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Astrocytes shape the plastic response of adult cortical neurons to vision loss

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Running title: Astrocytes shape cortical plasticity

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Main points:

- Visual deprivation induces a fast and persistent upregulation of Aldh1l1-positive astrocytes
- Acute fluorocitrate-induced silencing of astrocytes impedes neuronal reactivation
- G_i but not G_q GPCR astrocyte activation promotes adult cortical plasticity

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Abstract

Astrocytes are vital for preserving correct brain functioning by continuously sustaining neuronal activity and survival. They are in contact with multiple synapses at once allowing the expansion of local synaptic events into activity changes in neuronal networks. Furthermore, cortical astrocytes integrate local sensory inputs and behavioral state. From an anatomical, molecular and functional perspective, astrocytes are thus ideal candidates to influence complex large-scale brain mechanisms such as plasticity. We collected evidence for the astrocytic potential for plasticity modulation, using the monocular enucleation (ME) mouse model of visual cortex plasticity. The impact of one-eyed vision involves the functional recruitment of the deprived visual cortex by the spared senses within a 7-week time frame, reflecting a substantial change in sensory information processing. In visually deprived cortex a swift upregulation in Aldh1l1-positive astrocyte density lasts until maximal functional recovery is reached. Transient metabolic silencing of visual cortex astrocytes at the time of ME induction, through intracranial fluorocitrate injections, reveals that astrocytes are required on site to achieve adequate long-term neuronal reactivation. In addition, chronic stimulation by G_i but not G_q GPCR activation of local astrocytes boosts the cortical plasticity phenomenon. Hence, functional manipulation of protoplasmic astrocytes has long-lasting effects on the functional recovery of cortical neurons upon sensory loss, possibly by influencing the neuronal threshold to reactivate. Together, our results highlight an integral role for astrocytes in mediating adult cortical plasticity and unmask astrocyte specific G_i signaling as an interesting therapeutic pathway for brain plasticity regulation.

Keywords

Astrocytes – Visual cortex – Brain plasticity - Adulthood

Introduction

Astrocytes are one of the most abundant cell types in the mammalian cerebral cortex, where they are intermingling between the neurons in a tiled fashion (Bushong, 2002; Halassa, 2007). At the level of the tripartite synapse, astrocytes provide surrounding neurons with structural, metabolic and trophic support. Gliotransmission enables them to actively participate in synaptic transmission and to modulate neuronal function (Araque, 2014, 1999; Parpura, 2010). In addition, during development astrocytes possess synaptogenic capacities that remain latent in the adult brain (Christopherson, 2005; Singh, 2016; Stogsdill, 2017) (for review see (Baldwin, 2017)). Due to their specialized highly branched morphology, in cortex, protoplasmic astrocytes are able to contact multiple synapses at once, allowing them to exert local modulatory synaptic effects which can be expanded to influence coordinated neuronal circuit activity (Bushong, 2002; Halassa, 2007; Oberheim, 2009). Through lateral regulation of synapses, astrocytes can even serve as an information processing bridge between synapses with no direct neuronal connectivity (Covelo, 2016).

Astrocytes are thus ideally positioned and molecularly equipped candidates to mediate complex large-scale brain processes such as brain plasticity. This intrinsic ability of the central nervous system (CNS) allows it to structurally and functionally adapt to a continuously changing environment. CNS plasticity is crucial during early development and remains important throughout adult life, to facilitate learning, memory formation and even to functionally recover from traumatic events (Hensch, 2005; Hübener, 2014; Pascual-Leone, 2005).

However, in adulthood, the capacity for neural plasticity is more constrained making it more difficult for the adult brain to functionally recover from an injury or a neurological disorder. Discovering new strategies to reopen and modulate a window of heightened plasticity holds great promise for new therapeutic interventions for plasticity-related brain diseases. As neuronal circuit regulators, astrocytes present themselves as potential entry points for the fine-tuning of adult brain plasticity.

To test how and when astrocytes are involved in brain-region specific circuit adaptations in the context of neuronal plasticity, we performed three types of experiments in the monocular enucleation (ME) model of cortical plasticity. This mouse model of acquired

one-eyed vision leads to an extensive neuronal reactivation of both binocular and monocular territories within the deprived visual cortex contralateral to the lost eye, during a 7-week recovery period (7wME). The functional recovery is established through the recruitment of the deprived visual cortex by the spared senses. Potentiation of spared-eye inputs mainly leads to a reactivation of the binocular zone in the first weeks post-ME, whereas reactivation of the monocular zone in the visual cortex is somatosensory, whisker-driven, in nature (Nys, 2014; Van Brussel, 2011). Through immunohistochemical analysis of the pan astrocyte marker aldehyde dehydrogenase 1 family member L1 (Aldh1l1), we probed for astrocyte density changes in the visually deprived cortical areas. To test whether functional cortical astrocytes on site are necessary for adequate neuronal recovery we induced acute metabolic silencing of astrocytes in the deprived visual cortex at the time of ME induction, through direct fluorocitrate injection. In parallel experiments, we chronically modulated astrocyte activity through a chemogenetics approach to interrogate if enhancing G-protein coupled receptor (GPCR) associated G_i or G_q signaling in cortical astrocytes can boost neuronal reactivation after ME.

Materials and Methods

Animals

A total of 78 C57Bl6/J mice, obtained from Janvier Labs (Le Genest-St-Isle, France) were used for this study. All mice were housed under standard laboratory conditions under a 10/14-h dark/light cycle with food and water available *ad libitum*. All experiments were approved by the Ethical Research Committee of the KU Leuven and were in accordance with the European Communities Council Directive of 22 September 2010 (2010/63/EU) and with the Belgian legislation (KB of 29 May 2013). Every effort was made to reduce animal suffering and to reduce the number of animals used.

Monocular enucleation and tissue processing

Monocular enucleation (ME) was performed as previously described (Aerts, 2014). Briefly, P120 adult mice (n=51) were sedated by an i.p. injection of a mixture of ketamine hydrochloride (75 mg/kg, Dechra Veterinary Products, Eurovet) and medetomidine hydrochloride (1 mg/kg Orion Corporation, Janssen Animal Health). To prevent dehydration, eye ointment (Tobrex, Alcon) was applied on the left eye. The right eye was carefully removed and the orbit was filled with hemostatic cotton wool (Qualiphar, Bornem, Belgium) in case of bleeding. To prevent pain, analgesics were injected subcutaneously (Metacam, 2 mg/kg, 0.1 mL) and atipamezol hydrochloride was administered to reverse the anaesthesia (1 mg/kg i.p., Orion Corporation, Elanco Animal Health). Following enucleation, the mice were housed in their home cages under standard laboratory conditions for a 3-day to 7-week recovery period. At the end of the recovery period, mice (n=18) used for immunohistochemical analysis were deeply sedated by a lethal injection of Dolethal (1 ml/1.5 kg, Vétoquinol S.A., Belgium) before being transcardially perfused with a 1 % paraformaldehyde (PFA) solution in phosphate-buffered saline (PBS). Next, the brains were removed and post-fixed overnight in a 4 % PFA solution in PBS. Coronal brain slices of 50 μ m were prepared on a Vibratome (HM 650 V, Microm Microtech, France). For the analysis of *zif268* expression levels through radioactive *in situ* hybridization, the mice (n=33) were placed overnight in a dark room to reduce *zif268* expression to basal levels. The next day they were placed in a highly-lit

environment for 45 min to induce maximal sensory-driven *zif268* mRNA expression. Next, the mice were sacrificed through cervical dislocation after which the brains were carefully removed and immediately frozen in 2-methylbutane (Merck, Overijse, Belgium) at a temperature of -40 °C. All brains were stored at -80 °C until sectioning. Series of 25 µm-thick sections were prepared on a cryostat (HM 500 OM, Microm, Thermo Scientific, Walldorf, Germany), mounted on 0.1 % poly-L-Lysine-coated (Sigma-Aldrich) slides, and stored at -20 °C until further processing.

Aldh1l1 immunohistochemical analysis and quantification

To assess the ME-effect on astrocyte density, the pan-astrocytic marker Aldehyde Dehydrogenase-1 Family Member L1 (Aldh1l1) was used to visualize cortical astrocytes. Aldh1l1 diaminobenzidine (DAB) stainings were performed on 50 µm free-floating sections. First, a heat-induced epitope retrieval (HIER) was performed at 80 °C for 30 min in a 10 mM sodium citrate buffer (pH=6). The sections were then pretreated with H₂O₂ (0.3 % in Tris-buffered saline, TBS), rinsed in TBS and preincubated for 1 hour in blocking buffer (2 % milk (GE Healthcare, UK), 0.5 % Triton X-100 in PBS (1x)). Next, slices were incubated overnight at RT with rabbit anti-Aldh1l1 (1:200 in 5% bovine serum albumin (BSA; Sigma Aldrich), 0.5% Triton X-100 in PBS (1x)) after which detection was performed using biotinylated goat anti-rabbit IgG (1:500 in TBS, 30 min; Dako, Carpinteria, CA) and streptavidin horseradish peroxidase (1:500 in TBS, 1 hour; Dako). A glucose oxidase DAB-nickel method was used for detection, resulting in a black precipitate providing a higher contrast for cell counting compared to immunofluorescence-based stainings (Shu et al., 1998).

In order to correctly delineate the different areas in the visual cortex as well as the cortical layers for Aldh1l1 astrocyte counting, adjacent sections were Nissl-stained (1% Thionin, Fluka, Sigma-Aldrich) according to standard procedures. Pictures of the stained sections were obtained at 20X (NA: 0.8) with a light microscope (Zeiss Axio Imager Z1) equipped with an AxioCam MRm camera (1388 X 1040 pixels) using the software program ZEN (Zen Pro 2010, Carl Zeiss, Benelux).

