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Oncometabolite R-2 hydroxyglutarate aids the inflammatory transformation of peritumoral astrocytes

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ABSTRACT

Reactive transformation of astrocytes in IDH wild-type glioma contribute to anti-tumor immunity and support pro-oncogenic signaling. The role of intra- and peritumoral astrocytes in IDH1/2 mutated glioma, a prognostically beneficial subgroup with oncogene-driven enrichment of R-2-hydroxyglutamate (2-HG) remains sparsely explored. Here, we characterized the transcriptomic signature of IDH1/2-mutant glioma associated astrocytes and determined a unique inflammatory transformation, profoundly different to astrocytes in IDH wildtype glioma patients. IDH-mutated glioma inoculation into human neocortical sections or treatment with R-2-HG resulted in an oncometabolite-mediated transcriptional shift towards inflammation in astrocytes. This transcriptional shift was found to be mediated by myeloid cell polarization through R-2-HG in the tumor microenvironment, which was further confirmed by selective depletion of myeloid cells. Integrative analysis of bulk RNA-sequencing of purified microglia, single-cell transcriptomics and spatially resolved transcriptomics confirmed this *microglia-mediated* inflammatory activation of astrocytes. This inflammatory activation is accompanied by loss of neurotransmitter homeostasis. Further, treatment of cortical sections with RH2G results in increased neuronal activity and LFP activity, pointing to the excitotoxic nature of RH2G in the neural microenvironment, with a significant loss of neurons with chronic treatment. The presented findings provide insights into the role that R-2HG plays in the reactive transformation of astrocytes within the IDH-mutated tumor environment and is fundamentally different that seen in IDH wildtype glioma.

INTRODUCTION

Gliomas with a mutation in the isocitrate dehydrogenase enzyme *IDH1* or 2, has been shown to primarily affect younger patients, between 20 and 40 years of age. However, these gliomas are associated with significantly better prognosis compared to IDH wild-type patients. The mutation causes an accumulation of the oncometabolite R-2-HG which replaces physiologically synthesized alpha Ketoglutarate (α -KG). R-2-HG has been shown to cause epigenetic alterations in tumor cells, resulting in an accumulation of histone and DNA methylations, also referred to as the hypermethylated phenotype (Turcan et al., 2012). Recent studies suggest that this R-2-HG accumulation is not confined to the cytosol of tumor cells but is also highly enriched within the extracellular space (Fathi et al., 2016). This leads to the hypothesis that R-2-HG plays a key role in the various pathological alterations found within the microenvironment of IDH-mutated glioma. Recent reports revealed that R-2-HG plays an important role in the suppression of T-cell mediated anti-tumor immune response by disruption of the nuclear factor of activated T cells (NFAT) and polyamine biosynthesis (Bunse et al., 2018; Zhang et al., 2018). Others have described an increased inflammatory phenotype exhibited by macrophages and microglial cells within IDH-mutated gliomas (Poon et al., 2019).

The extent to which other cell types such as astrocytes and neurons are altered by R-2-HG remains poorly explored. In the context of IDH wildtype glioblastoma, we have previously reported that the reactive alterations of tumor associated astrocytes (TAA's) contribute to the evolution of an immunosuppressive microenvironment (Henrik Heiland et al., 2019) and further supports tumor growth (Wurm et al., 2019). In this work, we explored the transcriptional transformation of TAA's within the microenvironment of IDH-mutated tumors compared to wildtype glioma by means of RNA-sequencing of purified astrocytes. Using our recently established human neocortical GBM model, we were able to subsequently distinguish between the reactive transformation driven by IDH-mutated tumor cells or the oncometabolite R-2-HG. Our data revealed that R-2-HG leads to an inflammatory activation of surrounding astrocytes followed by complement activation. In addition to this astrocytic transformation, we also report that R-2-HG is able to evoke increased neuronal activity, significantly higher than seen by glutamate, which is generally enriched in IDH-wild type glioma. Our findings suggest that the role of R-2-HG is multifaceted within the IDH-mutated glioma microenvironment, with varying effects exerted on the multitude of cell types present within the system, driving complex alterations of the tumor microenvironment and potentially contributing to the high frequency of seizures reported clinically (Chen et al., 2017).

RESULTS

Tumor-associated astrocytes in IDH-mutated glioma marked by inflammatory transformation

