The influence of biomechanical properties and cannabinoids on tumor invasion

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ABSTRACT
Background: Cannabinoids are known to have an anti-tumorous effect, but the underlying mechanisms are only sparsely understood. Mechanical characteristics of tumor cells represent a promising marker to distinguish between tumor cells and the healthy tissue. We tested the hypothesis whether cannabinoids influence the tumor cell specific mechanical and migratory properties and if these factors are a prognostic marker for the invasiveness of tumor cells.

Methods: 3 different glioblastoma cell lines were treated with cannabinoids and changes of mechanical and migratory properties of single cells were measured using atomic force microscopy and time lapse imaging. The invasiveness of cell lines was determined using a co-culture model with organotypic hippocampal slice cultures.

Results: We found that cannabinoids are capable of influencing migratory and mechanical properties in a cell line specific manner. A network analysis revealed a correlation between a "generalized stiffness" and the invasiveness for all tumor cell lines after 3 and 4 d of invasion time: $r_{3d} = -0.88 [-0.52; -0.97]$; $r_{4d} = -0.90 [-0.59; -0.98]$.

Conclusions: Here we could show that a "generalized stiffness" is a profound marker for the invasiveness of a tumor cell population in our model and thus might be of high clinical relevance for drug testing. Additionally cannabinoids were shown to be of potential use for therapeutic approaches of glioblastoma.

KEYWORDS
cannabinoids; cell mechanics; glioblastoma; network analysis; slice cultures

Introduction
Cancer causes millions of death per year\(^1\) with increasing tendency.\(^2\) Thus there is a need of better detection techniques and therapeutic approaches that specifically target the differences between cancer and non-cancer cells. These differences refer to the internal structure, function and behavior of the whole cell or its components leading to different mechanical properties of tumor cells.\(^3-6\) essential for tumor progression.\(^7-9\) Due to high turnover and changes of their cytoskeleton cancer cells tend to be more responsive to external stress altering their biomechanical properties e.g. elasticity. Changes in elasticity can be determined and quantified by measuring the Young’s modulus. An atomic force microscope (AFM) represents a suitable instrument to analyze the elastic properties and the Young’s modulus by physically indenting the cells with a defined force. From all known tumor types the glioblastoma multiforme belongs to the most aggressive ones in terms of proliferation, intracerebral expansion and survival. It is highly resistant to radiation therapy and other treatments\(^10-14\) and thus of uttermost interest. One potential therapeutic target for glioblastoma might be the endocannabinoid (eCB) system. A clinical trial with glioblastoma patients in terminal phase revealed a potential anti-proliferative effect of cannabinoids.\(^15\) In human glioma reduced anandamide levels, a cannabinoid receptor (CB) 1 and CB2 agonist, were found while the level of the other main CB1 agonist 2-arachidonylglycerol was increased.\(^16\) This indicates that the eCB system is part of the (anti)tumor signaling. Most of the studies analyzing the effects of cannabinoids on tumor cells have focused on apoptosis\(^17-19\) or cell cycle arrest.\(^18,20\) Changes and disruptions of the cytoskeleton in a quantitative manner and thus mechanical parameters have been investigated only sparsely.

In this study we analyzed migratory and mechanical properties of single glioblastoma cell lines under the influence of specific synthetic CB1 and CB2 agonists, changes in FAK phosphorylation, as well as the invasive properties of several cell lines in organotypic hippocampal slice cultures (OHSC).
Results

Endocannabinoid receptors on glioblastoma cell lines

U138, LN229 and U87 cells expressed the CB1 receptor at mRNA and protein level, as shown in Figure 1A and B. Cnr2-mRNA was found in U138 cells only (Fig. 1A). The immunohistochemical staining of CB1 confirmed the results of the PCR and Western Blot analysis (Fig. 1C).

Single cell measurements – atomic force microscopy

Control experiments with solvents ethanol and DMSO showed that the measured properties were not affected (data not shown).