For quantification of the astrocyte density, Aldh1l1 positive astrocytes were manually counted using ImageJ (Fiji, US National Institutes of Health). Position of interareal

borders, delineations of monocular and binocular territory, and the cortical layers were transferred between pictures from the adjacent Nissl sections to the Aldh1l1-stained sections (Fig. 1b). Aldh1l1-positive astrocytes from the monocular and binocular visual cortex were counted in vertical rows of cortical bins perpendicular to the pial surface and spanning all cortical layers. Total number of counted cells was then normalized to the total cortical bin size and represented as astrocytes/mm². To assess differences in layer distribution of astrocytes, bins were assigned to cortical layers to then perform a frequency distribution analysis using the coordinates of the counted cells in the binocular and monocular bins. Per layer, counted astrocytes are represented as a percentage of the total number over all layers, to investigate whether density changes are layer-specific. Data were analyzed from six sections per animal and for all conditions 6 animals were used.

Stereotactic surgery

Mice were anesthetized through i.p. injection with a mixture of ketamine hydrochloride (75 mg/kg; Dechra Veterinary Products; Eurovet) and medetomidine hydrochloride (1 mg/kg; Orion, Janssen Animal Health) before being stereotactically injected through a craniotomy into the primary visual cortex (V1) of the left hemisphere: 3.4 mm posterior to bregma, 2.5 mm lateral to the midline, at a depth of 400 μ m (viral vectors) or 500 μ m (fluorocitrate) from the cortical surface (Franklin, 2013). Viral vectors or fluorocitrate were delivered via a glass capillary (20 μ m tip diameter) using a Nanoject II Auto-Nanoliter Injector (Drummond Scientific, Broomall, PA). A multiple injection approach per craniotomy was chosen to reach an optimal volume of transfected/affected cortex, based on a maximal distance of 300 μ m between injections along the A-P and M-L axis of the brain. After each injection, the capillary was left in place for 1 min before being slowly retracted. Following wound suturing, anesthesia was reversed by i.p. injection of atipamezol hydrochloride (1 mg/kg; Orion; Elanco Animal Health, Antwerp, Belgium).

Fluorocitrate preparation and intracranial injection

The fluorocitrate compound was used to induce an astrocytic metabolic silencing at ME onset. The fluorocitrate solution was prepared as previously described (Hayakawa, 2010). Briefly, 8 mg of DL-fluorocitric acid barium salt (Merck, Darmstadt, Germany) was

dissolved in 1 ml of 0.1 M HCl. Three drops of 0.1 M Na₂SO₄ were added to precipitate Ba²⁺. Next, 2 ml of 0.1 M Na₂SO₄ was added before centrifuging the solution for 5 min at 1000 g. The supernatant was further diluted with 0.9 % NaCl to obtain a final concentration of 1 nmol/μl (pH=7.4).

Intracranial injection of fluorocitrate was performed in P120 mice as described above. A total of 4 injections per craniotomy were performed, each consisting of a volume of 345 nL (in steps of 69 nL every 20 s). To validate specific metabolic silencing of astrocytes by fluorocitrate, mice were transcardially perfused one or three days (n=6) after intracranial injection for analysis. For characterization of the effect of astrocyte elimination in the ME model, mice were enucleated immediately after fluorocitrate or saline injection and were sacrificed by cervical dislocation after 7 weeks for *in situ* hybridization with probes specific for *zif268* (n=12) (Fig. 1a).

Viral vector injections

To evaluate if stimulating astrocyte activity could influence neuronal recovery after ME, a chemogenetic approach was implemented.

All used vectors (rAAV2/5-GFABCD1-hM3Dq-mCherry, rAAV2/5-GFABCD1-hM4Di-mCherry, rAAV2/5-GFABCD1-mCherry,) were produced at the Leuven Viral Vector Core as previously described (Van der Perren, 2011). Viral vectors were diluted to obtain a final genomic titer of 1×10^{12} genome copies /ml. The sequence coding for the fluorescent mCherry protein was added to all constructs for detection purposes. The combination of the vector serotype and promoter sequence was chosen to reach appropriate expression sufficiency and specificity in astrocytes only. The third sham-control vector expressing only mCherry (no DREADD construct) was used to create the control condition for correct comparative analysis. Intracranial injections of the viral vectors were performed in P105 mice as described above. Here triple injections were performed, each containing a volume of 200 nL (in steps of 50 nL every 20 s). To validate the astrocyte tropism of the vectors and the functionality of the DREADD constructs, a first set of mice (n=15) were intracardially perfused two weeks after vector injections for immunofluorescent-based analysis. For studying the DREADD effect in the ME model, mice (n=21) were enucleated

two weeks after vector injection, at P120. All mice then received daily CNO (5 mg/kg; i.p.; Tocris Bioscience, Bristol, UK) for 7 weeks (up to day 168) before being placed in darkness overnight, sacrificed and processed for *zif268 in situ* hybridization.

Immunofluorescence verification of fluorocitrate and viral vector specificity and functionality

Validation of fluorocitrate-induced astrocyte-specific suppression, confirmation of viral vector-promoter-based astrocyte-specific targeting and DREADD-construct functionality was assessed using free-floating immunofluorescence. Upon transcardiac perfusion with 1 % PFA in PBS, brains were dissected and postfixed overnight in 4 % PFA in PBS at 4 °C. Fifty µm-thick coronal sections were obtained using a Vibratome (HM 650 V, Microm Microtech, France). After washing with PBS, the sections were blocked for 60 min with normal goat serum (Merck Millipore, Overijse, Belgium) and overnight incubated at RT with the primary antibodies diluted in Tris-NaCl blocking buffer (TNB). The next day, slices were washed and then incubated for 2 h at RT with the secondary antibodies diluted in TNB buffer followed by a 15 min incubation with DAPI (2 µL/100 mL PBS, Sigma-Aldrich, St. Louis, MO, 3260). Sections were then mounted on glass slides and coverslipped using Mowiol solution. For the Aldh1l1 immunofluorescent stainings, a HIER pretreatment and adapted buffers were used as described above. Images were taken at 20X (NA: 0.8) with a fluorescence microscope (Zeiss Axio Imager Z1) equipped with an AxioCam MRm camera (1388 × 1040 pixels) and Apotome1 using the software program Zen (Zen Pro 2012, Carl Zeiss, Benelux).

Table 1. Overview of primary and secondary antibodies used for immunohistochemistry.

***In situ* hybridization**

High-throughput radioactive *in situ* hybridization (ISH) experiments for the immediate early gene (IEG) *zif268* were performed to obtain an elaborate molecular overview of neuronal activity patterns across all cortical layers in the different areas of the visual

cortex. The IEG *zif268* has previously been described as a predominant neuronal activity marker in the mammalian brain (Mataga, 2001). Furthermore previous studies have shown that increased *zif268* expression levels correspond with increased neuronal activation and can be used to assess alterations in cortical reactivation after ME (Lombaert, 2018; Nys, 2015; Scheyltjens, 2018; van Brussel, 2009; Van Brussel, 2011). In this study, the same *zif268* readout was used to characterize the effect of astrocyte modulation on the neuronal recovery in the ME model.

All *zif268* ISH experiments were performed on series of coronal brain sections between bregma levels -1.5 mm and -5 mm using a mouse-specific synthetic oligonucleotide probe (Eurogentec, Seraing, Belgium) with sequence 5'ccgttgctcagcagcatcatctctccagtttgggtagttgtcc-3'. Each probe was 3'-end labeled with ^{33}P -dATP using a terminal deoxynucleotidyl transferase (Invitrogen, Carlsbad, CA). MiniQuick Spin Oligo Columns (Roche Diagnostics, Vilvoorde, Belgium) were used to separate unincorporated nucleotides from the labeled probe. Cryostat sections were fixated, dehydrated and delipidated. The radioactively labeled probes were added to a hybridization cocktail (50% formamide, 4x standard saline sodium citrate (SSC) buffer, 1x Denhardt's solution, 10% dextran sulfate, 100 $\mu\text{g}/\text{ml}$ herring sperm DNA, 250 $\mu\text{g}/\text{ml}$ tRNA, 60 mM dithiothreitol, 1% N-lauroyl sarcosine, and 20 mM NaHPO_4 , pH 7.4) and applied to the cryostat sections (10^6 cpm/section). After overnight incubation at 37 °C in a humid chamber, the sections were rinsed in 1x standard SSC buffer at 42 °C, dehydrated, air-dried and exposed to an autoradiographic film (Biomax MR; Kodak, Rochester, NY). After 7 days, the films were developed in EMS replacement for Kodak developer D-19 (Electron Microscopy Sciences, Hatfield) and fixed in Rapid fixer (Ilford; Hypam; Kodak). Autoradiographic images of adjacent sections were scanned at 1200 dpi (CanoScan 9000F Mark II; Canon; Tokyo; Japan) and all images were similarly adjusted for brightness and contrast in Adobe Photoshop CS5 (Adobe Systems, San Jose, CA). Corresponding pseudocolor representations were made using ImageJ and represent a false color coding of the gray values ranging from black (0) to white (255); low gray values are represented in black/blue and high gray values in white/red.

