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STAT3-mediated astrocyte reactivity associated with brain metastasis contributes to neurovascular dysfunction

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Running title: Reactive astrocytes disrupt cerebrovascular function

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Abstract

Astrocytes are thought to play a pivotal role in coupling neural activity and cerebral blood flow. However, it has been shown that astrocytes undergo morphological changes in response to brain metastasis, switching to a reactive phenotype which has the potential to significantly compromise cerebrovascular function and contribute to the neurological sequelae associated with brain metastasis. Given that signal transducer and activator of transcription 3 (STAT3) is a key regulator of astrocyte reactivity, we aimed here to determine the impact of STAT3-mediated astrocyte reactivity on neurovascular function in brain metastasis. Rat models of brain metastasis and ciliary neurotrophic factor (CNTF) were used to induce astrocyte reactivity. Multimodal imaging, electrophysiology, and immunohistochemistry were performed to determine the relationship between reactive astrocytes and changes in the cerebrovascular response to electrical and physiological stimuli. Subsequently, the STAT3 pathway in astrocytes was inhibited with WP1066 to determine the role of STAT3-mediated astrocyte reactivity, specifically, in brain metastasis. Astrocyte reactivity associated with brain metastases impaired cerebrovascular responses to stimuli at both the cellular and functional level and disrupted astrocyte-endothelial interactions in both animal models and human brain metastasis samples. Inhibition of STAT3-mediated astrocyte reactivity in rats with brain metastases restored cerebrovascular function, as shown by *in vivo* imaging, and limited cerebrovascular changes associated with tumor growth. Together these findings suggest that inhibiting STAT3-mediated astrocyte reactivity may confer significant improvements in neurological outcome for patients with brain metastases and could potentially be tested in other brain tumors.

Significance

Findings demonstrate that selectively targeting STAT3-mediated astrocyte reactivity ameliorates the cerebrovascular dysfunction associated with brain metastasis, providing a potential therapeutic avenue for improved patient outcome.

Introduction

Neurovascular coupling matches local cerebral blood flow (CBF) to neuronal energy use and, thus, ensures normal functioning of the brain(1). Whilst neuronal, astrocyte and pericyte mediated pathways have been implicated in neurovascular coupling to varying degrees(2), the significant contribution of astrocytes remains the most widely recognised(1-3).

Astrocyte processes cover most of the cerebrovascular surface and connect neurons with blood vessels(3-5), playing essential roles in recycling ions and neurotransmitters(3), supplying energy substrates to neurons(6), maintaining endothelial tight junctions to support the blood–brain barrier (BBB)(7), and releasing vasoactive molecules that regulate vascular tone(1). However, in response to disease or injury, astrocytes become reactive, displaying hypertrophy, upregulation of intermediate filament proteins (e.g. GFAP)(8) and metabolic changes(9). The consequences of this astrocyte reactivity, however, on the control of CBF remains unknown(3).

Changes in astrocyte function may be particularly pertinent in brain metastasis, where tumour cells interact closely with blood vessels. Such perivascular growth enables tumour cells to use the brain's vasculature for nutrient supply(10-13), but also disrupts astrocyte-vascular coupling and BBB integrity(12). At the same time, brain metastases trigger astrocyte reactivity from the earliest stages(10-13), and gradually expel astrocyte end-feet to the border of the tumour(14). Notably, signal transducer and activator of transcription 3 (STAT3) mediated astrocyte reactivity, specifically, has been shown to be important in maintaining the vascular co-option profile of brain metastases(15). Consequently, brain metastases may impact on cerebrovascular function not only through mechanical dissociation of astrocyte end-feet from vessels(12), but also through astrocyte reactivity *per se* and, as such, may contribute to the neurological sequelae associated with brain metastases. However, a lack of

suitable animal models and imaging techniques that are able to separate these two processes, to date, have limited our ability to fully understand their relative contributions.

The aim of this study, therefore, was to determine the impact of reactive astrocytes, specifically, on cerebrovascular function using animal models of brain metastasis and ciliary neurotrophic factor (CNTF) induced astrocyte reactivity(16,17), together with human brain metastasis biopsies. The CNTF model induces astrocyte reactivity, as observed in disease, in the absence of any other confounding pathology. Subsequently, the potential of inhibiting STAT3-mediated astrocyte reactivity to maintain vascular function was determined, as a potential adjuvant therapy for brain metastasis. Cortical vascular and neuronal function were measured using a multimodal imaging strategy involving magnetic resonance imaging (MRI), laser speckle contrast imaging (LSCI) and local field potential (LFP) measurements, in combination with post-mortem immunohistochemical analysis.

Materials and methods

All animal experiments were approved by the University of Oxford Clinical Medicine Ethics Review Committee and UK Home Office (Animals [Scientific Procedures] Act 1986), and conducted in accordance with the University of Oxford Policy on the Use of Animals in Scientific Research, the ARRIVE Guidelines and Guidelines for the Welfare and Use of Animals in Cancer Research(18). No overt clinical signs or weight loss were evident, in any cohort, over the experimental period. For *in vivo* experiments, blood gases were within the normal ranges for animals under isoflurane anaesthesia (Supplementary Table I). No effects of WP1066 treatment on blood gases and mean arterial blood pressure were observed (Supplementary Table I).

Metastatic tumour cell line, lentivirus and drug

For the brain metastasis model, an ENU1564 tumour cell line was used (kind gift from Professor G. Stoica, Texas A&M University), which originated from an N-ethyl-N-nitrosourea-induced mammary adenocarcinoma in a female Berlin-Druckrey IX (BD-IX) rat, and is highly metastatic to brain and bone tissues. As a rat cell line, there is no publicly available short tandem repeat (STR) profile for the cells, thus they have not been externally validated. Cells were tested for mycoplasma immediately prior to freezing each stock vial using MycoAlert kit (Lonza, UK). Stock vials were revived, grown for up to one week in DMEM supplemented with 10% FBS and 1% 200mM glutamine, harvested by trypsinisation, washed and then injected, as described previously(19).

For the reactive astrocyte model, lentivirus-mediated gene transfer of ciliary neurotrophic factor (CNTF-Lv) was used to induce sustained production of CNTF by neurons, which is released and then gives rise to stable activation of astrocytes via the STAT3 pathway, as described previously(9,16,17). To this end, self-inactivated lentiviruses encoding either the human CNTF gene or the (control) β -galactosidase gene (LacZ-Lv), under the control of the mouse phosphoglycerate kinase I promoter, were used (9,17). In the CNTF-Lv model, transfected neurons display a normal phenotype in terms of morphology, expression of neuronal proteins and spontaneous electrophysiological activity(9,17).

For STAT3 inhibition, a potent inhibitor that can cross the BBB, WP1066 (Millipore, UK) was injected intraperitoneal daily (3mg/kg) using dimethyl sulfoxide (DMSO) as a vehicle.

Experimental models

All experimental procedures used in this study were approved by the United Kingdom Home Office. Two cohorts of female BD-IX rats (Charles River, France), 6-12 weeks old, were injected intracortically in the whisker barrel somatosensory cortex. One cohort of rats was injected with either 10^3 metastatic ENU1564 cells in PBS (n=8) or PBS alone (n=6). The second cohort of rats was injected with either CNTF-Lv (n=9) or LacZ-Lv (n=7) diluted in PBS at a concentration of 100ng p24/ μ l. The third cohort of rats was injected with 10^3 metastatic ENU1564 cells (n=10). Animals injected with ENU1564 cells were randomly distributed for intraperitoneal injection of either 50 μ l WP1066 in DMSO (3mg/kg, n=5) or 50 μ l DMSO (n=5).

All animals were transcardially perfusion-fixed under terminal anaesthesia at the end of the experiment for histological analysis. For full details please see Supplementary Methods.

Magnetic resonance imaging

All MRI experiments were performed on a horizontal bore 9.4T spectrometer driven by an Agilent DirectDrive™ console (Agilent Technologies, Santa Clara, CA, USA). Animals were anaesthetised with 1-2% isoflurane in 70%N₂/30%O₂ and positioned in a 72mm quadrature volume transmit coil with 4 channels phased array surface receiver (RAPID Biomedical GmbH, Rimpar, Germany). In some animals, a cannula was positioned in the tail vein prior to the MRI experiment for gadolinium-DTPA (Gd-DTPA) injection to identify potential blood-brain barrier breakdown.

Multimodal acquisitions included T₁- and T₂-weighted MRI and arterial spin labelling (ASL) MRI. For technical details please visit the Supplementary Methods section.

Laser Speckle Contrast Imaging (LSCI)

Through the cranial window, superficial (ascending venules and pial vessels) and deep blood vessels (penetrating arterioles and capillary beds) within the cortex were visible (0.5-1mm deep). Animals underwent LSCI on the day following MRI measurements. For full details regarding LSCI set up and surgery see Supplementary data. (20,21).

Local Field Potentials (LFPs)

In a subset of animals, LFPs were recorded to measure neuronal responses to the same electrical stimulation of the whisker pad (see above). In this case, burr holes were drilled over both barrel cortices and an electrode (0.155mm diameter, Teflon insulated platinum; Bilaney Consultants Ltd., Sevenoaks, UK) inserted to a depth of 0.5mm under stereotaxic control, as described previously(22). Electrode signals were recorded using a CED 1902 isolated pre-amplifier and signal processor (Cambridge Electronic Design limited, Cambridge, UK), which was controlled using Spike2.7 data acquisition and analysis package software (Cambridge Electronic Design limited, Cambridge, UK). Electrode signals were acquired with a waveform sampling rate of 10 kHz, 2 μ s per time unit resolution and without notch filtering. Low frequency noise was eliminated from the electrode signal using a 50/60Hz noise eliminator (Hum Bug, Quest scientific, Vancouver, Canada).

MRI data analysis

Signal intensity on T₁ and T₂ weighted images in the ipsilateral and contralateral cortices of animals was quantified using ImageJ software package (NIH, Bethesda, MD, USA)(23). CBF maps were quantified with nonlinear perfusion signal modelling(24) using the BASIL (Bayesian Inference for Arterial Spin Labelling MRI) toolset in FSL version 5.0 (FMRIB

Software Library, Oxford, UK). For more details regarding data analysis please see Supplementary data (24).

LSCI data analysis

LSCI datasets were redefined and down-sampled to 1Hz for data analysis. To quantify the time-course of the CBF response after electrical stimuli, datasets were averaged over 10 trials and regions of interest (ROI) drawn around areas corresponding to the whisker barrel cortex. The CBF response and its associated time-series were extracted from the ROIs in both ipsilateral (left) and contralateral (right) whisker barrel cortices. More information regarding data acquisition can be found in Supplementary data.

LFP data analysis

At the end of the LFP recording period, waveform and sampling times were converted into a MATLAB file using Spike 2.7 software. The data were aligned with the stimulation time-series and binned at 100Hz. LFP responses to whisker pad stimulation were extracted from 0 to 16s after stimulus using in-house MATLAB scripts. To quantify neuronal response magnitudes, amplitude (mV) was calculated from the average response to the first pulse in the stimulation train (over a 50ms period following stimulation onset), as described previously(22).

Immunohistochemistry and immunofluorescence

All animals were transcardially perfusion-fixed under terminal anaesthesia with 0.9% heparinised saline followed by periodate lysine paraformaldehyde (PLP) containing only 0.025% glutaraldehyde (PLP_{light}) at the end of LSCI and LFP measurements. The brains were post-fixed, cryoprotected, embedded in tissue-tek (Sakura Finetek Europe, Zoeterwoude, The

Netherlands) and frozen in isopentane at -40°C . Frozen, $20\mu\text{m}$ thick, serial sections spanning the somatosensory cortex were cut from fixed tissue and mounted on gelatin-coated glass slides. Immunohistochemistry was performed with antibodies against markers of activated astrocytes (1:300, GFAP, Dako, Ely, UK), STAT3 (1:300, STAT3 α , Cell Signaling Technology), neurons (1:500, NeuN, Millipore, Abingdon, UK), blood vessels (1:100, CD31, Abcam, Cambridge, UK), microglia (1:200, OX42 [cd11b/c], Abcam, Cambridge, UK), cyclooxygenase-1 and 2 (1:100, COX-1/2) (Abcam, Cambridge, UK), inducible isoform of nitric oxide synthetase (1:50, iNOS, Abcam Cambridge, UK), nitrotyrosine (1:400, NT, Millipore, Abingdon, UK), alpha-smooth muscle actin (1:100, α -SMA; Abcam Cambridge, UK), β -dystroglycan (1:200, Abcam, Cambridge, UK), Claudin-5 (1:200, Abcam, Cambridge, UK) and cytochrome p450-4A (1:100, Abcam Cambridge, UK), the enzyme responsible for the biosynthesis of 20-hydroxyeicosatetraenoic acid (20-HETE).

Immunofluorescent microscopy was performed to identify reactive astrocytes (GFAP), microglia (OX42), neurons (NeuN), blood vessels (CD31), cyclooxygenase-1 and 2 (COX-1/2), inducible isoform of nitric oxide synthase (iNOS), cytochrome p450-4A, α -SMA and β -dystroglycan and Claudin-5. More details about the performed protocols can be found in Supplementary Methods.