The only measurable effect of cannabinoids on the Young’s modulus of the used cells was observed for the U138 cells. The CB2 agonist JWH-133 led to softer U138 cells and reduced the modulus significantly from $E_{\text{CTL}} = 1692$ Pa to $E_{\text{JWH-133}} = 1270$ Pa, while ACEA had an opposing effect ($E_{\text{ACEA}} = 1817$ Pa). No significant effect was observed for LN229 ($E_{\text{CTL}} = 1584$ Pa; $E_{\text{JWH-133}} = 1676$ Pa; $E_{\text{ACEA}} = 1593$ Pa) and U87 ($E_{\text{CTL}} = 1079$ Pa; $E_{\text{JWH-133}} = 1094$ Pa; $E_{\text{ACEA}} = 1028$ Pa) cells treated with JWH-133 or ACEA (Fig. 2A). The measured adhesion energies, defined by the needed energy to detach the cantilever from the cell surface, were decreased after the incubation with cannabinoids in almost all conditions (Fig. 2B). For U138 cells the adhesion energy was reduced from $U_{\text{CTL}} = 8.4 \mu J/m^2$ to $U_{\text{JWH-133}} = 6.0 \mu J/m^2$ or $U_{\text{ACEA}} = 5.8 \mu J/m^2$ respectively. LN229 depicted a decrease from $U_{\text{CTL}} = 9.2 \mu J/m^2$ to $U_{\text{JWH-133}} = 5.5 \mu J/m^2$ or $U_{\text{ACEA}} = 4.4 \mu J/m^2$. U87 cells treated with JWH-133 showed a reduction in adhesion energy from $U_{\text{CTL}} = 9.0 \mu J/m^2$ to $U_{\text{JWH-133}} = 7.1 \mu J/m^2$. Only for U87 cells treated with ACEA no effect was observed ($U_{\text{ACEA}} = 8.2 \mu J/m^2$).

Single cell measurements – time lapse imaging

Measured parameters in time lapse microscopy were not significantly changed by the solvents ethanol and DMSO (data not shown).

The live cell experiments showed a decrease in speed for U138 cells treated with ACEA from $v_{\text{CTL}} = 0.32 \mu m/min$ to $v_{\text{ACEA}} = 0.29 \mu m/min$, while JWH-133 did not have any effect on this cell line ($v_{\text{JWH-133}} = 0.31 \mu m/min$). A similar behavior was observed for LN229 cells, displaying a decrease in speed under influence of ACEA from $v_{\text{CTL}} = 0.51 \mu m/min$ to $v_{\text{ACEA}} = 0.26 \mu m/min$, without being affected by JWH-133 treatment ($v_{\text{JWH-133}} = 0.47 \mu m/min$). In contrast, ACEA as well as JWH-133 led to a strong increase in speed in U87 cells from $v_{\text{CTL}} = 0.58 \mu m/min$ to $v_{\text{ACEA}} = 0.88 \mu m/min$ and...
The analysis of the contact area for each cell type and treatment depicted that both agonists had no significant effect on U138 cells (ACTL_9809 px; AACEA_10015 px). LN229 cells showed no response to the CB1 agonists with ACTL_4354 px and AACEA_3866 px, but a reduction of the contact area to AJWH-133_3092 px, when treated with the CB2 agonist. For U87 cells both agonists lead to a strong decrease in area (Fig. 2D) from an initial contact area of ACTL_6433 px to AJWH-133_3406 px and AACEA_4016 px respectively.

**Analysis of focal adhesion kinases (FAK)**

The amount of phosphorylated FAK (pFAK) and total amount of FAK (tFAK) was determined by Western Blot in all cell lines (Fig. 3A). After 24 hours of incubation with cannabinoids no significant changes were observed in the relative amount of pFAK for U138 cells (JWH133_0.97; ACEA_0.86), LN229 cells (JWH133_0.70; ACEA_0.49) or U87 cells (JWH133_0.88; ACEA_1.02) (Fig. 3A). We also determined whether the calculated ratio of pFAK/tFAK is correlated to any single cell parameter that is directly or indirectly related to adhesion. Cell speed, cell contact area, rupture force, total adhesion energy and normalized adhesion energy were tested. Thereby, a strong positive spearman was found between the ratio of pFAK/tFAK and the total adhesion energy (data not shown) and the normalized adhesion energy (Fig. 3B) with a slightly higher correlation for the normalized adhesion with r_0.90 and a 95% confidence interval of: [0.58; 0.98].

