely, cannabigerol (CBG), cannabigivarin (CBGV) and tetrahydrocannabivarin (THCV) proved to have similar potencies and efficacies to those of CBD (De Petrocellis et al., 2011), and were also capable of desensitizing TRPV1. Indeed, in neuropathic and inflammatory animal pain models (Costa et al., 2004, 2007; Comelli et al., 2008), these phytocannabinoids exhibited anti-hyperalgestic effects, most probably through desensitization of the channel, since the effects could be attenuated with CPZ treatment. CBD was also shown to have TRPV1-mediated effects on non-neuronal elements as it inhibited lung and cervical cancer cell invasion *in vitro* (Ramer et al., 2010a, b) and it induced myeloid-derived suppressor cells and subsequent amelioration of experimental autoimmune hepatitis *in vivo* (Hegde et al., 2011).

On TRPV2 channels, CBD was once again the first phytocannabinoid reported to act as an agonist both on rat recombinant TRPV2 overexpressing HEK-293 cells and on sensory neurons (where it caused CGRP release), and also resulted in desensitization (Qin et al., 2008). Interestingly, the efficacy and potency of CBD on human TRPV2 was found to be significantly lower; however, other phytocannabinoids that activate TRPV1 (CBG, CBGV, THCV and THC) were more efficacious and/or potent than CBD at both activating and desensitizing TRPV2. Nonetheless, CBD has been shown to sensitize glioblastoma cells to cytotoxic chemotherapeutic agents through TRPV2 (Nabissi et al., 2013).

Phytocannabinoids have also been tested on recombinant rat TRPV3 and TRPV4; TRPV3 was shown to be activated by CBD and THCV, while only the latter compound was truly efficacious on TRPV4. Interestingly CBGV and CBG, while having no activating effect on either channel, desensitized TRPV3 and TRPV4, respectively (De Petrocellis et al., 2012).

In relation to cold-sensitive channels, TRPA1 was shown to be activated by THC and cannabinol (CBN) (Jordt et al., 2004), while newer results also point to CBC and CBD as potent activators of the channel (De Petrocellis et al., 2008, 2011). Interestingly, the inhibitor of FAAH, URB597, was also shown to activate TRPA1, while TRPM8 on the other hand is inhibited, rather than activated by these compounds, with CBD, CBG, CBN and THC all behaving as antagonists at submicromolar doses (De Petrocellis et al., 2008, 2011).

Receptor	Reported ligands	Distribution	References
GPR18	N-arachidonoyl glycine Abnormal cannabidiol	Spleen, thymus, peripheral blood leukocytes, small intestine, appendix, lymph nodes, testis, ovary, uterus and the CNS	Vassilatis et al., 2003; McHigh et al., 2010; 2012
GPR55	2-AG PEA LPI AM251 Rimonobant THC CBD Abnormal cannabidiol	spleen, lung, adrenal gland, urinary bladder, uterus, adipose tissue, gastrointestinal tract, liver, bone, kidney, hippocampus, hypothalamus, cerebellum and frontal cortex	Ryberg et al., 2007; Pertwee, 2007; Lauckner et al., 2008; Whyte et al., 2009
GPR119	AEA OEA	β-cells of the pancreatic islets of Langerhans and in proglucagon positive cells of the small intestine	Chu et al., 2007; Reimann et al., 2008; Semple et al., 2008; Lauffer et al., 2009
TRPV1	AEA NADA CBD CBG CBGV THCV	Dorsal root ganglia, brain, kidney, pancreas, testes, uterus, spleen, stomach, small intestine, lung, liver and skin	Zygmunt et al., 1999; Bisogno et al., 2000; 2001; Huang et al., 2002; De Petrocellis et al., 2011
TRPV2	THC CBD CBG CBGV THCV	Skin, peripheral blood cells, retinal pigment epithelium, CD34+ hematopoietic stem cells, glioblastoma cells, various cancer cells	Qin et al., 2008; Nabissi et al., 2013
TRPV4	THCV	Trachea, kidney, liver, lung, spleen, skin.	De Petrocellis et al., 2012
PPARα	AEA PEA THC	Liver, muscle, heart and bone	Kozak et al., 2002; Sun et al., 2006, 2007
PPARγ	AEA PEA THC	Nearly ubiquitous	Rockwell and Kaminski 2004; Bouaboula et al., 2005; O'Sullivan et al., 2006; Rockwell et al., 2006; Gasperi et al., 2007

Table 2.: Summary of major ECS receptors

Nuclear receptors, i.e. the PPARs, a superfamily of nuclear hormone receptors, have also been shown to respond to cannabinoids. Two PPARs have been found to be activated by cannabinoids: PPARα and PPARγ. A number of cannabinoids show appreciable receptor binding affinity, such as THC, AEA and the 15-lipoxygenase metabolite of 2-AG, 15-hydroxyeicosatetraenoic acid glyceryl ether (Kozak et al., 2002; Sun et al., 2006, 2007). In behavioral studies, the inhibition of FAAH – and the concomitant increase in AEA and PEA – resulted in an anti-inflammatory effect which could be abolished by the application of a PPARα antagonist (Jhaveri et al., 2008). Although CB1 was not investigated in this study, Costa et al., (2008) concluded that the anti-hyperalgesic effects of PEA was mediated with both the CB1 receptor and PPARα. PPARγ was also shown to be activated by AEA, 2-AG and nanomolar concentrations of THC (Rockwell and Kaminski 2004; Bouaboula et al., 2005; O'Sullivan et al., 2006; Rockwell et al., 2006; Gasperi et al., 2007); both endocannabinoids suppressed the secretion of interleukin (IL)-2 in a PPARγ-dependent manner.

The ECS in the skin

As the above section shows, the ECS comprises a truly wide-ranging collection of ligands, enzymes and receptors. It is not surprising therefore that the ECS as a whole has been implicated in numerous regulatory functions in both health and disease. In the past decade, a plethora of research (not in small part from our own workgroup) has highlighted the largest organ of the body, the skin, as an important site of action of the ECS.

Classically, the main function of the skin has been considered to form a passive physico-chemical barrier against environmental challenges. However, more recent research has highlighted the complexity of the skin and its' adnexal components (i.e. hair follicles, sebaceous and sweat glands), thereby proving that it functions as an active neuro-immuno-endocrine organ (Roosterman et al., 2006). The skin can be considered a "stratified" organ, since it can be split

into three separate layers each with their own resident cell types and specific roles in skin functions (Figure 11.).

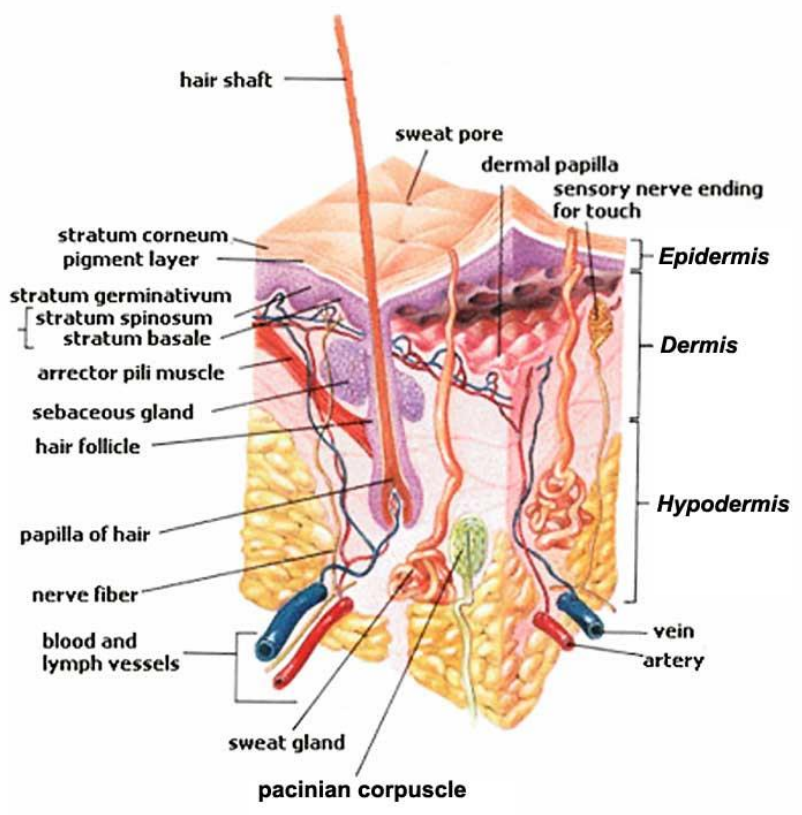


Figure 11.: The structure of human skin
(source:http://en.wikibooks.org/wiki/Human_Physiology/Integumentary_System)

The outermost layer of the skin, the *epidermis*, is composed of keratinocytes (responsible for acting as a barrier to infection and water-loss), Merkel cells (mechanoreceptors that take part in the sensation of touch and pressure), melanocytes (which determine the skin color) and Langerhans cells (one of the professional antigen-presenting cells of the skin immune system). Sensory nerve endings may also reach multiple layers of the epidermis, where they recognize touch, pressure, temperature as well as pain and itch. Underneath this layer is a dense connective tissue composed of collagen, elastic and reticular fibers, which are produced by dermal fibroblasts. The *dermis* is supplied by blood and lymphatic vessels and numerous nerve endings (both sensory afferents and motor efferents). The previously mentioned skin appendages (i.e. hair follicles, sweat glands and sebaceous gland) also reside in this layer. The final layer of the

skin, the *hypodermis*, is comprised of adipocytes, fibroblasts and macrophages as well as being well-supplied by vessels and nerve fibers (Kanitakis, 2002).

These components of the skin provide the basis of the complex multicellular communication network that is responsible for fine-tuning the barrier, sensory, motor, transport, thermoregulatory, regenerative, endocrine and exocrine functions of the skin. A telling example of this complex interplay is shown in the signaling cascade initiated upon the activation of sensory nerve endings located in the skin; not only does this result in antidromic signaling to the central nervous system, but also the orthodromic release of certain neuropeptides (e.g.: substance P, and calcitonin gene related peptide; Ansel et al., 1997; Luger et al., 2002). These mediators then activate non-neuronal cells and exert local immuno-endocrine effects, since almost all skin cell types have been shown to produce pro- and/or anti-inflammatory mediators that can then fine-tune the local immune response (Luger, 2002; Paus et al., 2006b; Rossterman et al., 2006). Production of various hormones by multiple cell types may also influence (via paracrine or autocrine action) the regulation of cellular metabolism and function of other cutaneous cell populations. The life-long rejuvenation of cutaneous cells and mini-organs, which is required for both the passive and active functions of the skin detailed above, is also greatly dependent on the well-orchestrated, local release of soluble mediators (growth and trophic factors, cytokines and chemokines) from skin cells (Ansel et al., 1997; Luger, 2002; Paus et al., 2003, 2006b; Roosterman et al., 2006).

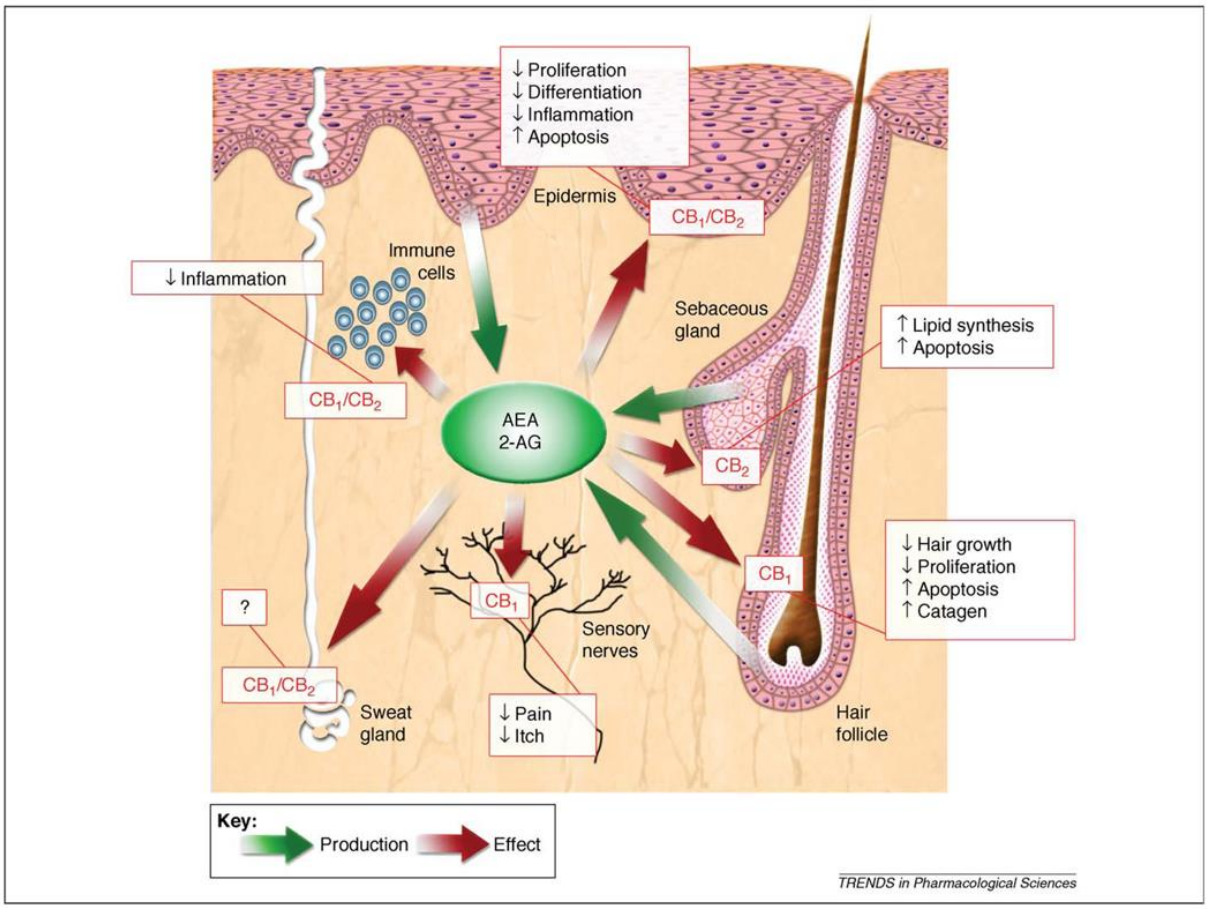


Figure 12.: The cutaneous ECS (from: Bíró et al., 2009)

It is not surprising therefore that in this varied and far-reaching network of signaling molecules the ECS has also emerged as a key regulatory determinant, in the skin as well (Figure 12.). The classical ECS receptors (CB1 and CB2) are differentially expressed in the human epidermis, with higher expression found in the more differentiated (i.e.: the granular and spinous) layers, which suggested their involvement in the regulation of differentiation and proliferation of these cells (Casanova et al., 2003; Ständer et al., 2005). Indeed, both phytocannabinoids and synthetic CB agonists inhibited the proliferation of cultured transformed human epidermal keratinocytes, although this effect proved to be independent of CB1 and CB2 (Wilkinson and Williamson, 2007). In contrast, on murine cells the growth-inhibitory effect (which was accompanied by apoptosis) could be prevented by relevant CB1 and CB2 antagonists (Casanova et al., 2003). Interestingly, synthetic CB1 and CB2 agonists had no appreciable effect on modulating cell growth of cultured normal human epidermal

keratinocytes (NHEKs) and non-tumorigenic human (HaCaT) and murine (MCA3D) keratinocytes (Casanova et al., 2003). Our own workgroup found however, that AEA markedly and dose-dependently caused cell death in cultured human keratinocytes, while inhibiting proliferation and inducing apoptosis *in situ*. These actions were found to be dependent on both CB1 and TRPV1 expressed by these cells, through a sequential CB1→TRPV1→Ca^{2+} influx signaling pathway (Tóth et al., 2011).

AEA has also been shown to regulate human epidermal differentiation, since locally produced AEA causes the transcriptional downregulation of keratin 1, keratin 5, involucrin and transglutaminase-5. These changes were mediated by increasing DNA methylation through MAPK-dependent pathways triggered by CB1 activation (Paradisi et al., 2008). In terms of skin barrier function, Roelandt et al. (2012) showed that CB1 and CB2 knockout mice display markedly different rates of barrier recovery after tape stripping; CB1 deficient mice exhibited delayed recovery, while this was increased in CB2 knockout mice, mainly due to increased lamellar body secretion. Markers for epidermal differentiation (filaggrin, loricrin and involucrin) and terminal differentiation (TUNEL assay and caspase-14) also changed in parallel with barrier recovery, i.e. they were respectively decreased and increased in CB1 and CB2 deficient mice. CB1 activation was also shown to cause downregulation of keratin 6, an important psoriasis-related marker, while also decreasing the proliferation of keratinocytes *in situ* (Ramot et al., 2013). Previously mentioned phytocannabinoids, CBD and CBG also decrease the expression of differentiation markers (e.g.: keratins 1 and 10, involucrin and transglutaminase 5), while CBDV had no such effect. Interestingly, although both CBD and CBG increased the methylation of the keratin 10 gene, only CBD increased global DNA methylation by enhancing the expression of DNA methyltransferase 1 (Pucci et al., 2013).

The ECS also influences the immune functions of keratinocytes. Mice with a keratinocyte-specific deletion of CB1 receptors developed increased and prolonged contact

hypersensitivity responses, while the same keratinocytes were shown to release increased C-X-C motif chemokine 10 (CXCL10) and chemokine (C-C motif) ligand 8 (CCL8) after interferon-γ (IFNγ) stimulation. The level of both cytokines was also increased in the contact allergic ear tissue, with CXCL10 recruiting CXCR3-expressing effector T cells, and CCL8 recruiting CCR2-expressing myeloid immune cells (Gaffal et al., 2013a). In line with these results, a subsequent study found that topical application of THC in a similar experimental setup effectively reduced ear swelling in wild type and CB1/2 receptor deficient mice by inhibiting IFNγ and IFNγ-induced CCL8 production, suggesting that in this case THC acts on CB1/2 independent pathways (Gaffal et al., 2013b).

The ECS has also been implicated in melanocyte-keratinocyte interactions, since UVB irradiation on melanocyte-keratinocyte co-cultures increased the CB1 receptor expression of melanocytes, in parallel to increasing endocannabinoid levels in keratinocytes. In the same study, activation of CB1 receptor using a selective agonist was shown to decrease melanogenesis, which data collectively suggest that UVB induced endocannabinoid production negatively regulates melanin synthesis in a paracrine fashion (Magina et al., 2011).