Immunohistochemistry and immunofluorescence analysis

Photomicrographs of each brain section were obtained by scanning each section with Aperio[®] Image Analysis (Leica, Germany), or using a Nikon Microscope (Nikon E800, Japan) coupled to a RoHS camera (RoHS, UK). Subsequently, images were analysed using ImageScope (Leica, Germany) and Qcapture Pro software (Qimaging, UK), respectively.

Immunofluorescent images were acquired using an inverted confocal microscope (LSM-710, Carl Zeiss Microimaging, Jena, Germany) or Leica DM IRBE (Leica, Germany). Detection ranges were set to eliminate crosstalk between fluorophores: 409-485nm for AMCA, 494-553nm for Alexa Fluo 488 and 561-595nm for Cy3/Texas Red. Images were analysed using in ImageJ software package (NIH)(23). For full information regarding the colocalisation studies please see Supplementary data.

Statistical analysis

All data are given as mean \pm SEM. Statistical analysis was performed using GraphPad Prism (GraphPad Software, Inc., La Jolla, CA, USA). Unpaired or paired *t*-tests were used to compare data between ipsi- and contralateral cortices. Unpaired *t*-tests were used to identify differences between DMSO and WP1066 groups. The relationships between ASL-CBF maps and areas of reactive astrocytes were tested by a bivariate correlation analysis, including calculation of the Pearson's correlation coefficient (*r*).

Results

Perivascular growth of focally-induced brain metastases

One week after intracortical injection of ENU1564 cells, 94% of all brain metastases were associated with blood vessels (Figure 1(a-c); $p < 0.001$ compared to non-associated), indicating that focally-induced metastases were predominantly perivascular. Of the blood vessels associated with ENU1564 cells, the majority (74%) were capillaries or small veins ($< 8.5\mu\text{m}$ diameter(25); Figure 1d), rather than arterioles, arteries and large veins ($> 8.5\mu\text{m}$ (25); $p < 0.001$ small vs. large).

Neurovascular coupling is disrupted in areas of brain metastasis

LSCI measurement of CBF responses to electrical stimulation of the whisker pad *in vivo*, revealed a focal region of hyperaemia in the contralateral (normal) cortex during stimulation, but a considerably reduced response in the ipsilateral (metastasis bearing) cortex of ENU1564-injected animals (Figure 1e). Quantitatively, the CBF response was significantly reduced in the ipsilateral *vs.* contralateral cortex (~50%; Figure 1f; $p < 0.05$). Simultaneous LFP recordings of neuronal responses showed no significant differences between ipsi- and contralateral cortices (Figure 1g). No significant differences between ipsi- and contralateral cortices were seen in PBS-injected animals for either CBF or LFP responses (Supplementary Figure S1(a-c)). Immunohistochemically, no differences in the number of NeuN-positive neurons between cortices in either group (excluding metastatic foci) were evident (Supplementary Figure S1d). Together, these findings suggest that neurovascular coupling is disrupted in the presence of brain metastases.

Brain metastases alter BBB-astrocyte morphology and induce reactive astrocytes

Within the metastatic foci, astrocyte end-feet appeared to be displaced from blood vessels owing to the perivascular growth pattern of the metastatic cells (mean displacement = $31.3 \pm 2.4 \mu\text{m}$; Figure 1h (i)). At the same time, a widespread area of reactive astrocytes was evident around the metastatic foci in the ipsilateral cortex (Figure 1h (ii), dotted line, $0.54 \pm 0.11 \text{mm}^2$). Moreover, a significant decrease in co-localisation between β -dystroglycan, GFAP and CD31 was evident throughout the area of reactive astrocytes in ENU1564 animals (Figure 1i, Table I; $p < 0.05$), suggesting detachment of astrocyte end-feet from the vascular endothelium unrelated to the direct mechanical effects of the metastatic cells. No decrease in co-localisation was observed in PBS-injected animals (Figure 1i, Table I).

Despite the apparent dissociation of astrocyte end-feet from the vasculature, BBB integrity remained intact, as determined by post-Gd-DTPA T₁-weighted MRI (Supplementary Figure S1e) and immunohistochemical detection of Claudin-5, a key tight junctional protein in BBB paracellular permeability (Supplementary Figure S1f-g). Moreover, although blood vessels associated with metastatic cells were abnormal in appearance (Supplementary Figure S1h), no change in blood vessel number was evident in either metastatic foci or areas of reactive astrocytes (Supplementary Figure S1i).

CNTF-induced reactive astrocytes

To assess the effect of reactive astrocytes *per se* on neurovascular function, we used a model of CNTF-induced reactive astrocytes(17) (Figure 2a). Six weeks after intracortical injection, an area of reactive astrocytes was observed in CNTF-Lv animals (Figure 2b; 0.46 ± 0.09 mm²), whilst negligible astrocyte reactivity was observed in control LacZ-Lv animals (Figure 2b). Reactive astrocytes induced by CNTF-Lv did not alter BBB integrity, as determined by post-Gd-DTPA T₁-weighted MRI (Figure 2c) and Claudin-5/CD31 (vessel marker) co-localization (Supplementary Figure S2a), neuronal number (Supplementary Figure S2b) or blood vessel numbers (Supplementary Figure S2c).

Neurovascular coupling is disrupted in areas of reactive astrocytes

LSCI measurements in CNTF-Lv injected animals indicated a significant reduction in the amplitude of the CBF response to whisker barrel stimulation in the ipsilateral cortex (~70%) compared to the contralateral cortex (Figure 2(d-e); $p < 0.01$). No significant difference was observed in control LacZ-Lv injected animals (Supplementary Figure S2(d-e)). As for the metastasis model, LFP responses to electrical stimulation were unaltered in both groups of

animals (Figure 2f, Supplementary Figure S2f), supporting the concept that neurovascular coupling is disrupted when astrocytes become reactive.

Reactive astrocytes alter BBB-astrocyte morphology

Again, a significant decrease in co-localisation of β -dystroglycan, GFAP and CD31 was observed (Figure 2g, Table I; $p < 0.05$), which was not seen in LacZ-Lv animals (Figure 2g, Table I). When assessing co-localisation of β -dystroglycan and GFAP alone, no change was found in either ENU1546 or CNTF-Lv models (Table I), indicating that reactive astrocytes exhibit reduced adhesion to the vascular endothelium rather than reduced expression of β -dystroglycan *per se*.

Inhibition of STAT3 reduces astrocyte reactivity in response to brain metastasis

Animals injected with ENU1564 cells and treated with WP1066 (Figure 3a), a selective inhibitor of the JAK/STAT3 pathway mediating astrocyte reactivity(15), showed a significant decrease in the area of astrocyte reactivity compared to vehicle (DMSO)-treated animals for both GFAP (~55%; Figure 3b-c; $p < 0.05$) and STAT3 staining (~77%; Figure 3b-c; $p < 0.05$). However, no significant reduction was evident in the area of metastatic foci (0.63 ± 0.12 vs. $0.55 \pm 0.27 \text{mm}^2$; Figure 3c). In WP1066-treated animals, nuclear STAT3 expression in reactive astrocytes was decreased compared to DMSO-treated animals (Fig 3b).

Inhibition of STAT3 in reactive astrocytes improves cerebrovascular function

As expected, (*cf.* Figure 1f), DMSO-treated ENU1564 animals, showed a significantly reduced (~48%) CBF response to electrical stimulation in the metastasis-bearing cortex

compared to the contralateral cortex (Figure 3d; $p < 0.05$). In contrast, WP1066-treated ENU1564 animals showed a much smaller and non-significant reduction (~26%) in CBF response compared to the contralateral hemisphere (Figure 3e). As above, LFP responses to whisker pad stimulation were unaltered in both groups (Figure 3(f-g)).

Subsequently, we demonstrated that cerebrovascular responses to hypercapnia(26) were significantly reduced in both ENU1564 (~44%) and CNTF-Lv (~40%) models, but not in their respective control groups (PBS and LacZ-Lv; Supplementary Figure S3; $p < 0.001$ and $p < 0.05$, respectively). Again, STAT3-inhibition improved the hypercapnia-evoked vascular responses in ENU1564-injected animals, with no significant difference evident between cortices (Supplementary Figure S3 (a-l)).

Together these findings indicate that inhibition of STAT3-mediated astrocyte reactivity can, at least partially, reverse cerebrovascular dysfunction associated with brain metastases.

Reactive astrocytes reduce basal cerebral blood flow

Measurements of basal CBF using ASL-MRI showed a significant reduction compared to the contralateral cortex of animals injected with either ENU1564 cells or CNTF-Lv (Figure 4(a-d), $p < 0.01$). Notably, the area of reactive astrocytes as a percentage of the somatosensory cortex was much larger than the metastatic foci (40% vs. 3%, respectively), and correlated spatially with the region of reduced CBF for both ENU1564 and CNTF-Lv models (Figure 4e; $p < 0.05$; Pearson's coefficient correlation $r^2 = 0.33$). In contrast, no changes in basal CBF were observed in PBS or LacZ-Lv control groups (Figure 4(f-g)).

STAT3 inhibition with WP1066 largely reversed the reduction in basal CBF observed in ENU1564 animals (Figure 4(h-i)), with a significant difference compared to DMSO-treated

animals ($p < 0.05$). Given that the area of reactive astrocytes showed a much greater reduction after WP1066 treatment (~55%) than the metastatic foci (~12%), we suggest that reactive astrocytes, rather than the presence of metastases *per se*, underlie the reduced basal CBF in metastasis-bearing animals.

STAT3-mediated reactive astrocytes regulate vessel diameter and vasoactive pathways

Large vessels ($> 8.5 \mu\text{m}$) showed significant vasoconstriction in the area of reactive astrocytes within the ipsilateral vs. contralateral cortex, in both ENU1564 and CNTF-Lv models (Figure 5(a-b); $p < 0.05$). Moreover, co-localisation between cytochrome p450-4A, an endothelium-dependent vasoconstrictor(27,28), and α -smooth muscle actin (α SMA), a marker of smooth muscle cells on large blood vessels(29), was significantly greater in ipsilateral vs. contralateral cortex for both models (Figure 5(c-d), Supplementary Table II; $p < 0.05$). No differences were evident in control animals (Figure 5(c-d), Supplementary Table II). No changes in cytochrome p450-4A levels were observed in either astrocytes or microglia (Figure 5(e-f)).

In contrast, a significant increase in the diameters of smaller vessels ($< 8.5 \mu\text{m}$ diameter) in the region of astrocyte reactivity was evident in both ENU1564 and CNTF-Lv animals (Figure 5(g-h)), compared to controls (PBS and LacZ-Lv; Figure 5(g-h); $p < 0.001$). Co-localisation of the vasodilatory molecule iNOS with astrocytes was significantly greater in ENU1564 and CNTF-Lv animals compared to controls (Figure 5i; $p < 0.05$ and $p < 0.01$; respectively), together with increased cortical levels of nitrotyrosine (NT), a marker of nitric oxide(30) (Figure 5j). Similarly, astrocytic levels of COX-1, a key enzyme in the arachidonic acid vasodilatory pathway(1), were significantly greater in ipsilateral vs. contralateral cortex of ENU1564 and CNTF-Lv groups (Figure 5k; $p < 0.05$ and $p < 0.01$, respectively), but not in

controls (PBS and LacZ-Lv; Figure 5k). COX-1 showed greater co-localisation with astrocytes than any other cells (Supplementary Table III), suggesting they are the dominant cell population driving COX-1-mediated vasodilation, whilst both astrocytes and endothelial cells showed the highest levels of iNOS co-localisation. A significantly higher number of OX42-positive pixels was found in the ENU1564 group compared to PBS, CNTF-Lv or LacZ-Lv injected groups (Supplementary Table IV), confirming an inflammatory microglia/macrophages response in presence of brain metastases(31,32), but no contribution of microglia/macrophages in the CNTF-Lv model(9,17).

No changes were observed in the levels of COX-2 in astrocytes, iNOS in endothelial cells, or iNOS/COX-1 in microglia in any of the groups (Supplementary Figure S4 (a-d)), suggesting that vasodilation of small vessels reflects activation of the nitric oxide and arachidonic acid pathways primarily in astrocytes. A significant decrease in both iNOS/GFAP and COX1/GFAP co-localisation was evident in WP1066- vs. DMSO-treated ENU1564 animals (Figure 5l). However, neither iNOS nor COX-1 was altered in microglia, endothelial cells or smooth muscle cells following WP1066 treatment (Supplementary Figure S5 (a-d)). Additionally, expression of iNOS and COX-1 was not altered in microglia (OX42) in the ipsilateral cortices of ENU1564 animals treated with WP1006 (Supplementary Figures S6 (a-c)). These results indicate that STAT3 inhibition is primarily limited to reactive astrocytes within the metastatic microenvironment.