**Tumor invasion measurements**

All tested cell lines showed invasive behavior in OHSC and formed tumors. The tumor cells were scattered over the slice cultures forming network like structures (Fig. 4D). The invasiveness of each tumor was determined by the area covered by tumor cells in relation to the area of OHSC, normalized to the respective control measurement. The U138 cells showed a reduced invasive potential after the treatment with ACEA and 3 d of invasion time (AACEA_3d_0.56), but the effect disappeared...
after 4 d ($A_{\text{ACEA}}^{4d} = 0.87$). Application of JWH-133 drastically increased the invasiveness for both 3 and 4 d of invasion time to $A_{\text{JWH-133}}^{3d} = 2.05$ and to $A_{\text{JWH-133}}^{4d} = 1.85$ respectively (Fig. 4A). Opposing results were observed for LN229 cells with a reduced invasion after 3 and 4 d to $A_{\text{JWH-133}}^{3d} = 0.79$ and to $A_{\text{JWH-133}}^{4d} = 0.80$. 

Figure 3. Measurement of the phosphorylation of focal adhesion kinases (FAK) for each cell line. (A) Top: No significant change of the phosphorylation ratio of FAK was observed for the used cells after 24 h of cannabinoid treatment (U138: n$_{\text{CTL}} = 4$, n$_{\text{JWH133}} = 4$, n$_{\text{ACEA}} = 4$; LN229: n$_{\text{CTL}} = 5$, n$_{\text{JWH133}} = 5$, n$_{\text{ACEA}} = 5$; U87: n$_{\text{CTL}} = 7$, n$_{\text{JWH133}} = 7$, n$_{\text{ACEA}} = 7$). Values are given as mean with SEM. Bottom: representative Western Blots with pFAK, FAK and GAPDH. (B) Plot of the normalized adhesion energy and the normalized relative FAK phosphorylation. An increase in FAK phosphorylation is associated with an increase in normalized adhesion energy, with a spearman correlation coefficient $r = 0.90$. 
CB1 activation via ACEA did not significantly affect the invasion behavior of LN229 cells after 3 (AACEA 3d $D_0.85$) or 4 d (AACEA 4d $D_0.87$) (Fig. 4B). For U87 cells neither JWH-133 (A_JWH-133 3d $D_0.92$; A_JWH-133 4d $D_0.84$) nor ACEA (A_ACEA 3d $D_0.94$; A_ACEA 4d $D_0.86$) had a significant influence on the tumor invasion (Fig. 4C). Therefore only ACEA led to a decrease in invasiveness in a cell line independent manner.

Network analysis of single cell properties and composite parameters

The obtained parameter groupings are summarized in Figure 5. The mutual information approach described in the methods section was used for calculating the network edges.

The live cell image analysis revealed that the persistence speed and the mean speed had a share of information, while the other measured quantities were independent of each other (Fig. 5A).

For the AFM measurements 3 clusters of parameters were observed (Fig. 5B): the indentation depth (parameter 1) clustering with the Young’s modulus (parameter 9), the adhesive properties (parameter 2, 4 and 5) and 2 parameters describing the occurrence of discrete jumps – energy and force – in the retract curve of the AFM measurements (parameter 3 and 6).

Maximizing the modularity of the obtained network according to Newman$^{21}$ lead to a community grouping that directly corresponds to the obtained clusters and can be described as composite parameters $M^C$. Calculating the composite parameters $M^C$, as described in the “methods” section, reduced the number of parameters from 16 to 11 for further analysis (Fig. 5).

The parameters persistence speed and mean speed that form a community in the live cell measurements describe velocities and express different aspects of cell motion, thus the resulting composite parameter can be interpreted as a “generalized” velocity.

In the AFM network a community is formed by the combination of indentation and Young’s modulus. These two quantities describe the cells response to external stress, the Young’s modulus in form of the cells stiffness and the indentation gives the model-free deformation of the cell under the applied load. Therefore, the obtained composite parameter can be understood as an extended form of cell “stiffness.” The three adhesive properties, normalized adhesion energy, total adhesion energy and rupture force form a composite...
parameter describing adhesive properties of the cell. The two parameters, jump energy and jump force, are related to each other via definition because for the force \( F \) and the energy \( E \) it holds: \( E = \int F \, ds \), with the length \( s \) of one jump. Thus, this composite parameter describes the force and energy landscape of the occurring discrete rupture processes.

A spearman correlation analysis between all composite parameters and the invasiveness after 3 and 4 d revealed a strong link between the composite parameter “stiffness” (Young’s modulus and indentation depth) and the normalized invasiveness \( r_{3d} = -0.88 [−0.52;−0.97]; r_{4d} = −0.90 [−0.59;−0.98]; \) Fig. 6), while other parameters showed no significant correlation. Notably, this correlation could not consistently be observed when analyzing the un-normalized invasiveness (not normalized to the control of the respective cell line; see supplemental information). The values represent the correlation coefficient with its respective 95% confidence interval.