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293 Histology and delineation of visual areas based on Nissl patterns

294 Following ISH, sections were Nissl counterstained (1 % Thionin; Fluka, Sigma-Aldrich)
295 according to standard procedures to visualize cortical layers and the boundaries between
296 the different visual areas, to aid in the interpretation of the *zif268* expression patterns.
297 Images of the Nissl sections were obtained at 5X (NA: 0.16) using a light microscope
298 (Zeis Axio Imager Z1) equipped with an AxioCam MRm camera (1388 × 1040 pixels)
299 using the software program Zen (Zen Pro 2012, Carl Zeiss, Benelux). Immunofluorescent
300 mCherry stainings were performed on post-fixed (4% PFA in PBS) cryostat sections
301 adjacent to the Nissl-stained ISH sections to confirm vector-mediated expression in V1.
302 This staining as well as the image acquisition was performed as described above.
303 The stereotactic mouse brain atlas was implemented to delineate the areal borders as
304 described previously (Franklin, 2013; Nys, 2015; van Brussel, 2009). In all figures, the
305 interareal borders are demarcated by black arrowheads. From lateral to medial five
306 subregions are defined: the lateral extrastriate cortex (V2L), which is subdivided into a
307 monocular (V2Lm) and a binocular (V2Lb) zone; the primary visual cortex (V1), which is
308 subdivided into a monocular (V1m) and a binocular (V1b) zone; the medial extrastriate
309 cortex (V2M) (Fig. 1b).

310 Top view and line graph representations of cortical neuronal activity

311 To evaluate the extent of the neuronal reactivation based on *zif268* expression levels in
312 the different experimental paradigms, a quantification of the optical density (OD) values
313 across different visual areas was performed using a custom-made Matlab (Matlab R2016;
314 Mathworks) script as previously described (Nys, 2015; Vreysen, 2017). Top view
315 representations of *zif268* expression levels across the primary and secondary visual
316 areas were created to obtain a spatial overview of the neuronal recovery in the different
317 conditions. Hereto, *zif268* sections from bregma levels -2 until -4.5 (100 µm spread
318 between sections) were loaded in the Matlab script. The region of interest in the left
319 hemisphere was demarcated by selecting the top and bottom edge of the cortex and the
320 border between the supra-granular (II/III and IV) and the infragranular (V and VI) layers.
321 The region of interest between the rhinal fissure and the medial border of V2M was

divided into 50 equal segments from lateral to medial to create two lattices, corresponding to layers II–IV and layers V–VI (Fig. 1b,c). To adjust for background signal, a small rectangular reference area was demarcated in the white matter. Per segment, the mean OD value was calculated and normalized to the reference region and then projected onto a horizontal planar projection. The horizontal projections per segment and per animal were used to create the top view image.

Line graph representations of the neuronal reactivation pattern were created to obtain a more detailed visualization of the averaged *zif268* expression levels per visual cortex area. Three sections near the injection site (bregma levels; -3.4, -3.5, -3.6) were selected per animal for every condition and loaded into the Matlab script. The region of interest in the left hemisphere was again delineated by selecting the top and bottom edge of the cortex, as well as the border between the supra-granular and the infragranular layers. The region of interest was divided into 27 (supra-granular layers) or 28 (infragranular layers) equal segments from lateral to medial to create two lattices corresponding to layers II–IV and layers V–VI (Fig.1c). Per segment the OD value was calculated and normalized as described above for the top view representations. Results are presented in subregion-specific bar graphs illustrating the neuronal activity patterns for the different analyzed conditions.

Statistics

Statistical analysis was performed using GraphPad Prism 5.01 (GraphPad Software, Inc). All cell counting data and relative OD-values of *zif268* sections are presented as mean $\pm$ SEM. Normal distribution and equal variance between groups was tested. A one-way Anova with Bonferroni post hoc tests was used for pairwise comparisons of the Aldh111-astrocyte cell counting experiments. For the analysis of the ISH data, a non-parametric test (Mann-Whitney-U) was applied for pairwise comparison. For all used tests, a probability level of < 0.05 was accepted as statistically significant (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results

Monocular enucleation impacts cortical astrocyte density in a time- and brain region-dependent manner

In order to study the astrocyte potential for cortical plasticity modulation, we first assessed if our visual deprivation paradigm affects astrocytes, and in which time frame they are responding to the loss of visual inputs. Transcriptome analysis previously revealed that the metabolic enzyme Aldh1l1 is highly specific for astrocytes and has a broader expression pattern than other commonly used markers such as Glial fibrillary acidic protein (GFAP) (Cahoy, 2008). We therefore applied immunohistochemical analysis of the expression of this pan astrocyte marker to investigate the effect of ME on visual cortex astrocytes from 3 days up to 7 weeks post ME. Aldh1l1-positive astrocytes are clearly detected across all cortical layers and display their typical star-shaped morphology (Fig. 2a,b). We counted the immunopositive astrocytes separately for the monocular and the binocular subdivisions of the deprived visual cortex on sections of control (normal sighted), 3-day enucleated (3dME), 3-week enucleated (3wME) and 7-week enucleated (7wME) mice. In the monocular cortex, astrocyte density is already significantly increased at the 3dME time point compared to the control mice, indicating that astrocytes are responding early on to the visual deprivation. This upregulation of Aldh1l1-positive cortical astrocytes remains apparent for weeks, suggesting that this density increase is not transient but related to the entire cortical recovery period (Fig. 2c). A layer distribution analysis of the astrocytes from the monocular visual cortex revealed uniform astrocyte density changes across all cortical layers (Fig. 2d). A similar increase in Aldh1l1-positive astrocytes occurs in the binocular zone of the deprived visual cortex after 3 days, 3 weeks and 7 weeks ME compared to the control mice (Fig. 2e). However, astrocyte density at the 7wME time point is already decreased compared to the earlier 3dME time point, which suggests that, at least in the binocular zone, astrocyte density may return to basal levels over time (Fig 2e). Evaluation of the astrocyte layer distribution pattern shows a more pronounced impact on layer V of the binocular cortex already at 3dME, possibly at the expense of layer VI since the astrocyte percentage in layer VI of 3dME mice is decreased compared to the control condition. This altered distribution pattern of astrocytes may

indeed suggest the occurrence of a relative astroglial redistribution across cortical layers in the binocular zone.

Overall, the detailed analysis of the ME-effect on the spatial distribution of Aldh1l1-positive astrocytes in visual cortex revealed a swift astrocyte response to the loss of visual input that persists throughout all assessed time points, indicating that early, long-lasting astrocyte contributions might be crucial to modulate the neuronal reactivation outcome of sensory-deprivation induced cortical plasticity. In addition, ME resulted in a redistribution of visual cortex astrocytes in favor of infragranular layer V in the binocular cortical territory.

Cortical fluorocitrate injection leads to a selective, reversible silencing of visual cortex astrocytes

Considering that cortical astrocytes show an early response to the drop in neuronal activity by upregulating their density swiftly, their initial presence might be necessary to modulate the cortex towards long-term neuronal recovery. We hypothesized that by experimentally blocking the early astrocyte response in V1, the plasticity-induced neuronal reactivation would be hampered. To test this theory, fluorocitrate was intracranially injected into V1 in order to first assess in normal mice if this approach can locally metabolically silence visual cortex astrocytes. Figure 3 shows a clear drop in Aldh1l1-positive astrocytes at one day post-injection, localized to the fluorocitrate injection site (Fig. 3a, white rectangle). This is not resulting from tissue damage, since the nuclear marker 4',6-diamidino-2-phenylindole (DAPI) signal remains largely intact along all cortical layers (Fig. 3a). At one day post-injection, this metabolic suppression is highly selective for astrocytes since no apparent changes could be observed in the microglial (TMEM119) or neuronal (NeuN) cell density (Fig. 3b, Supplementary Fig. 1), in line with the DAPI findings. This fluorocitrate-induced astrocyte depression appears transient as the effect is already largely reversed three days post-injection, when Aldh1l1-positive astrocytes are again detected at the injection site (Fig. 3c). The coincident induction of GFAP expression in cortical astrocytes is partially due to the intracranial surgery itself but seems to be more pronounced than in saline injected mice (Supplementary Fig.2). Most likely, this reawakening of visual cortex astrocytes is accompanied by a compensatory reaction leading to a heightened GFAP expression.

Early transient silencing of astrocytes impairs functional recovery of the deprived visual cortex

As astrocytes are capable of modulating neuronal activity, their early onset rise in density after ME might be necessary for the plastic neuronal recovery of the deprived visual cortex. To assess to what degree the early astrocytic response is contributing to the neuronal reactivation, we next injected fluorocitrate intracranially into the deprived visual cortex moments before performing ME (Fig. 1a). Mice were left to recover for 7 weeks in their home cages, a period in which ME-induced plasticity normally leads to extensive recovery of neuronal activity in the visual cortex. To compensate for possible effects of the stereotactic surgery, we injected sham control ME mice with the same volume of saline for comparison. Immediate early gene *zif268* expression levels were used as the readout for experience-induced neuronal activity and consequently the extent of plasticity-mediated recovery from ME. Considering that ME differently affects neuronal activity across cortical layers and taking into account the heterogeneity of cortical astrocytes, supra-granular (II/III and IV) and infragranular (V and VI) layer activity were separately evaluated (Bayraktar, 2019; Clarke, 2018; Xin, 2018; Zhang, 2010).