Reactive astrocytes are associated with vasoactive pathways in human brain metastasis

Reactive astrocytes were evident in human brain metastasis biopsies (Oxford Brain Bank: OBB-SH-1088-2013-) primarily in the areas surrounding tumour colonies (Figure 6a). As in the animal models, co-localisation between cytochrome p450-4A and α SMA staining was

seen on blood vessels associated with brain metastases (Figure 6b), and both iNOS and COX-1 staining were present in astrocytes (Figure 6(c-d)). The percentage of α SMA- and cytochrome p450-4A-positive blood vessels was greater around metastatic foci compared to control brain (Supplementary Table II). Significantly greater levels of both iNOS and COX-1 per GFAP-positive pixel, and cytochrome p450-4A per α SMA-positive pixel, were found in the area of reactive astrocytes around metastatic foci compared to non-tumour tissues (Figure 6e; $p < 0.001$ and $p < 0.05$, respectively).

Discussion

In a rat model of brain metastasis, we have demonstrated impairment of cerebrovascular function, disruption of astrocyte-vascular connections and widespread reactive astrocytes. Studies using a model of CNTF-induced astrocyte reactivity, indicated that reactive astrocytes *per se* can compromise cerebrovascular function, whilst inhibition of STAT3-mediated astrocyte reactivity partially reversed the cerebrovascular dysfunction associated with brain metastases. These findings suggest that STAT3-mediated astrocyte reactivity in brain metastasis may contribute to cerebrovascular, and hence neurological, dysfunction in patients.

In glioma, tumour cells displace astrocyte end-feet from blood vessels enabling tumour growth in the perivascular space(12,33). Similarly, we and others have shown that brain metastases grow in this co-optive fashion, particularly during the micrometastatic stages(10,11,34). Here, we show that not only are astrocytes displaced from the vasculature mechanically, but that reactive astrocytes distant from the metastatic foci dissociate from the vasculature. Watkins *et al.*(12) hypothesised that displacement of astrocytes from the vasculature by invading glioma cells prevents vasoactive molecules from reaching

endothelial cells, thus disrupting neurovascular coupling(12). Although this may also be partly the case in brain metastasis, our findings suggest that widespread reactive astrocytes, outwith the spatially restricted metastatic foci, contribute significantly to disruption of vascular function. Critically, a similar compromise of cerebrovascular function was observed in the CNTF-Lv model, in which astrocytes were activated to a reactive phenotype for a prolonged period, whilst neuronal activity was preserved(17). Similarly, CBF responses to hypercapnia were compromised in both models. To date, only two studies have suggested that vasoactive pathways in astrocytes are involved in CBF responses to hypercapnia(35,36). Our data support this concept and suggest that astrocyte reactivity may compromise autoregulation.

One possible explanation for the reduced CBF responses to both neuronal activation and hypercapnia is that vasodilation across the vascular bed leads to reduced vascular reserve(37,38). However, although we found vasodilation in the microvascular network, the arteriolar vessels appeared to be constricted and basal blood flow was reduced, in keeping with restricted arteriolar flow. Moreover, we found more cytochrome p450-4A, the enzyme responsible for the biosynthesis of vasoconstrictor 20-HETE(27,28), on smooth muscle cells at the arterioles. Consequently, we speculate that suppression of CBF responses to stimuli in the presence of reactive astrocytes may reflect either basal upregulation of vasoconstrictory pathways in the arteriolar bed and/or upregulation of enzymes that mediate vasodilation (iNOS and COX-1) downstream of the arterioles and reduced vascular reserve within the microvascular bed. A recent study has suggested that astrocytes regulate CBF at the capillary and arteriole levels through two distinct mechanisms(39), reinforcing the concept that reactive astrocytes exert differential effects on arterioles and capillaries.

Importantly, upregulation of the above vasoactive mediators was mirrored in all human brain metastasis samples studied, typically at the leading edge of the tumour. Recent studies have identified the JAK/STAT3 pathway in reactive astrocytes as a novel target for neurological diseases(40), brain metastasis(15) and glioblastoma(41). Together with significant anti-tumor effects(15), data suggest that inhibition of STAT3 may also reduce the immunosuppressive environment of brain tumours induced by cross-talk between astrocytes and microglia (15,42), implicating this inflammatory microenvironment as a potential target for treatment in brain metastasis(43). Given our data showing that STAT3 inhibition restores impaired cerebrovascular function in brain metastasis, we propose that therapy suppressing astrocyte reactivity may also be effective in reducing metastasis-associated neurocognitive effects and in improving the response of current therapies that are limited by tumour-associated hypoxia and abnormal vasculature (44,45). Notably, reduced blood flow has been linked to the neurological symptoms associated with brain metastases, such as seizures(46-48) and stroke(49), and this could be investigated in ongoing brain tumour clinical trials with STAT3 inhibitors (e.g. NCT01904123).

With regards to limitations of the current study, although we have shown that reactive astrocytes are the dominant cell type producing prostaglandin mediated vasodilation via COX-1 activity, NO-mediated vasodilation is not solely driven by reactive astrocytes. Thus, separating the contributions of different stromal cell populations to the production of vasoactive mediators requires further investigation, potentially using genetically encoded calcium indicators, transgenic techniques or viral constructs, in combination with *in vivo* recording of haemodynamic response. Additionally, only one brain metastasis model was used in this study. Given that ENU model is well-established(19,50-52), rather than using a second brain metastasis model we considered it to be more relevant to use a model of CNTF-induced reactive astrocytes in order to determine the effect of reactive astrocytes specifically,

in the absence of metastases, on neurovascular function. At the same time, the immunohistochemical data obtained from human brain metastasis samples lend further support to our experimental findings. Finally, we did not fully suppress astrocyte reactivity in our models via STAT3 inhibition, suggesting that astrocyte activation may be complex and heterogeneous(53). For example, the NF κ B (nuclear factor kappa-light-chain-enhancer of activated B cells) signalling pathway has been shown to contribute to the neurotoxicity of a subpopulation of reactive astrocytes induced by activated neuroinflammatory microglia(54) and, thus, may be an important additional pathway of astrocyte activation in brain metastasis that warrants further investigation.

In summary, this study is the first empirical demonstration that STAT3-mediated astrocyte reactivity associated with brain metastasis contributes to acute cerebrovascular dysfunction. STAT3 inhibition may, therefore, limit detrimental neurological symptoms associated with disease progression and improve outcome for patients with brain metastases.

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Tables:

% colocalization	CD31/ β DG		GFAP/ β DG		GFAP/CD31	
	ipsi	contra	ipsi	contra	ipsi	contra
ENU cells	13 \pm 2*	23 \pm 4	71 \pm 2	72 \pm 2	40 \pm 7	46 \pm 3
PBS	29 \pm 2	32 \pm 3	41 \pm 5	47 \pm 6	41 \pm 6	37 \pm 4
Lv-CNTF	18 \pm 3**	40 \pm 5	66 \pm 9	51 \pm 4	45 \pm 2*	57 \pm 3
Lv-LacZ	42 \pm 5	45 \pm 3	44 \pm 5	34 \pm 10	35 \pm 5	31 \pm 3

Table I: Percentage co-localisation of CD31, GFAP and β -dystroglycan staining in ipsilateral (ipsi) and contralateral (contra) cortices of animals injected with either ENU1564 cells (top; N=7) or PBS (bottom; N=3); 4 sections per animal; paired *t*-test; mean \pm SEM; **p*<0.05,***p*<0.01.

Figure 1. Brain metastasis growth disrupts both neurovascular coupling and astrocyte-blood vessel interaction. (a) Schematic of the experimental design. (b) Photomicrograph showing immunohistochemical detection of blood vessels (CD31, brown) in a rat injected with 10^3 ENU1564 cells (arrows), in both cortices (ipsilateral and contralateral). (c) Graph showing percentage of metastatic ENU1564 cells attached to the vasculature as quantified by presence of CD31 staining within a metastasis. N=4 animals; unpaired *t*-test (mean \pm SEM; ****p*<0.001). (d) Graph showing classification of vessels in the tumour area according to the 8.5 μ m diameter threshold in animals injected with ENU-1564 cells (N=4 animals; 4 sections per animals; unpaired *t*-test; mean \pm SEM; ****p*<0.001). (e) Laser speckle contrast images showing cortical blood flow in an animal injected with ENU1564 cells before and during whisker-barrel stimulation (*injection site; anterior-to-posterior (A) and rostral-to-caudal (R) axes). (f) Graphs showing CBF response and Δ CBF amplitude (arbitrary unit [a.u]; see online methods) in both cortices of ENU1564-injected animals (N=8; mean \pm SEM; paired *t*-test, **p*<0.05). (g) Graphs showing local field potential (LFP) responses and LFP amplitude (mV; see online methods) to whisker-barrel stimulation in both cortices of ENU1564-injected animals (N=6; mean \pm SEM). (h) Representative immunofluorescence images showing dissociation of astrocyte end-feet from blood vessels co-opted by perivascular metastases (arrows in (i)), and at lower magnification changes in cellular morphology of astrocytes in the area associated with brain metastases (within dotted line in (ii)). (i) Immunofluorescence images taken from ipsilateral and contralateral cortices of animals injected with either ENU1564 cells or PBS and showing astrocytes (GFAP, blue), blood vessels (CD31, red) and β -dystroglycan (β DG, green). To assess the interaction of astrocytes with blood vessels, quantitation of red and blue pixels within the green channel was measured in both cortices. Graphs showing percentage co-localisation of CD31, GFAP and β -dystroglycan staining both sides of animals injected with either ENU1564 cells (top; N=7) or PBS (bottom; N=3); 4

sections per animal; paired *t*-test; mean \pm SEM; **p*<0.05. Ipsilateral and injected cortex represented by (I) and contralateral (C) cortex used as control.

Figure 2. Reactive astrocytes disrupt both neurovascular coupling and astrocyte-blood vessel interaction. (a) Schematic of the experimental design. (b) Photomicrographs showing immunohistochemical detection of reactive astrocytes (GFAP; brown) in the ipsilateral cortex of animals injected with either CNTF-Lv (left) or the control LacZ-Lv (right). (c) T₂- and gadolinium-enhanced T₁ weighted magnetic resonance images showing that BBB integrity remained intact in the cortex of animals injected with CNTF-Lv at 6 weeks post-injection. Graph shows signal intensity in the cortices (paired *t*-test; mean \pm SEM, n=3). Box indicates region of reactive astrocytes. (d) Laser speckle contrast images showing cortical blood flow in an animal injected with CNTF-Lv pre- and post- stimulation of the whisker-barrel (*injection site; anterior-posterior (A) and rostral-to-caudal (R) axes). (e) Graphs showing CBF responses and Δ CBF amplitudes (arbitrary unit [a.u]; see online methods) to whisker-barrel stimulation in cortices of CNTF-Lv-injected animals (N=8; mean \pm SEM; paired *t*-test; ***p*<0.01). (f) Graphs showing LFP responses and LFP amplitudes (mV; see online methods) to whisker-barrel stimulation in both sides of CNTF-Lv-injected animals (N=7; mean \pm SEM). (g) Immunofluorescence images of animals injected with either CNTF-Lv or the control LacZ-Lv showing astrocytes (GFAP, blue, AMCA), blood vessels (CD31, red, Texas Red) and β -dystroglycan (β -DG, green, A488). Graphs show percentage co-localisation of CD31, GFAP and β -dystroglycan staining of animals injected with either CNTF (top; N=7) or control LacZ-Lv (bottom; N=4); (4 sections per animal; paired *t*-test; mean \pm SEM; **p*<0.05). Ipsilateral/injected cortex represented by (I) and contralateral (C) cortex used as control.

Figure 3. Inhibition of STAT3 in reactive astrocytes improves cerebrovascular function.

(a) Schematic of the experimental design. (b) Photomicrographs showing immunohistochemical detection of GFAP (top panel) and STAT3 (bottom panel) positive cells (brown) in the ipsilateral cortices (dotted line) of ENU1564-animals treated with DMSO (control) or WP1066 (STAT3 inhibitor). Arrows show STAT3. (c) Graphs showing area of GFAP (top left panel; n=5), STAT3 (bottom left panel; n=5) and tumour cells (right panel) in ipsilateral cortices. Unpaired *t*-test (mean± SEM; *p<0.05). (d) Laser speckle contrast images showing cortical blood flow in an ENU1564-animal treated with DMSO (control) before and during whisker-barrel stimulation (top panel; *injection site; anterior-posterior (A) and rostro-caudal (R) axes). Graphs showing CBF response and Δ CBF amplitude (arbitrary unit [a.u]; see online methods) in animals treated with DMSO (control) (bottom panel; N=4; mean ± SEM; paired *t*-test, *p<0.05). (e) Graphs showing local field potential (LFP) responses and LFP amplitude (mV; see online methods) to whisker-barrel stimulation in animals treated with DMSO (control) (N=3; mean ± SEM). (f) Laser speckle contrast images showing cortical blood flow in animals treated with WP1066 before and during whisker-barrel stimulation (top panel; *injection site; anterior-posterior (A) and rostro-caudal (R) axes). Graphs showing CBF response and Δ CBF amplitude (arbitrary unit [a.u]; see online methods) in animals treated with WP1066 (bottom panel; N=4; mean ± SEM). (g) Graphs showing local field potential (LFP) responses and LFP amplitude (mV; see online methods) to whisker-barrel stimulation in animals treated with WP1066 (bottom panel; N=4; mean ± SEM). For all graphs, cortices of ENU1564-animals are referred as ipsilateral (I) and contralateral (C).