**Discussion**

**Cannabinoids alter the adhesiveness and stiffness of tumor cells**

In this study we analyzed single cell properties of glioblastoma cell lines under the influence of CB1 or CB2 agonists. The data revealed a significant reduction of the measured unspecific adhesion energies for all cell lines and treatments except for U87 cells treated with ACEA. This is in agreement with previous studies in other systems that analyzed the phosphorylation of focal adhesion kinase (FAK). In mammary carcinoma cell lines a CB1 dependent decrease in phosphorylation was reported under the influence of cannabinoids.\(^{22,23}\) FAK was co-localized with focal adhesions and thus strongly associated with unspecific adhesions ensuing tumor progression.\(^{24}\) In lung carcinoma cells an opposing effect was observed using the CB1/CB2 agonist THC leading to an increase in FAK phosphorylation. Whether the effects were mediated by CB receptors remained unclear.\(^{25}\)
Since FAK is related to unspecific adhesions the results obtained in our study are not only plausible, but show for the first time that cannabinoids are directly capable to alter unspecific adhesions on single cell level.

Further changes of the cytoskeleton through cannabinoids have been reported. In myeloma cell lines a reduction of β-actin und β-tubulin protein levels were found after inhibition of CB2.26 A reorganization of cytoskeletal components was observed in various cell types treated with cannabinoids without reporting precise measurements of changes of biomechanical parameters.27-30 Here, we have shown that stimulation with cannabinoid receptor agonists can have a (non-)receptor specific impact on cell stiffness. Both LN229 and U87 express CB1 receptors but display no change in elasticity when treated with ACEA in contrast to the U138 cells. This might be due to the fact that the downstream signaling pathways related to CB1 differ significantly in some cell lines with varying results regarding the reorganization of the cytoskeleton and stiffness. A similar mechanism was observed in PC12 cells.29,30 Depending on the status of differentiation the amount of microtubules and microfilaments in PC12 was altered.

**Focal adhesion kinases is associated with cell adhesion**

In our experiments the pFAK/FAK ratio was not significantly altered in the used cell lines after the application of CB2 agonist JWH133 or CB1 agonist ACEA. This seems to be contradictory to the results published earlier demonstrating a decrease of adhesion after CB1/2 treatment.22,25 Another study in fibroblasts showed an involvement of FAK in initial adhesion, with an increase in adhesiveness after 15 minutes by inducing an integrin binding response,31 which is in accordance with our AFM measurements only taking short term interactions into account. Our results showed further that the measured adhesiveness is proportional to the pFAK/FAK ratio. This hints toward FAK being involved in rapidly forming (few seconds) unspecific adhesions.

**Figure 6.** Plot of the composite parameter “stiffness” versus the invasiveness after 3 (top) or 4 (bottom) days of invasion time. One can see that a decrease in the parameter “stiffness” is strongly correlated with an increase in invasiveness for all cell types and treatments. The respective spearman correlation coefficient is $r_{3d} = 0.88$ for 3 d of invasion time and $r_{4d} = 0.90$ for 4 d of invasion time.
These findings indicate that the used approach of directly measuring adhesive properties with an AFM can further be used to relate molecular properties with physical quantities. Its special advantage is its speed and much higher accuracy, compared to other methods.

On the other hand FAK did not show any correlation with either cell speed or cell area. The cell speed is among others dependent on the cell-substrate adhesion, but the functional relation between the 2 parameters is given in such a way that it has a global maximum and tends to zero for 0 or infinite adhesion.\textsuperscript{32} It is also well known that the cell speed is not only dependent on cell adhesion, but also on the cytoskeletal remodeling times, so that a strong correlation between speed and FAK phosphorylation cannot be expected. A similar argumentation may count for the cell area, which is partially dependent on the cell-substrate adhesiveness, but also on the cells volume and its surface tension, indicating a certain independence of FAK.