We recently reported that TAA's exhibit a distinct anti-inflammatory transcriptional shift which aids in the evolution of an immunosuppressive environment in IDH1/2 wildtype glioblastoma (Henrik Heiland et al., 2019). In order to investigate the transcriptional alterations of tumor-associated astrocytes in IDH1/2 mutated glioma, we purified and transcriptionally profiled astrocytes from 9 patients with confirmed IDH1-R132H mutation, by means of RNA-sequencing. These transcriptional profiles were then compared against our previously established dataset containing transcriptomic data from astrocytes obtained from 8 non-malignant controls (Access tissue from epilepsy surgery, metastasis or glioma-IDH-wildtype patients) and 7 IDH-wildtype glioblastoma, **Figure 1a**. Unsupervised clustering revealed a clear separation between astrocytes acquired from non-malignant specimens and tumor-associated astrocytes, **Figure 1b-c**. In order to further represent the astrocytes along a developmental trajectory (fetal/undifferentiated to mature/differentiated phenotype) versus their major types of reactive transformations (inflammatory to alternative transformation), we established a 2D representation as recently published (Henrik Heiland et al., 2019), **Figure 1d**. This 2D projection summarizes different transcriptional subtypes reported by multiple authors (Clarke et al., 2018; Escartin et al., 2021; Liddelov and Barres, 2017; Liddelov et al., 2017; Zhang et al., 2014, 2016b). This characterization reconfirmed our previous findings suggesting that reactive astrocytes in glioma further referred to as "tumor-associated astrocytes, TAA" exhibited a fetal transcriptional profile, **Figure 1c**. Our data suggests, that within the group of tumor-associated astrocytes, the transcriptional programs which lead to reactive transformation differ between glioma entities. Compared to TAA in IDH-wild-type glioma where Interferon gamma activation and JAK/STAT3 pathway were highly enriched, TAA's derived from IDH-mutated glioma revealed an inflammatory activation, including significant up-regulation of genes coding for the factors of complement system ($p_{adj}<0.001$), hallmark inflammatory response ($p_{adj}<0.001$), and Tumor Necrosis Factor alpha ($TNF\alpha$, $p_{adj}=0.02$). Immunostainings from IDH mutated tumors at the tumor edge (infiltrating border) confirmed the accumulation of complement component 3 (C3) in astrocytes, **Figure 1e**. To further investigate the spatial distribution of inflammatory astrocytes within IDH-mutated glioma, we extracted gene expression signatures and projected them into our recently published spatially resolved transcriptomics datasets (Ravi et al., 2021) from both IDH mutated and wildtype glioma, using a Non-negative matrix factorization (NMF) for deconvolution (Elosua et al., 2020). Our

analysis confirmed that the distinct IDH-mutation related TAA signature is more frequently observed within mutated than in wild-type glioma, **Figure 1f**.

Figure 1: a) Workflow of transcriptional profiling of astrocytes from IDH mutated and wildtype tumors. b) Heatmap of transcriptional profiles. High gene expression is visualized in red, low expression in blue. The bars at the bottom display sample origin. c) Gene Set enrichment analysis of multiple pathways (MsigDB) is displayed at the right side. P-value was determined by Kolmogorov Smirnov (K-S) test and corrected by the False-Discovery Rate (p_{adj}). d) 2D representation of different reactive states of astrocytes as recently established. The color depicts the sample origin. e) Immunofluorescence staining of IDH R132H mutation (tumor cells) GFAP (astrocytes and tumor cells) C3 (complement cascade) and DNA (DAPI). f) Spatial enrichment of astrocyte transformation signatures of IDH-mutated (upper) and IDH wildtype samples (bottom). Spotlight was used to integrate ranked gene signatures into space.

R-2-Hydroxyglutarate activates inflammatory signaling in tumor-associated astrocytes

Motivated by the findings pointing to R-2-HG mediated alterations in T cells (Bunse et al., 2018), we aimed to investigate the extent to which the reactive transformation of astrocytes is driven by the tumor-cells or the oncometabolite directly. For this purpose, we used our human neocortical GBM model, either inoculated with a primary IDH-mutated cell line or treated with R-2HG, **Figure 2a**. Post 7 days of tumor growth, we extracted and performed RNA-sequencing from astrocytes present within the cultured sections, **Figure 2b**. Unsupervised clustering and differential gene expression analysis revealed a

similar inflammatory activation in astrocytes from both conditions. The reactive transformation was driven by complement related genes (*C3* and *C1Q*) in combination with increased expression of common inflammatory interleukin genes (*IL1A/B*), **Figure 2b-c**, which mirrors the findings in clinical samples. However, to our surprise, we identified that astrocytes extracted from sections only treated with R-2HG exhibited increase inflammatory activation compared to those from tumor inoculated sections, as seen from our 2D representation, **Figure 2d**. Immunostainings confirmed the increased complement activation (*C3*) in astrocytes from sections treated with R-2HG, **Figure 2e**. In summary, these findings suggest that the oncometabolite R--2-HG contributes directly to the inflammatory transformation of astrocytes.

Figure 2: a) Workflow of transcriptional profiling of astrocytes from slices with IDH-mutated tumor injection or R--2HG treatment. b) Heatmap of transcriptional profiles. High gene expression is visualized in red, low expression in blue. The bars at the bottom reveal the sample origin. c) 2D representation of different reactive states of astrocytes as recently established. The color determines the sample origin. d) River plot of gene expression changes (Inflammatory response) in different conditions. e) Immunofluorescence staining of IDH1 R132H mutation (tumor cells) GFAP (astrocytes and tumor cells) C3 (complement cascade).