Figure 4. Reactive astrocytes reduce basal cerebral blood flow. (a) T₂ weighted-MRI showing site of injection (arrows) of either metastatic ENU1564 cells or CNTF-Lv and quantitative CBF maps obtained from arterial spin labelling (ASL) MRI. (b) Photomicrographs showing immunohistochemical detection of activated astrocytes (GFAP; brown) in the cortical region of reduced basal CBF and in the contralateral cortex of animals injected with either ENU1564 cells (top) or CNTF-Lv (bottom). (c) Graph showing basal CBF values in ipsilateral (I) and contralateral (C) cortices of ENU1564-injected animals (N=7; mean ± SEM; *p<0.05). (d) Graph showing basal CBF values in ipsilateral (I) and contralateral (C) cortices of CNTF-Lv-injected animals (N=6; mean ± SEM; **p<0.01). (e) Graph showing a significant correlation between reduced basal CBF and area of reactive astrocytes, as measured by GFAP staining in both ENU1564 and CNTF-Lv-injected animals. (r²=0.33; *p<0.05, N=13). (f) Quantitative CBF maps obtained from ASL-MRI in animals injected with either PBS or LacZ-Lv; arrows indicate injection site. (g) Graphs showing CBF values animals injected with either PBS or LacZ-Lv (N= 4-7; mean ± SEM). (* injection site; anterior-posterior (A) and rostral-to-caudal (R) axes). Injected cortices referred as ipsilateral (I) and contralateral (C). (h) T₂ weighted-MRI showing site of injection (arrows) of metastatic ENU1564 cells and quantitative CBF maps obtained from ASL-MRI. (i) Graphs showing basal CBF values in DMSO animals (bottom panel; N=4; mean ± SEM; paired *t*-test, *p<0.05) or with WP1066 (top panel; N=4; mean ± SEM).

Figure 5. Reactive astrocytes are associated with changes in vasoactive pathways. (a) Immunofluorescence images showing co-localisation (arrows) of cytochrome p450-4A-positive cells (green), αSMA-positive cells (red) and nuclei (DAPI, blue) in CNTF-Lv-injected animals. (b) Graphs showing percentage of co-localisation between cytochrome p450-4A and αSMA staining in ipsilateral (black bar) and contralateral (white bar) cortices of

all four experimental groups. (c) Immunofluorescence images showing co-localisation of iNOS (green), astrocytes (GFAP, red) and nuclei (blue; DAPI) in both cortices of CNTF-Lv-injected animals. (d) Graph shows percentage co-localisation of iNOS and GFAP in ipsilateral (black bar) and contralateral (white bar) cortices of all experimental groups. (e) Immunofluorescence images showing co-localisation of COX-1 (green), astrocytes (GFAP, red) and nuclei (blue; DAPI) in ipsilateral (black bar) and contralateral (white bar) cortices of CNTF-Lv-injected animals. (f) Graph shows percentage co-localisation of COX-1 and GFAP. All quantitative analysis performed using N=4 animals; 4 sections per animal, unless stated otherwise; mean \pm SEM; paired *t*-test, **p*<0.05, ***p*<0.01). (g) Graphs showing total number of iNOS/GFAP (left panel) and COX1/GFAP (right panel) positive pixels in the ipsilateral cortices of DMSO (control) or WP1066 experimental groups. N=4 animals; unpaired *t*-test (mean \pm SEM; ***p*<0.01). For all graphs, cortices of ENU1564-animals are referred as ipsilateral (I) and contralateral (C).

Figure 6. Reactive astrocytes are associated with changes in vasoactive pathways in human brain metastasis tissue. (a) Photomicrographs showing immunohistochemical detection of reactive astrocytes (GFAP; brown) surrounding brain metastases (*) in human tissue. (b) Immunofluorescence images showing co-localisation (*) of cytochrome p450-4A-positive cells (green), α SMA (red) and nuclei (blue; DAPI). (c) Immunofluorescence images showing co-localisation of iNOS (green), astrocytes (GFAP, red) and nuclei (blue; DAPI) in the blood vessels surrounding the tumour colonies. Insets (i) and (ii) with * indicates iNOS/GFAP co-localisation. (d) Immunofluorescence images showing co-localisation of COX-1 (green), astrocytes (GFAP, red) and nuclei (blue; DAPI). Insets (i) and (ii) with * indicates COX-1/iNOS co-localisation. (e) Graph shows percentage co-localisation of iNOS and GFAP, COX-1/GFAP and p450/ α SMA in intratumoral (I, black bar) and extratumoral (E,

white bar) regions of human tissue (3 brains; 4 sections per brain; mean \pm SEM; unpaired *t*-test, **p*<0.05, ****p*<0.001).