Ionotropic cannabinoid receptors (i.e.: thermo-sensitive TRP channels), as Ca^{2+} permeable channels, significantly modulate cellular Ca^{2+} homeostasis (Szallasi and Blumberg, 1999; Caterina and Julius, 2001; Clapham, 2003; Dhaka et al., 2006; Nilius and Mahieu, 2006; Ramsey et al., 2006; Nilius et al., 2007; Vriens et al., 2009) which is a key regulatory process that affects proliferation, differentiation and mediator production of various skin cells (Hennings et al., 1980; Lansdown, 2002; Proksch et al., 2008; Tóth et al., 2011). The expression of TRPV1 has been proven on numerous non-neuronal skin cells: epidermal and hair follicle keratinocytes, mast cells, Langerhans cells, sebocytes and endothelial cells (Bíró et al., 1998; Birder et al., 2001; Denda et al., 2001; Inoue et al., 2002; Southall et al., 2003; Amantini et al., 2004; Bodó et al., 2004, 2005; Ständer et al., 2004; Basu and Srivastava 2005; Tóth et al.,

2009a, 2011). On cultured human keratinocytes, stimulation with capsaicin or heat treatment induced TRPV1-dependent Ca^{2+} influx (since the concomitant application of the TRPV1 antagonist capsazepine could effectively block these changes), proving that the channel is functionaly expressed on these cells (Inoue et al., 2002; Southall et al., 2003; Bodó et al. 2004, 2005; Radtke et al., 2011). This elevation of intracellular Ca^{2+} decreased proliferation of cultured NHEKs and induced apoptosis (Tóth et al., 2011). It is not surprising therefore that TRPV1 activation delayed barrier recovery after tape stripping, which effect could also be blocked by CPZ (Denda et al., 2007). Oral administration of PAC-14028, another TRPV1 antagonist, also accelerated barrier recovery, highlighting the importance of this channel in skin physiology (Yun et al., 2011). TRPV1 also has important roles in the modulation of skin inflammation since its activation results in the release of numerous proinflammatory mediators (e.g.: IL-1β, IL-8, prostaglandin E2 [PGE2], transforming growth factor-β 2 [TGFβ2], matrix metalloproteinase [MMP]-1) from keratinocytes (Southall et al., 2003; Li et al., 2007; Lee et al., 2008; Jain et al., 2011). TRPV1 also takes part in the pro-inflammatory response induced by UV irradiation of cultured epidermal keratinocytes (Lee et al., 2009) and in mice *in vivo* (Lee et al., 2011).

TRPV4 activation, another thermosensitive channel activated by cannabinoids (see above), also accelerates barrier recovery in mice (Denda et al., 2007). In addition, deficiency of this channel causes leaky cell-cell junctions, pathophysiological actin arrangements, and insufficient stratification which leads to impaired barrier functions (Sokabe et al., 2010; Sokabe and Tominaga, 2010). The importance of this channel is further supported by *in vitro* data, since TRPV4 is functionaly co-expressed with junctional proteins such as β-catenin and E-cadherin (Kida et al., 2012). The activation of the channel augmented the expression of the junctional proteins claudin-4 and occludin, increased transepithelial electrical resistance and decreased

paracellular diffusion of labelled molecules through keratinocyte sheets, all of which point to a strengthening of the tight-junction barrier (Akazawa et al., 2013).

Interestingly, cold-sensitive TRP channels (TRPA1 and TRPM8) have been shown to behave similarly to TRPV4; i.e. their activation also prompts hastened barrier repair (Denda et al., 2010a, b). TRPA1 activity also increases the secretion of lamellar bodies, and, importantly, the blockade of this channel lead to the delay of barrier healing, which suggests that it is constitutively active in regulating the barrier homeostasis (Denda et al., 2010a). In cell cultures, the activation of either channel lead to marked changes in the expression of certain adhesion and extracellular matrix proteins as well as differentiation markers (Atoyan et al., 2009).

Cutaneous inflammation was also shown to be influenced by TRPA1; indeed, its activation enhanced ear swelling response and dendritic cell migration in a contact hypersensitivity model, while the blockade or genetic deletion of the channel decreased skin oedema, keratinocyte hyperplasia, leukocyte infiltration and scratching behavior in mice (Shiba et al., 2012). In agreement with these *in vivo* findings, TRPA1 stimulation of human keratinocytes *in vitro* evoked the production of IL-1α and IL-1β (Atoyan et al., 2009).

The pilosebaceous unit, compromised of the hair follicle and the sebaceous gland, influences numerous biological functions of the skin such as immunomodulation through the release of various cytokines and regeneration by acting as a reservoir of stem cells (Paus et al., 2006a, 2006b; Roosterman et al., 2006). We have shown that elements of this mini-organ constitutively produce AEA and 2-AG. Furthermore, AEA and THC (but not 2-AG) dose-dependently inhibited the proliferation of hair matrix keratinocytes and suppressed hair shaft elongation (Telek et al., 2007). Cannabinoids also induced characteristic signs of catagen transformation (intraepithelial apoptosis and premature hair follicle regression) in a CB1 dependent manner. The fact that CB1 expression fluctuates in a hair cycle-dependent manner also supports the idea that the CB1-mediated endocannabinoid signaling system in the follcilce

might act as a negative regulator of human hair growth (Telek et al., 2007). Indeed, a murine study demonstrated that CB1 receptor antagonists induce hair growth in mice (Srivastava et al., 2009). The activation of the ionotropic cannabinoid receptor TRPV1 on outer root sheath keratinocytes and human hair follicles showed a similar effect, as it inhibited hair shaft elongation and caused apoptosis on ORS keratinocytes (Bodó et al., 2005). Importantly, this phenomena could also be confirmed on TRPV1 knockout mice (Bíró et al., 2006).

In the case of sebocytes using the best available *in vitro* model system, i.e. the human, immortalized SZ95 sebocyte cell line (Zouboulis et al., 1999), we have also shown that these locally produced endocannabinoids, acting through a CB2 receptor → extracellular-signal-regulated kinase (ERK)1/2 MAPK → PPAR pathway, play a key role in the maintenance of the basal sebaceous lipid synthesis. On the other hand, endocannabinoid-treatment of the sebocytes resulted in dramatically elevated lipogenesis (Dobrosi et al., 2008). Knowing that one of the most common human skin diseases, acne vulgaris, is characterized by (among others) elevated lipogenesis (seborrhea) of the sebaceous glands (Kurokawa et al., 2009), based on the above findings, we concluded that the cannabinoid signaling and especially the local "endocannabinoid tone" generated by the sebocytes themselves, might be promising targets of novel anti-acne therapeutic approaches.

Intriguingly, this idea was further supported by our recent (Oláh et al., JCI, under revision) observations. In brief, we found that CBD exerted complex and remarkable cellular anti-acne effects, since (i) it normalized excessive lipogenesis induced by pro-acne agents both in a quantitative and qualitative manner; (ii) it reduced proliferation of the sebocytes; and (iii) it showed universal anti-inflammatory activity. These beneficial anti-acne effects (among which lipostatic and anti-proliferative activities were also evidenced *ex vivo* in full thickness human skin organ culture) were found to be mediated via two "unconventional" cannabinoid signaling pathways. Namely, the lipostatic and anti-proliferative actions developed upon

activation of TRPV4 ion channels, the subsequent inhibition of the pro-lipogenic ERK1/2 MAPK pathway, and the down-regulation of the "pro-lipogenic" target gene, nuclear receptor interacting protein-1 (NRIP1). On the other hand, the anti-inflammatory action of CBD was related to an adenosine A2a receptor → cAMP ↑ → tribbles homolog-3 (TRIB3) ↑ ┤ nuclear factor kappa-light-chain-enhancer of activated B cells (NFκB) axis, again highlighting the complexity of the cutaneous cannabinoid signaling (Figure 13.).

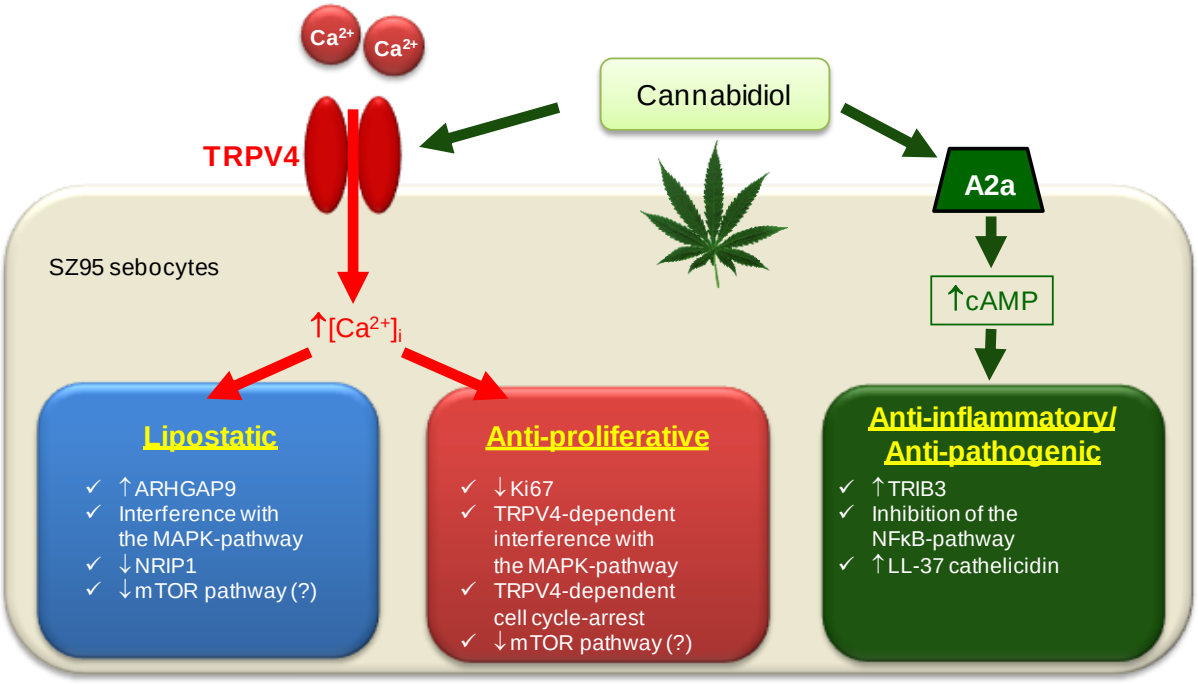


Figure 13.: Schematic overview of the cellular "anti-acne trinity" of CBD and its proposed mechanism of action (from Oláh et al., JCI, unpublished results)

In light of the above findings, it is not surprising at all that cutaneous (or at least sebaceous) cannabinoid signaling may very often lead to unexpected cell physiological results. Indeed, within the confines of another study (aiming at describing the biological role of the enzymes engaged with the regulation of the local endocannabinoid tone in the sebaceous glands), we found that application of certain FAAH-inhibitors (which were theorized to elevate the endocannabinoid tone, and hence further increase lipid synthesis) decreased the AEA-induced lipogenesis of the sebocytes, and exerted "CBD-like" complex anti-acne effects. In this case, our preliminary results suggest that the excessive "endocannabinoid-overload" led to

subsequent down-regulation of CB2 receptor, and therefore "switched off" this key lipogenic signaling pathway.

Similar to TRPV4, the activation of TRPV1 inhibited basal and induced lipogenesis, while also suppressing expressions of multiple genes involved in cellular lipid homeostasis (Tóth et al., 2009a). Interestingly, and in contrast to its effect on keratinocytes (see above), stimulation of TRPV1 suppressed the release of IL-1β (Tóth et al., 2011).

The ECS (and most especially CB2) has been conclusively proven to influence many aspects of both innate and adaptive immunity, and the overview of these results falls outside of the scope this current work. Overall, the ECS promotes mainly anti-inflammatory processes (for a comprehensive review see: Kendall and Nicolaou, 2013), and its role in the inflammatory processes of skin cells has been described above. On the immune cells of the skin, data is relatively sparse in relation to the ECS. Our own workgroup has shown however that monocyte-derived human dendritic cells (DC) express TRPV1 through the differentiation of monocytes to immature DCs (iDC), with a ten-fold increase in mRNA levels during this process. TRPV1 channels expressed on iDCs and mature DCs (mDCs) could be activated by the application of capsaicin, arguing for the functional expression of these proteins. Interestingly, the long-term application of capsaicin (5 days) did not induce either the differentiation of monocytes, or the activation of iDCs, in contrast to previous data reported on mice (Basu and Srivastava, 2005). Likewise, capsaicin did not significantly alter the viable cell number nor did it induce cell death of any form even at relatively high concentrations. One of the most important functions of iDC is the phagocytosis of foreign material (Banchereau and Steinman, 1998; Shortman and Liu, 2002; Gogolák et al., 2007), which was also inhibited by both long- and short-term capsaicin treatment (5 days and 2 hrs, respectively) in a TRPV1-dependent manner. Although capsaicin by itself did not initiate the maturation of iDCs, it did significantly inhibit the overexpression of maturational markers C-C chemokine receptor type 7 (CCR7) and CD83 induced upon

cytokine treatment). Moreover, capsaicin, via TRPV1, significantly yet differentially modulated the production and release of selected cytokines (i.e. increasing IL-10 while decreasing IL-6 and IL-12) from mDCs (Tóth et al., 2009b). All of these effects point to a rather anti-inflammatory effect of TRPV1 activation on human monocyte-derived DCs.

Goals

The overview presented above shows the ECS is not only complex in its heterogeneity and interactions with signaling systems previously thought to be unrelated to cannabinoids, but it also influences an ever-expanding array of physiological processes in practically all organs of the body. The overarching research goal of our workgroup is the investigation of the role of the ECS in the physiological processes of the skin. As part of our investigations, our research area presented in this thesis, on one hand, focused on elucidating the expression and putative functionality of the ECS on eccrine sweat gland cells, since the ECS was shown to have important regulatory roles on the biological processes on sebaceous glands, the other exocrine organ of the skin. On the other hand, we aimed at further elucidating the role of thermosensitive TRP channels on dendritic cells; since TRPV1 was shown to take part in the transduction of heat shock signaling in keratinocytes (Li et al., 2007) we aimed at determining whether it plays a similar role on iDCs.

Specifically, on eccrine sweat gland-derived NCL-SG3 epithelial cell, we aimed at investigating:

- The effect of endocannabinoids on the
    - Viability, apoptosis and necrosis of the cells
    - Differentiation of the cells
    - Secretory activity of the cells
- The expression of members of the ECS

- The signaling pathways involved in putative endocannabinoid effects

On human monocyte-derived DCs, we aimed at assessing:

- The expression of thermosensitive TRP channels
- The effect of heat shock on endocytotic activity of iDCs
- The involvement of thermo-TRP channels in mediating the putative heat shock effects

Materials and methods

Materials

Throughout the experiments, the following agents were used: AEA, 2-AG (Cayman, Ann Arbor, MI); AM251, CPZ, GF109203X, Ruthenium Red (RR) (Sigma-Aldrich, St. Louis, MO); AM630 (Tocris, Ellisville, MO); PD098059, Wortmannin (Calbiochem, Nottingham, UK). HC 067047 (Maybridge Ltd., Cambridge, UK).

NCL-SG3 cell cultures

Human eccrine sweat gland-derived NCL-SG3 epithelial cells were cultured in William's Medium E medium (Invitrogen, Paisley, UK) supplemented with 5 % fetal bovine serum, 10 μg/ml insulin-transferrin-selenium mixture, 20 ng/ml epidermal growth factor (all from Invitrogen), 2 mM L-glutamine, 10 ng/ml hydrocortisone, and antibiotic mixture (all from Sigma-Aldrich). Cells were passaged once they reached 80-90% confluence to avoid confluence-induced differentiation.

DC cultures

Thrombocyte-free buffy coats were acquired from the regional blood blank and centrifuged on Ficoll (GE Healthcare, Buckinghamshire, UK) gradient to separate the monomorphonuclear cells. Monocytes were subsequently isolated by immunomagnetic cell separation using anti-CD14-conjugated microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) according to the manufacturer's instructions. DC differentiation (which resulted in immature DCs, iDCs) was induced by supplementing AIMV medium (Invitrogen) with 80 ng/ml GM-CSF and 100 ng/ml IL-4 (both from Peprotech, London, UK). The same amount of cytokines was added at day 2, and the cells were cultured for another 3 days. Mature DCs

(mDCs) were generated by exchanging the culturing medium of iDCs with AIMV supplemented with a "pro-inflammatory cytokine cocktail" containing 80 ng/ml GM-CSF, 10 ng/ml TNF-α, 5 ng/ml IL-1β, 20 ng/ml IL-6 (all from Peprotech), and 1 μg/ml PGE2 (Sigma-Aldrich) on day 5 of culturing for one additional day. Cells were cultured at 37 °C, in a humidified environment containing 5% CO_2. All experiments were performed from a minimum of three independent donors.

Determination of cellular viability

The viability of the cells was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. Cells were plated in 96-well plates (10,000 cells/well) in quadruplicates, and were cultured for 2 days. Cells were then incubated with 0.5 mg/ml MTT for 2 hrs, and concentration of formazan crystals (as an indicator of number of viable cells) was determined colorimetrically at 550 nm. Results were expressed as percentage of vehicle controls regarded as 100%.

Determination of proliferation

Proliferation was assessed by the CyQuant Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's protocol. Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One) in quadruplicates and were treated with various compounds for the time indicated. Supernatant were then removed by blotting on paper towels, and the plates were subsequently frozen at -70˚C. The plates were then thawed at room temperature, and 200 μl of CyQuant GR dye/cell lysis buffer mixture was added to each well. After 5 min incubation, fluorescence was measured at 490 nm excitation and 520 nm emission wavelengths using a FLIPR (Molecular Devices, San Francisco, CA).

Determination of cytotoxicity (necrosis)

Necrotic cell death was determined by measuring the glucose-6-phophate-dehydrogenase (G6PD) release (G6PD Release Assay Kit, Invitrogen). The enzyme activity was detected by a two-step enzymatic process that leads to the reduction of resazurin into red-fluorescent resorufin. Cells (10,000 cells/well in the case of NCL-SG3 cells and 200,000 cells/well in the case of monocytes/iDCs) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One GmbH, Frickenhausen, Germany) in quadruplicates and treated with various compounds for 24-48 hrs (NCL-SG3 cells) or subjected to heat shock as described above. A 2x reaction medium was then prepared according to the manufacturer's protocol and added to the wells in 1:1 dilution. The fluorescence emission of resorufin was monitored by a Flexstation 3 fluorescent image plate reader (FLIPR; Molecular Devices, Sunnyvale, CA) at 545 nm excitation and 590 nm emission wavelengths. Results are presented as the percentage of the maximal G6PD release induced by detergent lysis of cells using undiluted Triton X-100 (Sigma-Aldrich).

Since the activity of the G6PD released from necrotic cells decreases over 24-36 hrs, the cytotoxic effects of long term treatment protocols were determined by Sytox Green staining (Invitrogen). The dye is able to penetrate (and then bind to the nucleic acids) only to necrotic cells with ruptured plasma membranes, whereas healthy cells with intact surface membranes show negligible SYTOX Green staining. NCL-SG3 cells were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One) and treated with various agents for the time indicated. Supernatants were then discarded and the cells were incubated with 1 μM SYTOX Green solution. Fluorescence of SYTOX Green was measured at 490 nm excitation and 520 nm emission wavelengths using a FLIPR (Molecular Devices).