R-2-Hydroxyglutarate induces reactive activation through myeloid cells

We and others have previously reported that the inflammatory transformation of astrocytes is predominantly driven by myeloid cells (Clarke et al., 2018; Henrik Heiland et al., 2019; Liddelow et al., 2017, 2020). To validate if this holds true for R-2HG mediated astrocytic transformation, we used our previously established chemical based ex-vivo myeloid cell depletion model (Ravi et al., 2019). For a positive control of inflammatory stimulation, we used lipopolysaccharide (LPS) which is known to drive an inflammatory reactive phenotype of astrocytes mediated by microglial activation (Clarke et al., 2018; Liddelow et al., 2017; Yun et al., 2018). Sections that underwent myeloid cell depletion were either inoculated with IDH-mutated GBM or were treated with R-2HG / LPS, **Figure 3a**. The absence of IBA1⁺ myeloid cells was validated by means of immunofluorescence post depletion for 72h, **Figure 3b**.

To determine the effect that each condition had on astrocytic activation, we estimated the number of C3⁺ astrocytes, **Figure 3c**. We observed a significant increase in C3⁺ astrocytes in all treatment conditions, with the highest number of C3⁺ astrocytes observed due to R-2HG treatment. No significant increase in C3⁺ astrocytes were observed when myeloid depletion was carried out, **Figure 3c**. These results confirm that astrocytic transformation due to R-2HG is primarily mediated by myeloid cells in the tumor microenvironment.

Figure 3: a) Workflow of the slice model with myeloid cell depletion and LPS/2HG stimulation. B) Immunofluorescence image of ctr and microglia/myeloid cell depletion after 3 days clodronate treatment with IBA1. A significant reduction of IBA⁺ cells are demonstrated ($p < 0.001$), data not shown. C) Imaging analysis pipeline combining trained segmentation algorithm (ilastik) to quantify the inflammatory marker (C3) in astrocytes from different conditions. Quantification demonstrated at the bottom. Alpha level was determined by Wilcoxon-Sum Rank statistics and illustrated by * $p < 0.01$, ** $p < 0.001$ and *** $p < 0.0001$. Each points represents single cells. D) Immunofluorescence image of ctr and microglia/myeloid cell depletion with C3 (red), GFAP (white) and tumor cells in green. Cellular shapes determined by the segmentation are illustrated at the bottom right.

Inflammatory myeloid cells drive inflammatory activation of astrocytes.

In order to comprehensively interrogate the transcriptional heterogeneity exhibited by myeloid cells within both the IDH wildtype and IDH mutated environments, we analyzed a recently published publicly available dataset (Friedrich et al., 2021), **Supp. Figure 1a**. Unsupervised clustering to decipher major regulatory programs revealed 5 major clusters, further summarized into 3 modules, based on partial similarity, **Supp. Figure 1b**. The first module represents homeostatic microglia with a large percentage of microglia from non-malignant samples. The remaining 2 modules show distinct activation patterns, namely non-inflammatory and inflammatory. The non-inflammatory activation pattern is marked by interferon gamma activation and expression of *CD163* and *TGFB*, **Supp. Figure 1c**. This module contained most of the microglial cells from IDH wild type tumors. The inflammatory module is marked by expression of common inflammatory cytokines such as *IL1B* and *TNF* and was composed of microglia

derived from IDH-mutated tumors, which is in line with our findings from the cortical GBM model, **Supp. Figure 1c-d**.

In order to confirm microglia activation patterns, we transcriptionally profiled purified myeloid cells (CD45⁺) from our human GBM model inoculated with either IDH mutated or wildtype glioma cells. The expression profiles were then projected into the PCA space of the single-cell dataset to align the myeloid activation from our cortical GBM model, **Figure 4a**. This approach helped us confirm the inflammatory character of myeloid cells from IDH-mutated vs IDH-wild type tumor microenvironment. In order to correlate the estimated co-activation of astrocytes and myeloid cells, spatial correlation was carried out by inferring the likeliness of neighborhood, **Figure 4b-c**. Our data suggests that astrocytic and myeloid activation was spatially co-localized only within IDH mutated tumor environment, **Figure 4d**.

Figure 4: a) Workflow of microglia analysis integrating sc-RNA-seq and RNA-sequencing of purified microglia. b) For integration a RNN trained on multidimensional PCA space was trained to predict the position of purified microglia (bulk-seq). c) For in-depth characterization of the spatial context, we estimated the spatial localization as explained in Figure 1. d) To estimate the co-localization of our predicted signatures we performed spatial correlation analysis. The scatter plots illustrate the spatial correlation

of IDH-specific signatures of astrocytes and microglia cells. e) Immunofluorescence image of slices with tumor injection and R-2HG treatment illustrate a loss of NeuN positive cells in both conditions. f) Quantification of cell counts demonstrate a significant loss of NeuN pos. cells compared to ctr slices. Alpha level was determined by Wilcoxon-Sum Rank statistics and illustrated by * $p < 0.05$, ** $p < 0.01$. Points in the bar plot represents biological replicates. g) Barplots of electrophysiology quantification of spikes in various conditions h) Example traces (MEA) from slices with Baseline, Glutamate and R-2HG stimulation.

