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ENDOCANNABINOIDS REGULATE GROWTH AND SURVIVAL OF HUMAN ECCRINE SWEAT GLAND EPITHELIAL CELLS

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To: Professor Paul. R. Bergstresser
Editor
Journal of Investigative Dermatology
Editorial Office

November 29, 2011

Dear Professor Bergstresser:

Please find enclosed our substantially revised manuscript (**JID-2011-0547R1**) which we now re-submit for publication to the *Journal of Investigative Dermatology*.

During the revision, we re-structured the manuscript according to the very constructive comments and suggestions of both the Editors and the Reviewers. In the below pages you find our point-by-point answers to the Reviewers. As requested, all changes are underlined in the revised text.

Both Editors emphasized to most adequately respond to the (otherwise positive) comments of Reviewer 4. Therefore, let me summarize how we addressed the issues raised by this Reviewer (a more detailed response can be found below in our responses to this Reviewer):

- It is quite accepted among researchers of the field that when one intends to define the actions of endocannabinoid substances on novel cellular targets (in our case, the human sweat gland epithelial cells), both the pharmacological effects **and** the expressions of components of the endocannabinoid system (ECS) (i.e. receptors, enzymes of synthesis and degradation) “must be” investigated.
- Yet, after carefully revision of the manuscript, we must admit that the Reviewer is right and the distinction between endogenous vs. exogenous effects are not carefully addressed. This is still true despite of the fact that we are (evidently) not in the position to define the actual “peak” concentrations of endocannabinoids around the producer or target cells and hence cannot exclude the possibility of reaching “pharmacological doses”.
- Therefore, we decided to modify the manuscript along the following lines:
    - We removed all “speculations” about endogenous autocrine/paracrine effects from the text.

    - We re-structured the manuscript by starting with describing the effects of endocannabinoids; then we continued by defining the mechanisms of action of the endocannabinoids.
    - Therefore, the presentation of expressions of components of the ECS is moved to Discussion section and the corresponding Figure is now shown in the Supplementary data.
- The modification resulted in a new order of Figures; new Figure parts (Figure 3b and 5d); new Suplementary Figures (S3 and S4); and new text parts.

These substantial changes in the presentation of our data made it quite difficult to address the issue raised by the Associate Editor to present immunohistochemical data on human sweat glands in situ. The way how we "solved" this problem is the following:

- We did perform the immunohistochemical analysis of expressions of ECS in the human skin in situ.
- We show these results only in the Supplementary materials (Supplementary Figure S4) and mention them in the Discussion section.

We hope that these extended revisions, along with our point-by-point answers to other comments of the other three Reviewers, are sufficient for considering our work for publication in your distinguished journal. Evidently, if further changes are required, we are happy to perform additional modifications.

Yours sincerely,

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Point-by-point Responses to the Reviewers

Responses to Reviewer 1

1. This is an interesting study written by experienced investigators that should be of strong interest to the readers of JID. For the most part the data is properly collected and adequately discussed. The methodology is sound.

→ Thank you very much for the positive evaluation of our work.

2. I have minor suggestions that should improve final presentation. General description would benefit by placing the endocannabinoid system in the wider context of skin neuroendocrine system (Endocrine Rev 21, 457-487, 2000; Drug Discovery Today: Dis Mech 5: e137-e144, 2008).

→ Very important suggestion. Although we were unable to extensively describe the novel neuroendocrine role of the cutaneous ECS due to strict wordcount limitations, we mention this concept in the revised Abstract (sentence 1), Introduction (sentence 1), and Discussion (sentence 2). In additon, the suggested references are now listed.

3. Also in Fig. 3e the reader would appreciate identifying arrows pointing at histochemical product as well as the bar indicating magnification.

→ The requested changes have been performed in the Figure (now it is Figure 2e) and in the corresponding legend.

Responses to Reviewer 2

In this manuscript, authors describe their finding of expression of ECS in the eccrine sweat gland derived NCL-SG3 cell line. Authors further show their involvement in differentiation, apoptosis and lipid synthesis of the cell line. The function of endocannabinoids was not mediated by their canonical receptors, though they exist in the cell line, and authors suggest that the MAKP pathway may play a role in endocannabinoid action. Expression of the neuromodulatory lipids in the sweat gland derived cells is an interesting finding; however, additional studies may be needed to have bigger impact.

1. The NCL-SG3 cell line was established decades ago, but its cellular origin (clear, dark or myoepithelial cells) is unclear. In sweat glands, K8 is expressed in the secretory cells (clear and dark), but K14 is expressed in the myoepithelial cells. However, the authors show simultaneous high expression of K14 and K8 in the cell line, which suggests that this cell line is severely transformed and deviates from specific sweat gland cell types. It would strengthen the study enormously if it were extended to in vivo. For instance, IHC or ISH with mouse footpad skin (if human skin is unavailable), can provide definitive ECS expression pattern (cellular or subcellular) in vivo. Also, functional analyses with endocannabinoids can be extended to in vivo by subcutaneous injections.

→ Very important suggestions. Actually, the Reviewer is „knocking on opened doors" since we have already started the evaluation of the effects endocannabinoids using an „in vivo-like" **human** model, the full-thicknes human skin organ-culture system. The special advantages of this model – which is kept in culture at the air-liquid interface – are that (i) functional properties (e.g. proliferation, differentiation) of human sweat gland epithelial cells can be investigated in situ within their natural

habitat; (ii) endocannabinoids can be applied either to the medium or to the top of the skin specimens, hence the respective systemic and topical application routes can be equally tested; (iii) long-term (10-14 day) effects of the endocannabinoids can be investigated; (iv) the in situ expressions of elements of the ECS on sweat gland cells can be assessed.

In relation to the last point, following the similar suggestion of the Associate Editor, we performed a pilot immunohistochemical labeling on human skin sections (the methodology is now mentioned in the Supplementary data section). As shown in the new Suplementary Figure S4, all elements of the ECS can be detected on human eccrine sweat gland cells in situ. In the future, we plan to assess the distribution of these molecule on eccrine vs. apocrine glands, and also on secretory vs. ductal cell types.

Since in our current study we specifically focused on **human** cells and tissues, we feel that animal experiments would be out of the scope of the work. Yet, we have recently launched a large project in which (by using wild-type and genetically modified mice) we aim at defining the role of the ECS in mouse skin in general. In this work, following the excellent suggestion of this Reviewer, we will also focus on the functional properties of sweat gland epithelial cells. We strongly hope that we would to be able to report on the results of these exciting experiments soon.

Finally, about the transformed "nature" of NCL-SG3 cells. We fully agree with the Reviewer that the keratin (and other marker) expression profiles (following the elegant description of Langbein et al, J Invest Dermatol 125:428-44, 2005) suggest a rather "mixed" feature of the cell line. In this study, our sole aim was to define the effects of endocannabinoids on human sweat gland cells; since we had only these cells available we had to make a compromise. We strongly hope that the aforementioned experiments on human skin organ-cultures as well as our ongoing trials to establish the culturing of **primary human** sweat gland epithelial cells (isolated from different regions of the glands) will clarify this issue.

2. The authors show that endocannabinoids do not work through their receptors, but activate MAPK in the NCL-SG3 cell line. Whether inhibition of MAPK will completely or partially block endocannabinoid action in the cell line requires further analysis.
→ We fully agree with this comment of the Reviewer. Actually, as we have shown in the original Figure 5a and b, effects of endocannabinoids were indeed almost completely blocked by the MAPK inhibitor PD098059. Although we cannot rule out the involvement of other signaling mechanisms (which are distinct from the PI-3K and PKC systems since their inhibitors did not modify the actions of endocannabioids, see the same figures), these data collectively argue for the central role of the MAPK system. Yet, to further support this concept, we have performed new experiments (and show the results in the new Figure 5d) in which we now present evidence that the Erk1/2 phosphorylation induced by the endocannabinoids can also prevented by the co-application of PD098059. Finally, we performed a minor modification in the text (last sentence of the Result section) – we removed the term "selective".

3. Sweat glands, especially eccrine sweat glands, do not secrete lipids in vivo, so the biological meaning of lipid secretion by endocannabinoids in this cell line needs to be discussed.

→ In this case, we respectfully disagree with the opinion of the Reviewer. The lipid secretion of sweat gland cells is surprising, although there are several reports on the existence of lipid granules in sweat gland cells (Bovell et al, (2001) Ultrastructure of the hyperhidrotic eccrine sweat gland, *Br J Dermatol*. 145: 298-301.; Montgomery et al. (1984) The effects of thermal stimulation on the ultrastructure of the human atrichial sweat gland. I. The fundus. *Br J Dermatol*. 110(4):385-97; Spicer et al. (1972) Ultrastructure of lipid inclusions and dense bodies in the human sweat gland. Lab Invest. 27(1):1-8.) and their appearence in the lumen of the eccrine gland. Another study also showed lipids in specially collected sweat, where the possibility of contamination from other sources (the stratum corneum and the sebum) was outruled. (Takemura et al. (1989) Free fatty acids and sterols in human eccrine sweat. *Br J Dermatol*. 120:43-47.). Furthermore, a recent paper by Porter (Porter (2001) Why do we have apocrine and sebaceous glands? *J R Soc Med.* 94(5):236–237.) hypothesizes that the lipids produced in apocrine sweat glands and sebaceous glands might have roles in thermoregulation, by acting as a surfactant for sweat, and thereby allowing it to form sheets instead of drops.

Responses to Reviewer 3

This is a very well carried out study, which investigated the effects of endocannabinoids on the growth and functions of the human sweat gland-derived immortalized NCL-SG3 cells. The authors concluded that endocannabinoids exert inhibitory effects on NCL-SG3 cell growth and promote apoptosis, induce differentiation and lipid synthesis. Moreover, the effects of endocannabinoids are likely do not involve the cannabinoid receptors or TRP receptors and occur via recruitment of the MAPK signalling pathway. The conclusions are supported by the variety of assays.

→ Thank you very much for the positive evaluation of our work.

Questions to the authors:

1. The Fig 1A demonstrates that expressions of CB1 and CB2 protein in NCL-SG3 cells harvested at various confluences are increased and decreased, respectively. Could authors speculate about physiological significance of these findings?