Determination of apoptosis

The determination of apoptosis of iDCs was performed using the "Mitochondrial Membrane Potential Apoptosis Kit with Mitotracker™ Red & Annexin-V Alexa Fluor® 488 – for Flow Cytometry" according to the experimental protocol supplied by the manufacturer (Invitrogen). Cells were subjected to heat shock treatment as described previously or left at 37°C, 5% CO_2 for 1 hr. Sequential double labeling was then performed by the MitoTracker Red/Alexa Fluor® 488 annexin-V Reagents and the cells were analyzed by a FACScan flow cytometer (BD Biosciences). Emissions of the dyes were measured at 530 nm and 585 nm, respectively. The assay measures two hallmarks of apoptosis, namely the decrease in mitochondrial transmembrane potential and the externalization of phosphatidylserine. Cells undergoing apoptosis are MitoTracker negative and annexin-V positive, while living cells show very little green fluorescence and bright red fluorescence.

A different combination of techniques was used for the determination of apoptosis in NCL-SG3 cells. Mitochondrial membrane potential of NCL-SG3 cells was determined using a MitoProbe™ DiIC1(5) Assay Kit (Invitrogen) where the decrease in fluorescence intensity may reflects apoptotic processes. Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One) in quadruplicates and were treated with various compounds for the time indicated. After removal of supernatants, cells were incubated for 30 minutes with DilC1(5) working solution (30 µl/well) and the fluorescence of DilC1(5) was measured at 630 nm excitation and 670 nm emission wavelengths using a FLIPR. In addition, apoptosis of NCL-SG3 cells was also determined by fluorimetric measurement of activation of pro-apoptotic caspases using a Poly Caspases Detection Kit (Invitrogen). The method is based on a fluorescent inhibitor of caspases (FLICA™) methodology. The reagent covalently interacts with the active centers of activated caspases via a caspase-specific recognition sequence. The FLICA also contains a carboxyfluorescein group (FAM) which results in a green fluorescence

labeling of activated caspases. NCL-SG3 cells were cultured in 96 well black-well/clear-bottom plates (Greiner Bio-One) and treated with various compounds for the time indicated. FLICA working reagent (30x) was prepared following the manufacturer's protocol. Cells were incubated with 1x FLICA reagent diluted in culturing medium for 1 hr. Cells were then washed twice and fluorescence was measured at 490 nm excitation and 530 nm emission wavelengths using a FLIPR.

Determination of endocannabinoid levels

Endocannabinoid levels were measured by our collaborators. Cultured NCL-SG3 cells were homogenized in 0.5 ml of an ice-cold solution of methanol: Tris buffer (50 mM, pH 8) 1:1 containing 7 ng of $^{2}H_4$-anandamide ($^{2}H_4$-AEA). To each homogenate 2 ml of ice-cold chloroform:methanol 1:1 and 0.5 ml of 50 mM Tris buffer, pH 8, was added. The homogenate was centrifuged at 4ºC (500 g for 2 min), the chloroform phase recovered and transferred to a borosilicate tube, and the water phase extracted two more times with ice-cold chloroform. The combined extract was evaporated to dryness at 32 ºC under a stream of nitrogen. The dried residue was reconstituted in 110 µl of chloroform, and 2 ml of ice-cold acetone was added. The precipitated proteins were removed by centrifugation (1,800 x g, 10 min) and the clear supernatant was removed and evaporated to dryness. The dry residues were reconstituted in 50 µl of ice-cold methanol, of which 35 µl was used for analysis by liquid chromatography/in line mass spectrometry, using an Agilent 1100 series LC-MSD, equipped with a thermostated autosampler and column compartment. Liquid chromatographic separation of endocannabinoids was achieved using a guard column (Discovery HS C18, 2 cm x 4.0 mm, 3µm, 120A) and analytical column (Discovery HS C18, 7.5 cm x 4.6 mm, 3µm) at 32 ºC with a mobile phase of methanol:water:acetic acid 85:15:0.1 (v/v/v) at a flow of 1 ml/min for 12 minutes followed by 8 min of methanol:acetic acid 100:0.1 (v/v). The MSD (model LS) was

set for atmospheric pressure chemical ionization (APCI), positive polarity, and selected-ion-monitoring (SIM) to monitor ions m/z 348 for AEA, 352 for $^{2}H_4$-AEA, and 379 for 2-AG. The spray chamber settings were: vaporizer 400ºC, gas temperature 350ºC, drying gas 5.0 l/min, and nitrogen was used as the nebulizing gas with a pressure of 60 psig. Calibration curves were produced using synthetic AEA and 2-AG (Cayman). The amounts of AEA and 2-AG in the samples were determined using inverse linear regression of standard curves.

Determination of intracellular lipids

For semi-quantitative detection of intracellular lipids, cells were cultured on glass coverslips, and treated with various compounds for 24 hrs. Cells were then washed in phosphate-buffered saline (PBS; 115 mM NaCl, 20 mM Na_2HPO_4, pH 7.4; all from Sigma-Aldrich), fixed in 4% paraformaldehyde/PBS (Sigma-Aldrich), washed again twice in PBS and once in 60% isopropanol, and stained in freshly prepared Oil Red O solution (in 60% isopropanol) (Sigma-Aldrich) for 20 minutes at 37°C. Nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich) for 20 s and coverslips were finally mounted in mounting medium (DAKO, Glostrup, Denmark).

Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One) in quadruplicates and were treated with compounds for 24 hrs. Subsequently, supernatants were discarded and 100 µl of a 1 µg/ml Nile Red (Sigma-Aldrich) solution in PBS was added to each well. Fluorescence was measured on a FLIPR and results are expressed as percentages of the relative fluorescence units (RFU) in comparison with the controls using 485 nm excitation and 565 nm emission wavelengths for neutral (mostly cytoplasmic) lipids, and 540 nm excitation and 620 nm emission wavelengths for polar (mostly membrane) lipids.

Immunocytochemistry

Monocytes were seeded onto glass coverslips and iDCs were generated as described above. iDCs were fixed with acetone for five minutes and permeabilized with PBS containing 0.1% Triton-X-100 (Sigma-Aldrich). Cells were subsequently washed with PBS and non-specific binding sites were blocked with PBS containing 1% bovine serum albumin (BSA; Sigma). Immunolabeling was performed by incubating the cells with polyclonal rabbit antibodies against TRPV1 [ab63083], TRPV2 [ab63149], TRPV3 [ab63148], TRPV4 [ab39260], TRPA1 [ab58844], TRPM8 [ab104569] (all used at 1:200 dilution, Abcam PLC, Cambridge, UK), CB1 and CB2 (1:200 dilution, Cayman) for 60 minutes. Coverslips were subsequently washed three times in phosphate buffered saline (PBS) and further incubated with a FITC-conjugated secondary antibody (1:200, Vector Laboratories, Burlingame, CA) for 60 minutes. Cell nuclei were stained with DAPI after three final washes with PBS. Cells were visualized using a Nikon Eclipse E600 fluorescent microscope (Nikon, Tokyo, Japan).

Western blotting

Cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (8% gels were loaded with 30 μg protein per lane), transferred to BioBond nitrocellulose membranes (Whatman, Maidstone, UK), and then probed with rabbit primary antibodies against CB1, CB2 (both 1:200**),** TRPV1, TRPV2 and TRPV4 (all used at 1:500 dilution, Abcam PLC**)**. A horseradish peroxidase-conjugated goat anti-anti IgG antibody (1:1000, Bio-Rad, Hercules, CA) was used as a secondary antibody, and the immunoreactive bands were visualized by a SuperSignal® West Pico Chemiluminescent Substrate enhanced chemiluminescence kit (Pierce, Rockford, IL) using LAS-3000 Intelligent Dark Box (Fuji, Tokyo, Japan). To assess equal loading, membranes were re-probed with an anti-cytochrome-C (1:50, Santa Cruz, Santa Cruz, CA) or anti-beta-actin antibody (1:1000, Sigma-Aldrich) and

visualized as described above. Where appropriate, immunoblots were subjected to densitometric analysis using the Image Pro Plus 4.5.0 software (Media Cybernetics, Silver Spring, MD).

Quantitative real-time PCR (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Carlsbad, CA) by using the 5′ nuclease assay. Total RNA was isolated using TRIzol (Invitrogen). One μg of total RNA were then reverse-transcribed into cDNA by using 15 U of AMV reverse transcriptase (RT) (Promega, Madison, WI) and 0.025 μg/μl random primers (Promega). PCR amplification was performed by using the TaqMan primers and probes (Assay ID: Hs00275634_m1 for human CB1; Assay ID: Hs00361490_m1 for human CB2; Assay ID: Hs00391374_m1 for human DAGLα; Assay ID: Hs00373700_m1 for human DAGLβ; Assay ID: Hs00200752_m1 for human MAGL; Assay ID: Hs00419593_m1 for human NAPE-PLD; Assay ID: Hs00155015_m1 for human FAAH; Assay ID: Hs00196158_m1 for human cytokeratin (CK) 1; Assay ID: Hs00818825_m1 for human CK7; Assay ID: Hs01595539_g1 for human CK8; Assay ID: Hs00166289_m1 for human CK10; Assay ID: Hs00265033_m1 for human CK14; Assay ID: Hs01651341_g1 for human CK18; Assay ID: Hs00761767_s1 for human CK19; Assay ID: Hs00846307_s1 for human involucrin (INV); Assay ID: Hs01894962_s1 for human loricrin (LOR); Assay ID: Hs00856927_g1 for human filaggrin (FIL); Assay ID: Hs00218912_m1 for human TRPV1; Assay ID: Hs00901640_m1 for human TRPV2; Assay ID: Hs00376854_m1 for human TRPV3; Assay ID: Hs00540967_m1 for human TRPV4; Assay ID: Hs00375481_m1 for human TRPM8; Assay ID: Hs00175798_m1 for human TRPA1; Assay ID, Hs99999905_m1 for human glyceraldehyde 3-phosphate dehydrogenase (GAPDH, internal control); all from Applied Biosystems) using the TaqMan universal PCR master mix protocol (Applied Biosystems). The relative gene expression was

determined using the ΔΔCt method (Applied Biosystems); expression was normalized to either the ΔCt of either positive control samples or to the sample showing the highest expression.

Gene silencing using small interfering RNA (RNAi) probes

iDCs or NCL-SG3 cells at 50-70% confluence were transfected on the second day of differentiation or culturing after passaging with specific Stealth RNAi oligonucleotides (40 nM, Invitrogen) against TRPV channels (trpv1: HSS111306, trpv2: HSS12144, trpv4: HSS126974) or classical cannabinoid receptors (cnr1: HSS102082, cnr2: HSS102087) using Lipofectamine 2000 Transfection Reagent (Invitrogen). For controls, RNAi Negative Control Duplexes (scrambled RNAi; Invitrogen) were employed. In all cases, 3 sequences supplied by the manufacturer were applied separately. The efficacy of RNAi-driven ‘‘knockdown’’ was evaluated daily (in the case of NCL-SG3 cells) or on day 3 after transfection (for iDCs) by Western blotting as described above. Optical density was normalized to β-actin (antibody from Sigma-Aldrich, 1:1000 dilution) and expressed relative to cells transfected with scrambled RNAi.

Heat shock treatment and determination of endocytotic activity

Heat shocked DCs were incubated at 43°C for 1hr, while control cells were kept at 37°C. Endocytotic activity was then measured by the internalization of FITC-labeled dextran (Sigma-Aldrich). DCs were incubated with 1 mg/ml FITC-dextran at 37°C for 1 hr after heat shock treatment (negative control cells were treated with 1% sodium azide and kept at 0°C). Afterwards, the cells were washed three times with ice-cold phosphate buffered saline (PBS; 115 mM NaCl, 20 mM Na_2HPO_4, pH 7.4; all from Sigma-Aldrich), and then stored on ice until the fluorescence intensity was measured by a FACScan flow cytometer (BD Biosciences Immunocytometry Systems, Franklin Lakes, NJ). When applicable, DCs were pre-incubated

with CPZ (Sigma-Aldrich) or HC 067047 (TRPV4 antagonist, Maybridge Ltd.) for 15 minutes before heat shock, or with the appropriate vehicle control (Dimethyl sulfoxide for both compounds).

Patch-clamp measurements

The standard whole-cell patch-clamp techniques were employed in voltage-clamp configuration. Whole-cell measurements were carried out using Axopatch-200A amplifiers connected to personal computers using Axon Instruments Digidata 1440 data acquisition boards (Axon Instruments, Foster City, CA). For data acquisition and analysis, the pClamp10 software package (Molecular Devices) was used. Pipettes were pulled from GC 150 F-15 borosilicate glass capillaries (Clark Biomedical Instruments, Pangbourne, UK) in five stages and fire polished to gain electrodes of 2-5 MΩ resistance in the bath. iDCs were identified and selected for patch-clamp recording in a Nikon TE2000 microscope. The normal bath or extracellular (EC) solution given in mM was as follows: 145 NaCl, 5 KCl, 1 $MgCl_2$, 2.5 $CaCl_2$, 5.5 glucose, and 10 HEPES (pH 7.35, 305 mOsm). The pipette solution was as follows: 140 CsCl, 5 EGTA and 10 HEPES (pH 7.20, ~295 mOsm). The temperature of the EC solution perfusing the DCs was maintained (TC2bip Temperature Controller, Cell MicroControls) at a constant level of either ~37°C or ~45°C. The bath solution was warmed up to ~37°C using a custom-made Petri dish. Thermal stimuli were applied using a preheated bath solution and temperature was monitored using a thermistor placed close to the cell. Bath perfusion around the measured cell was achieved using a gravity-flow perfusion system. Excess fluid was removed continuously. RR, CPZ and HC 067047 were applied by switching the perfusion system to a preheated bath solution containing the relevant antagonists.

Ca^{2+} imaging

Changes in $[Ca^{2+}]_i$ upon drug applications were detected by fluorimetric Ca^{2+} imaging. Cells were seeded in 96-well black-well/clear-bottom plates (Greiner Bio-One) at a density of 10,000 cells/well and then were incubated with culturing medium containing 2 μM of the cytoplasmic calcium indicator Fluo-4 AM (Invitrogen) at 37°C for 40 min. The cells were washed four times with and finally cultured in Hank's solution containing 1% bovine serum albumin and 2.5 mM Probenecid (both from Sigma-Aldrich) for 30 min at 37 °C. The plates were then placed in a FLIPR and changes in $[Ca^{2+}]_i$ (reflected by changes fluorescence; lEX=494 nm, lEM=516 nm) induced by various concentrations of the drugs were recorded in each well (during the measurement, cells in a given well were exposed to only one given concentration of the agent).

Immunoprecipitation

Cells were washed several times with ice-cold PBS and scraped into 1 ml of radioimmunoprecipitation assay (RIPA) buffer containing 50 mM Tris–HCl, 1% NP-40, 0.25% Na-deoxycholate, 1 mM NaCl, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 1 mM Na2VO4, 1 mM EDTA (all from Sigma-Aldrich), and were disrupted by sonication on ice. After mixing on an orbit shaker, the samples were incubated on ice for 15 min and then centrifuged in a microcentrifuge at 4 °C for 15 min at 15,000×g. The supernatant was removed, mixed with 100 μl of protein A/G Agarose (Sigma) and 5 μl of anti-TRPV2 antibody (Abcam), and then immunoprecipitation was performed by rotating the samples overnight at 4 °C. The samples were spun at 15,000×g at 4 °C for 5 min, the pellet was washed three times with RIPA buffer, and then resuspended in SDS-PAGE sample buffer and boiled for 10 min at 100 °C. All samples were then subjected to Western blotting as described above.

Statistical analysis

When applicable, data were analyzed using a two-tailed un-paired *t*-test and $P<0.05$ values were regarded as significant differences.

Results

1. Role of the ECS in sweat gland biology

Endocannabinoids cause mainly apoptotic cell death of human sweat gland cells

Since endocannabinoids have been conclusively proven to affect the life cycle of skin cells (see above), our initial question in relation to sweat gland cells was whether classical endocannabinoids, AEA and 2-AG, exert similar effects on the NCL-SG3 sweat gland cell line. Using colorimetric MTT and fluorimetric CyQuant assays, we found that both endocannabinoids dose-dependently suppressed the viable cell number and proliferation of NCL-SG3 cells (Figure 14.a, b). In an effort to differentiate between various forms of cell death, a series of functional assays was performed. Based on quantitative fluorimetric determinations, both endocannabinoids significantly decreased mitochondrial membrane potential (reflecting mitochondrial disturbance) (Figure 14.c) and induced the activation of pro-apoptotic caspases (Figure 14.d), hallmarks of apoptosis (Green and Reed, 1998; Susin et al., 1998; Tóth et al., 2011). In addition, higher concentrations of 2-AG significantly increased the release of G6PD (Figure 14.e) and Sytox Green accumulation to the cells (Figure 14.f), two complementary indicators of necrosis/cytotoxicity. AEA did not cause any observable necrosis. These findings suggested that the endocannabinoids suppressed cellular growth and induced apoptosis-driven cell death of human sweat gland cells, while causing minimal necrosis.