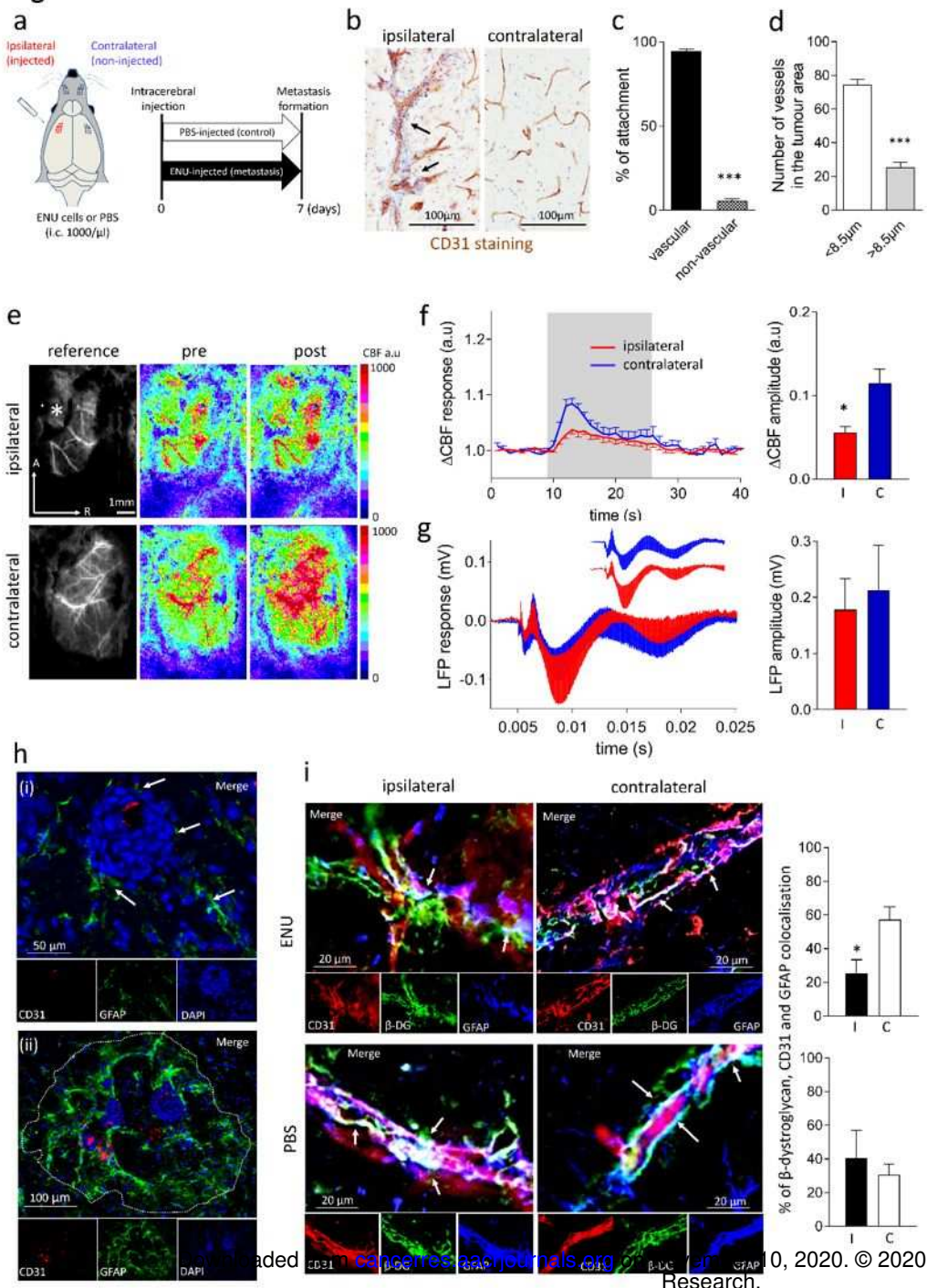
Figure 1

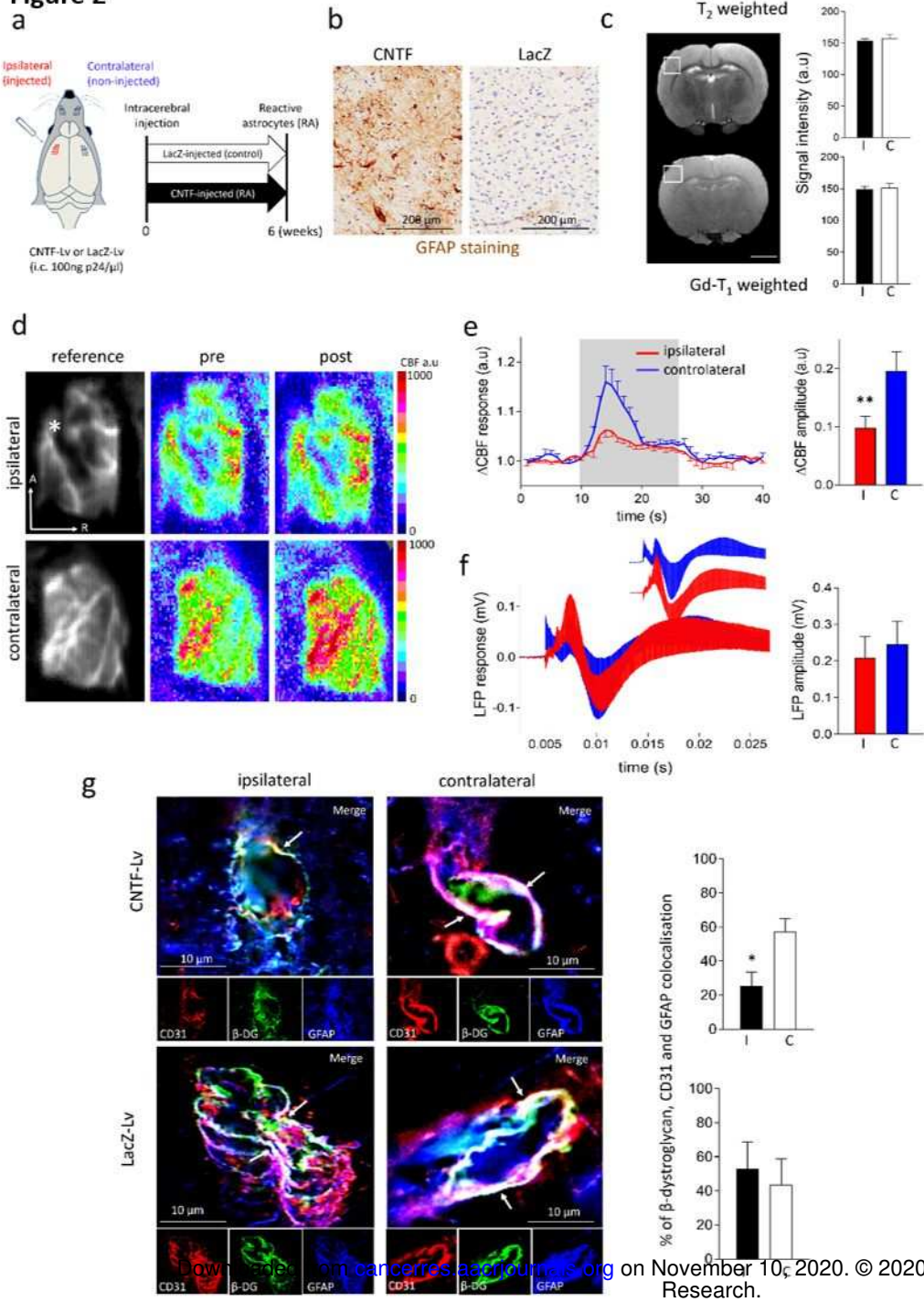
Figure 2

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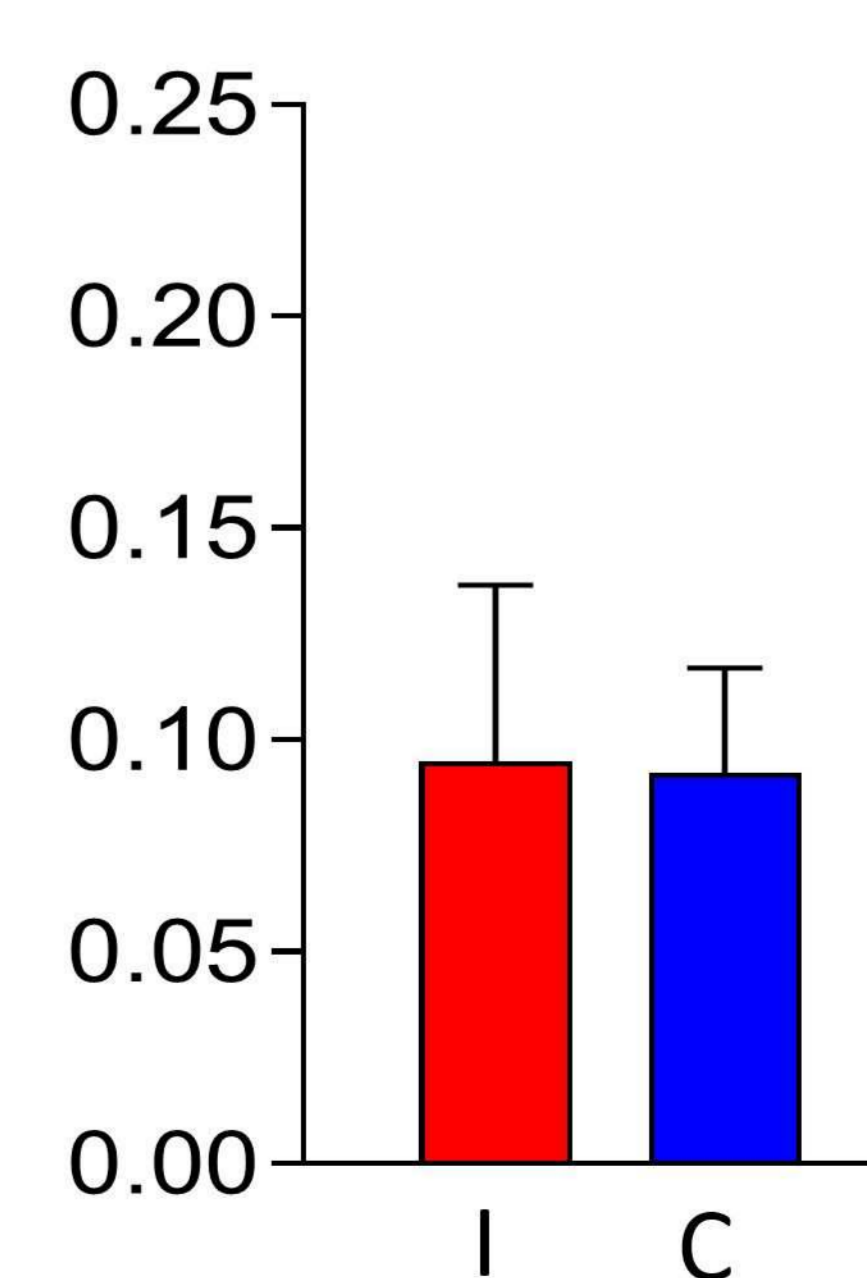