The creation of top view representations of the *zif268* expression pattern and levels across visual areas reveals differences in the spatial extent of the neuronal reactivation between the fluorocitrate and saline injected ME mice (Fig. 4a). The fluorocitrate condition shows an overall smaller spread in *zif268* expression in both the supra- and the infragranular layers (Fig. 4a, left and right panels). This lowered neuronal reactivation is most pronounced for the monocular visual cortex, indicating that the typical features of neuronal recovery (Fig. 1d), namely binocular zone expansion and monocular region reactivation, are hampered (Fig. 4a). In order to visualize the impact on neuronal reactivation in more detail, we also created line graph representations reflecting the neuronal activity levels of brain sections surrounding the injection site (bregma: -3.4). Figure 4b contains representative images of such sections processed for *zif268* mRNA expression, with their corresponding pseudocolor images, with black arrowheads indicating the boundaries between the analyzed visual cortical areas. Similar coronal sections from the group of mice from both conditions (n=6) were used to create the line

graphs representing the neuronal activity pattern based on averaged *zif268* expression levels across these anatomically delineated visual areas (dotted vertical lines represent interareal borders and correspond to the black arrowheads in figure 4b). The supra-granular layers (left line graph) clearly display lower *zif268* expression levels in the fluorocitrate condition (blue) compared to the saline condition (black) illustrating a reduced neuronal reactivation, similar to the observation made in the supra-granular top view. For the infragranular layers, the line graph (Fig. 4c, right graph) shows a complete overlap between the fluorocitrate (blue) and saline (black) condition, indicative of similar averaged *zif268* expression. Area-specific statistical analysis further revealed that the averaged *zif268* expression in the fluorocitrate condition is significantly lower in the supra-granular layers of V1 and the monocular region of the visual cortex, including V1m and V2M, compared to saline control. No significant differences could be observed in the supra-granular layers of the binocular zone (V2Lb and V1b) or in the infragranular layers (Fig. 4d).

These results show that an early and transient silencing of astrocytes in visual cortex impacts the neuronal reactivation process specifically in the supra-granular layers of the deprived visual cortex, thereby revealing that astrocytes are indispensable for plasticity following ME. The effect of fluorocitrate-induced astrocyte silencing is mostly concentrated in monocular visual cortex, where the loss of visual input due to ME is the greatest. This indicates that exactly where neuronal activity drops, due to an instant and complete loss of sensory input, a fast response of astrocytes is crucial for adequate functional recovery in the longer term.

Astrocyte specific targeting of AAV2.5-GFABCD1D vector and validation of the functionality of DREADD constructs

Astrocytes have been shown to be essential for synaptogenesis and subsequent modulation of synaptic function. We hypothesized that modulating their activity might therefore lead to a boost in neuronal recovery. To be able to chronically steer solely the activity of astrocytes, we implemented a chemogenetics approach. Via a recombinant adeno-associated viral vector approach, we first expressed hM3Dq and hM4Di DREADD constructs in normal visual cortex astrocytes. We obtained specific astrocyte expression,

by using an AAV2.5 viral vector with a modified GFABCD1D promotor and mCherry reporter gene. To validate the astrocyte targeting we performed immunofluorescent double stainings. Figure 5a shows the clear co-labeling between the mCherry reporter gene and astrocyte specific cytoplasmic (Aldh1l1, GFAP) as well as plasma membrane markers (Connexin43, Kir4.1) in visual cortex. The used viral vector construct shows no co-labelling with any of the other neuron (NeuN), interneuron (Parvalbumin), microglia (TMEM119) or oligodendrocyte (Olig2) cell-specific markers (Fig. 5b). Upon acute i.p. injection of CNO, the immediate early gene Fos is expressed only in hM3Dq- and hM4Di-expressing visual cortex astrocytes but not in astrocytes transduced with the mCherry control vector (Fig. 5c).

Chronic G_i but not G_q GPCR activation of astrocytes enhances neuronal plasticity in the ME model

In order to show that astrocytes are not only necessary for the occurrence of neuronal reactivation after ME but that they are capable of boosting the recovery process, we next applied the validated DREADD toolbox to reliably enhance their activity in the ME model. As our results indicated that astrocytes are fast responders to visual deprivation and that their increased detection lasts until the final phase of the plastic recovery, we opted to modulate their activity long-term until the end of the model. Therefore, mice of all conditions (mCherry, hM3Dq, hM4Di) received daily i.p. CNO (5mg/kg) injections for 7 weeks following ME. The *zif268* mRNA-based readout was again used to assess the neuronal reactivation pattern separately for layers II-IV and V-VI in the different areas of the deprived visual cortex.

Comparison of the top view representations of the spatial *zif268* expression patterns reveals a more confined expression pattern in the hM3Dq injected mice, and a broader expression pattern in the hM4Di injected mice compared to the mCherry controls. Again, a more pronounced impact is observed for the supra-granular layers of the visual cortex (Fig. 6a). The spatial *zif268* expression pattern of the hM4Di condition spreads further into the monocular territory of the visual cortex indicating that chronically enhancing G_i signaling in astrocytes can lead to a more pronounced reactivation of the monocular cortex (Fig. 1d). On the other hand, the spatial reactivation pattern of the hM3Dq

conditions seems to be more confined than what is seen in mCherry control mice, suggesting that chronic G_q signaling in astrocytes can hamper the spread of neuronal activity across the visual cortex. Representative images of *zif268* mRNA labeled sections from hM3Dq, mCherry and hM4Di expressing mice and their corresponding pseudocolor images, with black arrowheads representing the boundaries between the analyzed visual areas, are shown in figure 6b. Similar to the top view observation, the hM4Di condition shows higher *zif268* expression in V1 compared to the mCherry control. Figure 6b also illustrates correct vector localization in V1 based on mCherry reporter gene expression for the three experimental conditions. Line graph representations based on such sections surrounding the injection site (bregma: -3.4) were again created to obtain a more detailed overview of the neuronal reactivation pattern across visual areas. The supra-granular layers (left line graph) clearly show an overall lower averaged *zif268* expression in the hM3Dq condition (green) compared to the mCherry control (black) across the analyzed visual areas. Yet compared to the mCherry control (black), the hM4Di (red) condition is characterized by increased averaged *zif268* expression levels for the supra-granular layers in V1 as well as the monocular cortex. The infragranular layers display similar averaged *zif268* expression levels across all conditions (Fig. 6c). Statistical analysis of the relative *zif268* expression levels in the different visual areas revealed that the hM3Dq condition (green) is significantly less reactivated in the supra-granular layers of the binocular zone compared to the mCherry control (black). This suggests that G_q signaling hampers neuronal recovery by decreasing binocular zone expansion and monocular zone reactivation. hM4Di *zif268* (red) expression levels are significantly upregulated in the supra-granular layers of the monocular region of V1 (V1m) compared to the mCherry control (black). As such, modulation of astrocyte activity by enhancing G_i signaling is thus capable of boosting neuronal reactivation in the monocular zone of the deprived visual cortex. Comparison of hM3Dq and hM4Di averaged *zif268* expression levels reveals that the hM4Di condition is showing significantly higher neuronal activity levels in the supra-granular layers of the binocular and the monocular zone as well as in V1 (Fig. 6d).

In conclusion, besides being required on site for adequate neuronal reactivation to occur in deprived cortical territory, astrocyte stimulation even holds the potential to boost the

530 neuronal plasticity process, which further highlights their capacity as neuronal modulators
531 and plasticity mediators.

Discussion

Our results demonstrate how protoplasmic astrocytes actively contribute to large-scale reorganization of adult cortical function induced by sensory deprivation. The astrocyte density quickly increased in response to an instant drop in neuronal activity caused by loss of sensory inputs throughout the relevant cortical territories. Such changes in astrocyte density in the context of plasticity paradigms have already been described. Saur et al. reported how physical exercise, capable of positively affecting neuroplasticity, leads to a coincident increase in astrocyte density in the CA1 rat hippocampal region (Saur, 2014). On the other hand, a decrease in hippocampal astrocyte quantity has been identified in neurological disorders associated with disturbed neuroplasticity such as major depression disorder (Cantone, 2017; Cobb, 2016). Specifically, for the visual system Gonzalez et al. could show that ME leads to a long-term upregulation of astrocyte numbers in the rat lateral geniculate nucleus. They suggested that in subcortical visual nuclei such changes in astrocyte density (in response to ME) occur initially to facilitate removal of neuronal debris, followed at later points by synaptic reorganization (Gonzalez, 2006). We showed here that at the level of the visual cortex, which is not a direct retinal target region, a similar rise in the number of astrocytes already occurs in the first few days post injury. As no neuronal degeneration or reactive GFAP-positive astrocytes are detected in the visual cortex after ME (Nys, 2014; Supplementary Fig.3), this early cortical astrocyte response seems evoked by the acute drop in neuronal activity *per se*.

Besides alterations in astrocyte density, also shifts in astrocyte subpopulations have been observed in response to visual deprivation (Cornett, 2011). Our findings may support the possibility that normally active mouse visual cortex contains a silent, Aldh1l1-negative astrocytic subpopulation that gets triggered upon ME. However, as Aldh1l1 is a relatively 'new' astrocytic marker, more research is needed to reveal if Aldh1l1-negative astrocytes exist in mouse cortex under normal physiological conditions.

The astrocytic response to ME appeared heterogeneous throughout the mouse visual cortex. In the monocular territory, the rise in Aldh1l1 positive astrocytes occurred uniformly across all cortical layers and lasted the whole 7-week recovery period. In the binocular zone, the upregulation occurred with the same swiftness but started to downregulate towards the end of the model, when maximal functional recovery is

established (Van Brussel, 2011). Here, the increase in density was also more pronounced in the infragranular layers, especially in layer V of the visual cortex. These subregion-specific differences correlate with previous research from our lab showing that the impact of ME on neuronal activity in the monocular region is visible throughout all layers of the cortex, whereas in the binocular region the infragranular layers show the greatest loss (Van Brussel, 2011). These results thus indicate that astrocytes sense the region-specific loss of visual input and compensate accordingly. Neuronal activity has been shown before to affect and shape the surrounding astrocytic population. By altering the astrocytic transcriptome, neuronal activity can modify the astrocytes' functional, molecular and anatomical state (Farmer, 2016; Hasel, 2017). Furthermore, as visual stimulation elicits a response in mouse cortical astrocytes that can be influenced by the animal's behavioral state (Slezak, 2018; Sonoda, 2018), it is not surprising that also the loss of visual input would elicit a profound astrocytic response. As astrocytes are known to influence neuronal activity and synaptic transmission, specific alterations in astrocyte presence and functionality are most likely required for promoting brain region-specific synaptic rearrangement and reestablishment of neuronal functions in the visual cortex. Possibly, a reinstatement of latent synaptogenic abilities in astrocytes in the adult brain might induce excitatory synaptogenesis promoting functional cortical recovery (Baldwin, 2017; Singh, 2016).