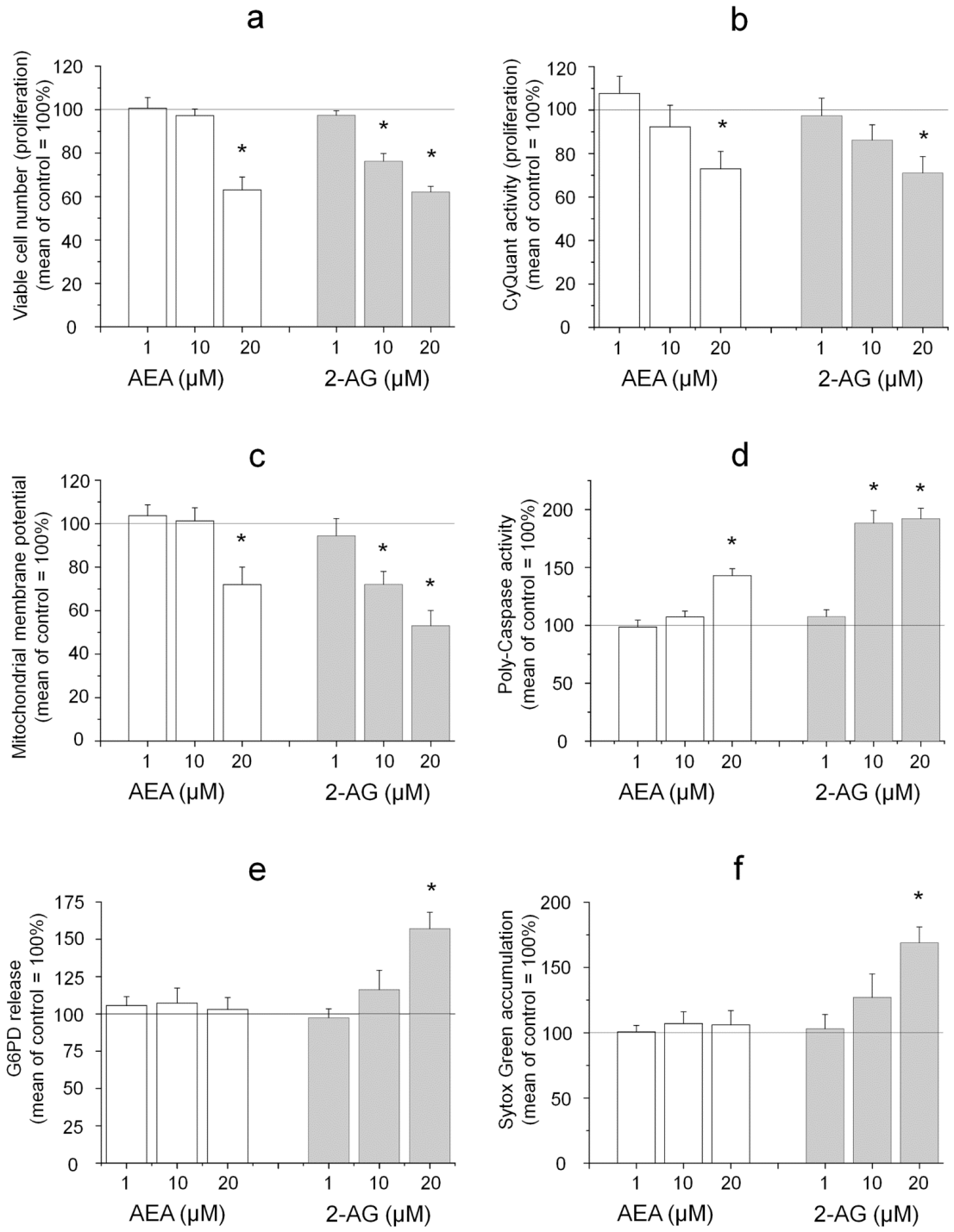


Figure 14.: *Endocannabinoids modulate cell growth and survival of NCL-SG3 cells:*
NCL-SG3 cells were treated by endocannabinoids (AEA, 2-AG) for 48 hrs. **a)** Determination of cell viability by colorimetric MTT cell viability assay. **b)** Determination of proliferation by fluorimetric CyQuant assay. Quantitative measurement of apoptosis by **c)** fluorimetric $DilC_1(5)$ apoptosis assay reflecting mitochondrial membrane potential and **d)** fluorimetric Poly-Caspase apoptosis assay reflecting activation of pro-apoptotic caspases. Quantitative measurement of necrosis by **e)** G6PD release assay and **f)** Sytox Green assay. Data (mean±SEM) are expressed as a percentage of the mean value (defined as 100 %, dotted line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the control group. n=4 in each group. Three-four additional experiments yielded similar results.

Endocannabinoids modulate expressions of cytoskeletal proteins and lipid synthesis of NCL-SG3 cells

Since most epithelial cells initiate their differentiation program with the onset of apoptosis (Candi et al., 2005; Proksch et al., 2008), we next wished to investigate whether endocannabinoids also take part in the regulation of this process on sweat gland cells. Although the expression of certain cytokeratins (CK) and cytoskeleton proteins (involucrin, filaggrin and loricrin) involved in the differentiation of skin cells have also been described on sweat gland epithelium (Ohnishi and Watanabe, 1999; Langbein et al., 2005; Tharakan et al., 2010), the exact details of the differentiation processes have not been investigated on cultured sweat gland cells.

Therefore, we analyzed the expression of various epithelial "differentiation markers" such as CKs (CK1, 7, 8, 10, 14, 18, 19) (Candi et al., 2005; Moll et al., 2008) as well as of involucrin, filaggrin, and loricrin in NCL-SG3 cells. Of the tested markers only CK1 and 10 were not detectable whereas all other molecules were identified (Figure 15.). Interestingly, expressions of certain CKs, namely CK7, 14, 18 and 19, were the highest in pre-confluent proliferating cultures, while CK8, involucrin filaggrin and loricrin were predominant in post-confluent, and presumably more differentiated cells. It appears, therefore, that expression profiles of these molecules in NCL-SG3 sweat gland cells, in part similar to those described e.g. in cultured epidermal keratinocytes (Papp et al, 2003, 2004; Candi et al, 2005; Langbein et al, 2005; Moll et al, 2008), are strongly affected by the growth rate (i.e. proliferation vs. growth arrest) of the cells.

We next investigated the effects of AEA and 2-AG on the expression of these markers. Pre-confluent (30-40%) NCL-SG3 cells were treated with AEA and 2-AG (10 μM each) for 48 hrs and the levels of the above markers were analyzed by Q-PCR. The expression of post-confluent markers (CK8, involucrin, filaggrin and loricrin) were significantly and differentially

increased by the two endocannabinoids. The expression of certain markers of proliferating cells were concomitantly suppressed upon endocannabinoid treatment (CK7 by 2-AG; CK14 by AEA and 2-AG).

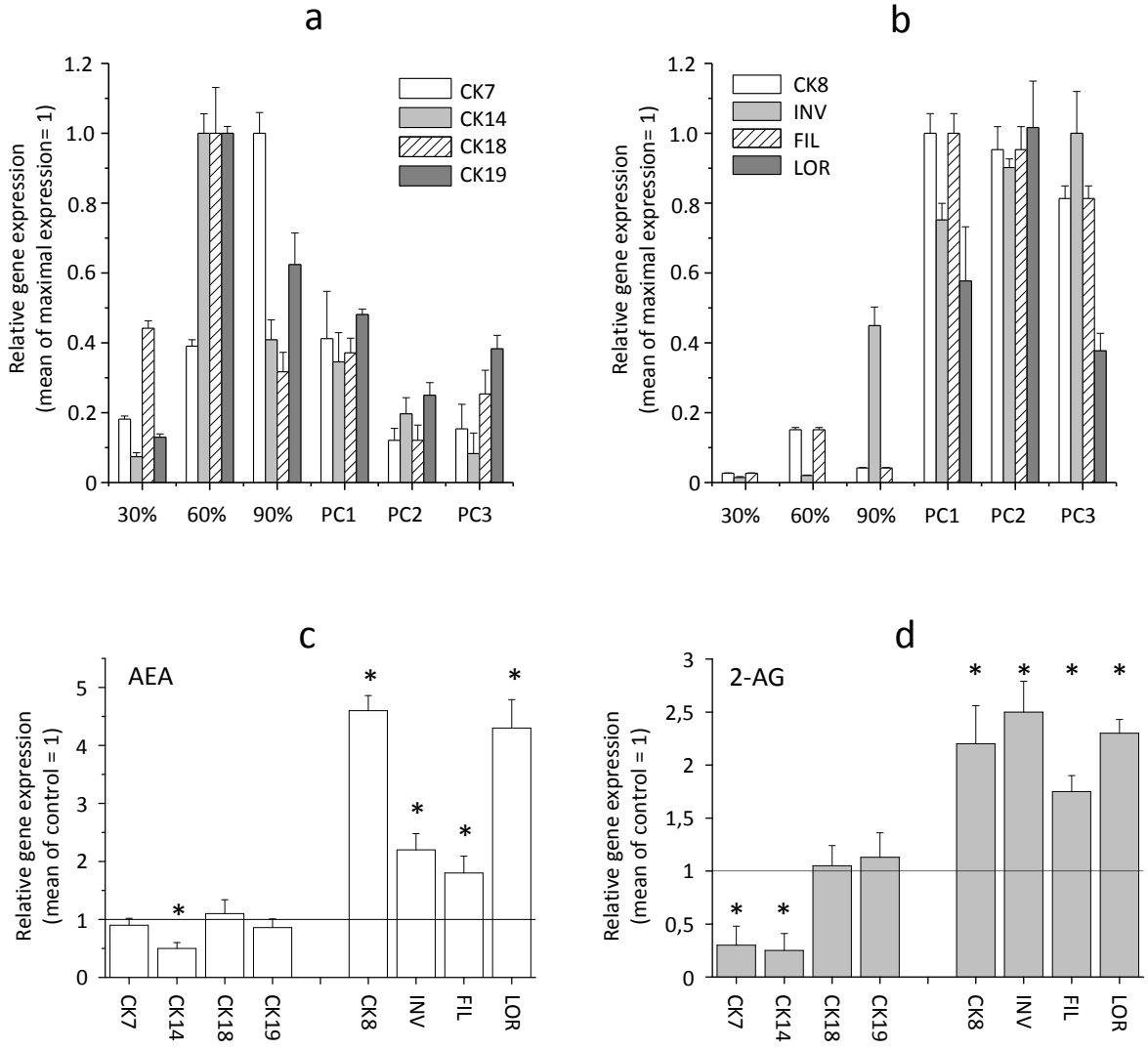


Figure 15.: *Endocannabinoids modulate expressions of cytoskeleton proteins and lipid synthesis of NCL-SG3 cells:*
a, b) Q-PCR analysis of various cytokeratins (CK7, 8, 14, 18, 19) and involucrin (INV), filaggrin (FIL), and loricrin (LOR) on NCL-SG3 cells at various confluences. PC1-3, 1-3 days at post-confluence. **c, d)** Q-PCR analysis of the above "differentiation markers" after treating NCL-SG3 cells with 10 μM AEA (**c**) or 2-AG (**d**) for 48 hrs. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100 %, straight line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the control group. n=4 in each group. Three-four additional experiments yielded similar results.

Endocannabinoids have previously been shown to markedly increase the lipid synthesis of cultured human sebocytes (Dobrosi et al., 2008). Since sweat gland epithelial cells were

shown to synthesize a wide-array of lipids (Takemura et al., 1989); we also assessed whether endocannabinoids have a similar effect on the lipid synthesis of NCL-SG3 cells. As measured by semi-quantitative Oil Red-O histochemistry and by quantitative Nile Red-based fluorimetry, both endocannabinoids (as early as 24 hrs after treatment) markedly and dose-dependently elevated neutral (but not polar) lipid synthesis of the cells (Figure 16.). Neutral lipids reflect the intracellularly stored, "de novo" synthesized lipid pool; as such, our data suggest that endocannabinoid treatment may exert a profound role in the regulation of secretory activity of human eccrine sweat gland cells by modifying the composition of produced sweat.

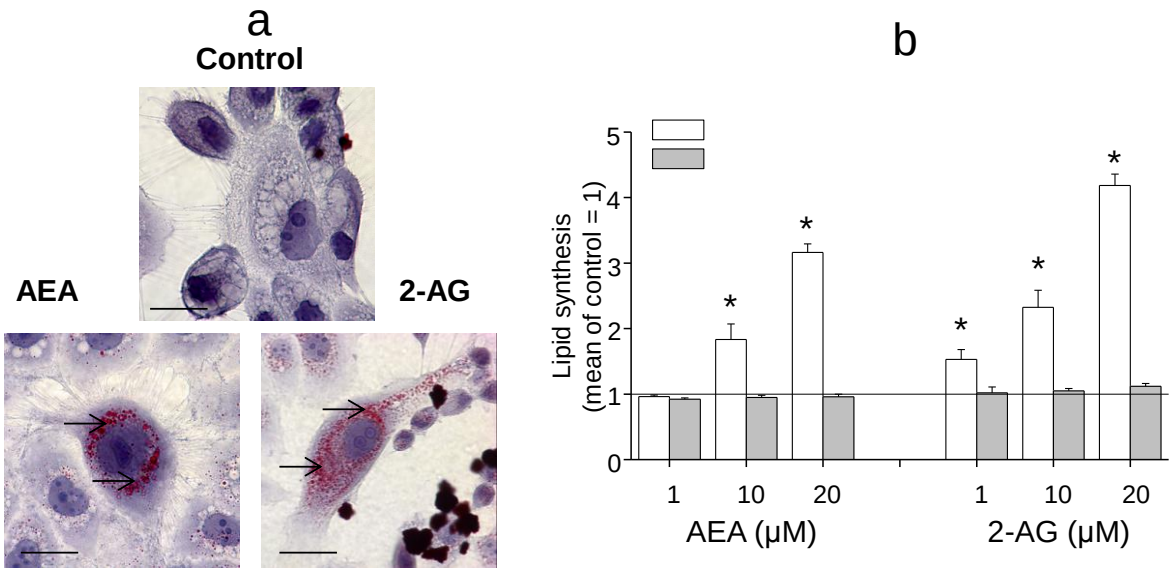


Figure 16.: *Endocannabinoids modulate expressions of cytoskeleton proteins and lipid synthesis of NCL-SG3 cells:*
a) Oil-Red O labeling after treating the cells by 10 μM AEA or 2-AG for 24 hrs. Arrows point to the relevant histochemical products. The scale bar marks 10 μm. **b)** Quantitative measurement of intracellular lipids as assessed by Nile red labeling followed by FLIPR measurement. Neutral lipids indicate intracellular lipids. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100 %, straight line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the control group. n=4 in each group. Three-four additional experiments yielded similar results.

Multiple members of the ECS are expressed by NCL-SG3 cells

AEA and 2-AG are both known to activate metabotropic and ionotropic cannabinoid receptors (see above). To determine which of these possible targets are present on sweat gland cells, we performed Q-PCR and Western blot analysis. CB1 and CB2 are expressed on both the mRNA and protein level; interestingly, the level of expression markedly altered in parallel with

the confluence of the cells (Figure 17.). Specifically, the two receptors behaved in opposite ways: CB1 expression was the highest in the post-confluent cultures, with the expression increasing during culturing, while CB2 levels were highest in pre-confluent cultures, and its expression decreased with culture time. These data suggest that cannabinoids might play a role in the regulation of growth and differentiation of human sweat gland-derived cells.

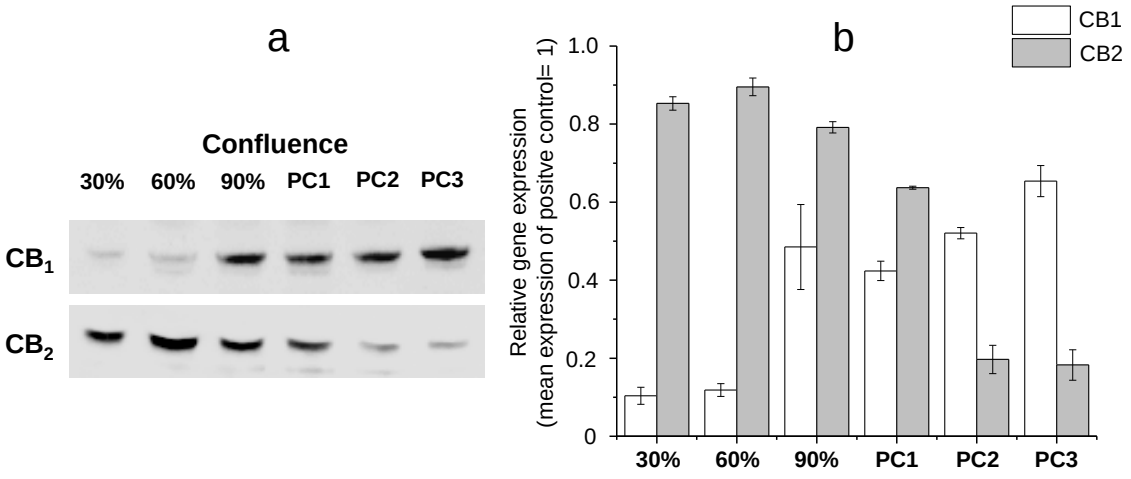


Figure 17.: *CB receptors and ECS enzymes are expressed in NCL-SG3 cells:*
a) Western blot analysis. Protein expressions of CB1 and CB2 were determined on cell lysates of NCL-SG3 cells harvested at various confluences. PC1-3, 1-3 days at post-confluence. **b)** Q-PCR analysis of mRNA transcript expression profiles of CB_1 and CB_2 at various confluences. Data (mean±SEM) are expressed as a fraction of the mean value of expressions (defined as 1) determined in cultured human epidermal keratinocytes (used as a positive control, Paradisi et al, 2008; Tóth et al, 2011). Three additional experiments yielded similar results.

The expression profile of ionotropic cannabinoid receptors (i.e.: thermosensitive TRP channels) was also determined by Q-PCR analysis. Our data shows that human sweat gland cells express TRPV1-4 (Figure 18.), while cold-sensitive channels TRPM8 and TRPA1 could not be detected (data not shown).

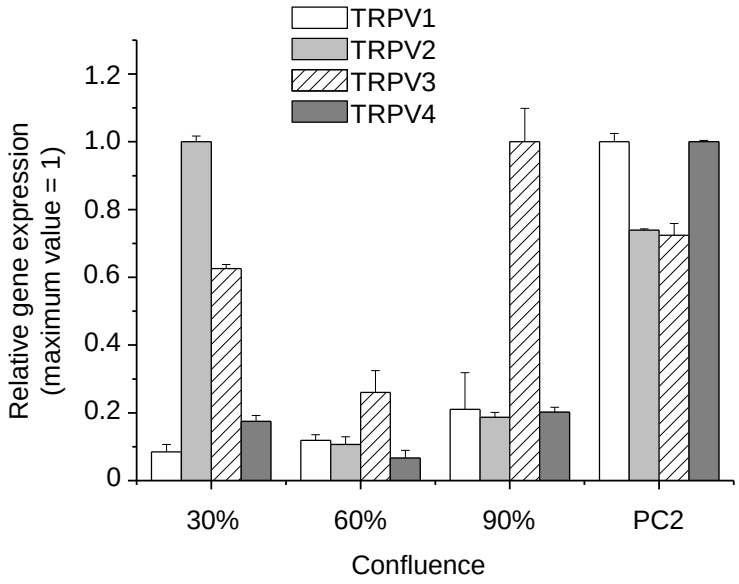


Figure 18.: *TRP channels are expressed on NCL-SG3 cells:*
Q-PCR analysis of mRNA transcript expression profiles of TRPV1, TRPV2, TRPV3 and TRPV4 at various confluences. Data (mean±SEM) are expressed as a fraction of the mean value of expressions (defined as 1) in the sample showing highest transcript levels for each gene. Three additional experiments yielded similar results.

Since numerous skin cells have been shown to produce endocannabinoids (see section: The ECS in the skin), with the help of our collaborators we also investigated whether sweat gland epithelial cells could also be considered a source of these lipid mediators. Mass spectrometry analysis by our collaborators revealed that NCL-SG3 sweat gland cells produce the prototypic endocannabinoids AEA and 2-AG, however, at a much lower concentrations than those reported in other skin cells (AEA, 15 fmol/10^6 cells; 2-AG, 0.2 pmol/10^6 cells in NCL-SG3 cells compared to AEA, 160 fmol/10^6 cells; 2-AG, 4.2 pmol/10^6 cells in human SZ95 sebocytes).