→ Very important note. The ECS has been shown to take part in the differentiation of numerous non-neuronal cell types of the skin (see summarized in Bíró et al, TIPS, 2009). Our workgroup has shown that the ECS has important functions in the physiological differentiation of sebaceous glands, one of the main skin appendages (Dobrosi et al, FASEB J2008). In addition, similar to the report of Stander et al, J Dermatol Sci, 2005, in the above study we have shown that the in situ expressions of CB1 and CB2 (measured by immunohistochemistry on human skin sections) were different on sebaceous gland cells with distinct differentiation statuses. Namely, CB2 was found to be more abundantly expressed on undifferentiated sebocytes whereas CB1 was mostly found on differentiated ones. Actually, in our *in vitro* model cells, very similar phenomena were observed; namely, CB1 expression was more prominent on the post-confluent (hence more differentiated) NCL-SG3 cells whereas CB2 was mostly identified in the pre-confluent (hence undifferentiated) ones. It is conceivable therefore that cannabinoid receptors are required for the differentiation and proliferation of sweat gland cells as well.

Minor comment: There is a typo in Figure legend 1: Q-PCR analysis should be labelled as b), but not a).
→ Thank you very much for the careful reading. The legend (which is now for Figure 3) has been corrected.

2. Could the authors explain the functional significance of their findings that endocannabinoids promote apoptosis and lipid synthesis at the same time?
→ Very interesting question. Honestly, we do not really know whether the simultaneous "appearance" of these two phenomena is a pure coincidence or there is causative relationship between them. We believe that there are 2 possibilities:

1. Data obtained with 2-AG suggest that here we most probably deal with a pure coincidence. Namely, as shown in Figure 2f (previously Figure 3f), 2-AG was able to significantly increase lipid production of cells at such low concentration as 1 uM. However, as shown in Figure 1a-f (previously Figure 2), this concentration of 2-AG did not compromise the viability of the cells. These data suggest that the endocannabinoid primarily targets lipid synthesis; however, when higher concentrations are applied, we can also detect its effect on the program of differentiation and apoptosis.
2. On the other hand, the coincidence of apoptotic events and lipid production may raise the possibility that, similar to as seen e.g. in sebaceous gland-derived sebocytes (Dobrosi et al, FASEB J, 2008), the terminal differentiation of these cells is followed by increased lipid accumulation.

3. What are the possible molecular mechanisms that link the endocannabinoids and MAPK signaling?
→ Another very interesting question. Although we do not have functional evidence, one possible explanation for the observed "non-CB1, non-CB2" effects could be the involvement of "novel" cannabinoid receptors. Among them one potential candidate could be GPR55 since this receptor was shown to be (key references of GPR55: Ryberg et al, *Br J Pharmacol*.;152(7):1092-101, 2007; Godlewski et al, *Prostaglandins Other Lipid Mediat*; 89(3-4):105-11, 2009. Henstridge et al, *Br J Pharmacol*.;160(3):604-14, 2010; Andradas et al, *Oncogene*;30(2):245-52, 2011)

- activated by both AEA and 2-AG, the latter being the more potent one
- coupled to various G-proteins such as e.g. $G\alpha_{q/11}$, $G\alpha_{12}$, $G\alpha_{13}$,
- capable of inducing such intracellular signal transduction pathways which are coupled to as e.g. RhoA , the MAPK system, the PKC isoforms, etc.

Evidently further studies are needed to clarify the existence of this novel signaling pathway.

Responses to Reviewer 4
This manuscript describes the presence of the elements of the endocannabinoid system in cultured sweat gland epithelial cells. It shows that exogenous addition of two endocannabinoids influences the differentiation and death of the cells. It characterizes markers of differentiation in these cells for comparison with other epithelial cells. Finally, it concludes that the endocannabinoids do not function through its previously characterized receptors. The paper is clearly written and may have therapeutic impact. The experiments seem well done.
→ Thank you very much for the positive evaluation of our work.

The paper, however, has a significant problem in presentation. It is presented as a study of the role of endocannabinoids on sweat gland cells. However, there was no basis presented that the levels of exogenous endocannabinoids added were relevant to the effects of endogenous cannabinoids. Further, the authors showed that the exogenous cannabinoids at the concentrations added did not act through typical cannabinoid mechanisms. Therefore, the paper has two somewhat separate parts. First, the authors show the presence of the elements of the endocannabinoid system but with no evidence that it is at a level to be of physiologically relevant. Second, they show that pharmacological doses of two compounds that in some other systems function as endocannabinoids have important effects (maybe of relevance for therapy) on these cells and they characterize these effects, also providing information on markers of differention in the sweat cells.

The efforts to link the two pieces of this paper have the potential to be misleading for the readers. The authors should modify the presentation to fit with the data that was obtained rather than try to fit it into the initial concept for the paper.

→ Thank you very much for this important and constructive comment. It is quite accepted among researchers of the field that when one intends to define the actions of endocannabinoid substances on novel cellular targets (in our case, the human sweat gland epithelial cells), both the pharmacological effects **and** the expressions of components of the endocannabinoid system (ECS) (i.e. receptors, enzymes of synthesis and degradation) "must be" investigated.

Yet, after carefully revision of the manuscript, we must admit that the Reviewer is right and the distinction between endogenous vs. exogenous effects are not carefully addressed in our manuscript. This is still true despite of the fact that we are (evidently) not in the position to define the actual "peak" concentrations of endocannabinoids around the producer or target cells and hence cannot exclude the possibility of reaching "pharmacological doses".

Therefore, we decided to modify the manuscript along the following lines:

- We removed all "speculations" about endogenous autocrine/paracrine effects from the text.
    - We re-wrote the Abstract
    - We removed misleading statements. Introduction: paragraph 3, sentence 1; Discussion: paragraph 1, sentence 2; paragraph 2, last sentence 1; paragraph 3, sentence 2; paragraph 4, sentence 1
- We fully re-structured the manuscript in which the emphasis is now on the effects of the endocannabinoids.
    - We now start with describing the effects of endocannabinoids. Hence the original Figure 1 is now positioned as Figure 3, and the original Figures 2 and 3 are now shown as Figures 1 and 2, respectively.
    - We then continue by defining the mechanisms of action of the endocannabinoids. Hence we removed all parts from the original Figure 1 (now Figure 3) about components of the ECS to the Supplementary section (see also below), and now we show only the expression of CB1 and CB2 (please, note that we constructed a new Figure 3b)
    - The presentation of expressions of components of the ECS is moved to Discussion section. We introduced a new paragraph (5) and modified the last paragraph (sentence 1 and 2)

- The corresponding Figure on the elements of the ECS is now shown in the Supplementary data as new Supplementary Figure S3. In addition, as requested by the Associate Editor and another Reviewer, we show their in situ expression in the new Suplementary Figure S4
- All modifications in the Figures are accompanied by corresponding changes in the text and figure legends.

We strongly hope that these changes in the presentation have substantially increased the clarity of the overall message of our manuscript.

ENDOCANNABINOIDS REGULATE GROWTH AND SURVIVAL OF HUMAN ECCRINE SWEAT GLAND EPITHELIAL CELLS

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Running title: Endocannabinoids inhibit growth of human sweat gland cells

Key words: endocannabinoids, sweat gland, proliferation, apoptosis, signal transduction

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ABSTRACT

The functional existence of the emerging endocannabinoid system (ECS), one of the novel neuro-endocrine players in cutaneous biology, is recently described in the human skin. In the current study, using human eccrine sweat gland-derived immortalized NCL-SG3 model cells and a wide-array of cellular and molecular assays, we investigated the effects of prototypic endocannabinoids (anandamide, 2-arachidonoylglycerol) on cellular functions. We show here for the first time that both endocannabinoids dose-dependently suppressed proliferation, induced apoptosis, altered expressions of various cytoskeleton proteins (e.g. cytokeratins), and up-regulated lipid synthesis. Interestingly, as revealed by specific agonists and antagonists as well as by RNA interference, neither the metabotropic CB receptors, nor the "ionotropic" cannabinoid receptor TRP ion channels, expressed by these cells, mediated the cellular actions of the endocannabinoids. However, the endocannabinoids selectively activated the MAPK 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. These results collectively suggest that cannabinoids exert a profound regulatory role in the biology of the appendage. Therefore, from a therapeutic point-of-view, up-regulation of endocannabinoid levels might help to manage certain sweat gland-derived disorders (e.g. tumors) characterized by unwanted growth.

INTRODUCTION

Recent bodies of evidence implicate that the endocannabinoid system (ECS) (Pacher et al, 2006; Di Marzo, 2008; Perwee et al, 2010), as one of the novel players in cutaneous neuroendocrinology (Slominski and Wortsman, 2000; Slominski et al, 2008), may play a significant role in controlling skin homeostasis (Bíró et al, 2009). Endocannabinoids such as anandamide (N-arachidonoylethanolamine, AEA) and 2-arachidonoylglycerol (2-AG) (Calignano et al, 1998; Karsak et al, 2007); enzymes involved in the synthesis and metabolism of these lipid mediators (Berdyshev et al, 2000; Maccarrone et al, 2003); as well as cannabinoid receptors (CB) were all identified on various skin cell populations (Casanova et al, 2003; Stander et al, 2005; Ibrahim et al, 2005; Blazquez et al, 2006; Karsak et al, 2007; Telek et al, 2007; Dobrosi et al, 2008; Tóth et al, 2011).

Of further importance, the cutaneous ECS was shown to regulate the well-balance growth and differentiation program of skin cells. Namely, AEA was reported to inhibit the differentiation of cultured human epidermal keratinocytes (Maccarrone et al, 2003; Paradisi et al, 2008) whereas we have recently shown that AEA suppressed growth and induced apoptosis of these cells (Tóth et al., 2011). Likewise, we have also presented that the locally produced AEA inhibited *in vitro* hair shaft elongation and induced apoptosis-driven premature catagen regression (Telek et al, 2007). Of further importance, using a human sebaceous gland-derived cultured sebocyte model, we have demonstrated that endocannabinoids (AEA and 2-AG), produced by these cells, constitutively enhanced lipid production and induced chiefly apoptosis-driven cell death (hallmarks of sebocyte differentiation and hence a model of holocrine sebum production) (Dobrosi et al, 2008).

Interestingly, although the *in situ* expressions of both CB_1 and CB_2 have been described on epithelial cells of human eccrine sweat glands (Stander et al, 2005), we lack *functional* data on the role of endocannabinoids ~~and the ECS~~ in the regulation of biology of the smallest appendage of the mammalian skin. Therefore, in the current study, we aimed at defining the effects of the most extensively studied endocannabinoids (i.e. AEA and 2-AG) on growth and survival of human eccrine sweat gland cells. By using cultured NCL-SG3 eccrine sweat gland cell culture model and by employing combined pharmacological and molecular approaches, we provide the first evidence that endocannabinoids markedly suppress cellular proliferation, induce chiefly apoptosis-driven cell death, and alter expression/production of various intracellular proteins (e.g. cytokeratins) and lipids in human sweat gland epithelial cells.