R-2-Hydroxyglutamate mediates neuronal excitotoxicity

Since R-2HG drives reactive transformation of astrocytes, there is a loss of normal homeostatic function (Escartin et al., 2021; Li et al., 2019), as seen in our data, **Supp Fig. 1e**. This reactive transformation is independent of the age of the specimens, **Supp Fig. 1f**. Since the reactive transformation has been shown to lead to a loss of homeostasis, which should be reflected in neurotransmitter profiles, we carried out metabolic profiling (LC-MS) from culture medium collected from both IDH-mutated glioma inoculated and R-2HG treated sections. Our findings showed that there is no significant increase in extracellular glutamate in IDH-mut GBM inoculated section, whereas treatment with R-2HG led to elevated levels of extracellular glutamate ($p = 0.005$), **Supp Fig 2a**, which is generally neurotoxic. Imaging results complement these results, where we observed a significant reduction in neuronal cells in sections that are treated with R-2HG, compared to IDH inoculated and control sections, **Fig. 4e-f**. In addition to the role that plays in neuroinflammation, the chemical structure of R-2HG is highly similar to glutamate, which could potentially induce excitotoxicity by means of increased glutamatergic activation. To determine if neuronal activity can be influenced by secreted R-2HG, we exposed cultured sections to both glutamate (100 μ M) and R-2HG (50 μ M). Glutamate exposure resulted in a significant increase in spiking events, with increased spontaneous LFP activity, **Figure 4g**. Further exposure of the same sections with R-2HG post washout resulted in further increase in both spike rates in addition to the frequency and amplitude of LFP events. These results suggest that chronic exposure to R-2HG could result in excitotoxicity, culminating in neuronal death, **Figure 4h**.

DISCUSSION

IDH1/2 mutated glioma are a distinct group of brain tumors that differ from IDH-wildtype glioma in several aspects besides clinical prognosis (Han and Batchelor, 2017; Huang et al., 2019; Waitkus et al., 2016). A crucial factor for the growth and invasive behavior of glioma is the tumor microenvironment and its supportive or inhibitory impact on the tumor. We have demonstrated that both astrocytes and microglia engage in non-inflammatory transformation in IDH wild-type gliomas which promote an immunosuppressive milieu (Henrik Heiland et al., 2019). Consequently, anti-tumor immunity is suppressed and concurrently prooncogenic stimuli from myeloid cells and astrocytes promote tumor growth (Bunse et al., 2018; Zhang et al., 2016a). Some of these prooncogenic effects have been shown to be mediated by the oncometabolite R-2HG in IDH-mutated tumors. Most recently, the impact of R-2HG on T cells was investigated, confirming that the R-2HG has a major impact on various cells of the IDH mutated tumor microenvironment (Friedrich et al., 2021). Motivated by this evidence, we explored the role of R-2HG on glial activation. In this study, we made use of a human neocortical slice model which was established recently to study the tumor microenvironment without interspecies bias. Since there are considerable differences within species with regards to the reactive transformation of astrocytes (Ravi et al., 2019), we believe that the human model has a decisive advantage. Our data suggests that both IDH tumor inoculation into tissue sections and R-2HG treatment were able to activate inflammatory transcriptional programs in astrocytes, mediated by the presence of microglia. Further, by spatially mapping the transcriptomic profiles of purified microglia, we were able to confirm that microglia also demonstrate inflammatory activation in IDH mutated glioma. This inflammatory astrocyte transformation is associated with a loss of neurotransmitter homeostasis, which is seen in our data with disrupted levels of glutamate in the treated sections, as has been previously reported in IDH mutated tumors (Nagashima et al., 2016). Additionally, R-2HG increased neuronal spiking rate in, pointing to potential excitotoxicity. This was shown to be true when the sections were incubated with 50µM R-2HG resulted in a significant reduction of neurons compared to controls.

In summary, our work provides a crucial contribution towards understanding the role of R-2HG in the IDH mutant glioma microenvironment and sheds light on the significant microenvironmental differences to IDH wild-type glioma.

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Author contributions

The study was designed and coordinated by DHH and VMR. Purification of astrocytes and FACS was performed by SPB, JG and NN. FACS analysis was checked by MF. Mass Spectrometric analysis was performed by LH. Tissue material was provided by OS, PF and CF. Neuropathological analysis was performed by RS and MP. Organotypic slice culture and Immunostainings was performed by VMR and KJ. Spatial transcriptomics data collection was performed by VMR and PW. Electrophysiology recording was performed by KJ. Bioinformatical analysis was performed by DHH and JK. Manuscript writing editing and figure design was performed by DHH, VMR, KJ, PF. UGH, OS and JB helped oversee the project.