Based on these data we next examined the expression of the enzymatic machinery required for their synthesis and degradation. Using Q-PCR we were able to determine that not only are the synthesizing (NAPE-PLD and DAGLα and β) and degrading enzymes (FAAH and MAGL) present on the cells (Figure 19.), but their expression level fluctuates in parallel with the confluence of the cells; this further suggests that there is a possible role for the ECS in the regulation of growth and most probably differentiation of these cells.

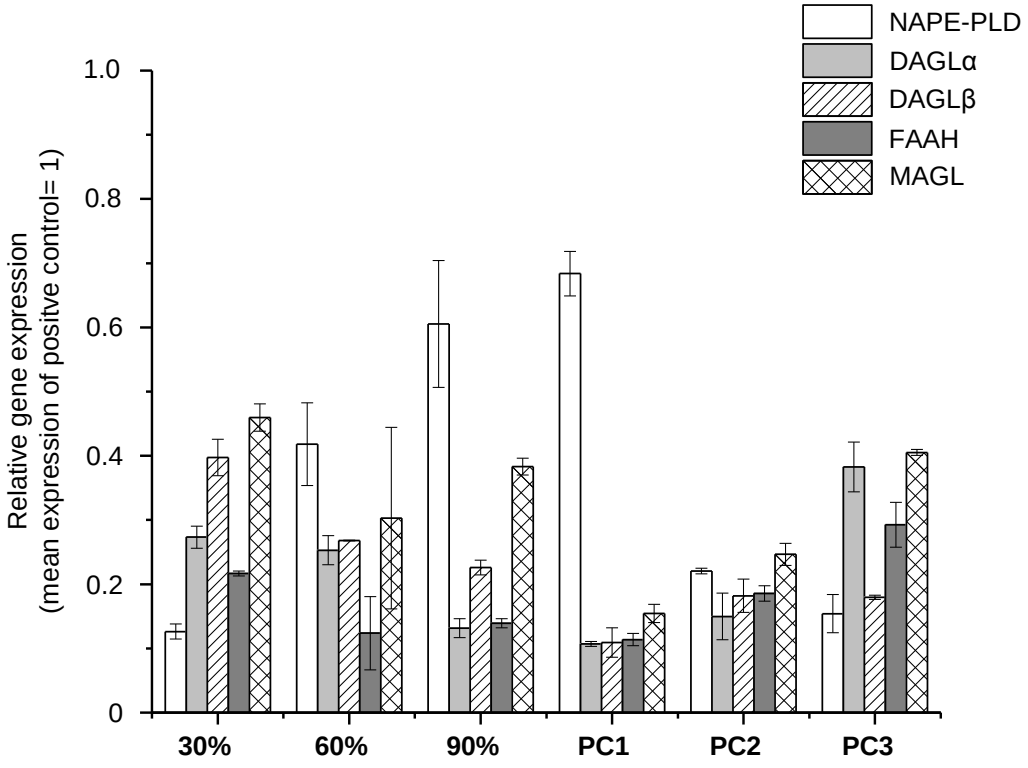


Figure 19.: *NCL-SG3 cells express enzymes of the ECS:*
Q-PCR analysis of various endocannabinoid synthesizing (DAGLα, DAGLβ, NAPEPLD) and degrading enzymes (MAGL and FAAH) on NCL-SG3 cells at various confluences. PC1-3, 1-3 days at post-confluence. Data (mean±SEM) are expressed as a fraction of the mean value of expressions (defined as 1) determined in cultured human epidermal keratinocytes (used as a positive control). Three additional experiments yielded similar results.

To support these results we also performed immunohistochemical labeling on human skin sections; importantly, we were able to identify the enzymatic machinery of the ECS on eccrine sweat glands *in situ* (Figure 20.).

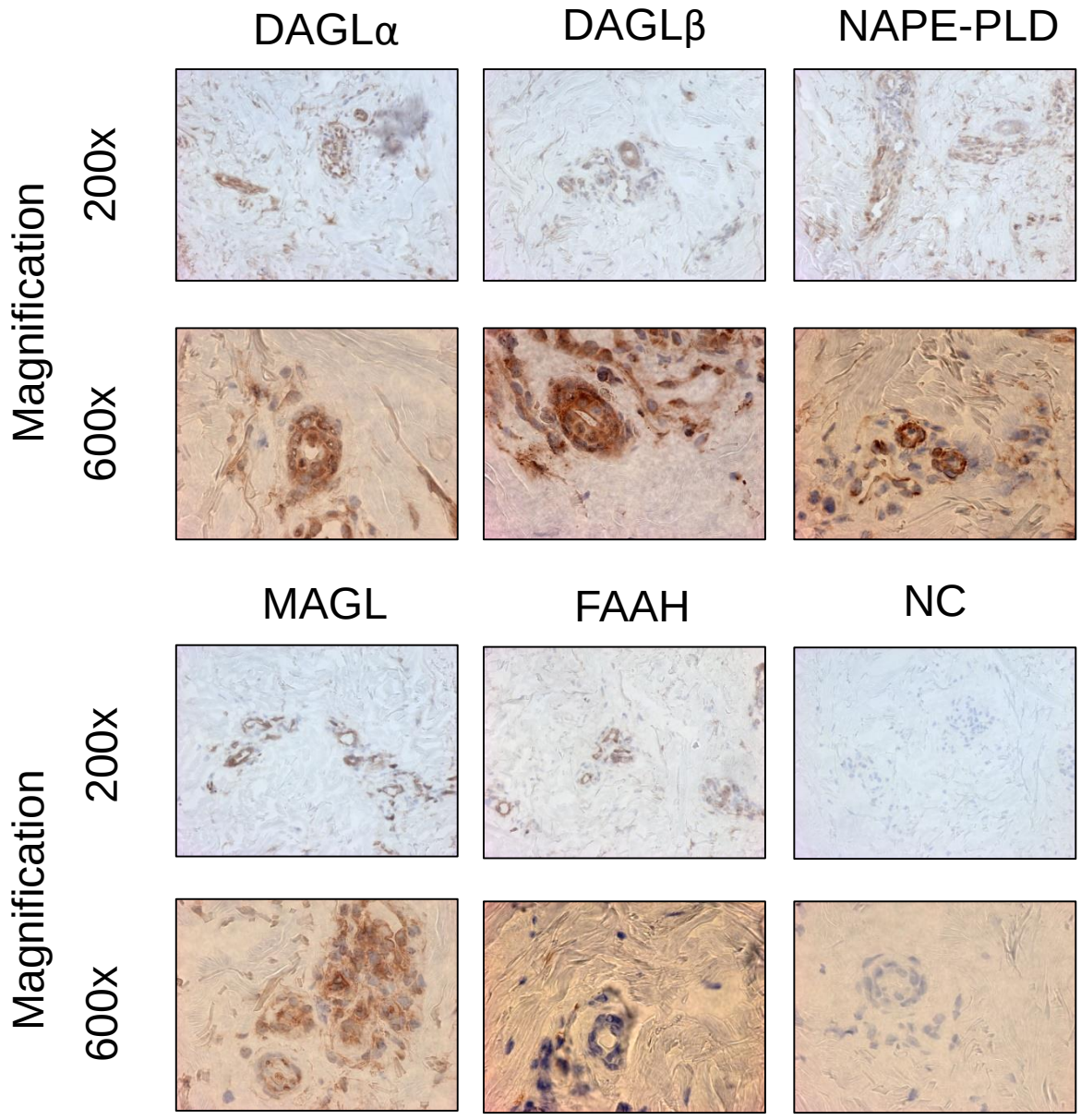


Figure 20.: *Human sweat glands express components of the ECS in situ* Immunohistochemical analysis of various endocannabinoid synthesizing (DAGLα, DAGLβ, NAPE-PLD) and degrading enzymes (MAGL and FAAH of as revealed by EnVision technique (brown staining), on human sweat gland epithelial cells *in situ*.

Endocannabinoid effects are not mediated by classical metabotropic receptors or TRP channels

After demonstrating that endocannabinoids (i) cause apoptotic cell death; (ii) increase the expression of differentiation-associated markers; and (iii) modulate the composition of secreted

sweat, we next wished to elucidate the putative involvement of the cannabinoid receptors expressed by these cells.

To assess the role of classical metabotropic cannabinoid receptors CB1 and CB2, we employed various antagonists and the RNAi technique on the endocannabinoid effects on cellular growth, survival and secretory activities (MTT assay) and lipid production (Nile Red fluorimetry). AM251 and AM630, inhibitors of CB1 and CB2 receptors, respectively, had no effect when applied independently of AEA or 2-AG. Importantly, both endocannabinoids were still able to suppress cellular viability and stimulate lipid synthesis in the presence of either antagonist (Figure 21.).

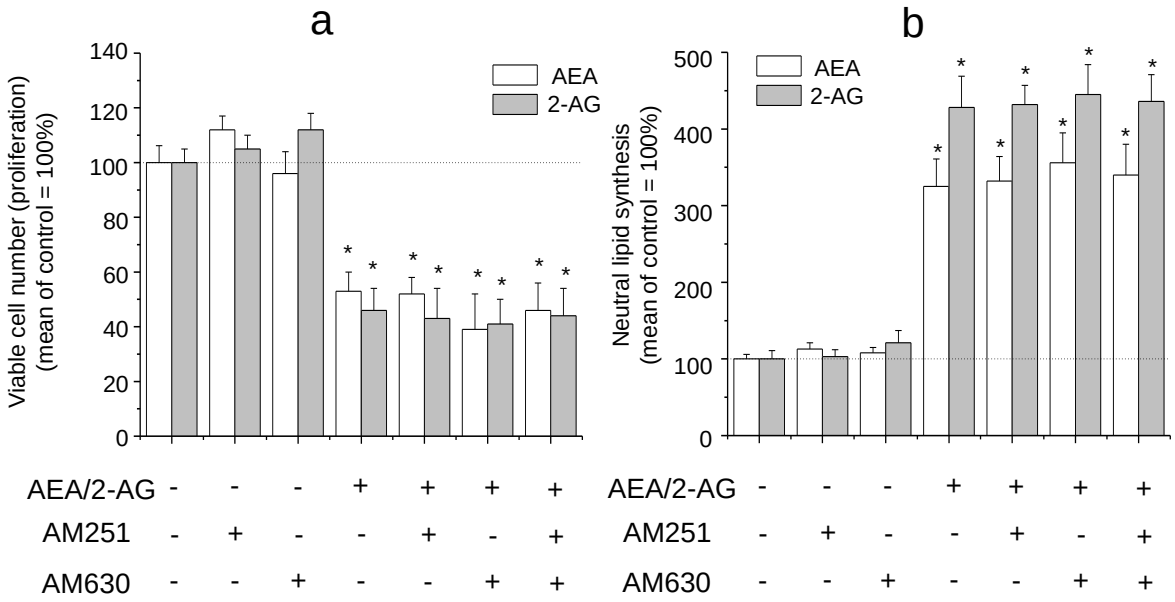


Figure 21.: *Pharmacological antagonists of CB1 and CB2 have no effect on endocannabinoid-dependent cellular processes on NCL-SG3 cells:* **a, b)** Cells were treated with AEA (20 μM), 2-AG (20 μM), AM251 (5 μM), AM630 (5 μM), or the indicated combinations. **a**) MTT cellular viability/proliferation assay performed after 48 hrs. **b)** Quantitative assessment of neutral lipids by Nile Red labeling followed by FLIPR measurement after 24 hrs. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100%, dotted line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the vehicle-treated control group (**a, b).** n=4 in each group. Two-three additional experiments yielded similar results.

Since both inhibtors have been reported to act as inverse agonists, and this may compromise their specificities on CB receptors, RNAi experiments were carried out to ascertain the results detailed above. The efficacy of RNAi-dependent knockdown was verified on both mRNA and protein levels with Q-PCR and Western blot respectively (Figure 22.). The level of

CB1 and CB2 expression was significantly and specifically decreased at day 3 after transfection. Of further importance, this knockdown was reversible, since the expression returned on day 4; moreover, scrambled RNAi probes had no effect on the expression of either CB1 or CB2 (Figure 22.).

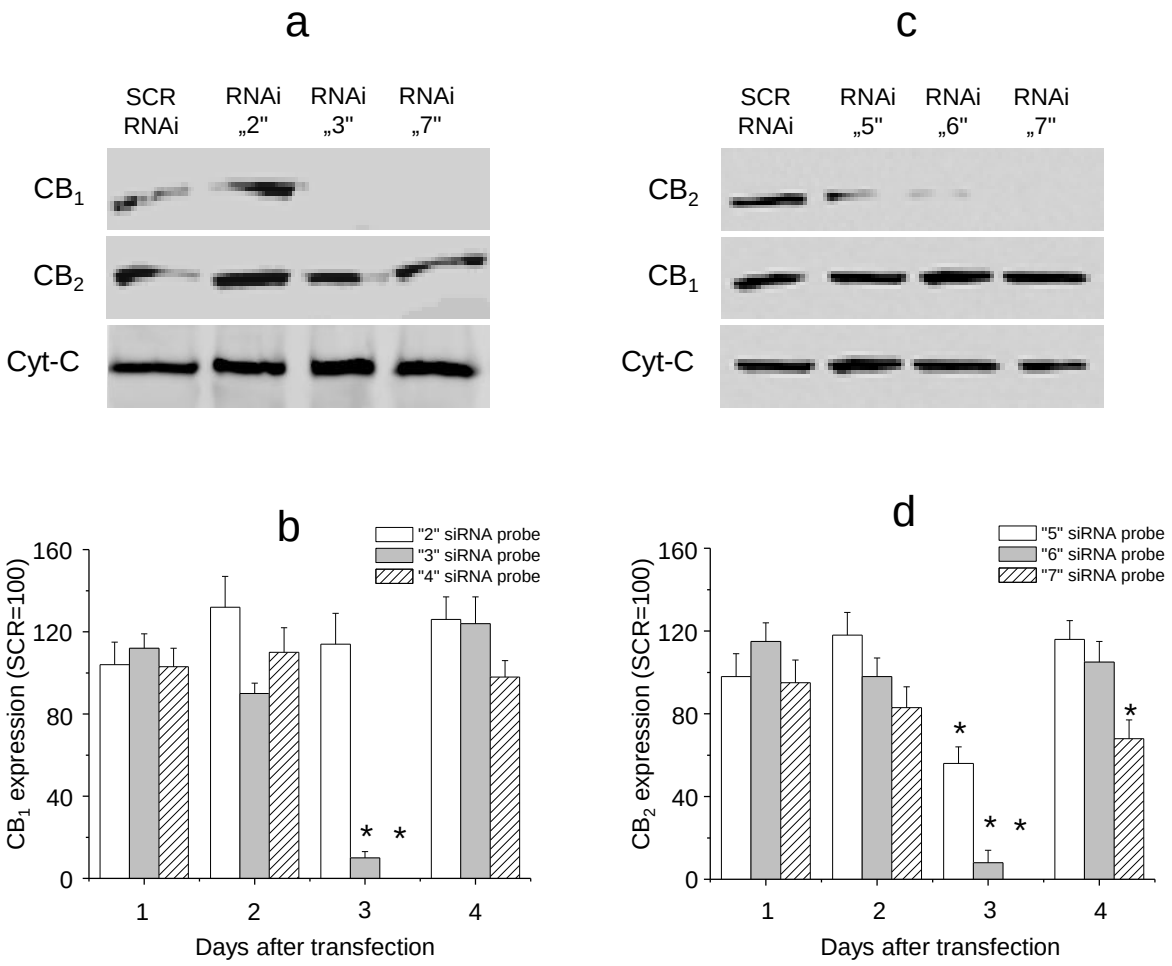


Figure 22.: *Assessment of efficacy of RNAi on NCL-SG3 cells:* Various RNAi probes against CB1 or CB2, as well as a scrambled RNAi probe (SCR), were introduced to cells by transfection. To evaluate the efficacy of this intervention, at days 1-4 after transfection, cells were subjected to Western blot analysis. **a, c**) Representative Western blot results at day 3 after transfection. As a house-keeping molecule, expression of Cytochrome-C (Cyt-C) was determined. **b, d**) Statistical analysis of Western blot data. Optical density (OD) values of specific immunosignals were determined at days 1-4 after transfection in 3 independent experiments. Normalized OD values (to Cyt-C) in each group were then averaged and expressed as mean ± SEM as the percentage of the averaged values of the respective SCR-treated groups regarded as 100%. * marks significant (P<0.05) differences compared to the SCR-treated groups.

We subsequently investigated the effects of AEA and 2-AG on NCL-SG3 cells with silenced CB1 or CB2. Similarly to the results obtained with pharmacological inhibitors, CB1 and CB2 knockdown could not prevent the growth-inhibitory and differentiation-promoting

cellular actions of the tested endocannabinoids (Figure 23.). These results, in line with the above data obtained with the antagonists, collectively argue that CB1 or CB2 coupled intracellular signaling mechanisms most probably do not participate in mediating the effects of endocannabinoids on human sweat gland epithelial cells.

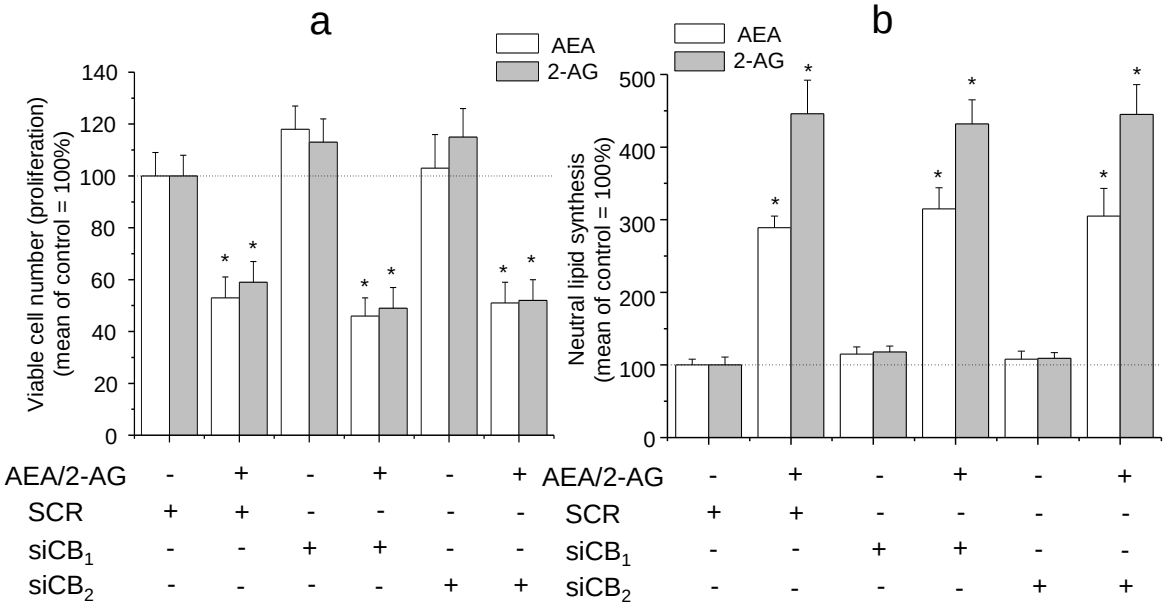


Figure 23.: *RNAi mediated knockdown of CB1 and CB2 has no effect on endocannabinoid-dependent cellular processes on NCL-SG3 cells:* **a, b)** Various RNAi probes against CB1 or CB2, as well as a scrambled RNAi probe (SCR), were introduced to cells by transfection. Gene silenced as well as SCR-transfected cells were then treated with AEA (20 μM) or 2-AG (20 μM). **b)** Quantitative assessment of neutral lipids by Nile Red labeling followed by FLIPR measurement after 24 hrs. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100%, dotted line) of the SCR group. * marks significant (P<0.05) differences compared to the vehicle-treated control group (**a, b).** n=4 in each group. Two-three additional experiments yielded similar results.