Our prediction was that blocking the initial astrocyte response to the sudden loss of sensory input would lead to impairment of neuronal recovery long-term in the deprived cortex. Acute fluorocitrate-induced metabolic suppression was clearly successful as we hardly detected any Aldh1l1-positive astrocytes in the immediate vicinity of the injection site at 1 day post-injection, an effect which had reversed at 3 days, comparable to previous studies on acute fluorocitrate exposure (Paulsen, 1987; Ronald Zielke, 2007; Zielke, 2009). We also demonstrated that fluorocitrate did not have a parallel overt effect on the relative amount or shape of the surrounding neuronal or microglial cell populations, consistent with previous findings showing activation of microglial cells related to astrocytic stress only following chronic fluorocitrate treatment (Kuter, 2018).

Furthermore, our results clearly indicate that when such an acute and selective metabolic silencing of astrocytes is applied to the deprived visual cortex of ME mice it is capable of impeding long-term neuronal recovery, confirming that astrocytes are responsive at the time of ME induction and act as an integral component in the plasticity process underlying the regain of cortical functionality.

Gliotransmission enables astrocyte-neuron communication at the level of the tripartite synapse. Astrocytes have the capacity to release a wide variety of molecules responsible for maintaining neuronal function and survival. Cortical astrocytes can, for instance, enhance excitatory synaptic transmission by releasing gliotransmitters such as glutamate and D-serine (Fossat, 2011; Navarrete, 2012). In addition, they supply surrounding neurons with the necessary energy and metabolic substrates for neuronal biosynthesis (Weber, 2015). Thus, by incapacitating astrocytes at ME onset, neurons will be stripped of critical building blocks to regain their function.

The initial brain state, determined by the excitation-inhibition balance, at the time of deprivation, can have long-lasting effects on functional recovery. Enhancement of inhibitory neurotransmission prior to ME, through either dark exposure or optogenetic activation of somatostatin interneurons, results in reduced functional recovery of the monocular region of the deprived visual cortex (Nys, 2015; Scheyltjens, 2018). Interfering with the natural astrocytic response to ME results in a similar phenotype. Such an early astrocyte response appears to be a necessary step in establishing a plastic state that allows full recovery of neuronal activity.

The DREADD technique allows time-dependent activation of visual cortex astrocytes (Armbruster, 2007). Durkee et al. recently revealed that both G_q GPCR and G_i GPCR activation leads to the cellular activation of astrocytes. Furthermore they showed, in acute hippocampal slices, that astrocyte activation through G_q and G_i signaling induces glutamate release, able to enhance neuronal excitability (Durkee, 2019). In our hands, acute CNO administration also activated both G_q - (hM3Dq) and G_i - (hM4Di) expressing astrocytes in the visual cortex, based on CNO-driven Fos expression in DREADD transduced astrocytes. In the ME model, long-term CNO-based cortical astrocyte activation through G_q GPCR activation resulted in a diminished local neuronal reactivation

compared to the control condition. G_q GPCR activation of hippocampal astrocytes induces the release of glutamate and D-serine which activates neuronal NMDA receptors leading to a heightened neuronal excitability (Adamsky, 2018; Durkee, 2019). We therefore expected that enhancing G_q signaling in cortical astrocytes would lead to a faster functional recovery of the deprived neurons. However, we must take into account the fact that the astrocyte population is highly heterogeneous between brain areas. Different subtypes of astrocytes exist across different areas all showing distinct functional, morphological and molecular profiles (Batiuk, 2019; Bayraktar, 2019; Clarke, 2018; Xin, 2018; Zhang, 2010). By activating G_q GPCR in visual cortex astrocytes we are thus potentially manipulating a molecularly different type of cell, leading to different effects on neuronal function compared to hippocampus. Furthermore, in our case, the G_q GPCR activated astrocytes find themselves in an environment of altered, sensory-deprived neurons which could further diversify their molecular composition compared to normal physiological conditions. In a model of addiction, glutamate release through G_q GPCR activation of astrocytes is, for instance, directed to the pre-synaptic mGluR2/3 receptor that, upon activation, prevents synaptic neurotransmitter release (Scofield, 2015). Likewise, visual deprivation also modifies the molecular architecture of synapses, by affecting glutamate release probability and composition of NMDA receptors, leading to enhanced NMDA-signaling, specifically in the supra-granular layers of the visual cortex (Yashiro, 2005). Glutamate, potentially released through G_q activation of astrocytes in the ME model, might thus be acting on glutamate receptors that block presynaptic release, such as mGluR2/3, leading to lowered neuronal excitability in the supra-granular layers of the visual cortex.

On the other hand, G_i GPCR activation of visual cortex astrocytes in the ME model had a positive influence on the plasticity process, generating a boost in neuronal reactivation in comparison to what happens in control or G_q DREADD ME mice. Brief stimulation of cortical astrocytes with GABA, which also induces G_i GPCR signaling, leads to long-lasting calcium oscillations in astrocytes, able to enhance synchronization of activity between pyramidal neurons (Mariotti, 2016). Stimulating plasticity with G_i GPCR astrocyte activation might thus have a longer lasting impact on surrounding neurons, which

confirms the potential of this pathway to positively influence long-lasting plasticity processes.

More recently, RNAseq of G_i GPCR activated striatal astrocytes revealed that stimulating this signaling pathway leads to the activation of thrombospondin 1 (TSP1), a latent synaptogenic cue in adult mice, and also important for intracortical synapse formation. As a result, this induced an increase in excitatory synapse formation, synaptic function and neuronal firing in mouse striatum (Nagai, 2019). Astrocyte-induced synaptogenesis is predominantly present in the developing mouse brain. Through the secretion of synaptogenic factors, such as TSP1, Hevin and TGF- β 1, that interact with specific neuronal receptors and adhesion molecules, astrocytes are involved in the formation and maturation of excitatory synapses (Baldwin, 2017; Singh, 2016; Stogsdill, 2017). In the adult brain, astrocytic expression of some of these factors is reduced, making mature astrocytes less synaptogenic (Christopherson, 2005). However, after brain injury or specific activation, such as with the G_i DREADD, mature astrocytes may reinstate their immature synaptogenic state to aid in neuronal recovery (Liauw, 2008; Nagai, 2019; Teng-nan, 2003). By stimulating G_i GPCR signaling in visual cortex astrocytes during the plastic recovery upon ME, we might thus have induced a reversal of local astrocytes into a more immature phenotype capable of inducing new excitatory synapse formation, responsible for the observed boost in neuronal reactivation. This interpretation appears in line with previous research on adult cat visual cortex showing that transplantation of immature astrocytes reintroduces critical period-like ocular dominance plasticity (Müller, 1989). More recently, Blanco-Suarez et al. showed that manipulating astrocytic synapse-modifying signals, by knocking out the chordin like 1 gene, is also capable of boosting visual cortex plasticity (Blanco-Suarez, 2018).

Functional manipulation of astrocytes in the ME model seems to mainly affect the supragranular layers of the deprived visual cortex, even though infragranular astrocytes are clearly also transduced. These results thus suggest that upper layer astrocytes are more capable of affecting the plasticity outcome. Previous research on astrocyte heterogeneity in the cortex also states that upper layer astrocytes are morphologically and molecularly

687 better equipped to modify excitatory synapse transmission and formation (Bayraktar,
688 2019; Lanjakornsiripan, 2018).

689
690 Finally, our observations of astrocyte contributions to experience-dependent brain
691 plasticity may be extrapolated to cortical reorganization processes observed after stroke
692 or traumatic brain injury, as in these cases astrocyte-mediated synaptic plasticity also
693 underlies functional recovery (Kim, 2018; Nikolakopoulou, 2016). Metabolic inhibition of
694 astrocytes, as in our study, worsens the behavioral outcome in a mouse model of stroke
695 (Hayakawa, 2010). Therefore, we propose G_i GPCR activation of astrocytes as a potential
696 therapeutic strategy to promote recovery of neuronal function not only after sensory
697 deprivation but also after direct cortical injury.

698
699 To conclude, our results show that astrocytes are central players in the plasticity-induced
700 functional reactivation of sensory-deprived visual cortex in the ME model. Furthermore,
701 stimulation of G_i signaling in cortical astrocytes actually boosted the neuronal reactivation
702 process, possibly by reverting astrocyte to a more immature and synaptogenic state. We
703 have, therefore, identified G_i GPCR activation of astrocytes as an interesting therapeutic
704 pathway to reopen plasticity windows in the adult mammalian cortex. Furthermore, this
705 study adds to the growing knowledge on astrocytes as neuronal regulators and mediators
706 of cortical plasticity.