Conflict of interests

No potential conflicts of interest were disclosed by the authors.

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Supplementary Figure 1:

Supplementary Figure 1: a) UMAP reduction showing transcriptional heterogeneity in myeloid cells using a single cell dataset from publicly available dataset. b) Unsupervised clustering showing 3 modules: Homeostasis, Non-inflammatory and Inflammatory. c-d) non-inflammatory modules showing the activation of TGF beta and CD163 and Inflammatory modules showing an upregulation for IL1beta and TNF e) Myeloid cells in the IDH-mutated glioma shows a loss of homeostatic function. (f) Geneset enrichment analysis shows that the enrichment for inflammatory response is independent of age.

Supplementary Figure 1: a) Barplots of LCMS from culture medium of various conditions :control, IDH-inoculated and R--2HG treated.

Methods:

[Ethical Approval and Resource Sharing](#)

The local ethics committee of the University of Freiburg approved data evaluation, imaging procedures and experimental design (protocol 100020/09 and 472/15_160880). The methods were carried out in accordance with the approved guidelines, with written informed consent obtained from all subjects. The studies were approved by an institutional review board.

[Cortical GBM model](#)

Tissue preparation: Capillaries and visibly damaged tissue were dissected away from donor tissue block submerged in preparation medium containing: Hibernate medium supplemented with 13 mM Glucose, 30 mM NMDG and 1 mM Glutamax. 300µm thick coronal sections were obtained using a vibratome (VT1200, Leica, Germany) which were retained in Carbogen (95% O₂/ 5% CO₂) bubbled ice-cold preparation medium for 10 min before plating to help removal of dead/damaged cells at the section surfaces. 3-4 sections were gathered per insert (PICM0RG50, Merck-Millipore, USA). The cultured sections were maintained in growth medium containing Neurobasal (L- Glutamine) supplemented with 2% serum free B-27, 2% Anti- Anti, 13 mM D+ Glucose, 1 mM MgSO₄, 15 mM HEPES (Sigma, H0887), and 2 mM Glutamax at 5% CO₂ and 37 °C. The culture medium was replaced at 24h post plating, and then every 48h for the course of the culture period. Culture medium was collected and stored at -20 °C for further LC-MS measurements.

[Tumor cell inoculation](#)

The injected tumor cell line is a patient derived, confirmed IDH-mutated GBM cell line. It was harvested and cultivated exclusively in the Universitätsklinik Freiburg since late 2018. Cells were cultured in serum-free conditions as described previously, Heiland et al. 2018. For mutation and growth analysis (electrophysiology) cells were plated on laminin coated dishes as described in previous manuscripts. In line with previous descriptions the cell line has shown a relatively slow growth rate. In order to monitor tumor invasion and proliferation profile, cells had to be fluorescently labeled using Thermo fishers Cell trace CFSE dye (Thermo Fisher Scientific, Waltham, Massachusetts, USA) prior to injection. Therefore, cultured cells were trypsinized, centrifuged and counted. Approximately one million cells were then resuspended in 1 mL of PBS. 1 µL of Celltrace CFSE dye is added. Cells were then incubated for 20 minutes at 37°C. In order to eliminate any unbound dye 5 mL of culture medium containing at least 1% of serum is added after incubation. Cells are then incubated 5 minutes and subsequently spun down. Cells were then resuspended in MEM media at 40,000 cells/µL. Cells were used immediately for injection onto tissue slices. A 10 µL Hamilton syringe was used to inject 1 µL into the white matter portion of the slice culture.

Slices with and without injected tumor cells were incubated at 37°C, 5% CO₂ for 7 days and fresh culture medium was added every 2 days. And, slices with R-2HG treatment were incubated in growth medium with a final concentration of 50 µM D-2hydroxyglutaric acid (Merck, Darmstadt Germany) for 7 days. After 7 days of culture, slices were harvested and processed to either single cell suspensions in the

above-described manner or fixed in 4% PFA for further immunofluorescence-based staining.

Isolation of cells of interest from the donor tissue/slice model

Viable single-cell suspensions from primary tumor tissue for subsequent MACs-sorting were generated by using a combined enzymatic-mechanical dissociation approach. Tissue was collected from surgery in phosphate buffered saline and placed in a sterile cell culture bench. All steps were performed under sterile conditions. After final dissociation the cell solution is passed through a 40µm strainer (BD Falcon #352340). The strainer is rinsed with 10ml of cold HBSS. After centrifugation (310g, 4°C; 6min), the remaining pellet is immediately processed for myelin removal via the Miltenyi debris removal kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Therefore, the pellet is resuspended in the appropriate amount of debris removal solution and PBS given by the manufacturers protocol. Suspensions are then centrifuged at 300xg for 10 minutes in a swing bucket centrifuge, in order to minimize cell loss. After successful myelin removal cells were counted and either frozen at -80°C or used in magnetic separation immediately.