**Cannabinoids change the cell motility and affect the cell area and invasiveness of tumor cells**

The mentioned cytoskeletal modulations induced by cannabinoid treatments are expected to have an impact on cell motility as well, as the cytoskeleton and especially actin is strongly correlated with migration.\textsuperscript{33-35} The application of a CB1 agonist led to a migratory phenotype, characterized by a rapid F-actin degradation, more stress fibers and a localization of F-actin at the edge of endometrial stromal cells.\textsuperscript{36} Our experiments validated these results for tumor cells by measuring the undirected migration speed of single cells that were altered by cannabinoids. Thereby we observed contrary effects after the application of the CB1 agonist that might be cell line specific. While the CB1 agonist slightly decreased the speed for LN229 and U138 cells, the incubation with ACEA caused a strong increase in velocity in U87 cells. Considering the contact area of the cells might explain this effect. While U138 and LN229 cells have shown no significant changes in cell area in time lapse imaging, U87 cells had a strongly reduced cell contact area when treated with ACEA. The measured adhesion energies displayed a strong decrease for U138 and LN229 cells but no significant changes for U87 cells. The AFM measurements done for quantifying the adhesion have been performed on a very short time scale in the order of seconds, governing only short-term interactions, while the time lapse imaging started 24 h after cell seeding thus also including long term interactions with the substrate. The change in contact area may therefore not exclusively be caused by a change in cell size but also by a decrease in long-term adhesiveness to the substrate making it also easier for the U87 cells to migrate.

All conducted single cell measurements are related to properties of the cytoskeleton so they may have an influence on the measured invasiveness. The addressed invasive properties of glioblastoma cell lines after cannabinoid treatment reflected the results obtained by other groups. Cannabinoids are in general associated with a reduced invasiveness for various tumor types.\textsuperscript{17,19,20,25,37} In contrast, one study reported an increased invasiveness after treatment with cannabinoids in a cannabinoid receptor independent way.\textsuperscript{38} The experiments conducted for the present study revealed differentiated effects of cannabinoids. While the CB1 agonist ACEA always led to a (in part non-significant) decrease of the invasiveness, the CB2 agonist resulted in a strong increase of tumor spreading in U138 cells and a (in part non-significant) decrease for the other 2 cell lines. Since U87 and LN229 did not express the mRNA for CB2 receptor it is likely that the effect of JWH-133 is not mediated via classical cannabinoid receptors for these cell lines.

**The CB2 receptor agonist JWH 133 displays CB2 receptor independent effects**

Since LN229 and U87 do not express CB2 receptors, but are influenced by the CB2 agonist JWH 133, it is likely that the observed effects are not mediated via the CB2 receptor. JWH 133 is known as one of the most selective synthetic cannabinoids.\textsuperscript{39,40} In vitro binding assays showed an affinity for CB2 (Ki = 3.4 ± 1.0 nM) and for CB1 (Ki = 677 ± 132 nM),\textsuperscript{41} but data about its affinity to other orphan receptors is missing. Thus a possible explanation for the behavior of LN229 and U87 cells treated with JWH 133 might be similar to AM251, which was known as selective antagonist for CB1 till its capability of activating GPR55 was proven.\textsuperscript{42}

Another possible explanation would be that JWH 133 effects on LN229 and U87 cells were mediated via the CB1 receptor. Based on this assumption JWH 133 and the CB1 agonist ACEA are expected to have the same effect on these 2 cell lines. For LN229 cells the CB1 and CB2 agonists showed a different effect for the measured speed, cell area and the invasiveness, while an identical behavior is observed for the adhesion energy only. In the case of U87 cells the application of CB1 and CB2 agonists results in the same effects for the measured cell speed and area. A different effect was observed for U87 cells for the adhesiveness only. Parameters where both agonists had no effect were not considered, because they cannot clearly be attributed to both agonists having the same effect. Thus it is likely that JWH 133 has to target at least a further receptor besides CB2 and CB1, supporting the hypothesis given above.
The composite parameter “stiffness” is a suitable marker for invasiveness

To relate the obtained single cell data with the tumor invasion data a network analytical approach was used. Thereby we observed an invasive behavior that is directly correlated with the measurement of the composite parameter “stiffness.” We showed that a decrease of this composite parameter is associated with an increased invasiveness and vice versa in each cell line separately. This is in good agreement with previously reported results for other cancer types.\(^{43-45}\) As far as the authors know this is the first time this was shown for glioblastoma cells. One has also to denote, that the overall invasiveness, spanning multiple cell lines and treatments cannot fully be explained with the composite parameter “stiffness” (see supplemental information). A reason might be, that the cell size, absolute cytoskeletal content (microtubule, actin, intermediate filaments), as well as cross linking proteins etc. varies between different cell lines and thus influence the measured stiffness.