RESULTS

Endocannabinoids inhibit growth and induce chiefly apoptosis-driven cellular death in NCL-SG3 cells

Using colorimetric MTT and fluorimetric CyQuant assays, we found that both AEA and 2-AG dose-dependently suppressed the viable cell number and proliferation of NCL-SG3 cells (**Figure 1a and b**). To define whether this effect was due to the induction of cell death (apoptosis and/or necrosis), a series of functional assays was performed. As measured by quantitative fluorimetric determinations, both endocannabinoids significantly decreased mitochondrial membrane potential (reflecting mitochondrial disturbance) (**Figure 1c**) and induced the activation of pro-apoptotic caspases (**Figure 1d**), 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 1e**) and Sytox Green accumulation to the cells (**Figure 1f**) two complementary indicators of necrosis/cytotoxicity. (Intriguingly, AEA did not induce necrosis.) These findings suggested that the endocannabinoids suppressed cellular growth and induced chiefly apoptosis-driven cell death of human sweat gland cells.

Endocannabinoids markedly alter expressions of certain cytokeratins and other cytoskeleton proteins involved in cutaneous differentiation

In most epithelial cells, the cessation of proliferation and the induction of apoptosis are accompanied by the onset of the differentiation program of the cells (Candi et al, 2005; Proksch et al, 2008). Expressions of various intermediate filament cytokeratins (CK) as well as of other cytoskeleton proteins (e.g. involucrin, filaggrin, loricrin),

which were shown to be involved in differentiation of certain skin cells (e.g. epidermal and hair follicle keratinocytes), have previously been described on human eccrine sweat gland epithelium (Ohnishi and Watanabe, 1999; Langbein et al, 2005; Tharakan et al, 2010). Yet, the exact details of the differentiation process have not been fully elucidated in cultured sweat gland epithelial cells.

Therefore, we first 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. We could not detect the expression of the epidermal differentiation markers CK1 and 10 (data not shown) whereas all other molecules were identified (**Figure 2**). Of great importance, however, we found that expressions of these markers differentially altered in parallel with the age of the cultures. Namely, whilst levels of CK7, 14, 18, and 19 were highest in the pre-confluent, highly proliferating cultures (**Figure 2a**), expressions of CK8, involucrin, filaggrin, and loricrin were predominant in the post-confluent, hence growth-arrested (and presumably more differentiated) ones (**Figure 2b**). 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 then investigated the effects of endocannabinoids. 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 quantitative "real-time" PCR. As seen in **Figure 2c and d**, both endocannabinoids significantly (yet differentially) elevated the

expressions of the "post-confluent" markers, i.e. CK8, involucrin, filaggrin, and loricrin. In addition, levels of some of the markers of the highly proliferation pre-confluent cells, i. e. CK7 by 2-AG and CK14 by both AEA and 2-AG, were markedly suppressed by endocannabinoid treatment.

Endocannabinoids stimulate lipid synthesis in NCL-SG3 cells

Another key function of sweat gland cells is to secrete various substances. Since we have previously shown that endocannabinoids markedly induced lipid synthesis in cultured human sebocytes (Dobrosi et al, 2008); and, furthermore, since sweat gland epithelial cells were shown to synthesize a wide-array of lipids (Barth et al, 1989); we also assessed whether endocannabinoids modulate 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 after 24 hrs treatment) markedly and dose-dependently elevated neutral (but not polar) lipid synthesis of the cells (**Figure 2e and f**). Since polar lipids reflect intracellularly accumulated "*de novo*" synthesized ones, our data argue for that endocannabinoids (besides modulating cell growth, survival, and expressions of various cytoskeleton proteins) may exert a profound role in the regulation of secretory activity of human eccrine sweat gland cells.

Cellular effects of endocannabinoids are not mediated by CB_1 or CB_2 expressed on NCL-SG3 cells

We then investigated the putative involvement of certain receptor-mediated signal transduction systems in mediating the cellular actions of endocannabinoids. Conforming previous data (Stander et al, 2005), we first identified the expressions of

both metabotropic CB subtypes (CB_1, CB_2) on sweat gland-derived cells using Western blot (**Figure 3a**) and immunocytochemistry (data not shown) techniques. In addition, transcription of genes encoding the above proteins was demonstrated by RT-PCR (data not shown) and by quantitative "real-time" PCR (**Figure 3b**). During the above analysis, we also observed that the expression of CB_1 and CB_2 markedly altered in parallel with the culturing of the cells (**Figure 3a and b**). Specifically, the level of CB_1 monotonously increased during culturing and reached its maximum in the post-confluent states. In contrast, the expression of CB_2 was the highest in the pre-confluent cultures whereas its level markedly decreased with reaching confluence of the cells. These data further suggested that the cannabinoids might play a role in the regulation of growth (and most probably of differentiation) of the human sweat gland-derived cells.

To assess the roles of the CB receptors, we have employed various antagonists and the RNAi techniques. In these experiments, to equally assess changes in cellular growth, survival, and secretory activities, we determined cellular viability/proliferation (MTT assay) and lipid production (Nile Red fluorimetry) of NCL-SG3 cells.

First, various inhibitors of CB subtypes (AM251 for CB_1, AM630 for CB_2) were employed. These inhibitors could not modify the actions of the endocannabinoids; namely, both AEA and 2-AG were still able to suppress cellular viability and stimulate lipid synthesis in the presence of either antagonist (**Figure 4a and b**).

Since the pharmacological natures of AM251 and AM630 (i.e. also acting as inverse agonists) (Pertwee et al, 2010) may compromise their specificities on the CBs, a

series of RNAi experiments against the CBs were carried out in accordance with the optimized techniques developed in our previous studies (Dobrosi et al, 2008; Tóth et al, 2009, 2011). Western blot analysis (**Supplementary Figure S1**) as well as Q-PCR determination (data not shown) revealed that expressions of both CB_1 and CB_2 were significantly and specifically "knocked-down" at day 3 after transfection by 2 out of 3 RNAi probes employed. However, this phenomenon was reversible since we observed a "return" of their expression at day 4. Scrambled RNAi probes had no effect on the expression of either CB_1 or CB_2.

We then investigated the effects of endocannabinoids on NCL-SG3 cells with "silenced" CB_1 or CB_2. Of great importance, in perfect agreement with the above pharmacological data, the molecular "knock-down" of CB_1 or CB_2 could not prevent the growth-inhibitory and differentiation-promoting cellular actions of AEA and 2-AG; namely, both endocannabinoids were able to suppress cellular viability and induce fat production (**Figure 4c and d**). Therefore, these results, in line with the above data obtained with the antagonists, collectively argue for that CB_1 or CB_2 coupled intracellular signaling mechanisms most probably do not participate in mediating the effects of endocannabinoids on human sweat gland epithelial cells.

Cellular effects of endocannabinoids are not mediated by TRP channels expressed on NCL-SG3 cells

Recent intriguing data from our laboratory and from others, however, suggest that cannabinoid compounds, besides CB_1 or CB_2, may also target other molecules (Pertwee et al, 2010). Among these, certain transient receptor potential (TRP) ion channels were implicated as "ionotropic cannabinoid receptors" (Di Marzo et al, 1998,

2001; Zygmunt et al, 1999; Akopian et al., 2009). These molecules, which function as highly Ca^{2+}-permeable ion channels, are the heat-sensitive TRPV1, TRPV2, TRPV3, and TRPV4 as well as the cold-activated TRPM8 and TRPA1 (Akopian et al., 2009; Vriens et al, 2009; Di Marzo and De Petrocellis, 2010; Pertwee et al, 2010). Hence, in the next phase of our experiments, we investigated the possible involvement of these molecules.

We first defined the expression profile of TRP channels on NCL-SG3 cells. As revealed by Q-PCR analysis, human sweat gland epithelial cells express all thermosensitive TRPV channels (**Supplementary Figure S2a**). However, we could not detect the expression of cold-sensitive TRPM8 and TRPA1 (data not shown).

We then investigated whether the TRP channel-mediated Ca^{2+}-influx to NCL-SG3 cells is involved in the action of cannabinoids. Using FLIPR-based Ca^{2+}-imaging we found that endocannabinoids (tested up to 50 μM concentration) did not modify the intracellular Ca^{2+}-concentration ($[Ca^{2+}]_i$) in NCL-SG3 cells (data not shown). In addition, we also observed that neither the "universal" TRP channel antagonists Ruthenium Red nor the suppression of extracellular $[Ca^{2+}]_e$ affected the cellular effects of endocannabinoids to suppress cellular growth and to induce lipid synthesis (**Supplementary Figure S2b and c**). These findings collectively suggest that TRP channels are most probably not involved in mediating the actions of cannabinoids in human sweat gland epithelial cells.

Endocannabinoids selectively stimulate the MAPK pathway in NCL-SG3 cells

Although we failed to identify surface membrane receptor/channel coupled mechanisms, we also aimed at identifying putative endocannabinoid-activated intracellular signaling pathways. Cannabinoids were shown to induce various signal transduction mechanisms, such as e.g. the mitogen-activated protein kinase (MAPK), protein kinase C (PKC) isoenzymes, phosphatidylinositide 3-kinase (PI-3K) (Howlett, 2005; Pacher et al. 2006); hence, we investigated the involvement of these mechanisms.

Using various pharmacological tools, we found that the general PKC inhibitor GF109203X and the PI-3K inhibitor Wortmannin did not affect the growth-inhibitory and lipid synthesis-promoting action of the endocannabinoids (**Figure 5a and b**). However, of great importance, the MAPK inhibitor PD098059 markedly (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 5c**), which effect was also abrogated by the application of the aforementioned antagonist (**Figure 5d**). These findings collectively argued for the crucial ~~(and selective)~~ involvement of the MAPK pathway in mediating the actions of endocannabinoids in human sweat gland epithelial cells.

DISCUSSION

In this study, we present the first evidence that prototypic endocannabinoids inhibit proliferation, induce cell death, and up-regulate secretory activity (lipid synthesis) in cultured human eccrine sweat gland epithelial cells. These data support the concept that human sweat glands may also function as novel targets for endocannabinoids, as emerging members of the skin neuroendocrine regulatory circuits (Slominski and Wortsman, 2000; Slominski et al, 2008).