We then made use of magnetic cell isolation, based on nano immuno-magnetic beads, combined with selective columns (MACS; Miltenyi Biotec). Astrocytes were tagged with 20 µl of biotin labelled anti-GLAST primary antibody (ACSA-1, Miltenyi Biotec, Germany), with 10^7 cells used as the input. Post incubation, we performed magnetic labelling with 20 µm beads. The tagged astrocytes were then isolated using a MACS column (MS Columns, Miltenyi Biotec) and washed. The resulting output was collected directly into Qiazol (79306, Qiagen, Germany) followed directly by RNA extraction and validation using a NanoDrop (2000c, Thermofischer Scientific, USA) Spectrophotometer.

Clodronate depletion model

Selective depletion of microglia in organotypic brain sections from IDH donors was carried out by supplementation of culture medium with 1 mg/ml Clodronate (Sigma, D4434) for 72h. Subsequently, the slice cultures were rinsed with growth medium to wash away any debris. Depletion success was monitored by means of immunofluorescence.

RNA extraction and purification

RNA was extracted using the PicoPure RNA extraction kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Due to the low amount of astrocytic RNA measured by Qubit (RNA high sensitivity assay, Thermo Fisher Scientific, Waltham, Massachusetts, USA) derived from as little as 0.1 gr of brain slices, amplification was needed. In order to amplify full length cDNA transcripts Smart-seq principals were used. Therefore, the reverse transcription reaction was performed using a mix of different anchored VN-primer as well as template switching oligo with SuperScript III (Thermo Fisher Scientific, Carlsbad, USA). Input RNA and enzymes were mixed according to the manufacturers protocol. After RT-primer annealing template switching oligo (....) was added. The sample was placed on a thermal cycler running the following program: 25°C for 10min, 50°C for 50 min, followed by 42°C for 10 min and 85°C for 5 min. In order to remove contaminating RNA, 1µl of RNase H (Thermo Fisher Scientific, Waltham, Massachusetts, USA) is added and incubated at 37°C for 20 min. The resulting cDNA concentration post-reverse transcription is analyzed via Qubit (High sensitivity ssDNA assay). To amplify resulting

cDNA a limited cycle PCR with a maximum of 10 cycles is performed. Primers complementary to the template switching oligo and to the reverse transcription VN-primers were added. Amplification progress was monitored by Evagreen dye (Biotium, Fremont, USA). After amplification Kappa pure beads (Roche Applied Science, Penzberg, Germany) are used for size selection (0,8x), excluding DNA fragments smaller than 300 base pairs. Subsequent DNA concentration is once again measured by Qubit analysis (High sensitivity dsDNA assay).

RNA sequencing

Purified cDNA from the amplification steps were prepared for sequencing using Nextera XT library preparation kit (Illumina, San Diego, USA). Input cDNA quantities are previously normalized to a starting concentration of 0,2 ng/µl. For each sample a unique combination of i5 and i7 indexes was used. After tagmentation and amplification, libraries are once more cleaned using Kappa pure beads (Roche Applied Science, Penzberg, Germany). Fragments with a length of over 300bp are selected. Using Qubits high sensitivity dsDNA Kit (Thermo) and the results of an Agilent bioanalyzer high sensitivity run, libraries are normalized to the concentration of 1nM and subsequently pooled. Sequencing was performed on Illumina Nextseq 550 using a high output 150 cycle Flow cell v2.5 (Illumina, San Diego, USA). The sequencing run was set up as an paired end run, splitting the number of cycles equally at 75 each between read one and two.

Analysis of RNA-seq:

BCL files were demultiplexed and converted into fastq files using the bcl2fastq converter (Illumina). We aligned the *.fastq files by the STAR aligner (STAR 2.7.9a, <https://github.com/alexdobin/STAR>, reference genome: GRCh38) resulting in a count matrix. Count data were processed by the RNA-seq pipeline (VisLab) as part of our in-house toolbox (https://github.com/heilandd/Vis_Lab1.5). Analysis of differentially expressed genes was performed using the DESeq2 package (Love et al., 2014). The algorithm mainly uses a generalized linear model with a negative binomial distribution. A detailed description is given in the R documentation.

Gene expression analysis of multiple groups and clustering:

Clustering and validation was performed as described recently (Henrik Heiland et al., 2019). First, we calculated the optimal number of clusters based on the “Partitioning Around Medoids” algorithm. A t-distributed stochastic neighbor embedding (tSNE) (Van der Maaten and Hinton, 2008) analysis and Consensus Cluster (Wilkerson and Hayes, 2010) was performed to confirm the chosen k. Next, we used either the PAMR (Hastie et al.) algorithm, a machine-learning based method, or a generalized linear model (McCullagh, 1984) to identify characteristic up/downregulated genes in each subgroup. The PAMR-score or the predictive-score extracted from the generalized linear model was used to identify subclass specific gene set enrichment. Pipeline and codes are available at <https://github.com/heilandd/AutoPipe> and CRAN AutoPipe, all analysis was described recently (Henrik Heiland et al., 2019).