Even though the obtained results suggest a strong relationship between single cell properties and tumor invasion one also has to denote that the composite parameter given here is obtained by measuring passive properties of single cells, neglecting cell-cell-interactions. Further investigations are needed to figure out how cannabinoids influence the collective behavior of glioblastoma cells in terms of migration and their mechanical properties. To verify whether the here obtained biomechanical findings under the effects of the eCB system are of more general relevance experiments with other substance classes, genetic manipulations or radiation will be needed.

Conclusion

In summary we showed that cannabinoids might be an additional therapy to reduce tumor progression in glioblastoma. We furthermore introduced a very quick method to predict qualitatively the effect of a drug on the tumor invasiveness in our model, based on its mechanical response to external stress that might facilitate drug testing in the future.

Materials and methods

Cell culture

U87 and LN229 cells were purchased from the American Type Culture Collection (U87: ATCC® HTB-147\(^{TM}\); LN229: ATCC® CRL-2611\(^{TM}\)) and U138 cells were obtained from Cell Lines Service (Cell Lines Service, 300363). LN229 and U87 cells were cultured using 89% (v/v) Roswell Park Memorial Institute medium (Lonza, BE12-115F), supplemented with 10% (v/v) fetal bovine serum (FBS, Gibco, 10500-064) and 1% (v/v) penicillin/streptomycin (P/S, Gibco, 15140-122). U138 were cultured in 87% (v/v) Low Glucose Dulbecco’s Modified Eagle Medium (Gibco, 31885-023), supplemented with 10% (v/v) FBS, 2% (v/v) non-essential amino acids (Biochrome, K0293) and 1% (v/v) P/S. Twenty four hours prior to the start of any experiment the culture medium of the cells was changed and in some groups cannabinoids (CB1 agonist ACEA solved in ethanol; Tocris, 12A/141371) or CB2 agonist JWH-133 (solved in DMSO; Tocris, 5B/97327) were added with a concentration of 10 \(\mu\)M.

Analysis of cannabinoid receptor expression on mRNA level

Confluent cells were removed from the flask by the use of Trypsin/EDTA (Biochrom, L2143), after isolation of mRNA, cDNA was synthesized (Reverse Transcription System, Promega Inc., A3500) and used for further analysis. qRT-PCR experiments were performed according to the protocol in Benz et al (2013).\(^{36}\) The reaction volumes contained 17 \(\mu\)l as follows: 10 \(\mu\)l PCR-MasterMix (Promega Inc., M7505), 0.5 \(\mu\)l of left and right primer (25 pM), 0.25 \(\mu\)l of Eva Green dye (Biotium, 31000), 4.75 \(\mu\)l of nuclease free water (Promega Inc., P1193) and 1 \(\mu\)l of cDNA. The PCR reaction was performed in a rotor-cycler (Rotor-Gene\(^\text{TM}\) RG 6000; Corbett Research, Pty Ltd) and consisted of 5 steps: initial denaturation at 95\(^\circ\)C, followed by 40 cycles of denaturation at 94\(^\circ\)C (3 s), annealing at 64\(^\circ\)C (30 s) ; followed by a stepwise decrease of 1\(^\circ\)C per cycle during the first 5 cycles down to an annealing temperature of 59\(^\circ\)C), elongation at 72\(^\circ\)C (30 s) and fluorescence detection at 80\(^\circ\)C (15 s). Products of the reaction were placed on a 1.5% agarose gel (PeqLab, 35-1020) containing GelRed (Biotinum, 41003) and were visualized using BioTek Synergy Mix (Biotek). The following primers were used: Cnrhum left: GCATCAGGAAAGGATGTA; Cnrhum right: CCG TTGTGTGTCTCATCCAC (product size: 250 bp); Cnrhum left: GCTCCCTCATCGTTGTTCC; Cnrhum right: TGACCATGGAGTTATGAGGC (product size: 125 bp); \(\beta\)-actin left: ACTTCCTACTGCTGGTTG; \(\beta\)-actin right: CAGGCAACACCGAAGATGGC (product size: 389 bp); gapdh left: ACCACATCCATGCCATCAC; gapdh right: TCCACCACCTGTGTGC TGTA (product size: 452 bp).