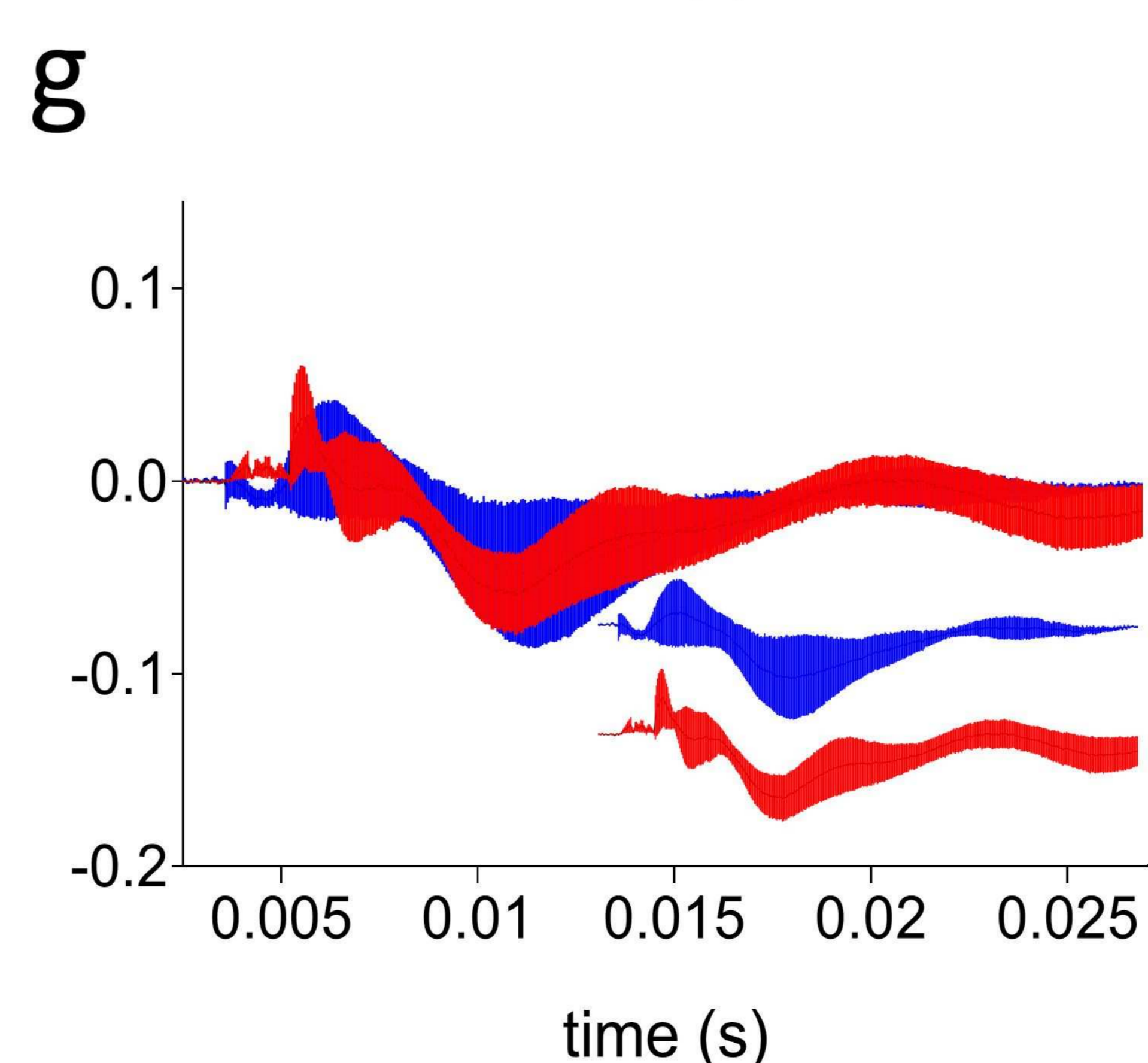
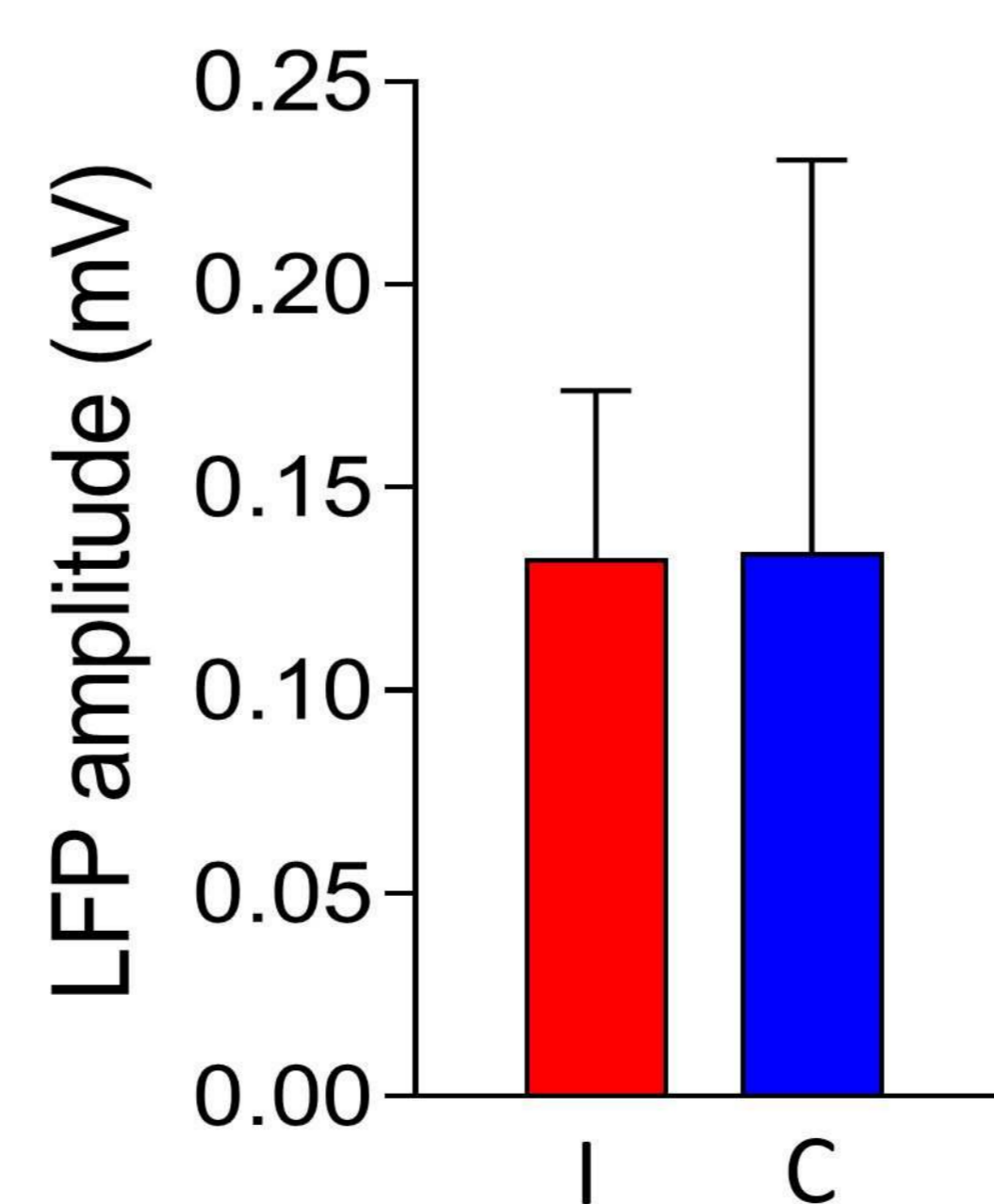
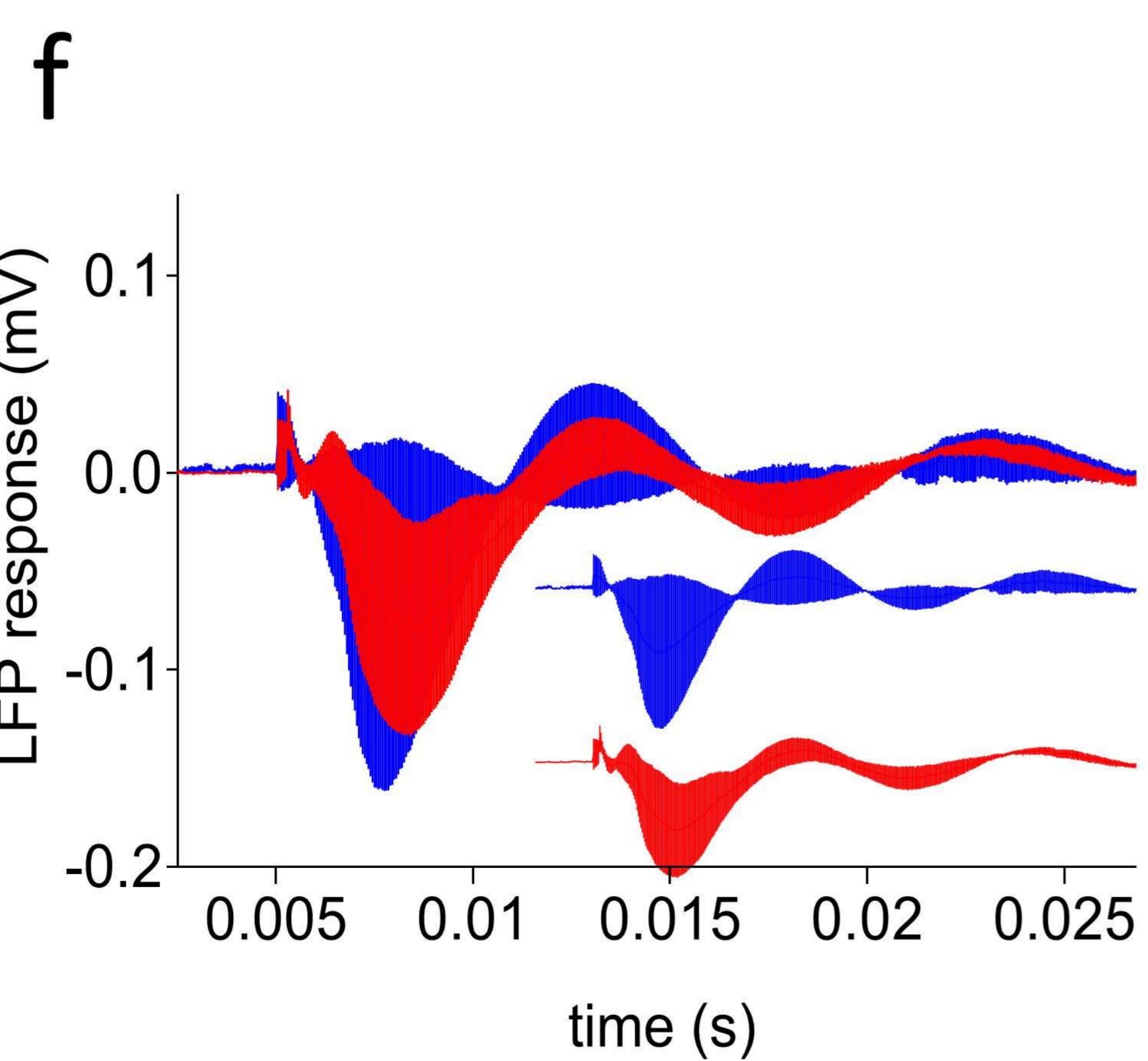
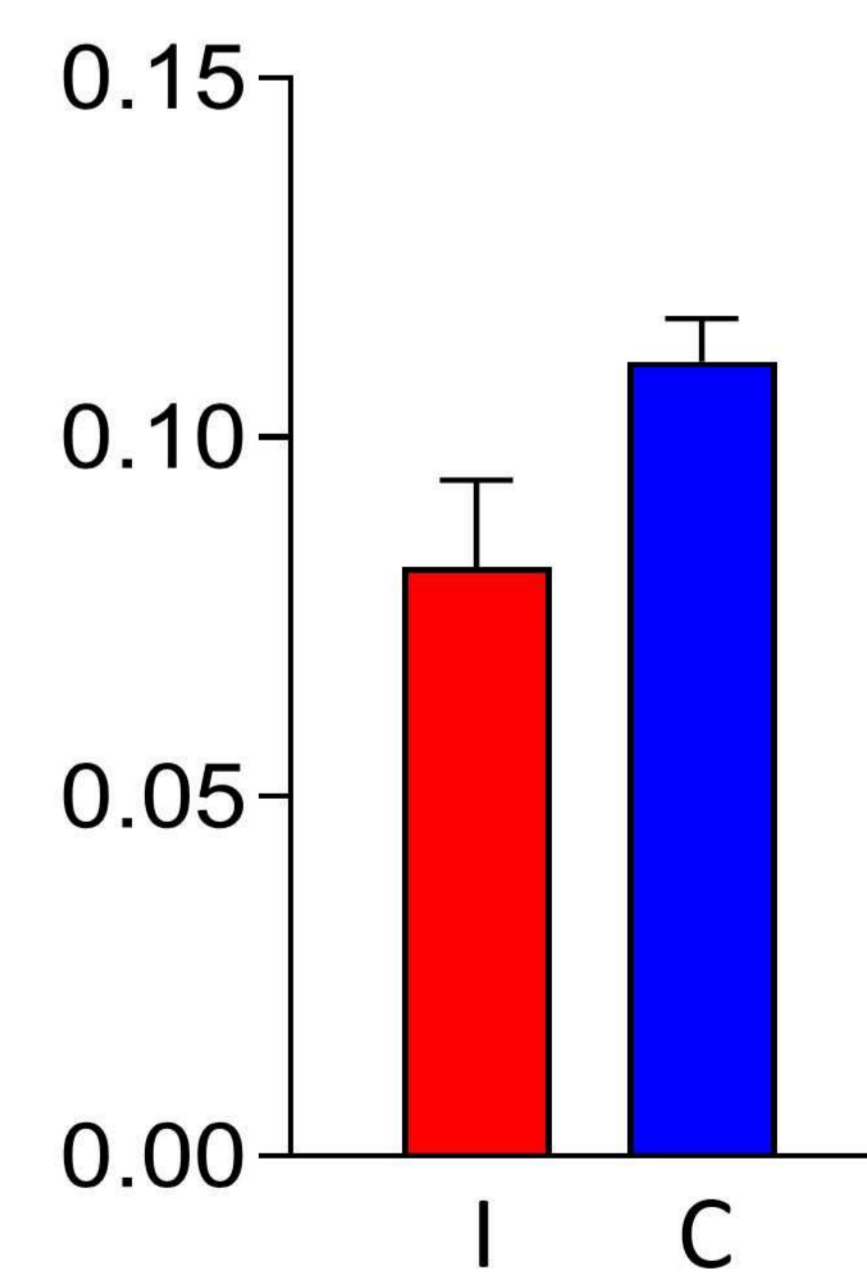
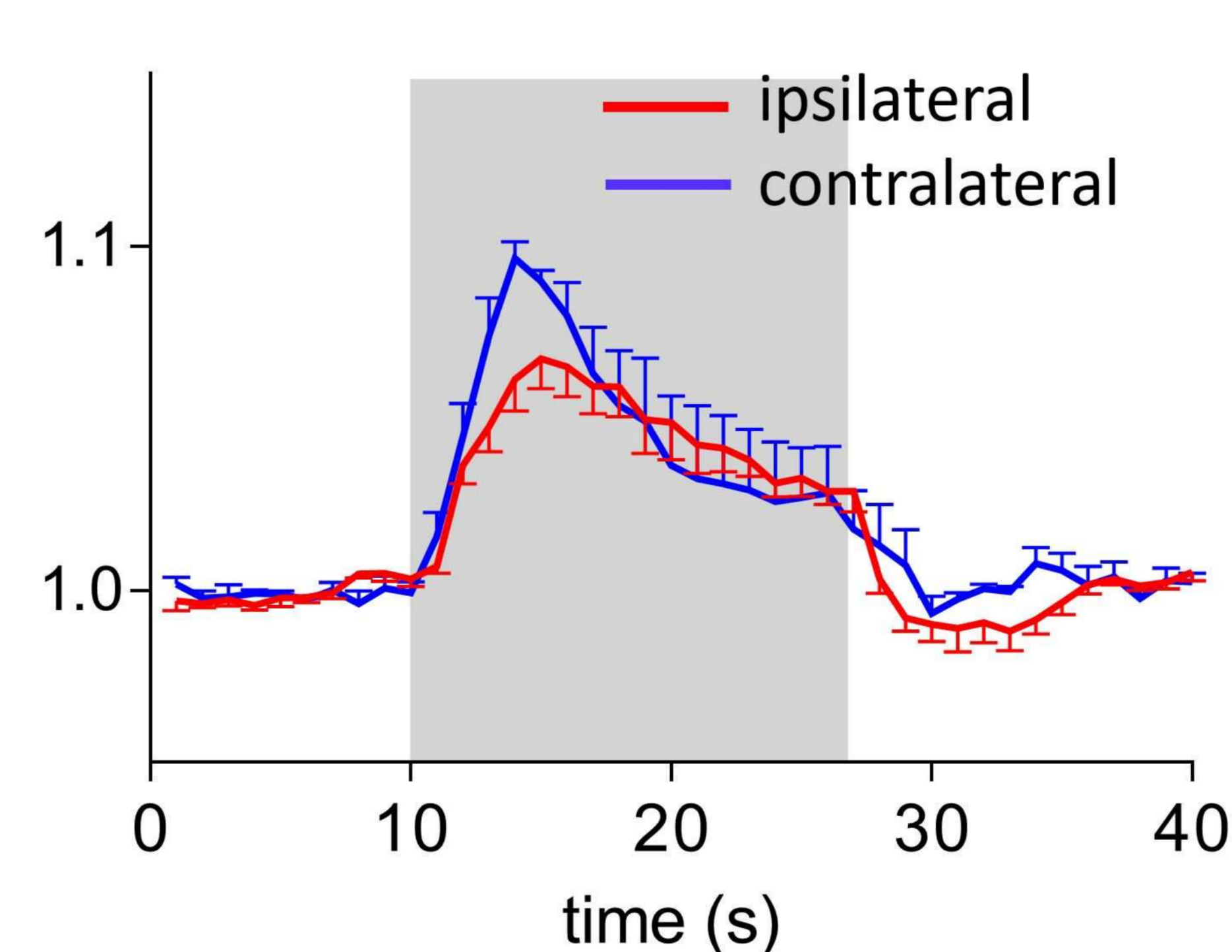