Since sweat gland cells also express TRP channels that have been implicated as ionotropic cannabinoid receptors, we next investigated whether Ca^{2+}-influx is involved in the action of AEA and 2-AG. Neither endocannabinoid caused any change in intracellular calcium concentration (data not shown). We found that neither the "universal" TRP channel antagonist Ruthenium Red nor the suppression of extracellular Ca^{2+} had any effect on the cellular growth suppression or lipid induction observed upon endocannabinoid treatment (Figure 24.).

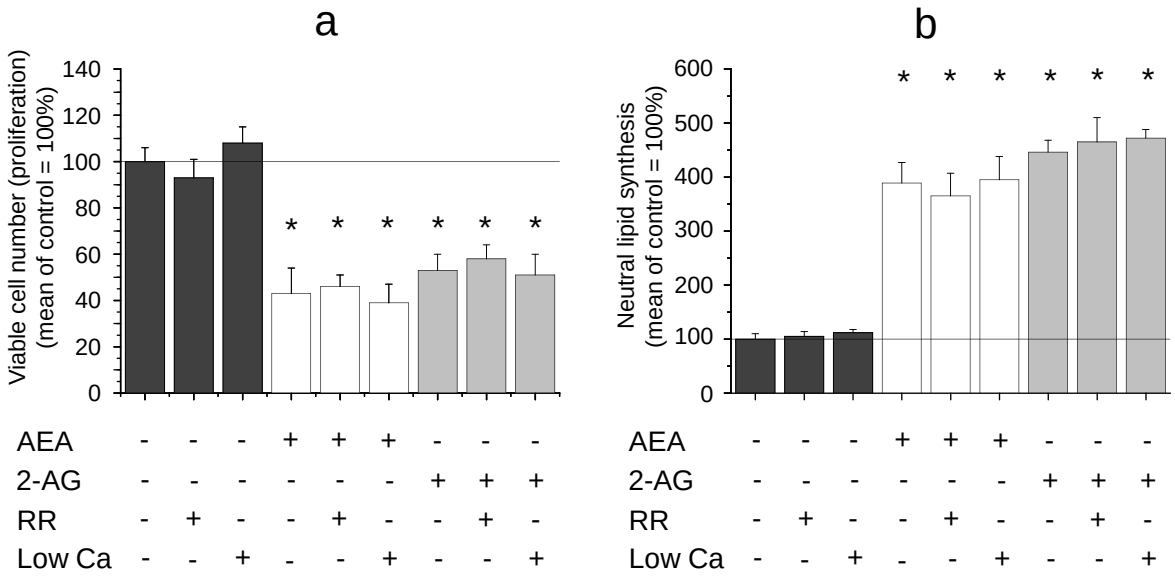


Figure 24.: *Effects of endocannabinoids are not mediated by TRP channels expressed on NCL-SG3 cells:* **a, b)** Cells were treated with AEA (20 μM), 2-AG (20 μM), Ruthenium Red (100 μM, RR), or combination. In addition, endocannabinoid treatment was performed when the extracellular Ca^{2+}-concentration was decreased from 1.8 mM to 0.2 mM (Low Ca). **b**) MTT cellular viability/proliferation assay performed after 48 hrs. **c)** Quantitative assessment of neutral lipids by Nile Red labeling followed by FLIPR measurement after 24 hrs. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100%, dotted line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the vehicle-treated control group n=4 in each group. Two additional experiments yielded similar results.

Taken together, these findings suggest that the effects of cannabinoids are not relegated through either metabotropic or ionotropic cannabinoid receptors, identified by us, in human sweat gland epithelial cells.

Endocannabinoids selectively stimulate the MAPK pathway in NCL-SG3 cells

Although our efforts to determine the receptor activated by endocannabinoids proved, sadly, fruitless, we also aimed at identifying the putative intracellular signaling pathways involved in the transduction of their effects. As described above, cannabinoids are known to activate a wide range of signaling pathways; hence we investigated whether the most common of these are involved in our current experiments. Therefore, the potential involvement of the MAPK, protein kinase C (PKC) isoenzymes and phosphatidylinositide 3-kinase (PI-3K) was assessed.

We found that pharmacological inhibitors of PKC enzymes and PI-3K (GF109203X and Wortmannin respectively) did not affect the growth-inhibitory and lipid synthesis-promoting actions of endocannabinoids (Figure 25.a, b). On the other hand, the MAPK inhibitor PD098059, almost completely prevented the effects of both AEA and 2-AG. Furthermore, the endocannabinoids also induced the transient phosphorylation of the MAPK Erk1/2 (p42/44) (Figure 26.a), which effect was also abrogated by the application of the aforementioned antagonist (Figure 26.b). These findings collectively argued for the crucial involvement of the MAPK pathway in mediating the actions of endocannabinoids in human sweat gland epithelial cells.

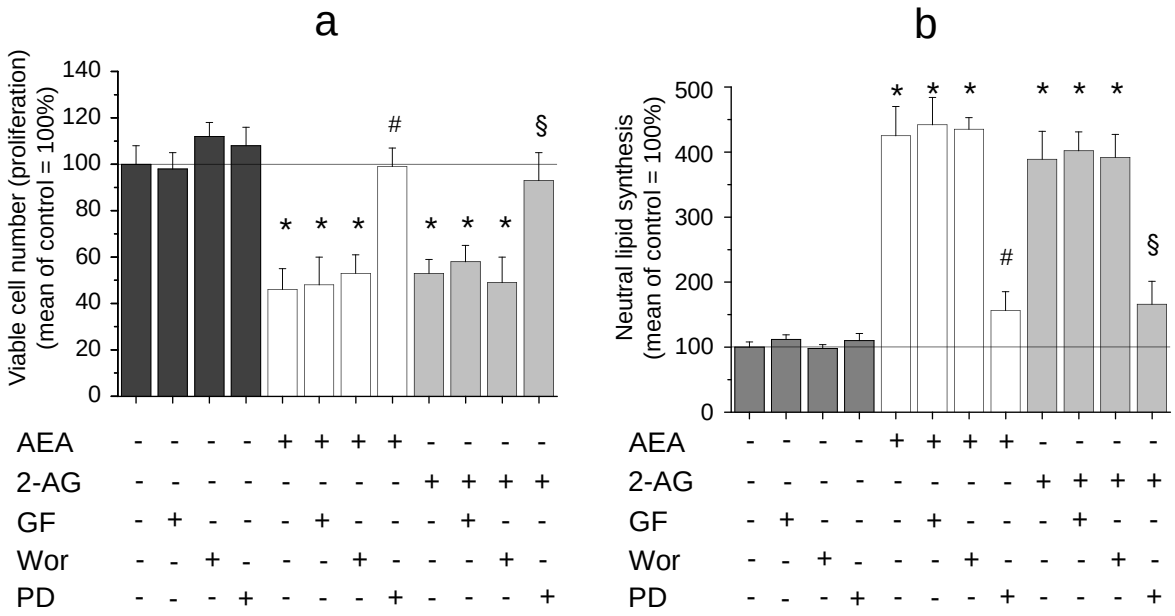


Figure 25.: *Functional effects of endocannabinoids are mediated by the MAPK pathway on NCL-SG3 cells:* Cells were treated with AEA (20 μM), 2-AG (20 μM), GF109203X (1 μM, GF), Wortmannin (0.5 μM, Wor), PD098056 (20 μM, PD), or combinations. **a**) MTT cellular viability/proliferation assay performed after 48 hrs. **b)** Quantitative assessment of neutral lipids by Nile Red labeling followed by FLIPR measurement after 24 hrs. Data (mean ± SEM) are expressed as a percentage of the mean value (defined as 100%, dotted line) of the vehicle-treated control group. * marks significant (P<0.05) differences compared to the vehicle-treated control group. # marks significant (P<0.05) differences compared to the AEA treated group. § marks significant (P<0.05) differences compared to the 2-AG treated group. n=4 in each group. Two-three additional experiments yielded similar results.

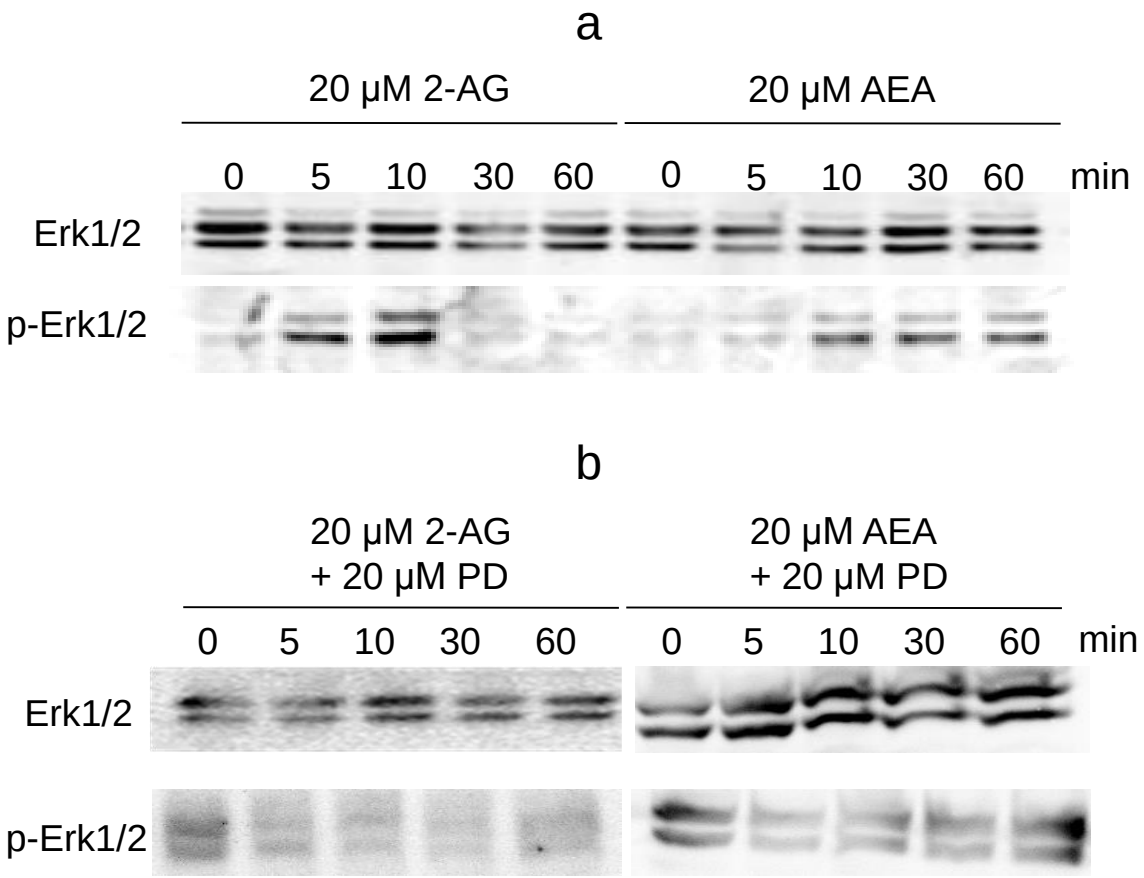


Figure 26.: *Endocannabinoid treatment activates the MAPK pathway on NCL-SG3 cells:*
a, b) Cells were treated by AEA and 2-AG (**a**) or in combination with PD (**b**) for the time indicated then Western blotting was performed to reveal expressions of the MAPK Erk1/2 (to assess equal loading) and its phosphorylated form (p-Erk1/2). Two-three additional experiments yielded similar results.

2. *Role of TRP channels in DC biology*

DCs express various heat-sensitive TRP channels

In the first step of our experiments, we aimed at elucidating the putative involvement of thermosensitive TRP channels in the transduction of heat shock on human monocyte-derived dendritic cells, we identified the expression of thermosensitive TRP channels on monocytes, iDCs and mDCs dendritic cells using QCPR. We found that all three cell types express TRPV1, TRPV2 and TRPV4 at the mRNA level, with the expression of all three increasing concomitantly with differentiation (Figure 27.). The protein level of these channels was also verified by Western blotting and (in the case of iDCs) with immunocytochemistry (Figure 28.). Interestingly, the expression of TRPV3, TRPM8 and TRPA1 was not identified on any of the tested cells by Q-PCR (Figure 27.) or immunolabeling (Figure 28.a).

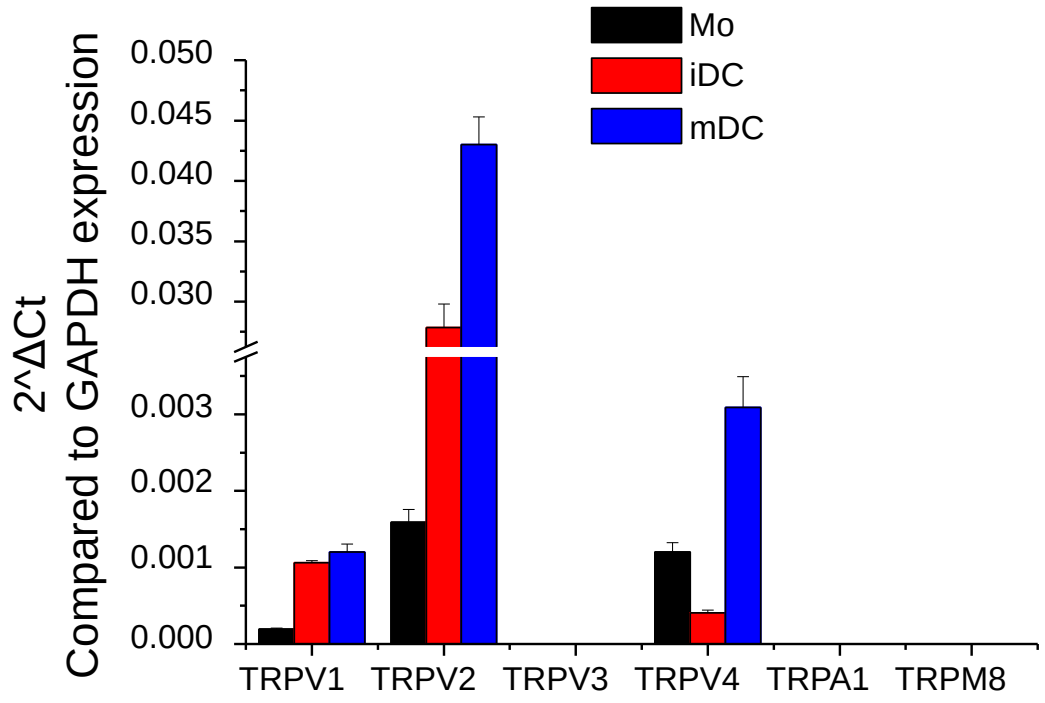


Figure 27.: *Human monocyte-derived DCs express TRPV1, TRPV2 and TRPV4 of the thermosensitive TRP channels at the mRNA level* Q-PCR analysis of thermosensitive TRP channels on monocytes, iDCs and mDCs. Data of channel expression was normalized to the level of GAPDH of the same sample and are expressed as mean±SEM (N=4).

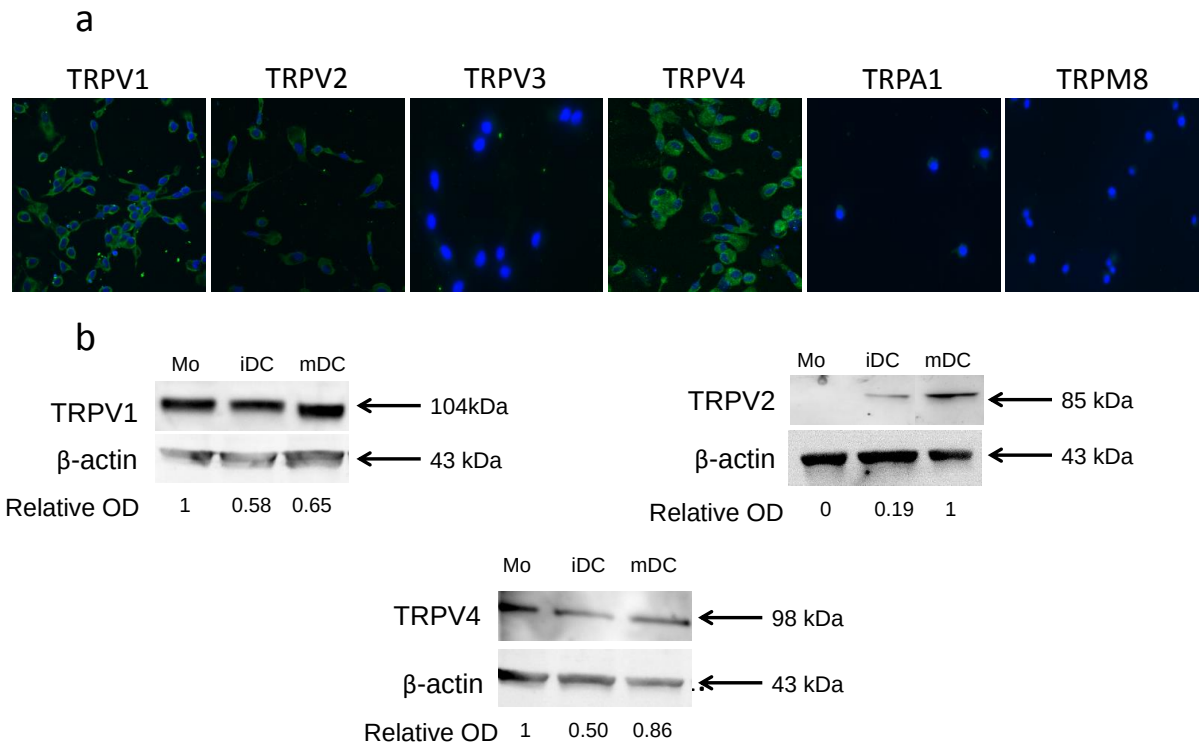


Figure 28.: *Human monocyte-derived DCs express TRPV1, TRPV2 and TRPV4 at the protein level* (a) TRPV1, TRPV2, TRPV3, TRPV4, TRPA1, and TRPM8 immunoreactivity on iDCs as determined by immunofluorescence (FITC, green). Nuclei were counterstained by DAPI (blue). Note the lack of signals in samples stained with TRPV3, TRPA1 and TRPM8 antibodies; since the isotype of all antibodies were identical these slides were used as negative control. Magnification: 400x. (b) Western blot analysis of TRPV1, TRPV2 and TRPV4. Optical density (OD) was normalized to β-actin and expressed as relative OD values compared to cells that showed the highest expression.