Analysis of cannabinoid receptor and phosphorylated focal adhesion kinases (pFak) expression by the use of western blot

Western Blot analysis was performed according to Benz et al (2013) and Ernst et al (2016).\(^{47}\) Using Trypsin/
EDTA cells were removed and transferred to reaction vessels, sonificated, cooked at 70°C and centrifuged at 4°C. After the protein concentration was measured (BCA Protein Assay Kit, Pierce; Life Technologies, 23225), 20 µg (for cannabinoid receptor 1) or 10 µg (for phosphorylated focal adhesion kinases) of protein was used for the electrophoresis. Gels were blotted onto nitrocellulose membranes (BA 85 6E Protein; Whatman plc/G E Healthcare Life Sciences), washed with washing buffer, blocked with Roti-Block (Roth, A151.2) for 30 min and incubated over night with primary antibody against the N-terminus of the CB1 receptor (0.5 µg/ml; Cayman Chemical, 101500) or p-FAK (1:1000 Cell Signaling, 3284S) following FAK (1:1000, Cell Signaling, 3285S) and GAPDH conjugated with HRP (Cell Signaling, 3683) diluted in Roti block at 4°C. Membranes were washed 3 times with washing buffer, incubated with peroxidase labeled anti rabbit IgG (H+L) (Vector Laboratories, PI1000) and washed 5 times. The signal detection was performed using Luminata Classico Western HRP Substrate (Merck Millipore, WBLUC00500). GAPDH was used as a reference. Image J was used for quantification.

The specificity of the antibody for CB1 has thoroughly been proven earlier. It is well accepted that the results obtained by available antibodies for CB2 are inconsistent and unclear. Therefore, immunohistochemistry for CB2 has not been performed.

**Analysis of the cannabinoid receptor distribution**

For immunohistochemical staining 50 000 cells were seeded on glass plates covered by poly-L-lysine (Sigma Aldrich, P1399) and fixed with 4% paraformaldehyde (Applichem, 141451). Like in Benz et al (2013) cells were treated with 3% (v/v) hydrogen peroxide – methanol solution for 10 min, 3 times washed with PBS/Triton and unspecific binding sides were blocked with normal goat serum (Jackson ImmunoResearch, 005-000-121). Overnight the samples were incubated with antibody against N-terminal CB1 (1.65 µg/ml, Cayman Chemical, 101500) diluted in 5% (w/v) bovine serum albumin at 4°C. After washing with PBS/Triton, samples were incubated with rabbit specific biotinylated secondary antibody (Sigma Aldrich, B8895), followed by horseradish peroxidase conjugated streptavidin (Sigma Aldrich, E2886) with PBS/Triton - wash steps in between. Staining was developed with diaminobenzidine (DAB, Sigma Aldrich, D8001) for 3 min and subsequent counterstain of nuclei was performed using Meyer’s haematoxylin (Hollborn und Söhne, H02-0500). Image acquisition was performed using a Zeiss microscope (Zeiss) equipped with an Axio-Cam digital camera (Zeiss) using a 40x objective.

**Time lapse microscopy**

For time lapse microscopy 1000 cells were seeded in a 6-well plate 24 hours prior to the start of experiments. Images were taken with a microscope (Zeiss) equipped with temperature (37°C) and CO2 regulation (5% (v/v)). For studying single cell motion one frame per 5 minutes was taken with a 10x phase contrast objective and recorded with a CCD Camera (Zeiss) for 24 hours. The obtained images were analyzed using a custom written MatLab (The MathWorks) script that determines the edge of the cell using a gradient operator and tracks each cell individually. The parameters cell area, mean squared displacement, directionality, persistence time, persistence speed and mean speed were obtained and analyzed (supplementary information, Table S2).

**Atomic force microscopy**

To determine the Young’s modulus an atomic force microscope (AFM; Bruker, Bioscope Catalyst, Billerica, USA) was used. Cells in suspension were seeded on a petri dish and allowed to slightly adhere for 15 min. Afterwards, single cells were indented with a tip-less cantilever (Nanoworld, Arrow-TL2) with a force of 3 nN. The Young’s modulus was determined using the Hertz model with the force F, the Young’s modulus E, the radius of the cell R, the Poisson ratio ν and the indentation δ0. Using the Derjarguin-Muller-Topolov model with the obtained Young’s Modulus the contact area between cantilever and cell and thus the adhesion energy can be estimated: a = \frac{F(1-\nu^2)}{E}\sqrt{R_0^2δ_0^2}, with the total adhesion energy E_{ad} and the contact radius a. The parameters indentation, normalized adhesion energy, jump energy, total adhesion energy, minimal force, jump force, slope of approach curve, cell radius, Young’s modulus and jump number were obtained for further analysis (supplementary information, Table S1).

**Organotypic hippocampal slice cultures (OHSC)**

All experiments involving animal material were performed in accordance with the directive 2010/63/EU of the European Parliament and the Council of the European Union (22.09.2010).