In the course of our experiments, a key goal was to identify how endocannabinoids affect the differentiation process of the sweat gland cells. However, as we have detailed above, the exact "differentiation marker pattern" of cultured sweat gland epithelial cells is not fully known. Therefore, we have investigated the expressions of those cytoskeleton proteins (e.g. various CKs, involucrin, filaggrin, loricrin) which, on the one hand, were shown to be expressed in human sweat glands; and, on the other hand, were found to be involved in differentiation of certain skin cells such as e.g. epidermal and hair follicle keratinocytes (Candi et al, 2005; Langbein et al, 2005). Our experiments resulted in several novel findings: (i) To our best knowledge, this is the first demonstration that levels of these molecules in cultured human sweat gland epithelial cells are strongly affected by the growth rate (i.e. proliferation vs. high cell density-induced growth arrest) of the cells. (ii) Moreover, we have also found that expression patterns of some of these markers were very similar to those described (by us and others) in human epidermal keratinocytes (Papp et al, 2003, 2004; Candi et al, 2005; Langbein et al, 2005; Moll et al, 2008). Namely, in both cell types, highest levels of the "differentiation markers" "involucrin, filaggrin, and loricrin were detected in the post-confluent (hence more differentiated) cultures. Nevertheless, it should

also be noted, that the cytoskeleton protein profile of sweat gland cells, by far, was not identical to those of epidermal keratinocytes since e.g. expressions of the "classical" epidermal markers CK1 and 10 were not detected in cultured sweat gland cells. (iii) Of further importance, we have also shown that endocannabinoids up-regulated the expression of the above "post-confluent" markers (i.e. involucrin, filaggrin, and loricrin) whereas suppressed expressions of some of those cytoskeleton proteins which were expressed at high levels in the pre-confluent (hence greatly proliferation) cultures. Taken together, these findings collectively argue for that ~~locally produced~~ endocannabinoids not only inhibit cell growth and induce cell death, but may also promote the differentiation process in the human sweat gland epithelium.

We also wished to uncover the signaling pathways that were involved in mediating the cellular actions of endocannabinoids. ~~Importantly, we have successfully identified elements of the ECS (i.e. receptors, enzymes) in NCL-SG3 cells.~~ Quite unexpectedly, we were unable to find evidence for the involvement of the "classical" metabotropic CB_1 and CB_2 receptors (lack of effects of CB_1 or CB_2 antagonists or RNAi-mediated silencing of these receptors), or the "ionotropic cannabinoid receptor" TRP channels (lack of elevation of $[Ca^{2+}]_i$ upon endocannabinoid challenge) expressed by these cells. Although these observations suggested a CB_1/CB_2/TRP-independent cellular action of the endocannabinoids – similar to as seen on various cell types including e.g. leukocytes, endothelial cells, etc. (Rockwell et al, 2006; McCollum et al, 2007) – we have intriguingly found that both AEA and 2-AG selectively stimulated the MAPK pathway (but not the PI3-K and PKC signal transduction mechanisms). Since the MAPKs are most often activated by receptor-coupled processes, further studies are

now invited to define the exact intracellular signaling of endocannabinoids in human sweat gland cells.

Comparison of the above findings with our previously published data obtained on other of human skin adnexal structures revealed another exciting feature of the cutaneous effects of cannabinoids. Namely, our presentations that endocannabinoids (i) inhibit human hair growth and induce apoptosis by activating of CB_1 (Telek et al, 2007); (ii) promote lipid synthesis and apoptosis in human sebaceous gland-derived sebocytes via CB_2-mediated signaling (Dobrosi et al, 2008); (iii) inhibit proliferation, induce cell death, and stimulate lipid synthesis and differentiation of human sweat gland epithelial cells by activating non-CB_1/CB_2-coupled signal transduction pathways; highlight the existence of cell type-specific and (most probably) receptor-selective regulatory endocannabinoid mechanisms in the human skin appendages.

Of further importance, sweat gland epithelial cells apparently are not only targets but sources of endocannabinoids. Indeed, mass spectrometry analysis revealed that NCL-SG3 sweat gland cells produce the prototypic endocannabinoids AEA and 2-AG. However, the concentrations of the endocannabinoids determined in NCL-SG3 cells (AEA, 15 fmol/10^6 cells; 2-AG, 0.2 pmol/10^6 cells) were much less than those found in other human cultures skin cells (e.g. AEA, 160 fmol/10^6 cells; 2-AG, 4.2 pmol/10^6 cells in human SZ95 sebocytes) (Telek et al, 2007, Dobrosi et al, 2008). In addition, we were also able to identify the expression of those enzymes which are involved in the synthesis (NAPE-PLD, N-acylphosphatidylethanolamine-hydrolyzing phospholipase D; DAGL, diacylglycerol lipase-α and -β) and degradation (FAAH, fatty acid amid hydrolase; MAGL, monoacylglycerol lipase) of the endocannabinoids

(**Supplementary Figure S3**). Interestingly, similar to CB_1 and CB_2 receptors (**Figure 3**), we also detected marked fluctuations in the expressions of the elements of the ECS further suggesting that the role of the ECS in the regulation of growth (and most probably of differentiation) of the human sweat gland-derived cells. Finally, by performing immunohistochemical labeling on human skin sections, we were able to identify the elements of the ECS on eccrine sweat glands *in situ* (**Supplementary Figure S4**) which findings perfectly complement the above PCR data obtained on NCL-SG3 cells.

Evidently, further explorative research efforts are needed to define whether alterations in the activities of the above enzymes can modify the endocannabinoid production (hence the endocannabinoid "tone") in the human sweat glands *in situ*. Nevertheless, our findings now warrant proof-of-principle clinical studies to test the therapeutic value of cutaneous ECS-targeted approaches in the clinical management of multitude of human skin diseases. Specifically, based on our current preclinical data in human sweat gland model cell cultures, it is envisaged that agents increasing the cutaneous endocannabinoid "tone" (such as employing endocannabinoids or molecules which up-regulate the production of endocannabinoids by e.g. stimulating their synthesis of inhibiting their degradation) (Di Marzo, 2008; Bíró et al, 2009) may be successfully applied in certain sweat gland disorders (e.g. benign or malignant tumors) characterized by unwanted cell growth – similar to as we have previously suggested 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).

MATERIALS AND METHODS

Materials

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

Cell culturing

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).

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 the with rabbit primary antibodies against CB_1 and CB_2 (both 1:200). 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 antibody (1:50, Santa Cruz, Santa Cruz, CA) 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) (Gönczi et al, 2088; Szegedi et al, 2008).

Immunocytochemistry

Cells were fixed in acetone, permeabilized by 0.1 % Triton-X-100 (Sigma-Aldrich), and then incubated with the above primary antibodies against CB_1 or CB_2 (1:200 dilution, Cayman). For fluorescence staining, slides were then incubated with fluorescein-isothiocytanate (FITC) conjugated secondary antibodies (dilution 1:200) (Bodó et al, 2005; Dobrosi et al, 2008; Tóth et al, 2009).

Quantitative Real-Time PCR (Q-PCR)

Q-PCR was performed on an ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA) using the 5' nuclease assay as detailed in our previous reports (Dobrosi et al, 2008; Tóth et al, 2009, 2011). PCR amplification was performed by using TaqMan primers and probes (Applied Biosystems) (see **Supplementary data** for Assay IDs).

~~*Determination of endocannabinoid levels*~~

~~Endocannabinoids were determined by liquid chromatography/in line mass spectrometry as described in our earlier reports (Telek et al, 2007; Dobrosi et al., 2008) (see **Supplementary data** for further details).~~

Determination of viable cell numbers and proliferation

The number of viable cells (hence the rate of proliferation) was determined by measuring the conversion of the tetrazolium salt MTT (Sigma-Aldrich) to formazan by mitochondrial dehydrogenases. In addition, proliferation was also assessed by the CyQuant fluorimetric Cell Proliferation Assay Kit (Invitrogen) according to the manufacturer's protocol (Bodó et al, 2005; Kiss et al, 2008; Szegedi et al, 2009) (see **Supplementary data** for further details).

Determination of apoptosis

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis. Mitochondrial membrane potential of NCL-SG3 cells was determined using a MitoProbe™ $DiIC_1(5)$ Assay Kit (Invitrogen) where the decrease in fluorescence intensity reflects apoptosis. In addition, apoptosis was also determined by fluorimetric measurement of activation of pro-apoptotic caspases using a Poly Caspases Detection Kit (Invitrogen) (Dobrosi et al., 2008; Tóth et al, 2010, 2011) (see **Supplementary data** for further details).

Determination of cytotoxicity (necrosis)

Necrotic cell death was determined by measuring the glucose-6-phophate-dehydrogenase (G6PD) release (G6PD Release Assay Kit, Invitrogen). Moreover, since the activity of the G6PD released from necrotic cells decreases over 24-36 hrs, the cytotoxic effects of long term treatment protocols were assessed by the fluorimetric determination of Sytox Green accumulation to nuclei of necrotic cells with ruptured plasma membranes (Invitrogen) (Tóth et al, 2010, 2011) (see **Supplementary data** for further details).

Determination of intracellular lipids

For semi-quantitative detection of cellular lipids, cells were fixed in 4% paraformaldehyde, washed in 60% isopropanol (both Sigma-Aldrich), and stained in freshly prepared Oil Red O solution (in 60% isopropanol) (Sigma-Aldrich). Nuclei were counterstained with Mayer's hematoxylin (Sigma-Aldrich) and coverslips were mounted in mounting medium (DAKO, Glostrup, Denmark). In addition, for quantitative measurement of lipid content, a Nile Red (Sigma-Aldrich) fluorimetric method was employed as described before (Wróbel et al, 2003; Alestas et al, 2006; Dobrosi et al, 2008; Tóth et al, 2009) (see **Supplementary data** for further details).

RNA interference (RNAi)

NCL-SG3 cells at 50-70 % confluence were transfected with specific Stealth RNAi oligonucleotides (40 nM, all from Invitrogen) against CB_1 (ID No. HSS102082) or CB_2 (ID No. HSS102087) using Lipofectamine 2000 transfection reagent (Invitrogen). For controls, RNAi Negative Control Duplexes (Scrambled RNAi, Invitrogen) were employed. The efficacy of RNAi-driven "knock-down" was daily evaluated by Q-PCR and Western blotting for 4 days (see **Supplementary Figure S1** and **Supplementary data** for further details).