Heat shock decreases endocytosis in a TRPV1-independent manner

To determine whether heat shock has similar effects to TRPV1 activation by capsaicin (previously reported by our workgroup; Tóth et al., 2009b) on human monocyte-derived DCs, we investigated its effect on one of the most important functions of iDCs, namely their endocytotic activity.

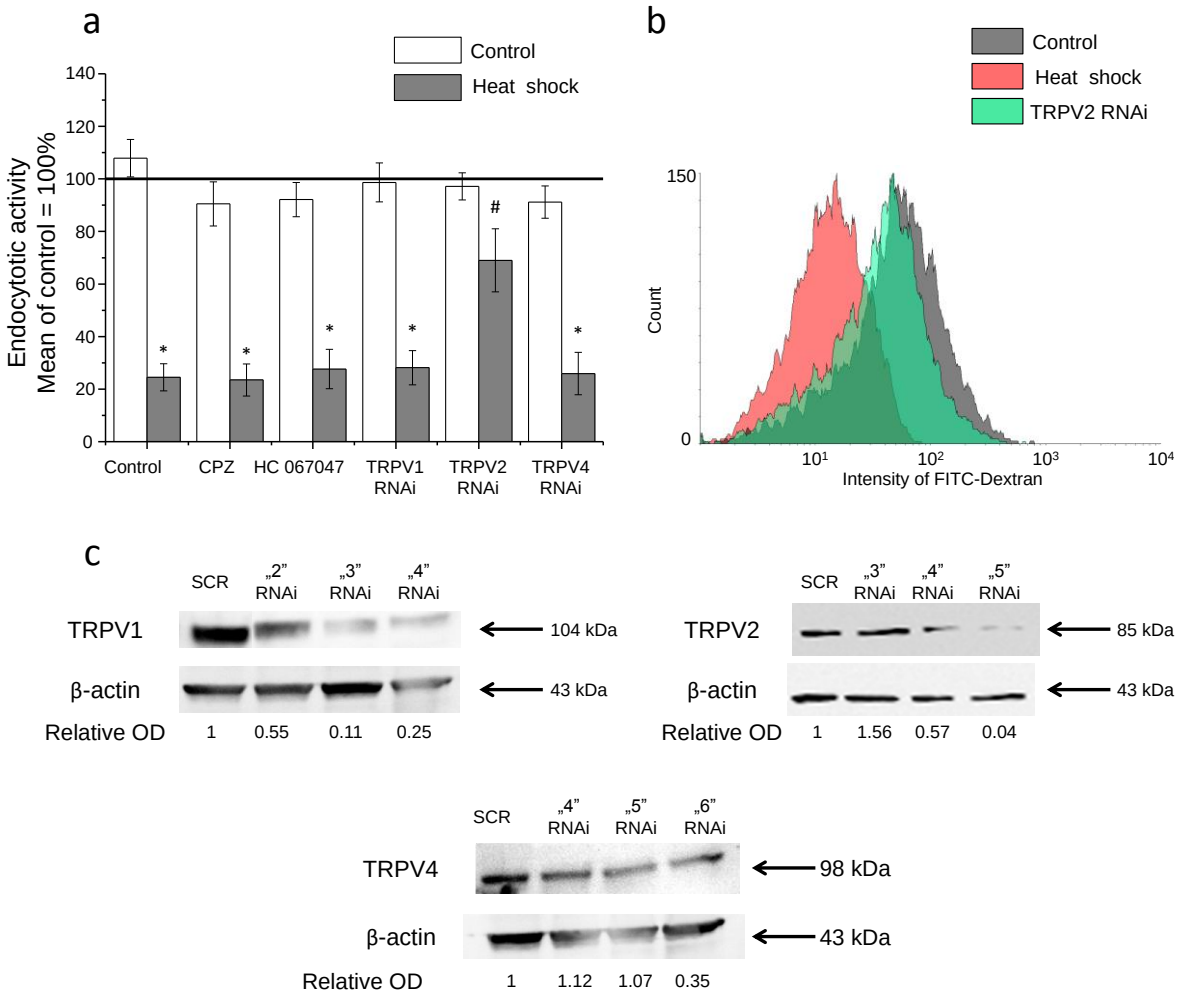


Figure 29.: *Heat shock decreases endocytotic activity of iDCs* (**a**) Endocytosis of iDCs as measured by FITC-dextrane internalization after heat shock (43°C for 1 hr). For the pharmacological studies, DCs were pre-incubated with 5 μM capsazepine (CPZ) or 1 μM HC 067047 for 15 minutes before heat shock. Control cells were kept at 37°C for 1 hr with or without the relevant antagonist(s). For RNAi experiments, cells were investigated three days after the transfection with the relevant probes. Data are expressed as mean±SEM of four independent donors as the percentage of the control (100%, solid line). * marks significant (P<0.05) differences compared to the control cells. # marks significant (P<0.05) differences compared to the heat shocked control. (b) Representative histogram of endocytosis of FITC-dextran after heat shock. (c) Evaluation of efficacy of RNAi by Western blotting. Optical density (OD) was normalized to β-actin and expressed as relative OD values compared to cells transfected with scrambled RNAi (SCR, control cells). Images present results of the three TRPV1-, TRPV2- and TRPV4-specific RNAi constructs ("2-4", "3-5" and "4-6" RNAi, respectively) employed.

We found that a short heat shock (43ºC for 1 hr) decreased the endocytosis of iDCs (Figure 29.a), while at the same time it did not induce significant necrotic (Figure 30.a) or apoptotic (Figure 30.b) cell death, which echoes our previous data regarding the activity of TRPV1 on DCs. In spite of the fact that the above temperature range is appropriate for the activation of TRPV1, and, moreover, previous reports have implicated TRPV1 in the transduction of heat shock effects on keratinocytes (Li et al., 2007), antagonism of TRPV1 by

CPZ was unable to abrogate the suppression of endocytotic activity (Figure 29.a). Likewise, RNAi mediated knockdown of TRPV1 (albeit efficacious, see Figure 29.c) did not protect DCs from the effect of heat shock. The application of a TRPV4 antagonist, HC 067047, and the RNAi mediated silencing of TRPV4 also failed to influence the effect of heat shock (Figure 29a.). RNAi mediated knockdown of TRPV2, on the other hand (as demonstrated in Figure 29c.), significantly prevented the action of heat shock (Figure 29.a and 29.b) (due to the lack of commercially available, highly selective TRPV2 antagonists, we were unable to perform pharmacological experiments).

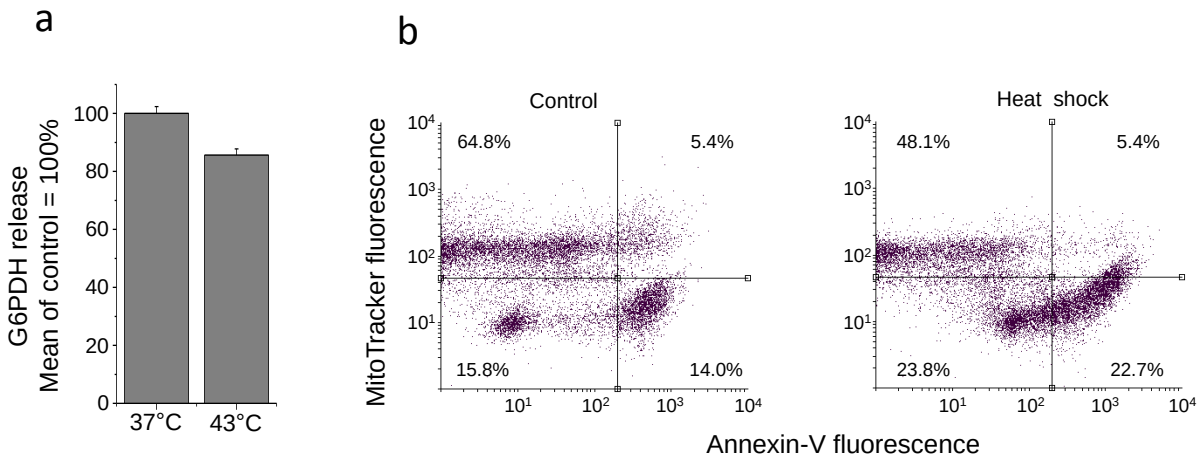


Figure 30.: *Heat shock decreases endocytotic activity of iDCs* (a) Necrosis assay. G6PDH release from heat shocked iDCs. Data are expressed as mean±SEM of four independent donors as the percentage of the control (100%). (b) Representative dot plot of apoptosis determination after heat shock. Cells were stained with MitoTracker Red ® and annexin-V dyes; Mitotracker positive, annexin-V negative cells are living cells, while Mitotracker negative, annexin-V positive cells are considered apoptotic.

Heat shock induces membrane currents which, most probably, are mediated by TRPV2

Since TRPV2 channels seems to be instrumental in transducing the effects of heat shock on iDCs, we next wished to examine the functionality of these molecules using patch-clamp experiments. Using a self-made thermostable patch-clamp setup, we found that heating the cells above 43°C induced a robust membrane current. This current could be reversibly antagonized by the application of the general TRPV antagonist RR, showing that the TRP channels described above could be responsible for the observed currents (Figure 31.a). Interestingly the

combined pharmacological antagonism of TRPV1 and TPRV4 did not significantly change the observed current. Importantly, the RNAi mediated knockdown of TRPV2 (Figure 31.b) completely abolished the heat-induced current, which points to its key role in the formation of the putative heat-activated channel.

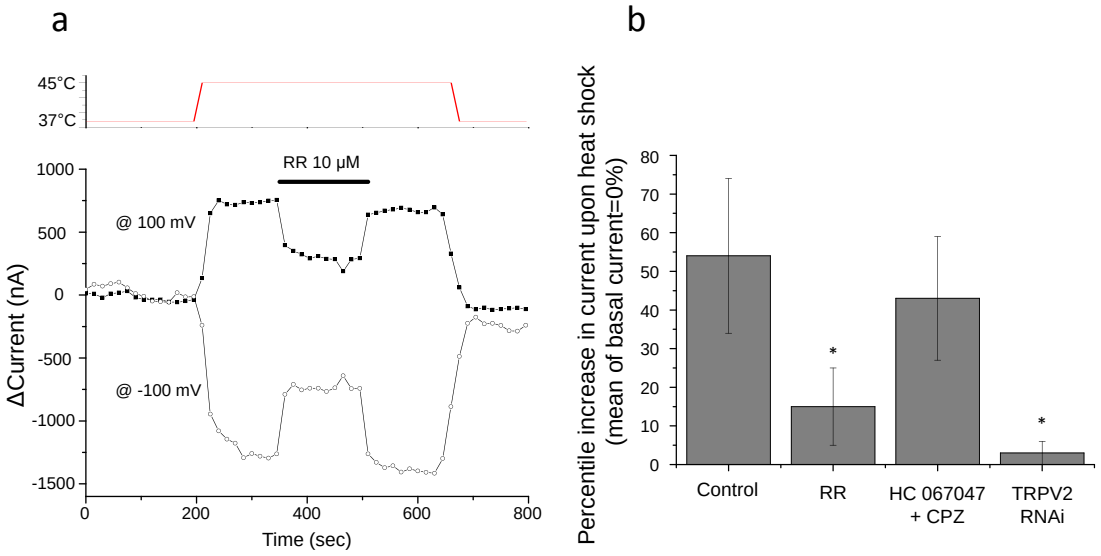


Figure 31.: *Heat shock induced membrane currents on DCs are mediated by TRPV2* (**a**) A representative trace of time-dependent changes of heat activated currents on iDCs at +100 and -100 mV holding potentials. Changes in current are normalized to average basal current measured at 37°C. The line marks application of 10 µM Ruthenium Red (RR). (**b**) Statistical analysis of heat-dependent currents on iDCs at +100 mV. Data are presented as mean ± SEM; N=9 for control measurements, N=4 for RR and RNAi groups, and N=8 for the HC067047+CPZ group (1 and 5 µM were applied, respectively). * marks significant ($p<0.05$) differences compared to the heat shocked cells without inhibitors or RNAi silencing (Control).

TRPV2 is forms heteromeric complexes with TRPV1 and TRPV4

The finding that TRPV2 is responsible for the heat activated current is surprising in the sense that this channel is reportedly activated at higher temperatures (Caterina et al., 1999; Greffrath et al., 2003). However, recent findings (Cheng et al., 2007) on heterologous expression systems that express multiple TRP isoforms may explain how this is possible. In the study mentioned above they found that TRP channels may also form heteromers, and that these heteromers show intermediate gating properties. Based on these intriguing findings, using immunoprecipitation, we aimed at determining whether the TRPV proteins on iDCs also formed common complexes. As seen in Figure 32. we found that the TRPV2-precipitate also

stains positively for TRPV1 and TRPV4, which supports the idea that TRPV2-TRPV1 and TRPV2-TRPV4 heteromeric complexes are present in human DCs.

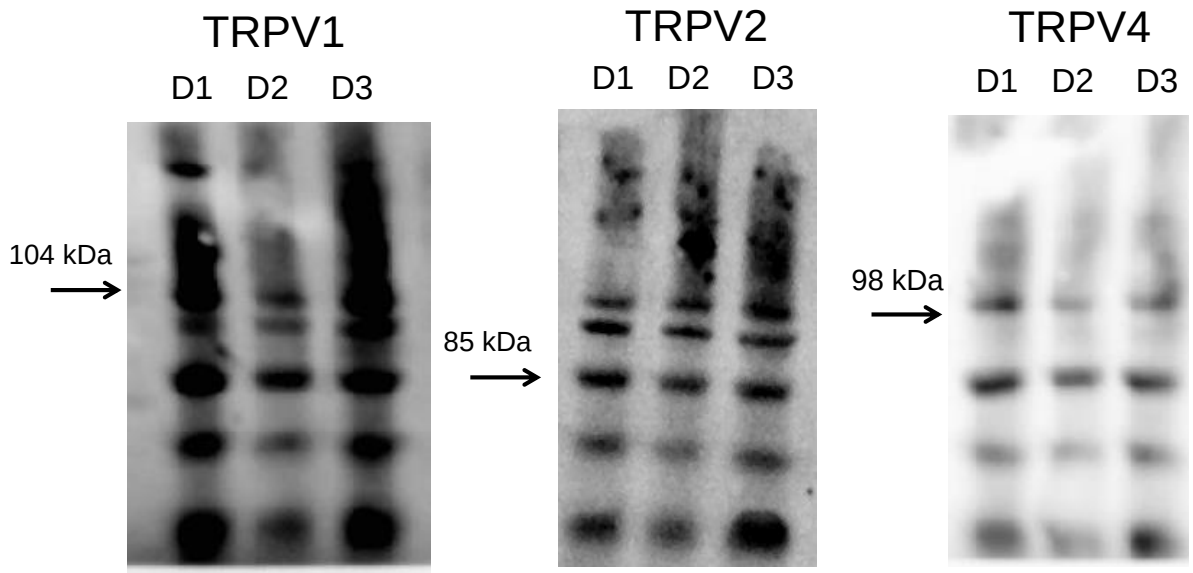


Figure 32.: *Immunoprecipitation of DC proteins* Protein samples prepared from iDCs were precipitated with a TRPV2 antibody. The precipitate was then assayed by Western blotting. Samples were stained using TRPV1 and TRPV4 antibodies, while a different TRPV2 antibody was used to validate the precipitation. Precipitation was performed on DCs obtained from three independent donors, marked D1-D3.

Discussion

As detailed in the Introduction, the ECS has recently emerged as one of the most important signaling systems which is fundamentally involved in the homeostatic regulatory processes of practically all organ-systems of the human body. With respect to the skin, the largest neuro-endocrine organ of the body, we and others have recently shown that the ECS, via locally produced endocannabinoids and by activating versatile receptor-mediated signal transduction pathways, plays a crucial role in controlling epidermal and adnexal growth, cell fate and survival, differentiation, lipogenesis, and inflammatory/immune processes (see reviewed in Bíró et al., 2009).

The ECS is involved in the regulation of human sweat gland biology

As part of our quest to obtain a deeper insight to the functionality of the cutaneous ECS, in the first part of our studies, we aimed at examining the effects of classical endocannabinoids AEA and 2-AG on the human sweat gland-derived NCL-SG3 cell line. Our results prove that both these mediators inhibit proliferation, induce cell death, and up-regulate secretory activity (lipid synthesis) of these cells. These data support the concept that human sweat glands may also function as novel targets for endocannabinoids, as important members of the skin neuroendocrine regulatory network (Slominski and Wortsman, 2000; Slominski et al., 2008).

Since the differentiation program of these cells has not been extensively detailed, we investigated the expression of various cytoskeletal proteins (various CKs, involucrin, filaggrin, loricrin) which are expressed in human sweat gland cells and are also known to be involved in the differentiation of certain skin cells such as the keratinocytes of the epidermis and the hair follicles (Candi et al., 2005; Langbein et al., 2005). In these experiments, we presented the first evidence that levels of these differentiation markers in cultured human sweat gland epithelial

cells are dependent on the proliferating state of these cells (i.e. proliferation vs. high cell density-induced growth arrest) of the cells. Although there are very close similarities between the expression pattern of some of these markers to that described in human epidermal keratinocytes (Papp et al., 2003, 2004; Candi et al., 2005; Langbein et al., 2005; Moll et al., 2008), namely the expression of involucrin, loricrin and filaggrin were highest in postconfluent (and more differentiated) cultures, key cytokeratins such as CK1 and CK10 showed no expression on these cells.

Once we have determined which markers might be useful to track the differentiational state of sweat gland epithelial cells, we aimed at determining whether endocannabinoid treatment had any effect on these proteins. AEA and 2-AG markedly increased the expression of the aforementioned post-confluent markers while concomitantly suppressing the expression of certain markers expressed dominantly in proliferating cells. These data point to the involvement of endocannabinoids in the regulation of cell growth and death in human sweat gland cells, while at the same time promoting their differentiation process.