Functional analysis by enrichment analysis:

For functional analysis, a permutation-based pre-ranked Gene Set Enrichment Analysis (GSEA) was applied. We used predefined gene sets downloaded from the Molecular Signature Database v7.1. The enrichment score of either differentially expressed genes (DE) or marker genes of clusters was calculated by the rank order of gene expression/fold change DE. For significant enrichment, p-values were adjusted by FDR. Gene Set Variation Analysis (GSVA) was performed with the GSVA package implemented in R-software (Subramanian et al., 2005).

[Integration of spatial transcriptomics](#)

To project our extracted gene signatures into space, we imported the Visium 10X datasets available from Ravi., et al 2021 including two IDH wild-type and two IDH mutated patients. The signature genes from the above defined clusters of glioma-associated astrocytes were used to fit a non-negative matrix factorization (NMF) (Elosua et al., 2020) implemented into the spotlight package. Surface plots were generated using the SPATA2 software tool (<https://github.com/theMILOlab/SPATA2>).

[Single-Cell RNA sequencing analysis](#)

Single cell RNA-seq were downloaded from the Gene Expression Omnibus under GSE166418 (human CEL-seq2 data). Postprocessing was performed by the MILO-pipeline for scRNA-seq (<https://github.com/theMILOlab/scPipelines>). Single cell analysis was performed by the Seuratv4.0 package and SPATA 1.0 package. After pre-processing of the data through Seurat, we imported the data into SPATA. Further analysis was performed as explained in the sections above.

[Deep-learning integration of scRNA-seq and cell-type RNA-sequencing](#)

To integrate RNA-sequencing from purified microglia into the latent space of the scRNA-seq, we trained a neural network to predict the first two eigenvectors based on gene expression. The trained model was used to fit our RNA-sequencing (microglia bulk) into the scRNA-seq latent space.

[Immunofluorescence and Imaging](#)

Immunostaining of slices with or without microglia and tumour injection was performed following the established protocol. Growth media was removed and exchanged for 1 ml of 4% paraformaldehyde (PFA) for 1 h and further incubated in 20% methanol in PBS for 5 min. Slices were then permeabilized by incubating in PBS supplemented with 1 % Triton (TX-100) overnight at 4 °C and further blocked using 20% BSA for 4 h. The permeabilized and blocked slices were then incubated in the following primary antibodies: anti- NeuN rabbit for neuronal nuclei (1:1000), anti-GFAP chicken for astrocytes (1:2000), Iba-1 (1:1000, pig) for microglia. After washing in PBS, slices were labelled with secondary antibodies conjugated with Alexa 488, Alexa 555 and Alexa 647 for 3 h at room temperature. Slices were then mounted on glass slides additionally using DAPI fluoromount (Southern Biotech, Cat. No. 0100–20). Fluorescence imaging was performed using a Fluoview FV10i confocal microscope from Olympus. All measurements and image processing were performed using the company's software. Optical magnification settings of ×10 and ×60 with oil were used.

8. LC-MS/MS based quantification of neurotransmitters from culture medium

Separation and detection of metabolites. This step was performed as previously reported (Maier et al., 2021; Xu et al., 2018), with modifications. Metabolites were separated on an AC 3 AQ column, 1.0 x 150 mm (HiChrom) using a gradient of solvents A (0.1% formic acid in water) and B (0.1% formic acid in MeOH) over 10 minutes as follows: 0-0.5 min 15 % B, 0.5-5 min 85 % B, 5-6 min 85 % B, 6-6.20 min 15 % B, 6.21-10 min 15 % B. The flow rate was 0.050 mL/min connected to a Nexera X2 LC system (Shimadzu). Metabolites were detected on a Sciex 6500+ ESI-tripleQ MS/MS on low mass mode (0–1000 Da), with curtain gas (CUR) at 40, collision gas (CAD) at 10, Ion spray voltage (IS) at 5000 Volts, temperature (TEM) at 400°C, ion source gas 1 (GS1) at 40 and ion source gas 2 (GS2) at 30. Each metabolite was optimized individually using chromatographic solvent conditions and exhibited optimal ionization in the positive mode (Table 1).

Table 1. Mass transitions and optimized mass spectrometry parameters for the neurotransmitters and internal standards in this study.