Ca^{2+} imaging

Changes in $[Ca^{2+}]_i$ upon drug applications were detected by fluorimetric Ca^{2+} imaging (Bodó et al, 2005; Gönczi et al, 2008; Tóth et al, 2009). Cells were seeded in 96-well black-well/clear-bottom plates (Greiner Bio One, Frickenhausen, Germany) at a density of 10,000 cells/well and then were incubated with culturing medium containing the cytoplasmic calcium indicator 2 μM 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 ℃. The plates were then placed to a FlexStation II^{384} fluorimetric image plate reader (FLIPR, Molecular Devices, San Francisco, CA) and changes in $[Ca^{2+}]_i$ (reflected by changes fluorescence; IEX=494 nm, IEM=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).

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. In addition, statistical differences were further verified using One-Way ANOVA with Bonferroni and Dunnett post-hoc probes, resulting in similar results (data not shown).

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FIGURE LEGENDS

Figure 1. *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 $DiIC_1(5)$ 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.

Figure 2. *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. **e**) 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. **f)** 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 %,

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.

Figure 3. *CB receptors are expressed in NCL-SG3 cells*

a) Western blot analysis. Protein expressions of CB_1 and CB_2 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, Maccarrone et al, 2003; Karsak et al, 2007; Paradisi et al, 2008; Tóth et al, 2011). Three additional experiments yielded similar results.

Figure 4. *Effects of endocannabinoids are not mediated by CB_1 and CB_2 expressed 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. **c, d**) Various RNAi probes against CB_1 or CB_2, 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). **a, c**) MTT cellular viability/proliferation assay performed after 48 hrs. **b, d)** 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 (**a, b**) or of the SCR group (**c, d**). * marks significant (P<0.05)

differences compared to the vehicle-treated control group (**a, b**) or to the SCR group (**c, d**). n=4 in each group. Two-three additional experiments yielded similar results.

Figure 5. *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 n=4 in each group. **c, d**) Cells were treated by AEA and 2-AG (**c**) or in combination with PD (**d**) 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.

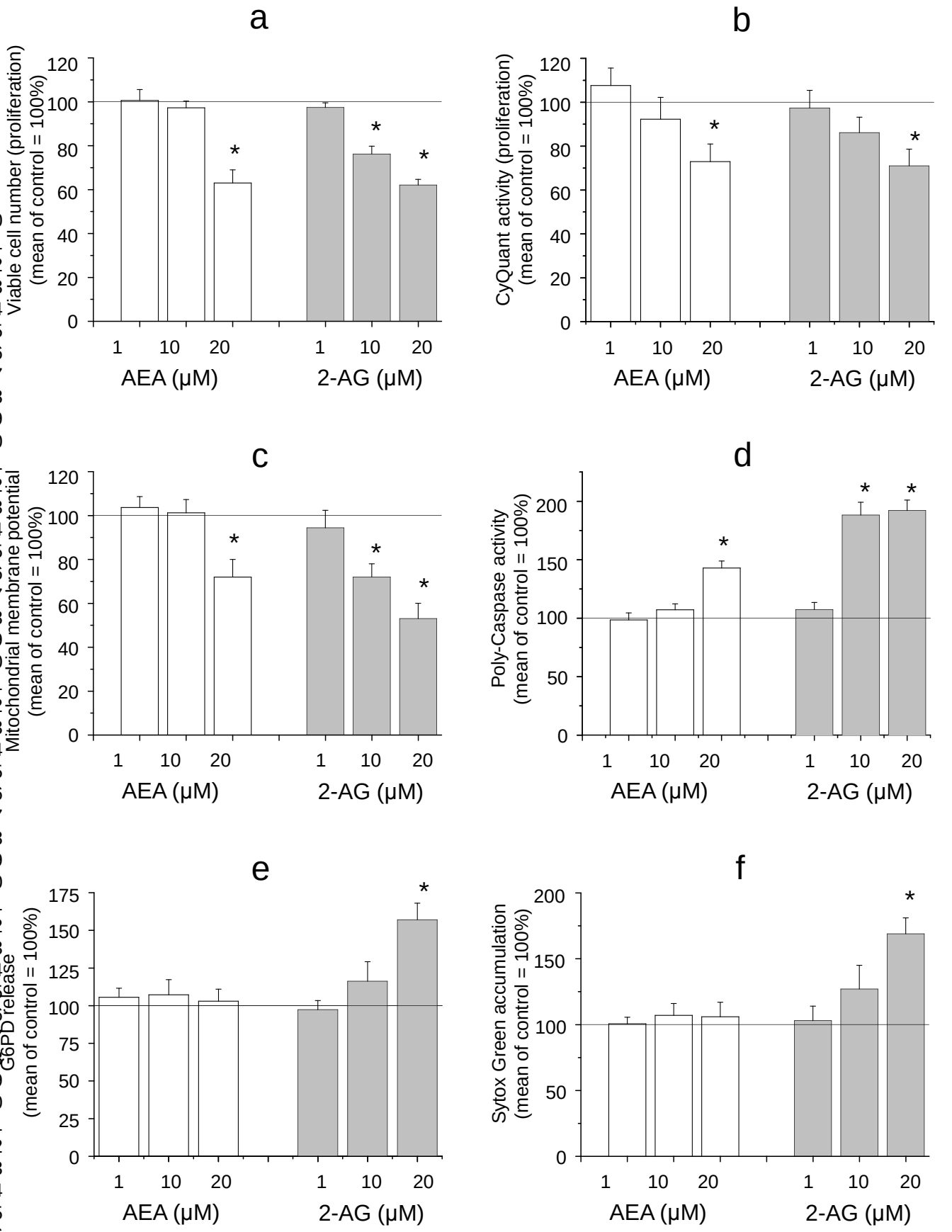

a
Viable cell number (proliferation)
(mean of control = 100%)
120
100
80
60
40
20
0
*
*
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)
b
CyQuant activity (proliferation)
(mean of control = 100%)
120
100
80
60
40
20
0
*
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)
c
Mitochondrial membrane potential
(mean of control = 100%)
120
100
80
60
40
20
0
*
*
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)
d
Poly-Caspase activity
(mean of control = 100%)
200
150
100
50
0
*
*
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)
e
G6PD release
(mean of control = 100%)
175
150
125
100
75
50
25
0
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)
f
Sytox Green accumulation
(mean of control = 100%)
200
150
100
50
0
*
1
10
20
1
10
20
AEA (μM)
2-AG (μM)

Figure 1

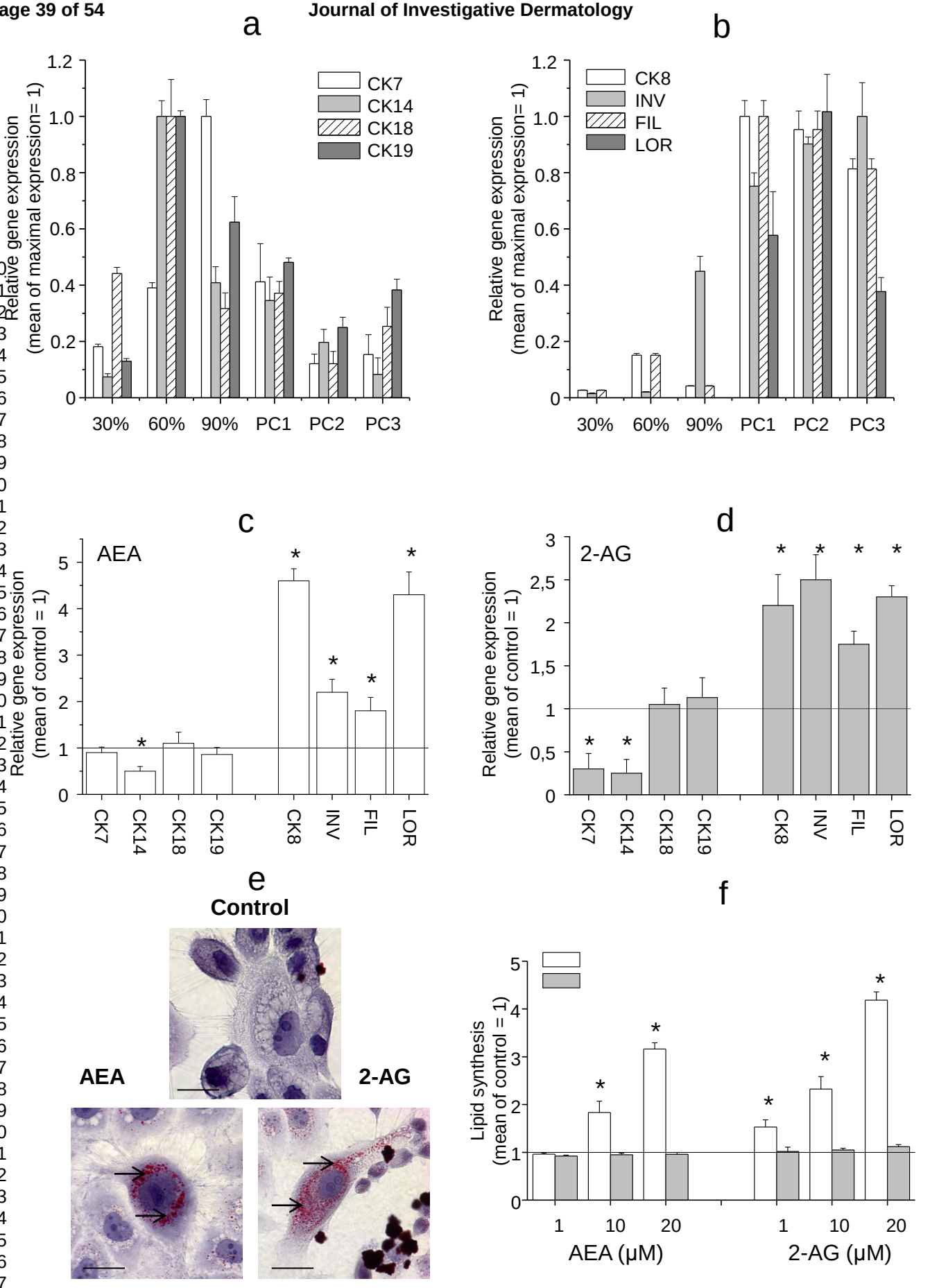

a
Relative gene expression (mean of maximal expression= 1)
CK7
CK14
CK18
CK19
30%
60%
90%
PC1
PC2
PC3
b
Relative gene expression (mean of maximal expression= 1)
CK8
INV
FIL
LOR
c
AEA
Relative gene expression (mean of control = 1)
CK7
CK14
CK18
CK19
CK8
INV
FIL
LOR
d
2-AG
Relative gene expression (mean of control = 1)
e
Control
AEA
2-AG
f
Lipid synthesis (mean of control = 1)
AEA (μM)
2-AG (μM)