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Figure legends

Figure 1. Illustration of experimental setup.

a. Overview illustrating the timeline of stereotactic injections and manipulations of all monocular enucleation (ME)-related experiments. All mice had normal vision until the age of P120 when ME was performed and the animals were left to recover for 7 weeks (7wME). For the fluorocitrate experiment, mice received a stereotactic injection of fluorocitrate or saline in V1 at P120 (arrowhead timeline), prior to ME. For the chemogenetics experiment, mice were stereotactically injected with the viral vector constructs in V1 at P105 (arrowhead timeline). Animals in the mCherry, hM3Dq-mCherry and hM4Di-mCherry group received daily injections of 5 mg/kg CNO for 7 weeks. **b.** Illustration of the interareal borders delineating the different visual cortex regions based on Nissl staining, and overlaid on the matching *zif268 in situ* hybridization pattern for sections of interest of each individual mouse. Delineated visual cortex regions from lateral to medial: V2L, V1, and V2M with the distinction between monocular (m) and binocular (b) segments. The binocular zone (Bz) consists of the V2Lb and the V1b area while the monocular zone (Mz) contains the V1m and the V2M area. Cortical layers are indicated with Roman numbers: I–VI. **c.** Strategy of segmentation of cortical *zif268*–mRNA labeling for OD calculation of the different visual cortex regions in the supra- and infragranular layers to create line graph representations and allow statistical analysis concerning regional changes in neuronal activity. Illustration of the transformation of OD values to a grayscale heat map used for top view representation of the spatial *zif268* expression pattern indicative of the neuronal reactivation. **d.** Supra- and infragranular area masks of

the primary (V1) and extrastriate visual regions (POR: Postrhinal area; LI: Laterointermediate area; LM: Lateromedial area; LLA: Laterolateral anterior area; AL: Anterolateral area; RL: Rostrolateral area; A: Anterior area; AM: Anteromedial area; PM: Posteromedial area) delineating the monocular (white) and the binocular (gray) zones. Red arrows indicate the direction of binocular zone expansion and monocular zone reactivation during the plasticity recovery phases following enucleation. Axis units in mm from midline (x-axis) and bregma (y-axis).

Figure 2. Time-dependent effect of monocular enucleation on the density and layer distribution pattern of Aldh1l1-positive astrocytes in the monocular and the binocular zone of the deprived visual cortex.

a. Representative images showing Aldh1l1-positive astrocytes across all layers of the visual cortex in control normal sighted (left) and 3-day enucleated mice (3dME) (right). Scale bar: 50 μm . **b.** High magnification picture showing clear protoplasmic astrocyte labeling based on typical star-shaped cellular morphology in control normal sighted (top) and 3dME mice (bottom). Scale bar: 10 μm . **c.** Astrocyte density in the monocular zone at different times post-enucleation represented as astrocytes/ mm^2 . One-way ANOVA, Bonferroni post hoc tests, *** p-value<0,001. **d.** Astrocyte layer distribution pattern in the monocular zone for all analyzed conditions. Two-way ANOVA, Bonferroni post hoc tests. **e.** Astrocyte density in the binocular zone at different times post-enucleation represented as astrocytes/ mm^2 . One-way ANOVA, Bonferroni post hoc tests, * p-value<0,05; ** p-value<0,01; *** p-value<0,001. **f.** Astrocyte layer distribution pattern in the binocular zone for all analyzed conditions. Two-way ANOVA, Bonferroni post hoc tests, * p-value<0,05; *** p-value<0,001. (n=6 for all conditions). 3dME, 3 days post-enucleation; 3wME, 3 weeks post-enucleation; 7wME, 7 weeks post-enucleation.

Figure 3. Fluorocitrate injection leads to a localized, selective and reversible silencing of visual cortex astrocytes. a. Intracranial injection of fluorocitrate leads to a local drop in Aldh1l1-positive astrocytes at one day post-injection in V1. White rectangle:

injection site, Blue: DAPI, Red: Aldh1l1. **b.** Fluorocitrate does not affect the surrounding microglia (TMEM119) or neurons (NeuN) at one day post-injection Blue: DAPI, Green: NeuN, Red: TMEM119. (a, b illustrate data from adjacent sections). **c.** Three days post-injection, the effect of fluorocitrate is largely reversed as Aldh1l1- and GFAP-positive astrocytes are again present at the injection site. Blue: DAPI, Green: GFAP, Red: Aldh1l1. Scale bar: 500 μ m.

Figure 4. Analysis of the long-term effect of a single fluorocitrate injection on neuronal recovery after monocular enucleation. **a.** Top view representations of the spatial neuronal reactivation profile based on *zif268* expression levels in 7-week enucleated (7wME), fluorocitrate treated or saline injected mice. Left: top view supragranular layers, Right: top view infragranular layers, *: injection site. **b.** Representative images of *zif268*-mRNA labeled coronal sections surrounding bregma level -3.50 mm and corresponding pseudocolor representations. Calibration bar: 0 (high) -255 (low), Scale bar: 1 mm. **c.** Line graph representations of the relative *zif268* expression measured as the averaged OD value per segment from lateral to medial for saline (black) and fluorocitrate (blue) injected 7wME mice. *Zif268* expression levels in the supragranular layers II/III and IV (left) and the infragranular layers V and VI (right) are displayed for the monocular (V1m, V2M) and binocular zone (V2Lb, V1b) (separated by dotted vertical lines, in register with black arrowheads in b). Error bars represent SEM. **d.** Comparison of averaged *zif268* OD values for the binocular, monocular and V1 region of the deprived visual cortex in fluorocitrate (blue) or saline (black) injected 7wME mice, separately analyzed for the supra- and infragranular layers. Mann-Whitney U test, * p-value<0,05, n=6 for all conditions.

Figure 5. Astrocyte specific expression and functional validation of the DREADD constructs. **a.** Coronal sections illustrating co-labeling between mCherry (GFABC1D-AAV2.5 viral vector) with astrocyte specific cytoplasmic (Aldh1l1, GFAP) and plasma membrane markers (Connexin43, Kir4.1) in V1. Blue: DAPI, Red: mCherry reporter gene, Green: cellular marker. **b.** No co-labeling was observed between the mCherry and

neuronal (NeuN), interneuronal (Parvalbumin), microglial (TMEM119) or oligodendrocytic (Olig2) cellular markers. Blue: DAPI, Red: mCherry reporter gene, Green: cellular marker.

c. Acute CNO administration leads to induction of Fos expression in hM4Di- and hM3Dq-expressing astrocytes but not in control (mCherry –expressing) astrocytes. White arrows: Fos expressing astrocytes, Blue: Fos, Red: mCherry reporter gene, Green: GFAP. Scale bar: 50 μ m.

Figure 6. Comparison of the neuronal reactivation patterns after long-term chemogenetic astrocyte activation following monocular enucleation.

a. Top view representations of the spatial neuronal reactivation pattern based on *zif268* expression levels in hM3Dq-mCherry, mCherry and hM4Di-mCherry expressing mice after 7 weeks of daily CNO injections following ME. Left: top view supragranular layers, Right: top view infragranular layers, *: injection site. **b.** Representative images of *zif268* - mRNA coronal sections surrounding bregma -3.50 mm with their corresponding pseudocolor representation and mCherry expression confirming V1 vector localization. Calibration bar: 0 (high) – 255 (low). Scale bar: 1 mm, 100 μ m (detail mCherry expression)

c. Line graph representations of the relative *zif268* expression measured as the averaged OD value per segment from lateral to medial for hM3Dq-mCherry (green), mCherry (black) and hM4Di-mCherry (red)-expressing mice. *Zif268* expression levels of the supragranular layers II/III and IV (left) and the infragranular layers V and VI (right) are displayed for the monocular (V1m,V2M) and binocular zone (V2Lb, V1b) (separated by dotted vertical lines, in register with black arrowheads in b). Error bars represent SEM. **d.** Comparison of the averaged *zif268* OD values in the measured regions for hM3Dq-mCherry (green), mCherry (black) and hM4Di-mCherry (red)-expressing mice. Only bar graphs showing significant differences are presented. Mann-Whitney U test, * p-value<0,05, ** p-value<0,01, n=7 for all conditions.

Table of contents image

Page 39 of 63	Primary Antibody	Company	Dilution
	rabbit anti-Aldh1l1	AB87117, Abcam (Cambridge, UK)	1:200
	rabbit anti-GFAP	PA5-16291, Thermofisher Scientific (Rockford, USA)	1:500
	mouse anti-GFAP	MA5-12023, Thermofisher Scientific (Rockford, USA)	1:500
	mouse anti-NeuN	MAB377, Milipore Corp (CA, USA)	1:200
	rabbit anti-TMEM119	AB209064, Abcam (Cambridge, UK)	1:500
	rabbit anti-Connexin43	71-0700, Thermofisher Scientific (Rockford, USA)	1:500
	rabbit anti-Kir4.1	APC035, Alomone Labs (Jerusalem, Israel)	1:500
	mouse anti-Parvalbumin	PV235, Swant (Marly, Switzerland)	1:10 000
	rabbit anti-Olig2	AB109186, Abcam (Cambridge, UK)	1:500
	mouse anti-RFP	200-301-379, Tebu-Bio (Boechout, Belgium)	1:2000
	rabbit anti-RFP	600-401-379, Tebu-Bio (Boehout, Belgium)□	1:2000
	RFP Booster	RBA594-100, ChromoTek (Planegg-Martinsried, Germany)	1:500
	rabbit anti-Fos	In house made	1:5000
	Secondary Antibody	Company	Dilution
	Goat anti-rabbit Alexa Fluor 488	AB150085, Abcam (Cambridge, UK)	1:250
	Goat anti-rabbit Alexa Fluor 594	AB150088, Abcam (Cambridge, UK)	1:250
	Goat anti-mouse Alexa Fluor 488	AB150117, Abcam (Cambridge, UK)	1:250
	Goat anti-mouse Alexa Fluor 594	AB150120, Abcam (Cambridge, UK)	1:250
	Goat anti-mouse Cy5	AB6563, Abcam (Cambridge, UK)	1:250

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Supplementary figure legends

Supplementary figure 1. Neuronal and microglial cells are not affected by fluorocitrate at one day post-injection. No differences in neuronal or microglial cell shape or distribution can be observed one day post-injection between saline- **(a)** or fluorocitrate- **(b)** injected mice. Red: TMEM119, Blue: DAPI, Green: NeuN. Scale bar: 50 μ m, 10 μ m.