Q1	Q3	Dwelling time (ms)	Metabolite	DP	EP	CE	CXP
177,1	160,2	20	Serotonine (5-HT)	38	10	10	7
148,2	84,1	20	Glutamate (Glu)	41	10	24	15
104,2	87,1	20	GABA (γ -aminobutyric acid)	26	14	15	16
154,1	137,2	20	Dopamine (DA)	37	10	13	8
205,2	188	20	Tryptophan	40	4	14	12
182,2	136,1	20	Tyrosine	52	8	25	7
212	152,2	20	Isoprenaline	46	8	20	9
76,1	30	20	Glycine (Gly)	6	7,6	19	14
158,1	141,2	20	D4-Dopamine	37	10	13	8
106,1	60	20	Serine (Ser)	6	10,5	15,5	7
78,1	31	20	^{13}C , ^{15}N -Gly	6	7,6	19	14
105,048	59,1	20	Choline	76	10	39	8
162,055	98,1	20	L-2-AAA	56	10	21	10

Calibrators and internal standards: All metabolites were quantified with respect to a calibration curve and selected internal standards. The dynamic range of calibrators was 0-5 μM for Glutamate, Serine, Dopamine, Serotonin and GABA, and 0-100 μM for Glycine, Tryptophan, Tyrosine, Choline and 2-L-aminoadipic acid. D4- dopamine was utilized as internal standard for the quantification of dopamine. Glycine-1- ^{13}C , ^{15}N was utilized as internal standard for the quantification of Glycine. All other metabolites were normalized by isoprenaline as the internal standard, as described in a published method validated according to FDA guidelines (REF). The internal standard mix contained D4-dopamine (5 μM), Glycine-1- ^{13}C , ^{15}N (50 μM) and isoprenaline (50 μM). A list of reagents is given in Table 2.

Table 2. List of reagents, supplier and product numbers		
Reagent	Supplier	Product number
D-Serine	Sigma	S4250-5G
L-Serine	Sigma	S4500-1G
Dopamine	Sigma	H8502-5G
Dopamine-D4 hydrochloride solution	Sigma	D-072-1ML
Serotonin hydrochloride	Sigma	H9523-100MG
GABA, g-amino butyric acid	Sigma	A2129-10G
L-Glutamate	Sigma	49621-250G
Glycine hydrochloride	Sigma	G2879-100G
Glycine-1- ¹³ C, ¹⁵ N	Sigma	299340-100MG
L-2-Aminoadipic acid	Sigma	A7275-250MG
Tryptophan	Sigma	T8941-25G
Isoprenaline	Sigma	I5627-5G
Water, CHROMASOLV™ LC-MS	Honeywell Research Chemicals	39253-1L
Methanol, CHROMASOLV™ LC-MS Ultra, tested for UHPLC-MS	Honeywell Research Chemicals	14262-1L
AC 3 AQ column 1.0 x 150 mm	HiChrom	ACE-116-1501

Sample handling-Culture medium: Culture media was collected and centrifuged at 9447 x g at room temperature for 10 min. The cleared culture media samples were stored at -80°C for further metabolomic analysis.

Sample handling-Tissue biopsies: Flash-Frozen tissue biopsies were stored at -80°C until used. Frozen tissue was quickly weighed before thawing on ice and resuspended with ice-cold lysis buffer (10 mL PBS + 100 µl protease inhibitor cocktail (P8340-5ML, Sigma)) to a ratio of 100 mg tissue per mL of lysis buffer. The tissue was homogenized on ice with a tissue disruptor (47747-370, VWR). An aliquot of this extract was diluted 1:10 with lysis buffer and utilized for metabolomics analysis and protein quantification by the bicinchoninic acid assay (23225, Thermofisher).

Sample preparation: Neurotransmitters were extracted according to a fast acidic methanol extraction procedure previously described for polar metabolite extraction (Behringer et al., 2019). Briefly, 20 µL of calibrator or biological sample of interest was mixed with 20 µL of internal standard mix and 20 µL of 0.1% formic acid in H₂O. Metabolites were extracted by addition of 100 µL of 0.1% formic acid in MeOH. Samples were vortexed, then centrifuged at 9447 x g at room temperature for 10 minutes.

Data analysis: Quantification of metabolites was carried out with Analyst® 1.6.3 software, 2015 AB Sciex.

9. Extracellular electrophysiological recording experiments

Recordings: Extracellular recordings were made as previously described (Ravi et al., 2019). Briefly, recordings were made using an MEA 1060 UP (Multichannel Systems, Reutlingen). Sections were placed onto the recording electrode (60PedotMEA200/30iR-Au-gr, Multichannel Systems, Reutlingen).

618 A slice grid (HD5, Ala Scientific) was further placed over the section to ensure optimal contact with the
619 electrode array. Recordings were carried out post stabilization (20 min) for a duration of 20 mins. Data
620 was sampled at 10 kHz, at 37°C (TC02, Multichannel Systems, Reutlingen). For experiments involving
621 the usage of multiple compounds, a peristaltic pump (PPS2, Multichannel Systems, Reutlingen) was
622 used at a flow rate of 3 mL/min. All recordings were carried out in the same culture medium as described
623 above.

624 Analysis: Raw data was analyzed using the MC_Rack software (Multichannel Systems, Reutlingen).
625 Recordings were either high-pass filtered (300Hz-3000Hz) for neuronal activity or low pass filtered (0-
626 150 Hz) to detect changes in local field potentials (LFP). For neuronal activity, the threshold was set to
627 4.5 x S.D. of the recorded signal. Data was further exported to the R computing environment for plotting
628 and analysis.
629