Figure 2

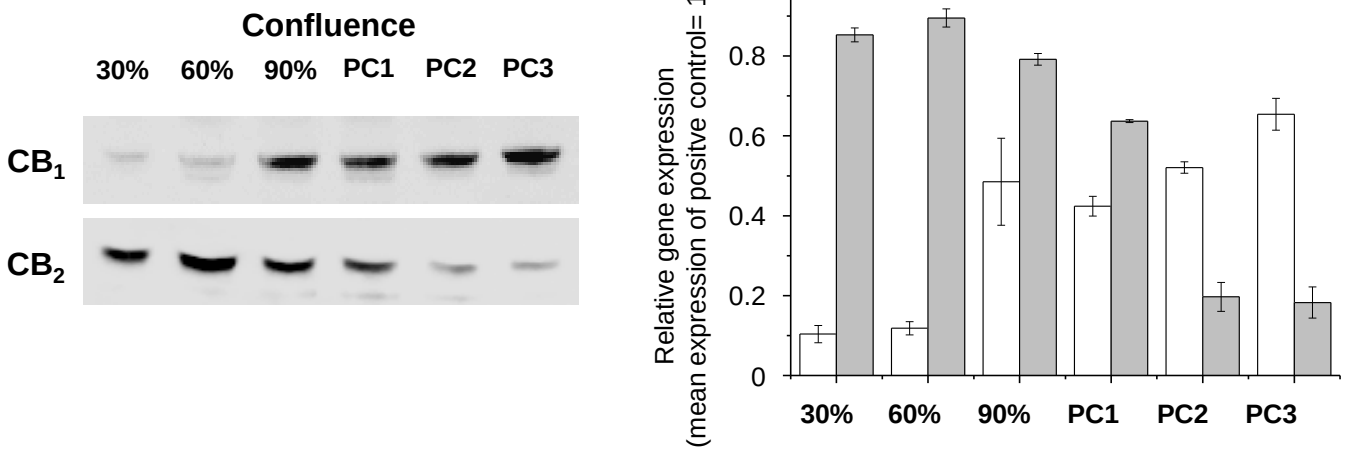

a
Confluence
30%
60%
90%
PC1
PC2
PC3
CB1
CB2
b
CB1
CB2
Relative gene expression
(mean expression of positive control= 1)
1.0
0.8
0.6
0.4
0.2
0
30%
60%
90%
PC1
PC2
PC3

Figure 3

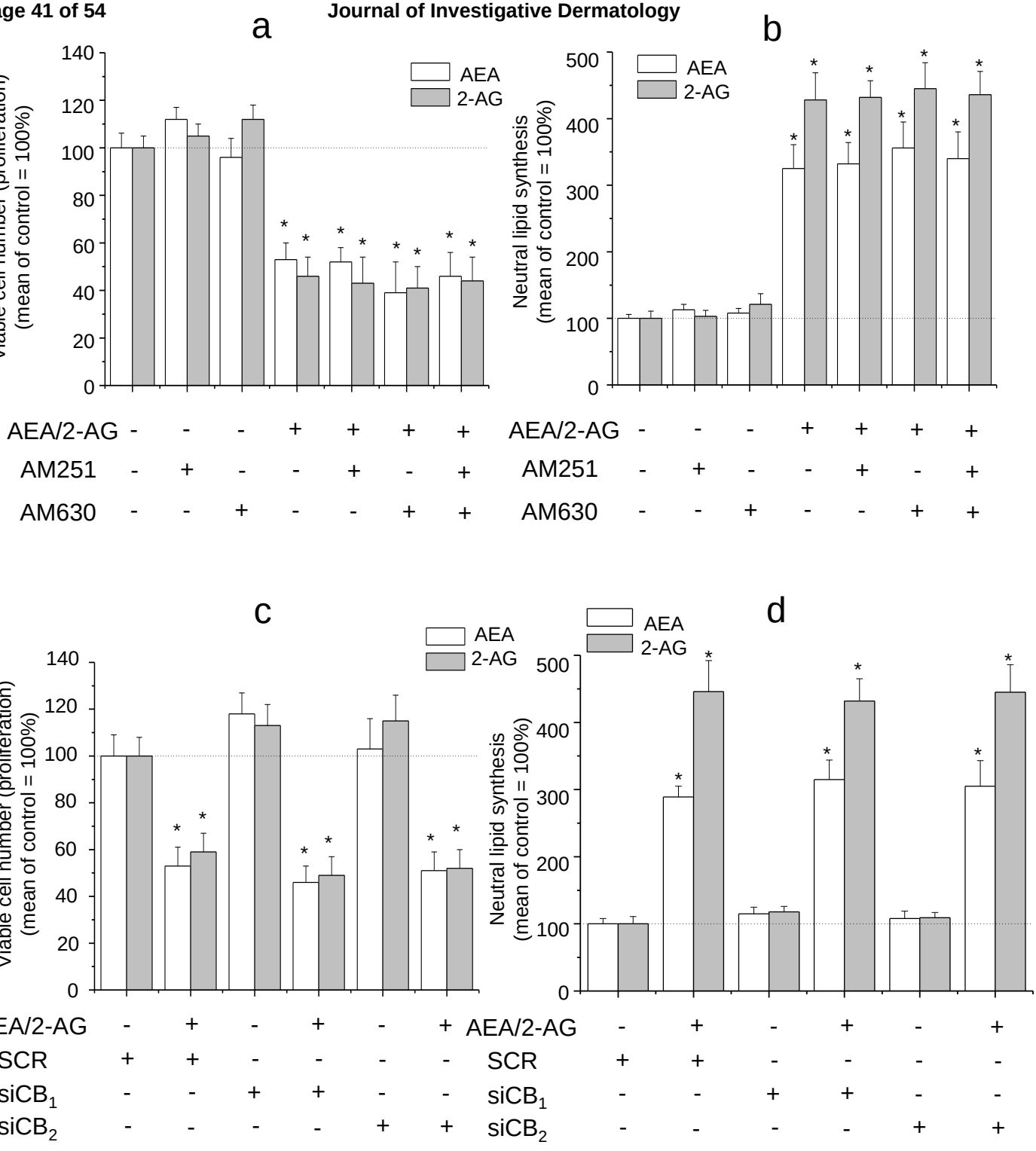
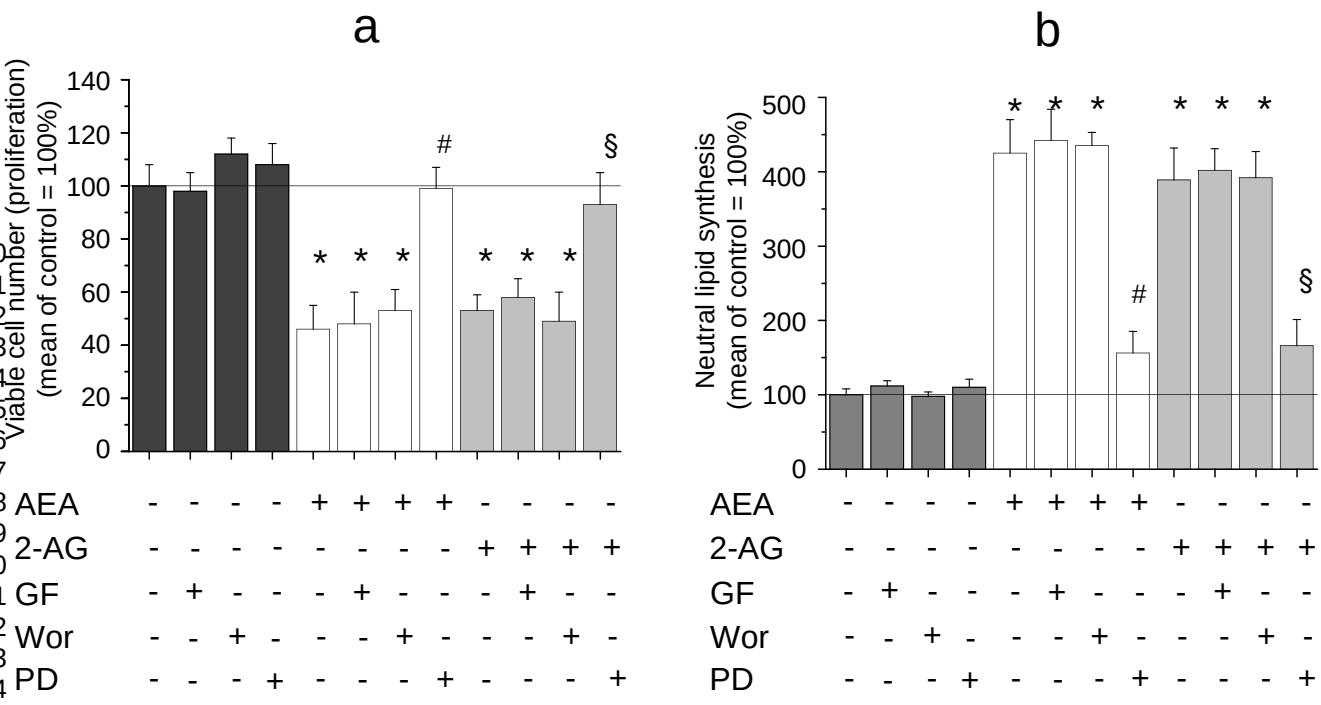
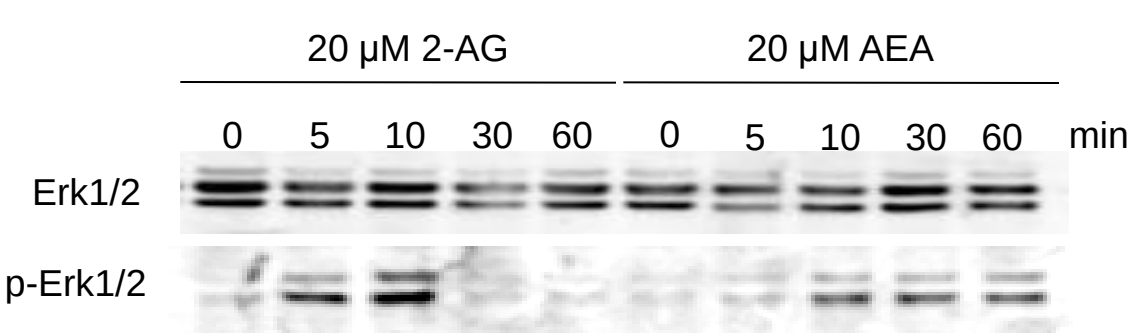

a
b
c
d
AEA
2-AG
Viable cell number (proliferation)
(mean of control = 100%)
Neutral lipid synthesis
(mean of control = 100%)
AEA/2-AG
AM251
AM630
SCR
siCB1
siCB2