Supplementary figure 2. Cortical saline injection does not suppress the surrounding Aldh1l1-positive astrocyte population. **a.** At one day post-injection Aldh1l1-positive astrocytes remain detectable at the injection site. White rectangle: injection site, Blue: DAPI, Red: Aldh1l1. **b.** GFAP-positive astrocytes are detectable in the visual cortex three days post-injection of saline as a result of the intracranial injection. Blue: DAPI, Red: Aldh1l1, Green: GFAP. Scale bar: 500 μ m.

Supplementary figure 3. Monocular enucleation is not inducing neuronal degeneration or reactive astrogliosis. **a.** Overview of the neuronal and reactive astrocyte population in visual cortex of control normal sighted P120 mouse. Red: NeuN, Green: GFAP. **b.** Three days post ME, the neuronal cell distribution remains intact and no reactive astrocytes are observed in the visual cortex. Red: NeuN, Green: GFAP. Scale bar: 500 μ m.

Leuven, February, 2020

Cover letter Revision

Dear editor,

Thank you for the opportunity to revise our manuscript: "Astrocytes shape the plastic response of adult cortical neurons to vision loss" - ID GLIA-00394-2019. We appreciate the thorough review and constructive suggestions that substantially improved our manuscript.

To meet the editors requests, we have included Main points to our manuscript as well as a summary graphic for the online table of contents. The manuscript was carefully proof-read and figures were adapted to match size requirements.

Following this letter you can find the reviewers comments marked in blue and our responses marked in black with reference to how and where the manuscript was edited. In our opinion, we have provided the reviewers with answers and additional figures to meet their requests.

We want to thank you and the Editorial board for your time and consideration.

Kind regards,

Lutgarde Arckens

Head Laboratory of Neuroplasticity and Neuropoteomics
Division of Animal Physiology and Neurobiology, Department of Biology
KU Leuven
Belgium

12-Dec-2019

Dear Miss Hennes:

Manuscript ID GLIA-00394-2019 entitled "Astrocytes shape the plastic response of adult cortical neurons to vision loss" has been reviewed. The comments of the reviewer(s) are included at the end of this letter.

There is interest in your manuscript, but as you can see from the reviews (below), substantial revisions are recommended and the paper must undergo further peer review. Therefore, I invite you to revise your manuscript, taking into account the suggestions made by the reviewers.

Before you resubmit your paper, please carefully proof-read the manuscript to minimize typographical, grammatical, and bibliographical errors. In addition, check to make sure that all abbreviations are defined.

In addition, please read the author guidelines carefully: <https://onlinelibrary.wiley.com/page/journal/10981136/homepage/forauthors.html>

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Once again, thank you for submitting your manuscript to GLIA and I look forward to receiving your revision.

Sincerely,
Prof. Helmut Kettenmann
Editor-in-Chief, GLIA

Point-by-point reply to the comments of the reviewers:

Reviewer: 1

Comments to the Author

This manuscript by Hennes et al. raises the interesting research question, if and how astrocytes mediate processes of neuronal plasticity in the deprived visual cortex after monocular enucleation (ME). The authors observed ME-induced upregulation in the density of Aldh1l1-positive astrocytes up to 7 weeks after the injury. To test, whether this increased density of astrocytes affects the functional reorganization processes, they intracranially applied fluororocitrate at the time of ME to metabolically silence this type of glial cells in the visual cortex. By analyzing the expression pattern of the neuronal activity marker Zif-286 through in situ hybridization experiments they showed that metabolic activity of astrocytes is required to observe the previously observed neuronal hyperactivity as measured by an increased Zif-286 expression. Using a chemogenetic approach the authors further showed that a chronic stimulation of the Gi- but not of Gq GPCR in astrocytes can boost cortical reorganization. The authors conclude that astrocytes play an important role for functional reorganization of the visual cortex after ME via Gi-signaling, and they suggest this mechanism as an interesting therapeutic pathway for brain plasticity and repair.

The manuscript is carefully written, methodically clean and the presented experiments are convincing. Since a peripheral, unilateral injury forces the visual system to perform its sensory tasks in an altered mode of using one-instead of two-eyes, the present ME model

is ideal to study the role of astrocytes for processes of functional recruitment and reorganization in the visual cortex. The results are new with respect to the visual cortex and scientifically very interesting.

Minor comments:

1. One minor comment refers to Fig. 3. I had some difficulties to understand the details of the immunohistochemical pictures in Fig. 3a, b and c. Normally, the antibody overlap "merged" photograph is shown on the right of each row as the summary picture of the single antibody data shown in the other pictures on the left of the same row (like in Fig. 3a.). However, in Fig. 3b,c it is presented here rather mixed and confusing. I suggest to redo the figure and always present the "merged" data on the right of each row.

We apologize for the confusion and adapted the figure in line with the reviewer's comment. For completeness we also added the new figure 3 and legend below.

Figure 3. Fluorocitrate injection leads to a localized, selective and reversible silencing of visual cortex astrocytes. **a.** Intracranial injection of fluorocitrate leads to a local drop in Aldh1l1-positive astrocytes at one day post-injection in V1. White rectangle: injection site, Blue: DAPI, Red: Aldh1l1. **b.** Fluorocitrate is not affecting the surrounding microglia (TMEM119) or neurons (NeuN) or microglia at one day post-injection Blue: DAPI, Green: NeuN, Red: TMEM119. (a, b illustrate data from adjacent sections). Blue: DAPI, Green: NeuN, Red: TMEM119. **c.** Three days post-injection, the effect of fluorocitrate is largely reversed as Aldh1l1- and GFAP-positive astrocytes are again present at the injection site. Blue: DAPI, Green: GFAP, Red: Aldh1l1. Scale bar: 500 μ m.

2. The presented data on the role of astrocytes for neuronal functions in the peripheral ME-model are also relevant in the context of direct injuries of the neocortex. The authors should add a paragraph to discuss their own findings of astrocyte mediated neuronal hyperactivity after ME with respect to functional reorganization processes after stroke and/or traumatic brain injury.

We would like to thank reviewer 1 for this nice suggestion. Findings about cortical plasticity induced by the ME-model can indeed be relevant in the case of stroke and traumatic brain injury (TBI) as astrocytes have been shown to be involved in the functional recovery processes after brain trauma. We have included a paragraph in the discussion, right before our conclusion, in which we explain how our results are related to findings in stroke and TBI and also how our G_i GPCR astrocyte activation approach could also be beneficial for boosting neuronal recovery in these conditions.
(Line 686 – 693)

Reviewer: 2

Comments to the Author

This manuscript reports upregulation in astrocytic density upon visual deprivation in the deprived visual cortex. “Metabolically silencing” astrocytes led to a decreased neuronal reactivation and recovery. Similarly, DREADD activation with G_i (and not of G_q) GPCR of astrocytes led to an amplification of cortical plasticity and recovery. While I appreciate the idea and background of the study, there are a number of issues with the work.

1. The difference between G_i and G_q GPCR activation is unexpected, yet interesting and calls for further experiments. The authors hypothesize the involvement of possible downstream signaling pathways, which they should pharmacologically probe to make this more than just an idea.

We would like to thank reviewer 2 for the thorough analysis of our manuscript and for providing us with valid comments. We fully agree that the difference between G_i and G_q GPCR activation in the context of ME-plasticity is intriguing and already considered to attempt to pharmacologically phenocopy the Gi-GPCR outcome. However, revealing how these differences arise in the ME model turned out near impossible for several reasons. Even though we mention in the manuscript which pharmacological targets are of interest based on previous research (see also below for related preliminary data), we still remain quite uninformed about what pharmacological validation to perform, and in addition are also faced with a number of technical limitations specifically arising from chronic pharmacological manipulation (7-week time frame).

Technical limitations: Astrocytes and neurons possess many of the same receptors for which we lack drugs to specifically target the astrocytes, potentially leading to off-target effects. Our DREADD approach leads to very local effects whereas a pharmacological

strategy would induce brain-wide effects. Considering that the neuronal reactivation in the ME model is partly mediated by cross-modal inputs, a brain-wide approach could therefore also impact neuronal activity in the regions surrounding the visual cortex, making interpretation of the results very complex. Another issue that complicates using pharmacology in the ME model is the duration of the recovery period. As the drugs would have to be given for 7 weeks, there is a high chance of inducing desensitization of the target receptor, which would again prevent us from correctly analyzing our data.

Preliminary data:

We already attempted to pharmacologically phenocopy our DREADD effect on neuronal recovery. As previously described in Durkee et al., G_i and G_q GCPR activation was shown to induce glutamate release from hippocampal astrocytes in acute slices (Durkee, 2019). We thus hypothesized that G_i and G_q GPCR activation of cortical astrocytes might be influencing plasticity processes by increasing extracellular glutamate levels. We therefore performed a chronic (7 weeks) pharmacological manipulation of the astrocyte-specific glutamate reuptake transporter GLT-1 through systemic delivery of dihydrokainate (DHK) antagonist with Alzet osmotic minipumps in ME mice. We have included the typical line graph analysis of the *zif268*-expression levels of DHK and vehicle (PBS) 7wME mice in which no statistically significant drug effect could be observed (Revision Figure 1). We decided not to include the DHK data in the manuscript since the lack of an effect might be attributed to a number of technical factors as explained above.

Revision Figure 1. Long-term systemic delivery of dihydrokainate (DHK) does not influence neuronal recovery after monocular enucleation. Line graph representations of the relative *zif268* expression levels measured as the averaged OD value per segment from lateral to medial for 7wME that received long-term systemic delivery of PBS (black) and DHK (purple) through osmotic minipumps. *Zif268* expression levels in the supragranular layers (II/III) and IV (left) and the infragranular layers V and VI (right) are displayed for the monocular (V1m, V2M) and binocular zone (V2Lb, V1b) of the visual cortex. Error bar represent SEM. PBS: n=4, DHK: n=5.