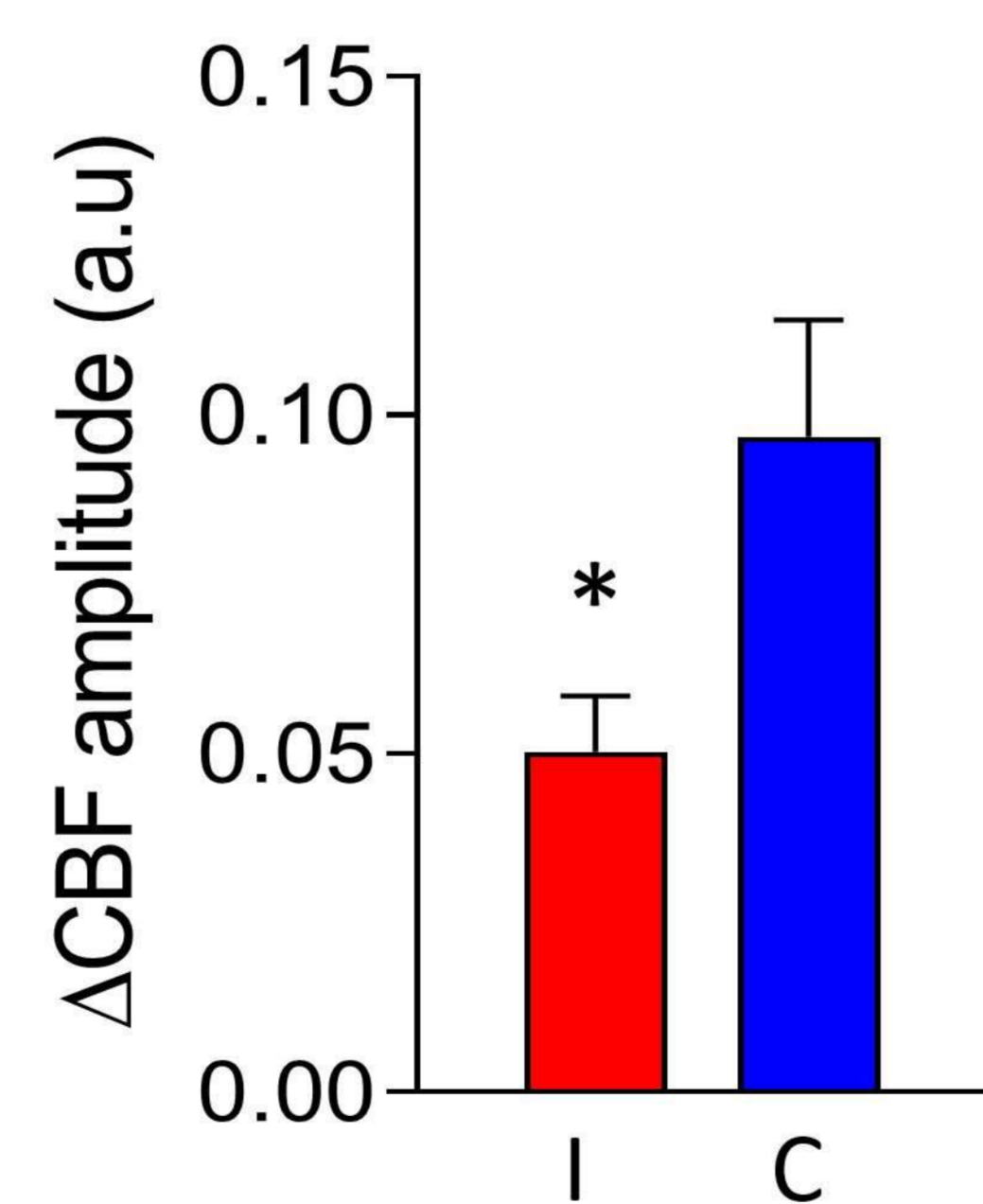
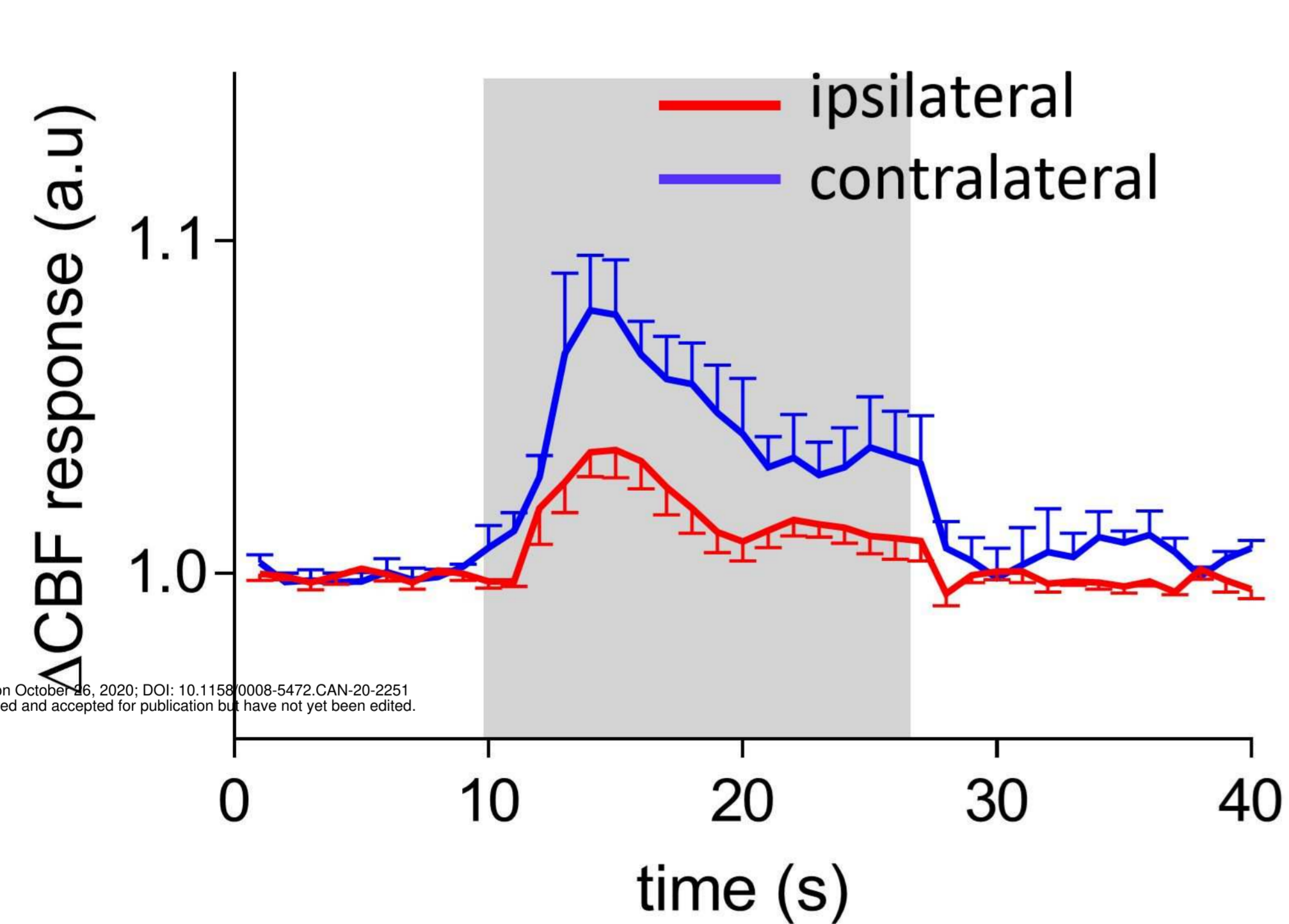
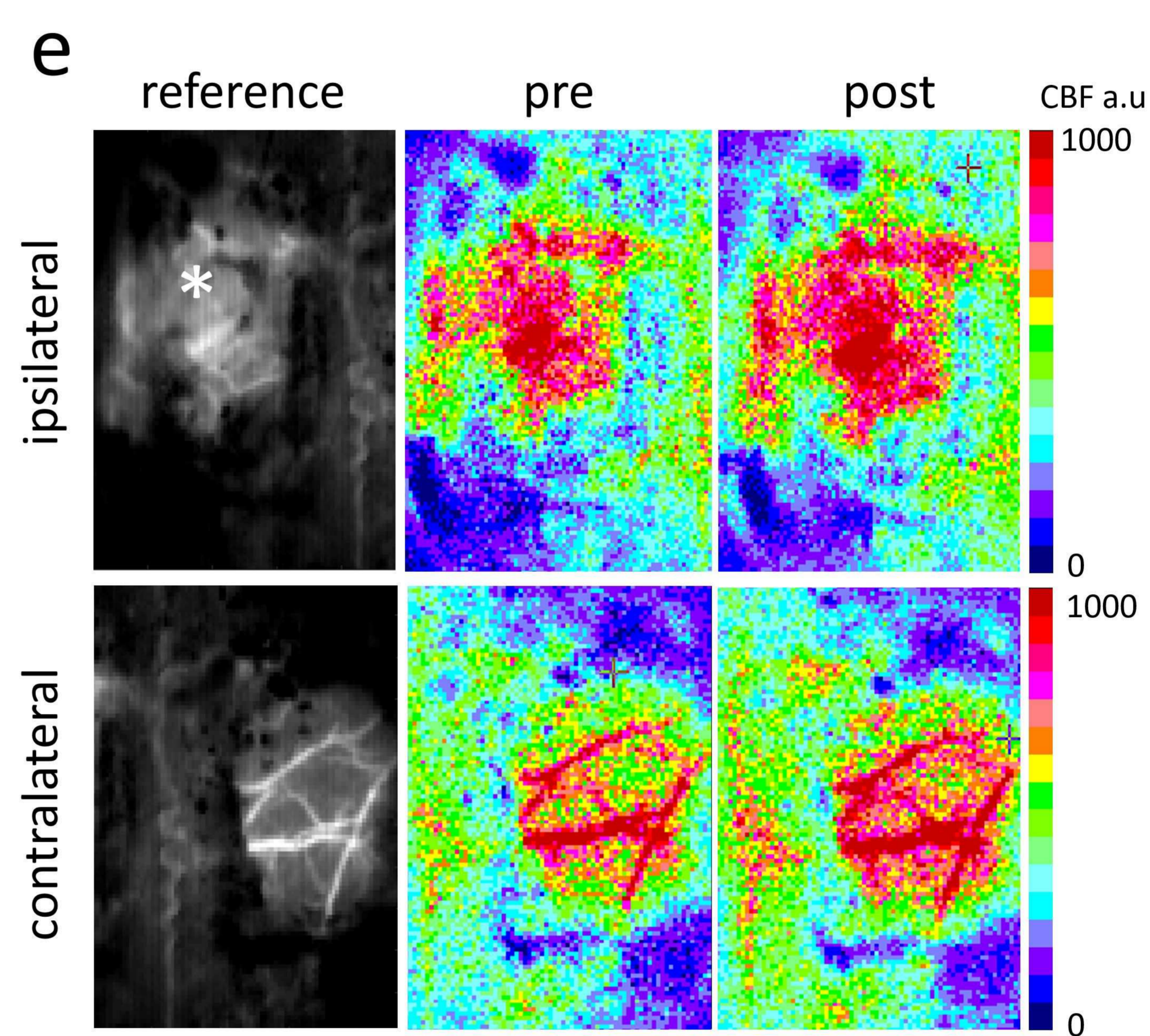
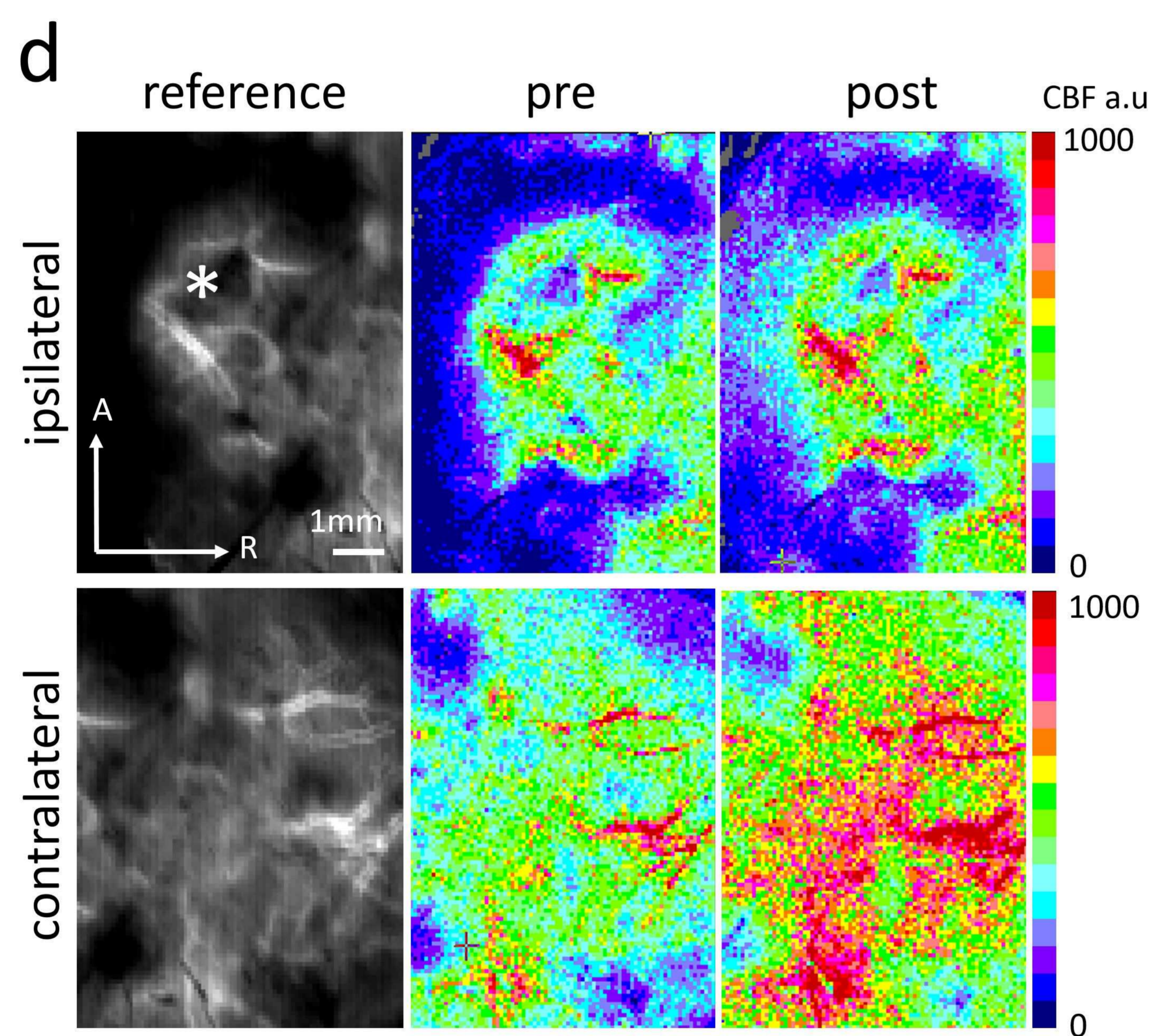
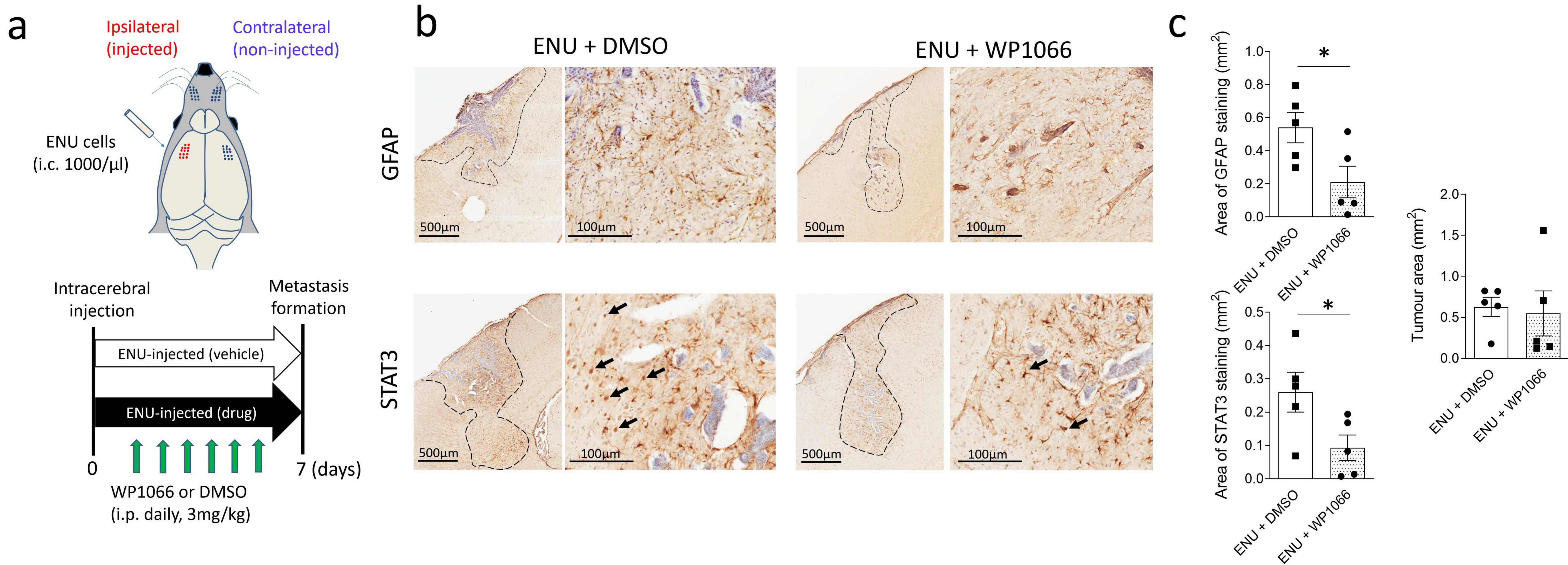