Interestingly, these effects were independent of classical metabotropic receptors CB1 and CB2, since neither pharmacological antagonists nor RNAi-mediated silencing of these receptors was capable of influencing the effects of endocannabinoids. We have also shown that NCL-SG3 cells express ionotropic cannabinoid receptors (TRP channels); however, AEA and 2-AG were unable to elevate the intracellular calcium concentration. As such, our efforts to find the receptor activated by these endocannabinoids was sadly unfruitful; however, we were able to determine that AEA and 2-AG selectively stimulated the MAPK pathway (while having no effect on the PI-3K and PKC secondary messenger pathways; see Figure 33.).

Although neither classical, nor ionotropic receptors played any part in the transduction of the endocannabinoid effects, it is conceivable that novel endocannabinoid receptors might play a role. Unfortunately the expression of these receptors on sweat gland cells has not been

investigated as of yet; however, our unpublished data show that all three (GPR18, GPR55 and GPR119) are expressed at the RNA level in NCL-SG3 cells. Since only GPR55 is activated by both AEA and 2-AG (Ryberg et al., 2007; Pertwee, 2007; Lauckner et al., 2008; Whyte et al., 2009), it is likely that, if novel endocannabinoid receptors are responsible for the observed effects, GPR55 could be the target.

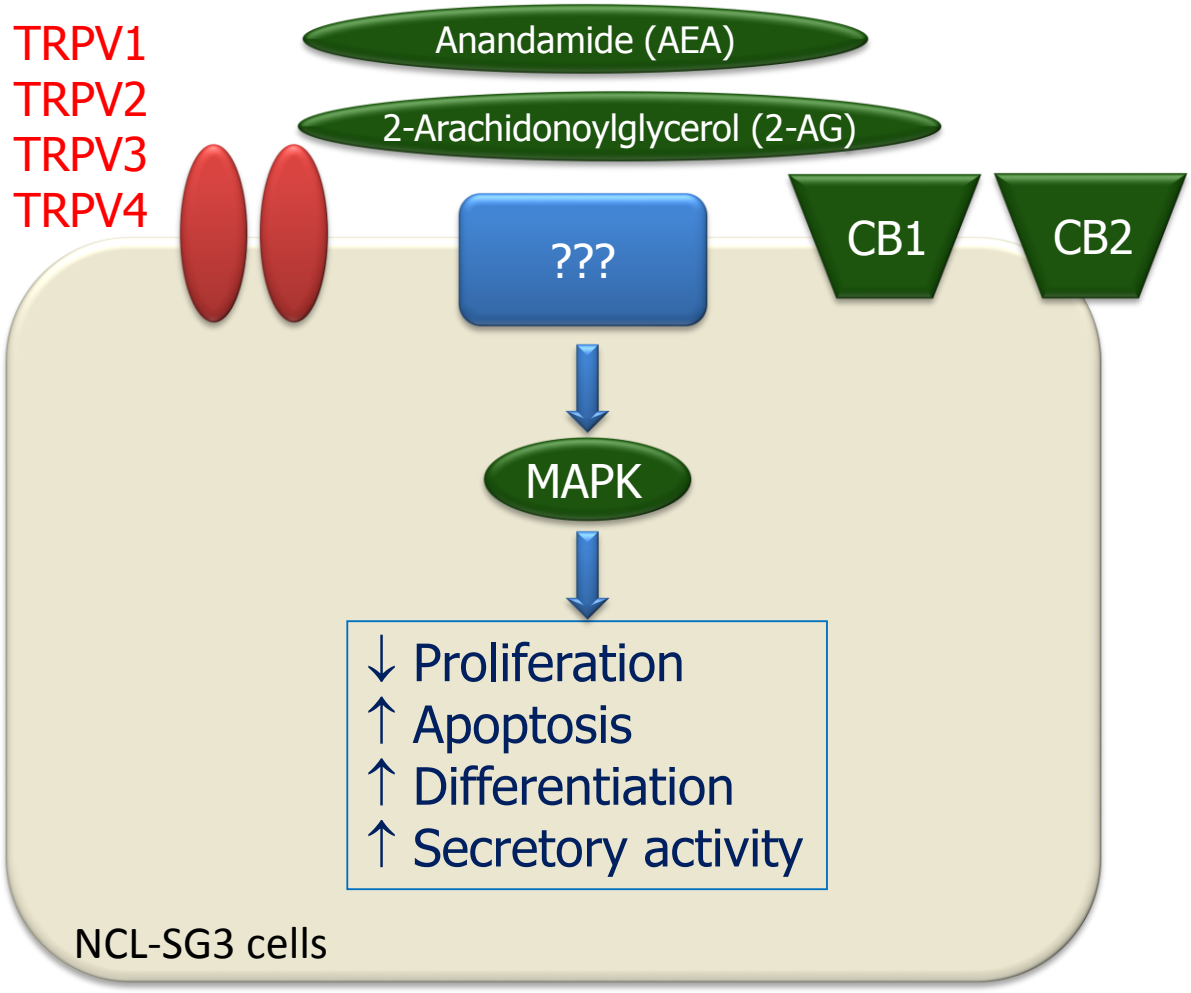


Figure 33.: Summary of effects endocannabinoids on NCL-SG3 cells.

Our workgroup has previously shown that endocannabinoids inhibit hair shaft elongation through CB1 (Telek et al., 2007) and promote lipid synthesis and apoptosis on human sebaceous gland-derived sebocytes via CB2 receptors (Dobrosi et al., 2008). We may now add sweat gland cells as a new target of endocannabinoids, since AEA and 2-AG inhibits proliferation, induces cell death, and stimulates lipid synthesis and differentiation of human sweat gland epithelial

cells by activating non-CB1/CB2-coupled signal transduction pathways. The actions ascribed to the ECS in lieu of the above results compared with previously published data on other skin appendages (i.e. hair follicles and sebaceous glands; Telek et al., 2007; Dobrosi et al., 2008) highlight the existence and importance of cell type specific and (most probably) receptor-selective regulatory endocannabinoid effects in the human skin.

TRPV2 is involved in mediating the effects of heat-shock in human dendritic cells

In the other part of our studies, we showed that of the thermosensitive TRP channels TRPV1, TRPV2 and TRPV4 are expressed on monocytes. iDCs and mDCs. We furthermore found that a short heat shock challenge (43°C for 1 hr) decreased the endocytotic activity of iDCs, without causing necrotic or apoptotic cell death. Interestingly, although TRPV1 channels were shown to be activated on human keratinocytes by heat shock (Li et al., 2007), on iDCs only the RNAi-mediated silencing TRPV2 was capable of abrogating the effect of heat shock, while neither pharmacological, nor molecular suppression of TRPV1 and TRPV4 had any such effect. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 "silencing" (whereas modulation of TRPV1 and TRPV4 activities, again, had no effect).

The above data strongly implicate that the cellular effects of heat shock on human DCs are mediated by TRPV2 ion channels. TRPV2 has been shown to have multiple functions on immune cells. Indeed, on the human mast cell line HMC-1, TRPV2 has been shown to mediate the effects of mechanical, heat and red laser-light stimulation to induce degranulation (Zhang et al., 2012). In murine macrophages, TRPV2 has been found to be instrumental for proper phagocytosis, particle binding, and bacterial lipopolysaccharide induced cytokine production, as well as in podosome assembly (Link et al., 2010; Yamashiro et al., 2010; Nagasawa et al., 2012). In rats, TRPV2 has also been described on macrophages, Langerhans cells and DCs,

although we lack functional data in this species (Shimohira et al., 2009). It appears, therefore, that TRPV2 ion channels are key players in processing the cellular actions of harmful signals on multiple immune cells.

There are multiple explanations as to how TRPV2, which is reported to be activated by temperatures higher than 52°C, mediates the actions of a much lower, 43°C heat challenge. First of all the temperature-gating properties of TRPV2 were originally described on sensory neurons and in heterologous systems (where host cells ectopically expressed the channels; Caterina et al., 1999). Since we lack information on the related biophysical properties of the naïve TRPV2 channels found on non-neuronal cells, we cannot exclude the possibility that it is different from those found on sensory neurons; hence, the DC-expressed TRPV2 can be activated at somewhat lower temperatures than its sensory neuron-localized counterpart. Actually, differential sensitivities/affinities for various pharmacological agonists of TRPV1 expressed by neurons and non-neuronal cells have already been reported (Kark et al., 2008; Kun et al., 2012). Another interesting data is that, in newer studies with heterologous expression systems, TRPV channels have been shown to form heteromers which show intermediate temperature-gating properties (Zhang et al., 2012). Indeed, we have shown that immunoprecipitates of TRPV2 also stain positively for TRPV1 and TRPV4, which suggests that TRPV2-TRPV1 and TRPV2-TRPV4 heteromerization may take place in human DCs. Nonetheless, it is apparent that TRPV2 is the most "active" component since i) it is the most abundant TRPV channel expressed by human DCs; ii) its molecular suppression fully prevented the actions of heat shock.

Taken together, although further biophysical and biochemical studies are invited to uncover the molecular assembly and gating properties of thermo-TRPs expressed by human DCs, our presented results strongly argue for the central role of TRPV2 in mediating the cellular action of heat shock on these cells.

Perspectives

Evidently, based on our presented current and previous pre-clinical findings, proof-of-principle clinical studies are now warranted to test the therapeutic value of cutaneous ECS-targeted approaches in the clinical management of multitude of human skin diseases. In the human sweat gland, it is envisaged that agents increasing the cutaneous endocannabinoid ‘‘tone’’ (such as employing endocannabinoids or molecules that upregulate the production of endocannabinoids by for example stimulating their synthesis of inhibiting their degradation) may be successfully applied in certain sweat gland disorders (e.g., benign or malignant tumors) characterized by unwanted cell growth. Likewise, modulators of the ECS and TRP channels, via influencing DC functions, may be effective tools in controlling the functions of the skin immune system. Actually, we have previously suggested similar approaches for the management of various growth and inflammatory conditions of the human pilosebaceous unit (e.g. hair growth problems, acne vulgaris) (reviewed in Bíró et al., 2009). Finally, from industrial and social point-of-views, it is noteworthy that the proper control of excessive sweating e.g. by modulating the activity of the endocannabinoid-coupled signaling in the sweat gland epithelium, will surely attract the intense interest of the cosmetics industry.

Summary

The endocannabinoid system (ECS) was first described following the investigation of the main psychotropic component of the *Cannabis sativa* plant. Since then, the ECS has grown to encompass numerous cannabinoid ligands, the transmembrane receptors (both metabotropic and ionotropic) that bind and transduce the effects of said ligands, and the enzymatic apparatus responsible for the synthesis and degradation of various endogenous cannabinoids. Although the ECS was first described in the central nervous system, it is now widely accepted that the ECS is expressed, and is functionally active, in almost all non-neuronal tissues as well.

Our own workgroup (among others) has shown that various skin cells (keratinocytes, sebocytes, etc.) not only express multiple members of the ECS, but many of these cells also actively produce endocannabinoids. Indeed, the ECS was found to play a profound role in the regulation of numerous biological processes in skin. In the present work, as part of our extensive research effort on cutaneous functions, we aimed at examining the ECS on two cell types, namely human monocyte-derived dendritic cells (DCs) and sweat gland cells.

Our results show that, on the human eccrine sweat gland-derived NCL-SG3 cell line, endocannabinoids dose-dependently suppressed proliferation, induced apoptosis, altered expressions of various cytoskeleton proteins (e.g., cytokeratins), and upregulated lipid synthesis. Interestingly, as revealed by specific agonists and antagonists as well as by RNA interference, neither the metabotropic CB cannabinoid receptors, nor the "ionotropic" transient receptor potential ion channels, expressed by these cells, mediated the cellular actions of the endocannabinoids. However, the endocannabinoids selectively activated the mitogen-activated protein kinase signaling pathway. Finally, other elements of the ECS (i.e., enzymes involved in the synthesis and degradation of endocannabinoids) were also identified on NCL-SG3 cells.

On human monocyte-derived DCs, our goal was to evaluate the effect of heat shock on the endocytotic activity of the cells, and to elucidate the role of themosensitive transient receptor potential (TRP) channels in the process. We have shown that heat shock decreased the endocytotic activity of the DCs and that this effect could be alleviated by the RNAi-mediated knockdown of TRPV2 but, importantly, not by the pharmacological (antagonists) or molecular (RNAi) suppression of TRPV1 and TRPV4 activities/levels. Likewise, the heat shock-induced robust membrane currents were selectively and markedly inhibited by TRPV2 "silencing" whereas modulation of TRPV1 and TRPV4 activities, again, had no effect.

Taken together, our pre-clinical data suggest that the targeted manipulation of the activity of the cutaneous ECS might be exploited in the future the clinical management of multitude of human skin conditions (including e.g. sweat gland-derived tumors, inflammation, excessive sweating, and other diseases of the human pilosebaceous unit and the related adnexal structures).

Összefoglalás

Az endocannabinoid rendszert (ECS) a *Cannabis sativa* növény hatásaiért felelős specifikus természetes anyagok (úgynevezett cannabinoidok) vizsgálata során fedezték fel. Az azóta eltelt időben az ECS számos egyéb endogén liganddal, az általuk aktivált metabotróp és ionotróp, transzmembrán receptorokkal, illetve a ligandok szintézisét és lebontását végző enzimapparátussal bővült. Bár az ECS-t elsőként a központi idegrendszerben írták le, napjainkra egyértelművé vált, hogy az ECS kifejeződik és funkcionálisan aktív a legtöbb nem-neuronális szövetben is.

Többek között saját munkacsoportunk is kimutatta, hogy a különféle bőr sejtek (keratinociták, szebociták, stb.) expresszálják az ECS több tagját, valamint képesek az endocannabinoidok termelésére is. Nem meglepő módon az ECS kulcsfontosságú szereppel bír a bőr számos biológiai folyamatainak szabályozásában. Jelen munkánkban, a bőr funkcióit feltáró széleskörű kutatásaink keretében, célunk az ECS vizsgálata volt humán verejtékmirigy sejteken és monocita-eredetű dendritikus sejteken (DC).

Eredményeink szerint a humán ekkrin verejtékmirigyből származtatott NCL-SG3 sejtvonalon sikerrel azonosítottuk az ECS legtöbb elemét (endocannabinoidok, receptorok, az endocannabinoidokat szintetizáló és lebontó enzimek). Bebizonyosodott az is, hogy az endocannabinoid kezelés dózisfüggő módon csökkentette a sejtek proliferációját, apoptózist indukált, megváltoztatta bizonyos citoszkeletális proteinek (pl. citokeratinok) expressziós mintázatát és fokozta a sejtek lipid szintézisét. Érdekes módon sem a sejteken kifejeződő klasszikus cannabinoid receptorok, sem az ionotróp tranziens receptor potenciál (TRP) csatornák farmakológiai illetve siRNS-el történő gátlása nem befolyásolta az endocannabinoidok hatását. Ugyanakkor az endocannabinoidok szelektíven aktiválták a mitogén-aktivált protein kináz másodlagos hírvivő útvonalat.

Humán monocita-eredetű DC-en célunk a hősokk hatásainak vizsgálata volt, illetve az ezen hatások közvetítésben szerepet játszó termoszenzitív TRP csatornák feltérképezése. Kimutattuk, hogy a hősokk gátolja az DC-ek endocitotikus aktivitását, illetve specifikus áramkomponenst indukál. Ezen hatást a TRPV2 géncsendesítése teljes mértében képes volt kivédeni, ugyanakkor a TRPV1 és TRPV4 csatornák farmakológiai illetve siRNS-el történő gátlása nem befolyásolta azt.

Összességében pre-klinikai vizsgálataink azt sugallják, hogy a bőr ECS-ének célzott befolyásolása számos humán bőrbetegség kezelésében nyújthat ígéretes új terápiás lehetőséget (pl. verejtékmirigy-eredetű tumorok, gyulladás, túlzott verejtékezés és a bőrfüggelék egyéb betegségei).

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Keywords

Endocannabinoid system

Sweat gland

Dendritic cell

Transient receptor potential vanilloid

Skin

Heat shock

Mitogen-activated protein kinase

Kulcsszavak

Endocannabinoid rendszer

Verejtékmirigy

Dendritikus sejt

Tranziens receptor potenciál vanilloid ioncsatornák

Bőr

Hősokk

Mitogén-aktivált protein kináz

Acknowledgements

I would first of all like to thank my PhD advisor, **Dr. Tamás Bíró** for his inexhaustible patience, immeasurable help and irreplaceable guidance over the years.

I owe special thanks to **Dr. László Kovács** and **Dr. László Csernoch**, the onetime and current director of the Physiology Department for providing me with the opportunity to work in the Laboratory for Cellular and Molecular Physiology.

I would like to express my gratitude towards our collaborational partners for their help and expertise. I would like to thank **Dr. György Panyi** and **Dr. Ferenc Papp** for their immense efforts on behalf of our joint projects, as well as our industrial partners from L'Oreal Research, specifically **Julien Demaude**, **Charbel Bouez**, **Lionel Breton**, and **Odette Jammayrac**.

I would like to thank thank my close colleagues **Gabriella Czifra**, **Erika Lisztes**, **Attila Oláh**, **Nikolett Vasas**, **Lídia Ambrus**, **Ágnes Angyal**, **Dóra Bodnár**, **Norbert Balogh**, **Imre Lőrinc Szabó**, **Balázs István Tóth**, and **Nóra Zákány** for their day-to-day help and for providing an extremely positive atmosphere that made coming to work every day a joyous occasion.

For their invaluable technical support I would like to thank **Mrs. Ibolya Varga, Erika Hollósi, Renáta Papp** and **Lilla Furin**.

Finally I would like to express my gratitude towards **all of my colleagues, PhD students and predoctoral students** in the Department of Physiology who have helped my work over the years.

This research was supported by the **European Union** and the **State of Hungary, co-financed by the European Social Fund** in the framework of TÁMOP 4.2.4.A/2-11-1-2012-0001 'National Excellence Program' as well as the Baross Gábor Program: REG-EA-09-1-2009-0010, and the "Lendület" LP2011-003/2013 grant.

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List of publications related to the dissertation

1. **Szöllősi, A.G.**, Oláh, A., Tóth, I.B., Papp, F., Czifra, G., Panyi, G., Bíró, T.: Transient receptor potential vanilloid-2 mediates the effects of transient heat shock on endocytosis of human monocyte-derived dendritic cells.
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   DOI: http://dx.doi.org/10.1016/j.febslet.2013.03.027
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List of other publications

3. Tóth, I.B., Oláh, A., **Szöllősi, A.G.**, Bíró, T.: TRP channels in the skin.
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DOI: http://dx.doi.org/10.1016/j.febslet.2009.04.031
IF:3.541

Total IF of journals (all publications): 50.593

Total IF of journals (publications related to the dissertation): 9.775

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06 June, 2014

Appendix:

Publications related to the dissertation