In our discussion we also hypothesize about the possibility of reverting astrocytes, through G_i signaling, towards a more synaptogenic phenotype capable of promoting

plasticity. As an example we mention that in striatal astrocytes G_i GPCR activation has been found to promote the release of thrombospondin-1 (TSP1), capable of enhancing excitatory synapse formation (Nagai, 2019). However, this study was focused on striatal astrocytes, which are anatomically, molecularly and functionally different from cortical astrocytes (Matias, 2019). G_i GPCR manipulation has also been shown to elicit a different response in astrocytes of different brain regions (Chai, 2017). Even though we thus hypothesize that G_i GPCR activated cortical astrocytes become more synaptogenic, it may well be through another molecular strategy.

If we would still attempt to pharmacologically test the TSP1 involvement, we would choose to counteract the DREADD-induced TSP1 release with gabapentin (TSP1 receptor blocker) injections. Based on gabapentin's half-life (6 hours) we would have to administer the drug on a daily basis during the full 7 week recovery period (Kim, 2016). However, Yang (2016) showed that insensitivity to gabapentin starts from the second week of administration. In addition, gabapentin also affects GABA and glutamate levels in the cortex, thereby influencing plasticity, again preventing us from making any conclusions about the mode of action of G_i DREADD on the plasticity outcome (Cai, 2012; Cunningham, 2004).

In conclusion, based on the existing information and pharmacological tools we are not capable of elucidating G_i and G_q GPCR differences in the ME model, at this time and thus apologize to the reviewer for not being able to provide him with the requested information and hope our explanation as to why is satisfying.

2. Methods: After washing with PBS, the sections were blocked for 60 min with normal goat serum (Merck Millipore, Overijse, Belgium) and overnight incubated with the primary antibodies diluted in Tris-NaCl blocking buffer (TNB). Please add the temperature at which the sections were incubated.

Thank you for pointing this out, the sections were incubated at room temperature. We have now corrected this in the manuscript. (Line 240)

3. Figures 2B: Unclear which one is ME and which one ctrl.

As figure 2B was only intended to indicate that our Aldh1l1 immunostaining is clearly labeling protoplasmic astrocytes, based on their typical star-shaped morphology, both pictures were of control animals. We appreciate the comment/suggestion and have adapted this figure to show one control (top picture) and one ME (bottom picture) example of Aldh1l1+ astrocytes. We adapted the figure legend accordingly.

Figure 2. Time-dependent effect of monocular enucleation on the density and layer distribution pattern of Aldh1l1-positive astrocytes in the monocular and the binocular zone of the deprived visual cortex.

a. Representative images showing Aldh1l1-positive astrocytes across all layers of the visual cortex in control normal sighted (left) and 3-day enucleated mice (3dME) (right). Scale bar: 50 μm . **b.** High magnification picture showing clear protoplasmic astrocyte labeling based on typical star-shaped cellular morphology in control normal sighted (top picture) and 3dME mice (bottom picture). Scale bar: 10 μm . **c.** Astrocyte density in the monocular zone at different times post-enucleation represented as astrocytes/ mm^2 . One-way ANOVA, Bonferroni post hoc tests, *** p-value<0,001. **d.** Astrocyte layer distribution pattern in the monocular zone for all analyzed conditions. Two-way ANOVA, Bonferroni post hoc tests. **e.** Astrocyte density in the binocular zone at different times post-enucleation represented as astrocytes/ mm^2 . One-way ANOVA, Bonferroni post hoc tests, * p-value<0,05; ** p-value<0,01; *** p-value<0,001. **f.** Astrocyte layer distribution pattern in the binocular zone for all analyzed conditions. Two-way ANOVA, Bonferroni post hoc tests, * p-value<0,05; *** p-value<0,001. (n=6 for all conditions). 3dME, 3 days post-enucleation; 3wME, 3 weeks post-enucleation; 7wME, 7 weeks post-enucleation.

4. “Metabolically silencing” astrocytes with the injections of fluorocitrate is not a well-established approach and seems unspecific. The authors need to take a step back with the interpretation of these data. There can be countless effects of this intervention.

We understand the reviewer’s concern that using a gliotoxin to silence astrocytes might be unspecific. Several recent publications exist in which the fluorocitrate approach was also used for *in vivo* silencing of astrocytes (Ishihara, 2019; Sobrinho, 2017; Wang, 2018; Zhang, 2019). We are not aware of any other/better method currently available that can be implemented to acutely induce a widespread inhibition of astrocytes *in vivo*.

The specificity of fluorocitrate for astrocytes stems from its preferential uptake into astrocytes, similar to exogenous citrate, where it inhibits aconitase and thereby blocks the TCA cycle (Fonnum, 1997; Hassel, 1992; Paulsen, 1988; Westergaard, 1994). In the context of too high a dose of fluorocitrate, the possibility exists that it could be taken up by other cells leading to off target effects. We therefore used existing literature to choose an optimal dose that elicits inhibitory effects only on astrocytes, and did tests with different concentrations in order to obtain a concentration that induces an extensive silencing of astrocytes whilst not affecting surrounding neuronal and glial cells, as illustrated in figure 3 of the original manuscript.

Prolonged administration can also lead to off target effects: chronic fluorocitrate infusion will lead to astrocyte death and, as a consequence, reactive microglia are observed surrounding the injection site (Kuter, 2018). Even though our data as well as current literature shows fluorocitrate to be highly specific for astrocytes, we acknowledge the possibility that at a later time point, as a secondary response, microglia will be affected. In sum, our fluorocitrate manipulation consisted of one single injection and did not induce a high degree of cell death (Revision Figure 2) reducing the chance for such secondary effects substantially.

We still understand the issue of the reviewer and have adapted the text in order to clarify to the reader that other cell types might still be affected as a secondary response to the astrocytic silencing. (Line 586)

Revision Figure 2. A single fluorocitrate injection does not induce extensive cell death. Only a few apoptotic cells could be observed at the fluorocitrate injection site at one day post-injection. Green: Tunnel assay-labeled apoptotic cells. Scale bar: 200 μm , 50 μm .

5. TMEM119 is not really the optimal antibody to monitor changes in microglia. While Iba1 shows an increase in immunoreactivity after LPS injection, TMEM119 does not show gross changes (this is the initial paper characterizing the antibody <https://www.pnas.org/content/113/12/E1738>, Figure3D).

There a no ctrls shown for NeuN and microglia. It is therefore not really possible to claim that neurons and microglia are not affected after fluorocitrate injections. The NeuN antibody was raised in mouse. It shows this increased background at the site of fluorocitrate, indicating that the secondary antibody recognizes some upregulated immunoproteins (possibly due to microgliosis?) in the tissue.

We agree with the reviewer that a TMEM119 staining will label the entire microglial population, making it difficult to separate reactive from non-reactive microglia, and that, in order to study microgliosis, IBA1 is the antigen target of choice. The reason why we opted for TMEM119 is to observe if fluorocitrate induced an apparent effect on the microglial cell density comparable to what we observed for the Aldh111+ astrocyte population (please compare Figure 3a,b – red channel). We therefore chose to use a pan microglial marker as we did for the astrocytes with Aldh111. We reasoned TMEM119 to be a better choice than IBA1 since it specifically labels microglial cells and not blood-derived macrophages (Sato, 2016). Considering that we deliver both fluorocitrate and saline through intracranial injections, the damaged tissue will most probably be infiltrated by macrophages preventing us from correctly interpreting differences in the relative number of microglia should we have used IBA1. The reviewers concern about the anti-mouse NeuN antibody creating background due to immunoproteins is in our opinion also resulting from the damage induced by the stereotactic injections.

To respond to the reviewer’s concern, we have performed an additional IBA1-staining (mouse antibody) on sections of 1-day post-injection, saline- (a) or fluorocitrate- (b) injected animals. We observed an intensive background labeling at the injection site in both conditions, preventing us from analyzing alterations in the microglia population. We also provide additional images from nearby the injection site where the IBA1+ microglia can be clearly distinguished, thereby showing that our protocol for IBA1 immunostaining is working (Revision Figure 3). In addition these images also show that IBA1+ microglia

nearby the injection site have a ramified appearance instead of an amoeboid one, indicating that they are not reactive (Davis, 1994).

Revision Figure 3. IBA1 immunoreactivity in response to saline and fluorocitrate at one-day post injection.

No differences can be observed between IBA1 labeling of saline- (a) or fluorocitrate- (b) injected animals at (left panels) or nearby (right panels) the injection site on the same section. Blue: DAPI, Red: IBA1, Scale bar: 50µm.

6. Figure 3: Please provide high resolution images to be able to judge the condition of the different cell types, comparable to the pictures in figure 5.

We prepared a new figure, including the requested high-resolution images, to allow the reviewer to judge the shape and relative number of the neurons and the microglia in both saline- and fluorocitrate-injected animals at one day post-injection (New Supplementary Figure 1). We are confident that from this figure it is clear that no apparent changes in the neuronal and microglial population can be observed between saline and fluorocitrate-injected mice. We have adapted the main body of the text in accordance. We added the new supplementary figure and its legend to the revised Supplement. (Line 586)

Supplementary figure 1. Neuronal and microglial cell are not affected by fluorocitrate at one day post-injection. No differences in neuronal or microglial cell shape or distribution can be observed one day post-injection between saline- (a) or fluorocitrate- (b) injected mice. Red: TMEM119, Blue: DAPI, Green: NeuN. Scale bar: 50 μ m, 10 μ m

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