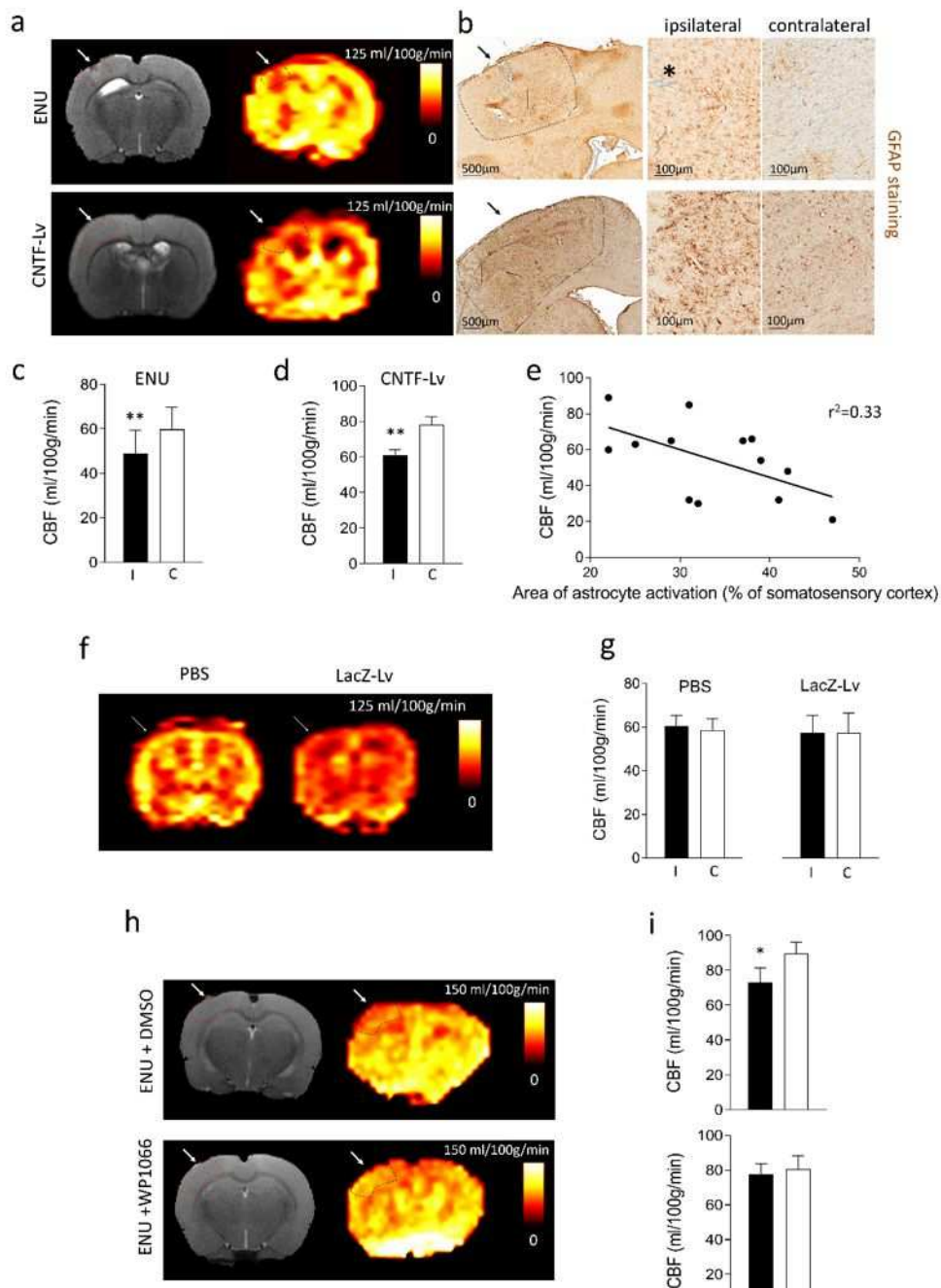
Figure 4

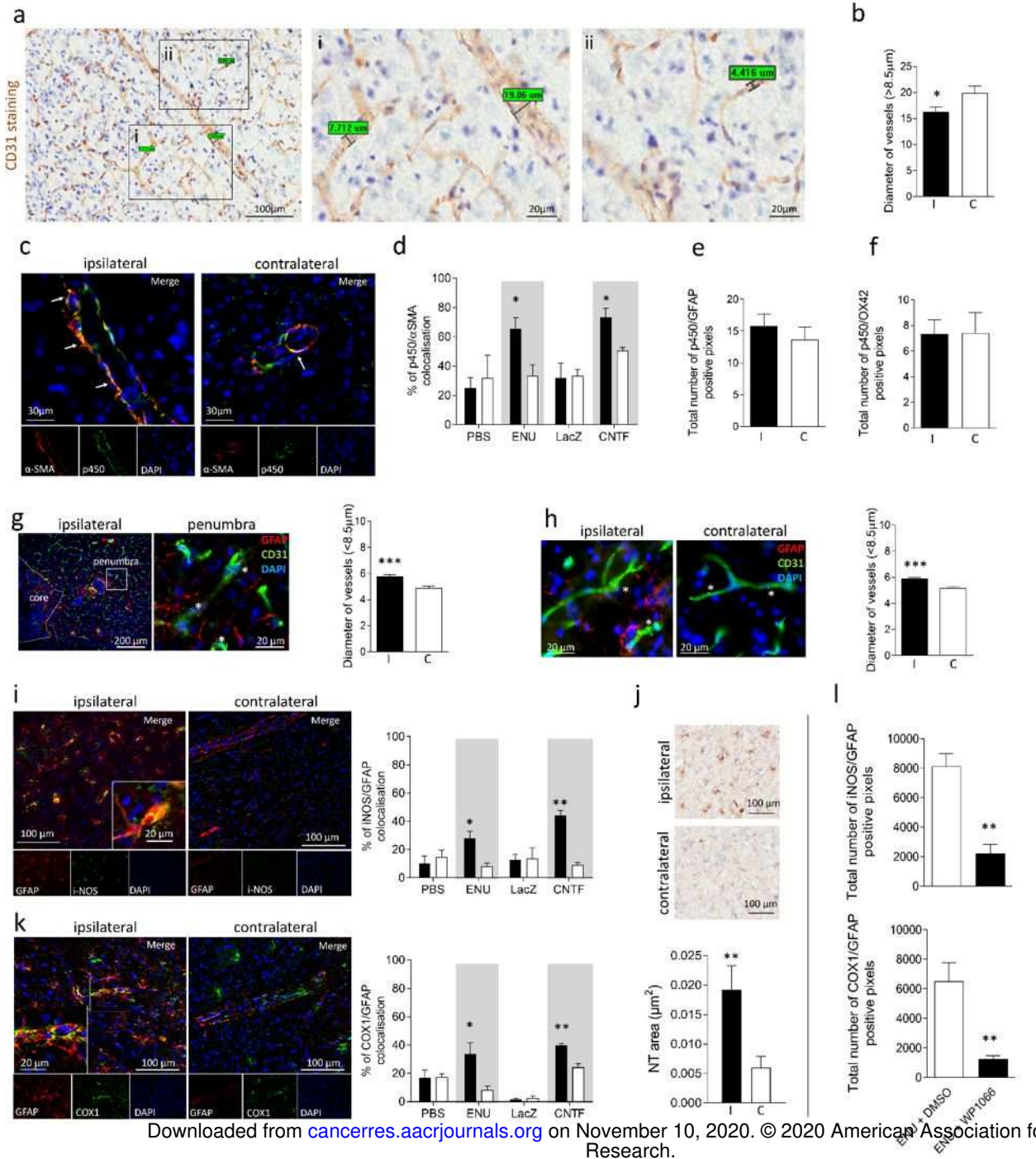
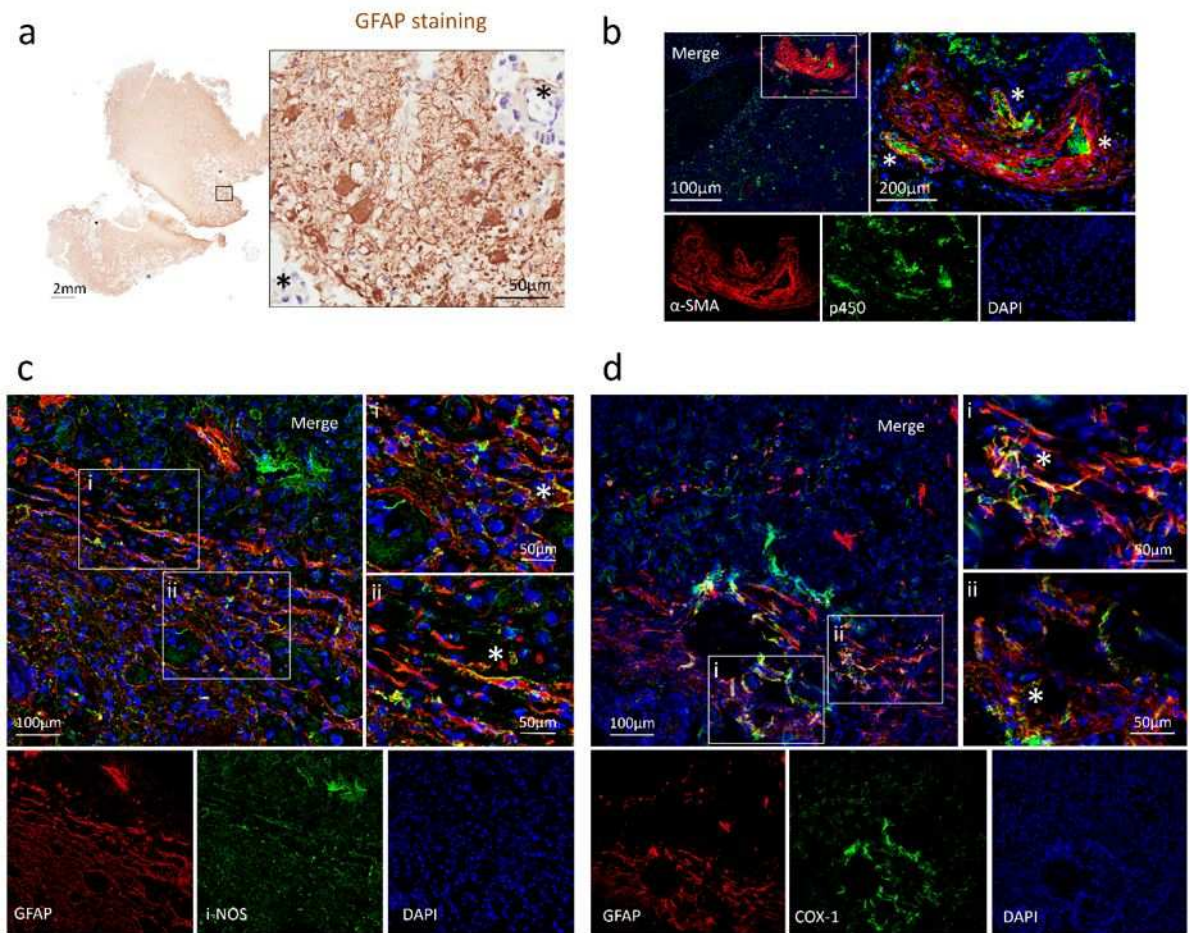
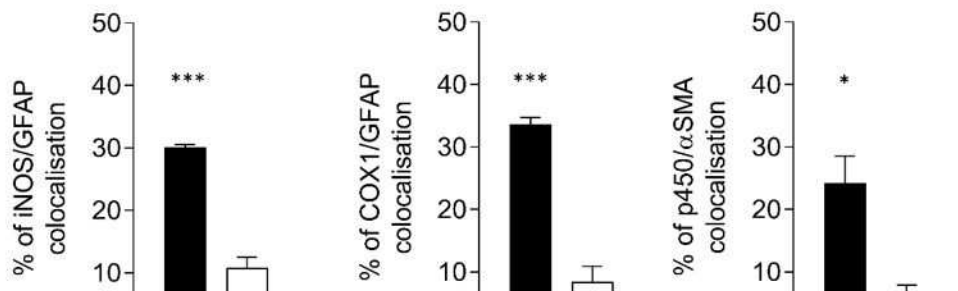
Figure 5

Figure 6



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STAT3-mediated astrocyte reactivity associated with brain metastasis contributes to neurovascular dysfunction

Manuel Sarmiento Soto, James R. Larkin, Chris Martin, et al.

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