Figure 4


a
Viable cell number (proliferation)
(mean of control = 100%)
140
120
100
80
60
40
20
0
*
#
§
AEA - - - - + + + + - - - -
2-AG - - - - - - - - + + + +
GF - + - - - + - - - + - -
Wor - - + - - - + - - - + -
PD - - - + - - - + - - - +
b
Neutral lipid synthesis
(mean of control = 100%)
500
400
300
200
100
0
*
#
§
AEA - - - - + + + + - - - -
2-AG - - - - - - - - + + + +
GF - + - - - + - - - + - -
Wor - - + - - - + - - - + -
PD - - - + - - - + - - - +


c
20 µM 2-AG
20 µM AEA
0 5 10 30 60 0 5 10 30 60 min
Erk1/2
p-Erk1/2

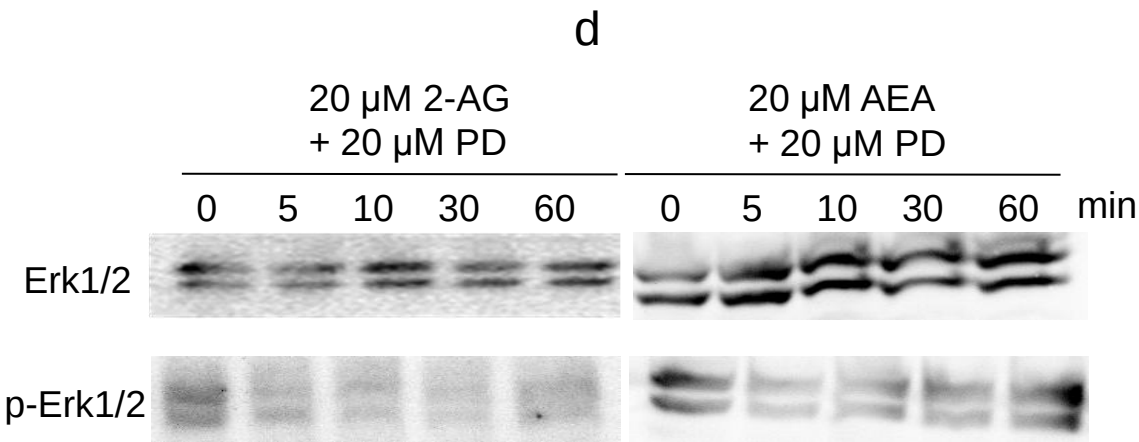

d
20 µM 2-AG
+ 20 µM PD
20 µM AEA
+ 20 µM PD
0 5 10 30 60 0 5 10 30 60 min
Erk1/2
p-Erk1/2

Figure 5

SUPPLEMENTARY DATA

Supplementary Materials and Methods

Taqman pimers and probes

For the Q-PCR determination, the following Taqman probes were used (Applied Biosystems). Assay ID: Hs00275634_m1 for human CB_1; Assay ID: Hs00361490_m1 for human CB_2; 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 CK1; 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 INV; Assay ID: Hs01894962_s1 for human LOR; Assay ID: Hs00856927_g1 for human 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).

Immunohistochemistry

The study was approved by the Institutional Research Ethics Committee and adhered to Declaration of Helsinki guidelines. Frozen skin blocks were obtained from normal female trunk skin samples, acquired during plastic surgery. Four µm-thick sections

were obtained and fixed in acetone. After blocking of the endogenous peroxidase activity, tissue sections were incubated with primary antibodies: FAAH, DAGLα and β (Santa Cruz Biotechnology Inc., Santa Cruz, USA) (dilution 1:50), NAPE-PLD, (Atlas Antibodies AB, Stockholm, Sweden), and MAGL (Abcam, Cambridge, United Kingdom) (dilution 1:50). Sections were then incubated with the EnVision+ System Labeled polymer-HRP Anti-Rabbit, Anti-Mouse or Anti-Goat (Dako, Glostrup, Denmark) with 3,3'-diaminobenzidine (DAB) visualization techniques. Cell nuclei were counterstained with haematoxylin and tissue samples were finally mounted in aqueous mounting medium (Dako).

Determination of endocannabinoid levels

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 x 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 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, Frickenhausen, Germany) 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 Molecular Devices FlexStation II^{384} Fluorescence Image Plate Reader (FLIPR) equipment (Molecular Devices, San Francisco, CA).

Determination of apoptosis

A decrease in the mitochondrial membrane potential is one of the earliest markers of apoptosis. Mitochondrial membrane potential of NCL-SG3 cells was determined using a MitoProbe™ $DiIC_1(5)$ Assay Kit (Invitrogen) where the decrease in fluorescence intensity reflects apoptosis. 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 $DiIC_1(5)$ working solution (30 µl/well) and the fluorescence of $DiIC_1(5)$ was measured at 630 nm excitation and 670 nm emission wavelengths using a FLIPR.

In addition, apoptosis 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 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. NCL-SG3 cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio One) in quadruplicates and treated with various compounds for 24-48 hrs. 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 the FLIPR device at 545 excitation and 590 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 FLIPR.

Quantitative determination of intracellular lipids

Cells (10,000 cells/well) were cultured in 96-well black-well/clear-bottom plates (Greiner Bio-One, Frickenhausen, Germany) 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 phosphate buffered saline (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.

Supplementary Figure Legends

Supplementary Figure S1. *Assessment of efficacy of RNAi on NCL-SG3 cells*

Various RNAi probes against CB_1 or CB_2, 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.

Supplementary Figure S2. *Effects of endocannabinoids are not mediated by TRP channels expressed on NCL-SG3 cells*

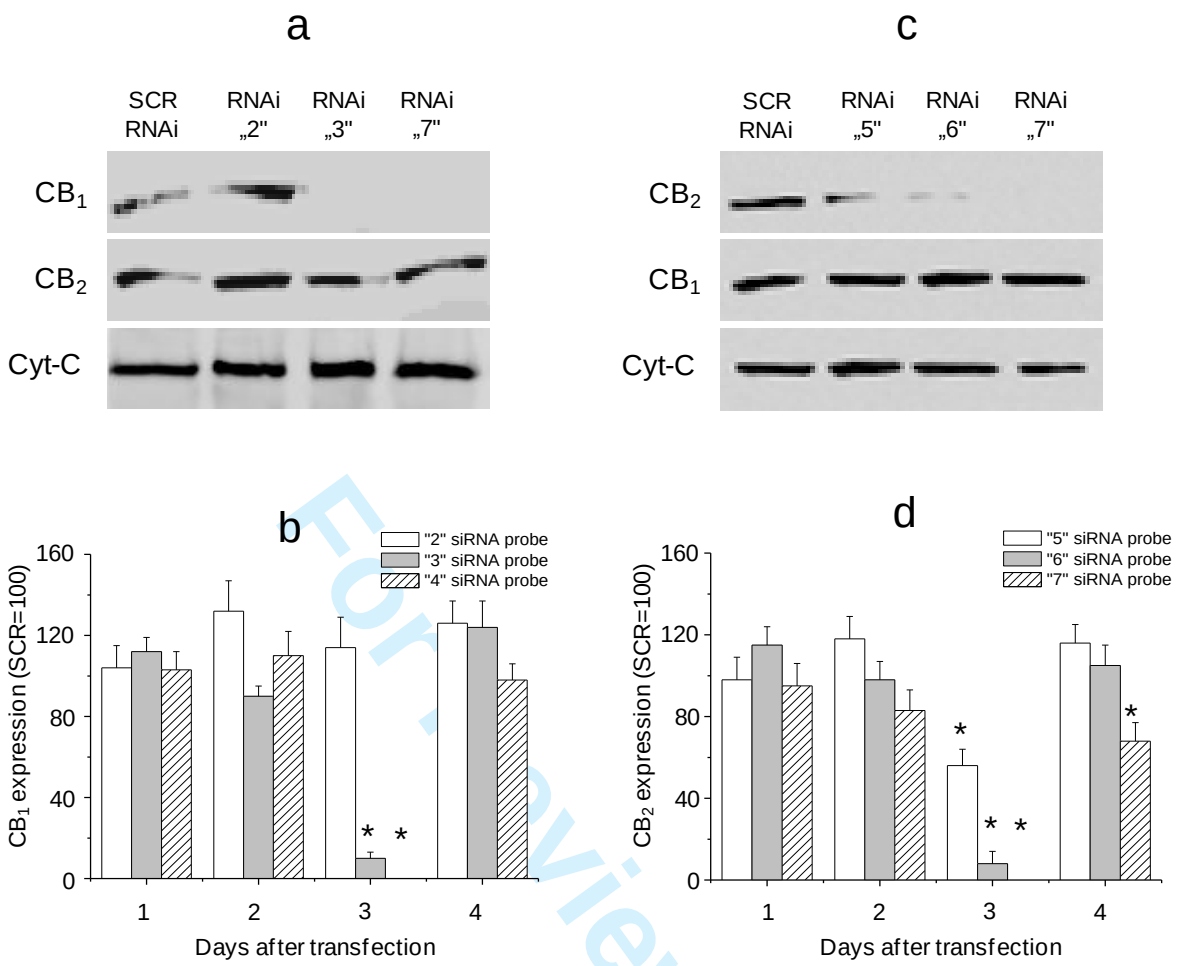
a) Q-PCR analysis of expression profiles of TRP channels at various confluences. PC2, 2 days after post-confluence. **b, c)** Cells were treated with AEA (20 μM), 2-AG (20 μM), Ruthenium Red (100 μM, RR), or combination. In addition., endocannabinoid treatment was performed when the extracellular Ca^{2+}-concentration 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.