OHSC were prepared from 5 day old C57 Black6/J mice as reported earlier and kept at 35°C in a fully humidified atmosphere with 5% (v/v) CO2. Culture medium was changed every other day. After 14 d in vitro the experiments were started.

The model of OHSC brings several advantages for analysis of invasion experiments. As out of one brain up
to 5 slices can be obtained, the number of animals used is considerably reduced. Furthermore, the conditions for different cell lines remain nearly constant. In addition, the slices show an unchanged morphology of the neuronal cells and their circuits remain intact after preparation resembling the in vivo situation. OHSC also allow the investigation of direct interactions between tumor cells and neuronal tissue without confounding factors from the bloodstream.\textsuperscript{50-54} Thus it becomes possible to control the tumor invasion process and the interaction with glial cells more precisely.\textsuperscript{55,56}

Tumor invasion experiments were conducted with the fluorophores carboxyfluorescein diacetate (CFDA; Gibco, 12883) for labeling the tumor cells and propidium iodide (PI; Merck Millipore, 537059) for visualization of the cytoarchitecture as previously reported.\textsuperscript{55,57}

Confocal laser scanning microscopy

The analysis of the fixed OHSC was performed using a confocal laser scanning microscope (Leica, TCS SP 2) with an excitation wavelength of 488 nm for CFDA and 543 nm for PI. Emission was detected using a band-pass filter sensitive for $\Delta \lambda = 510-550$ nm (CFDA) and $\Delta \lambda = 610-720$ nm (PI). The tumor invasion pattern was visualized with a 10x objective, as a z-stack with a step width of 2 $\mu$m. The obtained image stack was analyzed by regarding the maximal intensity projection and the application of a threshold algorithm to calculate the area of the OHSC covered by tumor cells.

Network analytical approach

Data obtained by AFM and live cell microscopy were used to quantify the relationship between several cell specific parameters. Therefore a network analytical approach was used. To quantify the relation between the parameters we introduced the mutual information ($MI$), which is given for 2 parameters $K$ and $I$ as follows:\textsuperscript{58}

$$MI(K,I) = \sum_{k \in K} \sum_{i \in I} p(k,i) \log \left( \frac{p(k,i)}{p(k)p(i)} \right)$$

With $p(;;)$ as the marginal probability of measuring value $i$ or $k$ when measuring parameter $I$ or $K$ and $p(;;)$ is the joint probability distribution that a measurement of $K$ and $I$ will give $k$ and $i$ simultaneously. To weight the obtained edges the $MI$ is weighted in a way introduced by Faith et al. (2007),\textsuperscript{59} modified according to Kießling et al. (2013)\textsuperscript{58} as z scores:

$$z_{IK} = \frac{MI(K,I) - MI_K}{\sigma(MI)_K}$$

The average and standard deviation is calculated over all $MI$ for the parameter $K$. The edge weight is than given as:

$$z[IK] = \sqrt{z^2_{IK} + z^2_{JI}}$$

To check whether certain parameters group in sub communities the modularity $Q$ was calculated:\textsuperscript{21}

$$Q = \sum_i \left( c_{ii} - O_i^2 \right)$$

Where $c_{ii}$ is the relative number of edges inside community $i$ and $O_i$ is the relative number of edges in community $i$ if edges were randomly distributed. This approach results in stable communities $C$.

To reduce the number of parameters for the later analysis composite parameters were introduced.\textsuperscript{58} The composite parameter $M_C$ is the “sum” of all parameters in community $C$ normalized regarding their mean and standard deviation:

$$M_C = \frac{1}{N_C} \sum_{K \in C} \frac{[m_K] - m_K}{\sigma(m_K)}$$

The $[m_K]$ are single measurements of parameter $K$ and $N_C$ is the number of parameters in community $C$.

Statistics

Statistics was performed using the 2-sided Mann-Whitney-Wilcoxon test and significance was chosen for $p < 0.05$. All p values refer to the respective controls of the same parameter of the same cell line.

Abbreviations

AFM atomic force microscopy
CB cannabinoid receptor
CFDA fluorophores carboxyfluorescein diacetate
CTL control
DAB diaminobenzidine
eCB endocannabinoid
FAK focal adhesion kinase
FBS fetal bovine serum
OHSC organotypic hippocampal slice cultures
PBS phosphate buffered saline
PI propidium iodide
P/S penicillin/streptomycin
THC tetrahydrocannabinol
Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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