Supplementary Figure S3. *NCL-SG3 cells express components of the ECS*

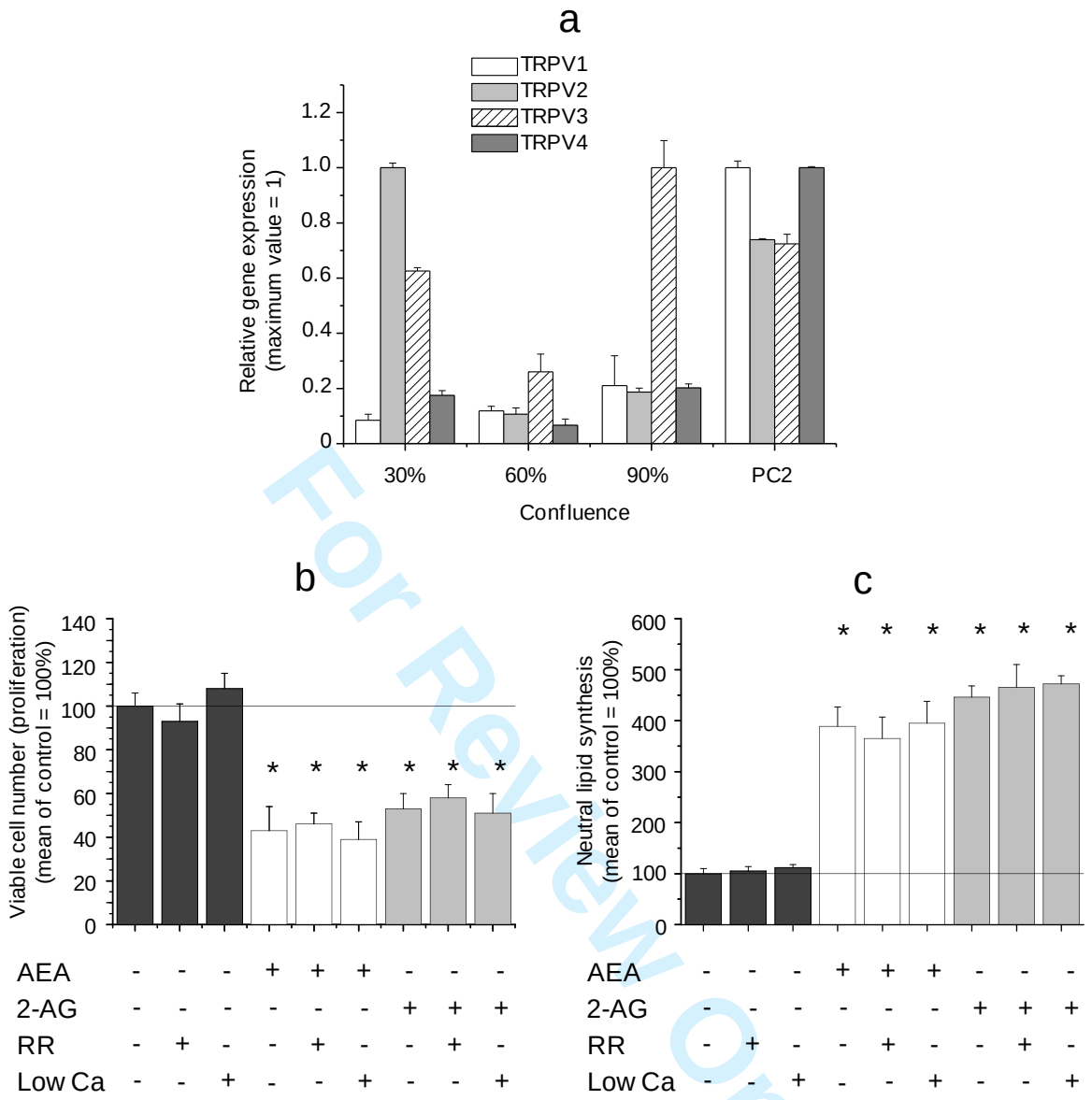
Q-PCR analysis of various endocannabinoid synthesizing (DAGLα, DAGLβ, NAPE-PLD) and degrading enzymes (MAGL and FAAH) on NCL-SG3 cells at various confluences. PC1-3, 1-3 days at post-confluence. Data (mean±SEM) are expressed as a fraction of the mean value of expressions (defined as 1) determined in cultured human epidermal keratinocytes (used as a positive control, Maccarrone et al, 2003; Karsak et al, 2007; Paradisi et al, 2008; Tóth et al, 2011). Three additional experiments yielded similar results.

Supplementary Figure S4. *Human sweat glands express components of the ECS in situ*

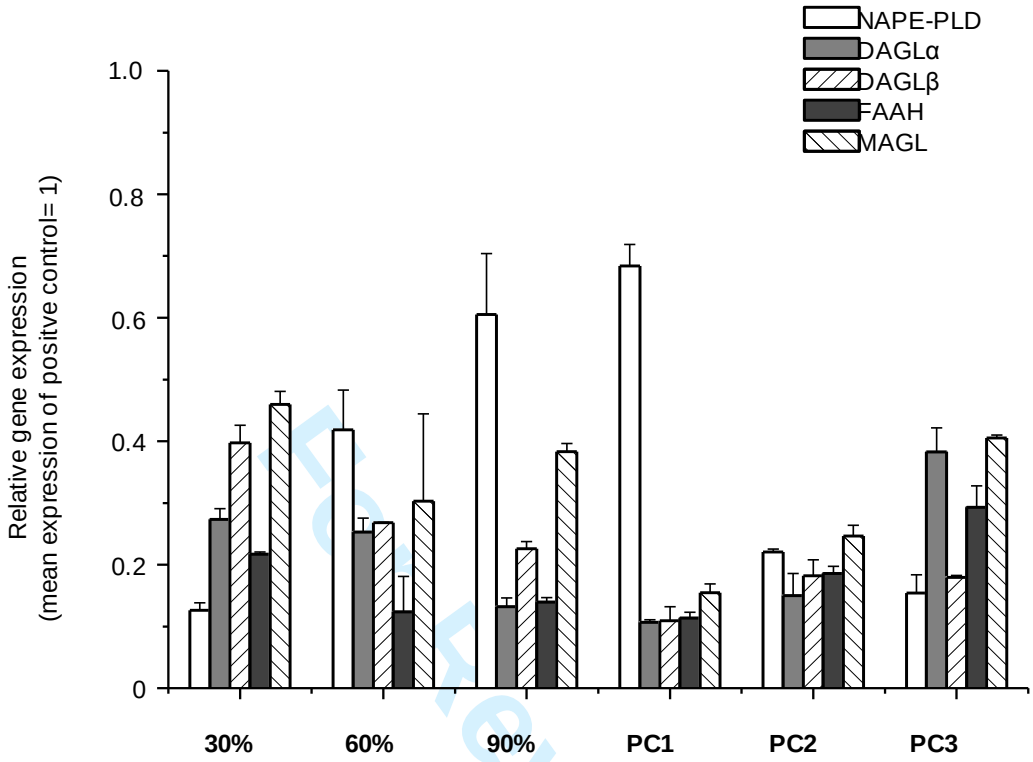
Immunohistochemical analysis of various endocannabinoid synthesizing (DAGLα, DAGLβ, NAPE-PLD) and degrading enzymes (MAGL and FAAH of as revealed by EnVision technique (brown staining), on human sweat gland epithelial cells *in situ*.



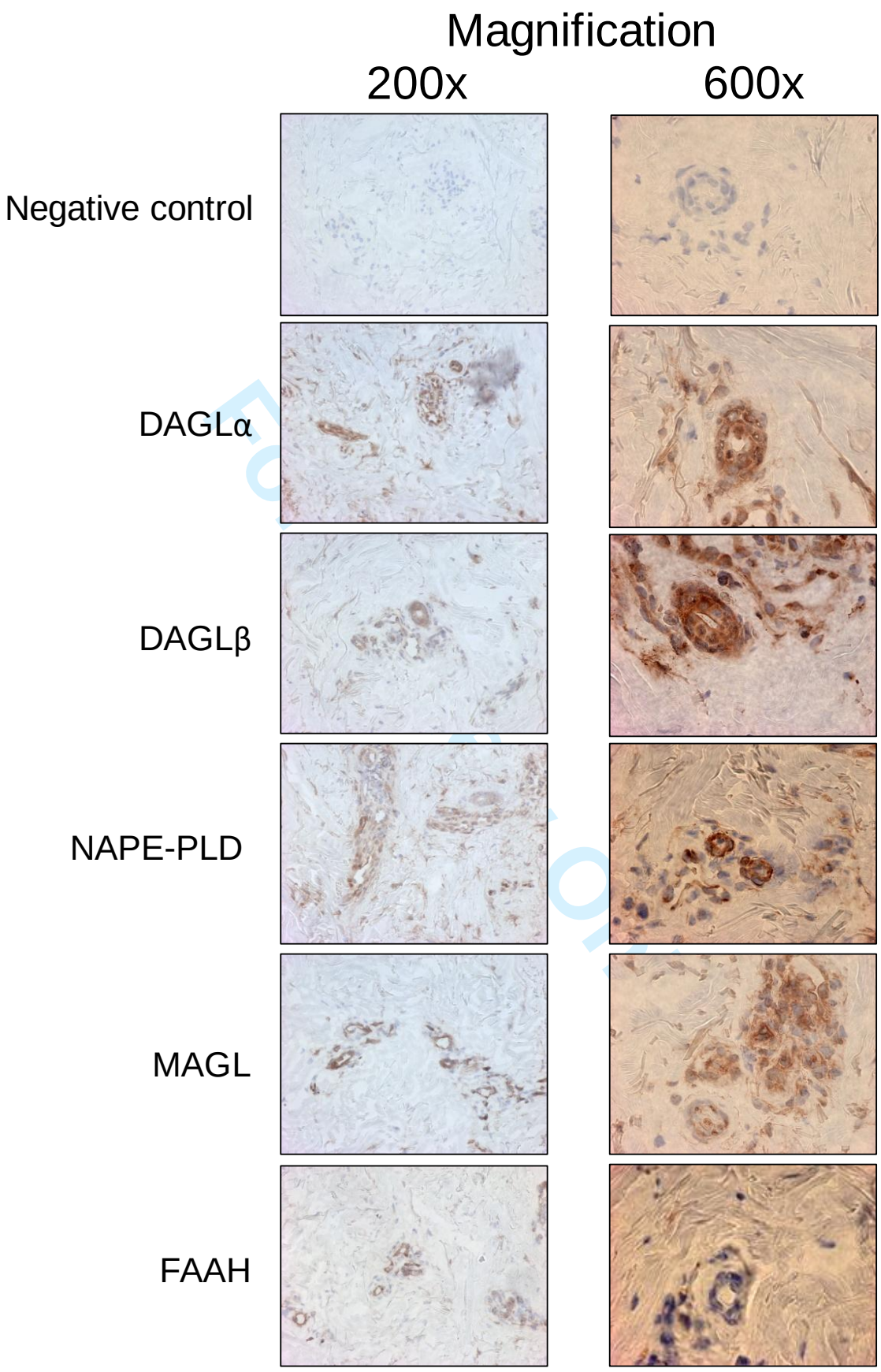
Supplementary Figure S1



Supplementary Figure S2



Supplementary Figure S3



Supplementary Figure S4