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CAPILLARY BLOOD FLOW AROUND MICROGLIAL SOMATA DETERMINES DYNAMICS OF MICROGLIAL PROCESSES IN ISCHEMIC CONDITIONS

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Abstract

Microglia are the resident immune cells in the brain. Under normal conditions resting ramified microglia constantly extend and retract fine processes while performing immunological surveillance. In ischemia, microglia become activated as demonstrated by morphological changes during deramification leading to transformation from ramified to amoeboid form. *In vivo* two-photon microscopy of EGFP-expressing microglia in mouse neocortex was used to examine microglial dynamics during the early periods of focal and global ischemia. A penumbra-like “area-at-risk” surrounded by a square-shaped area of severely hypoperfused tissue was created by laser-induced photothrombosis. The dynamics of microglial processes in the area-at-risk were strongly correlated with capillary blood flow (BF) measured within 10 μ m of microglial somata. Changes in BF around distal microglial processes (>30 μ m from somata) had no effect on microglial dynamics. A severe reduction of capillary BF near somata by $84\pm6\%$ resulted in initiation of microglial deramification, suggesting activation. A moderate decrease in BF near somata by $22\pm5\%$ or increase by $87\pm10\%$, reflecting a redistribution of capillary BF, had no effect on microglial morphology. Complete BF loss during cardiac arrest (CA) or transient bilateral common carotid artery occlusion (BCCAO) entirely stalled all microglial processes without structural changes. Reperfusion after BCCAO induced recovery of microglial dynamics to pre-occlusion values. These findings suggest that during ischemia, the severe drop in BF around microglial somata coincides with morphological activation. However, this activation requires some residual BF because complete perfusion loss (as during BCCAO and CA) did not support microglial deramification.

Keywords

microglia; dendrites; two-photon laser scanning microscopy; in vivo imaging; stroke; global ischemia

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Introduction

One of the first post-ischemic responses is activation of microglia (Kempermann and Neumann, 2003). Activation can either have a beneficial or detrimental effect on neuronal survival depending on the balance between secreted anti- or proinflammatory neuroactive compounds (Ekdahl et al., 2009; Hanisch and Kettenmann, 2007). In intact brain, microglia exhibit a “ramified” phenotype with highly dynamic processes that constantly survey surrounding brain parenchyma (Davalos et al., 2005; Nimmerjahn et al., 2005). Sensing motility of microglial processes requires actin polymerization (Hines et al., 2009). In response to microdamage, random probing quickly transforms to targeted outgrowth of larger processes to the site of injury to prevent damage expansion (Davalos et al., 2005; Haynes et al., 2006; Hines et al., 2009). This outgrowth involves functional chloride channels and is also actin-dependent (Hines et al., 2009). In response to larger injury as during stroke, microglia undergo a wide array of molecular adjustments paralleled by morphological transformations into “amoeboid” form existing at the far end of the spectrum of morphological activation (Hanisch and Kettenmann, 2007; Raivich et al., 1999). Change in phenotype follows stereotypical steps including a decrease in branching, reduction in total branch length, and withdrawal of ramified branches (Kurpius et al., 2007; Raivich et al., 1999; Stence et al., 2001). Finally, activated microglia become deramified with enlarged soma and few thickened, short processes. Since actin polymerization governs the sensing and fast process outgrowth of microglia in healthy tissue, it is also likely that the dynamic changes in actin cytoskeleton underlie the microglial transformation into “amoeboid” form.

Actin polymerization in motile cells is ATP-dependent (Atkinson et al., 2004). In focal ischemic stroke the core and penumbra are characterized by abrupt reductions in blood flow (BF). The immediate consequence is a profound drop in cellular ATP. Rapid activation of microglia during early periods of infarct evolution could increase neuronal survival (Imai et al., 2007; Kitamura et al., 2004; Lalancette-Hebert et al., 2007; Madinier et al., 2009). We hypothesize that the morphological manifestation of microglial activation is energy-dependent and will require the presence of residual blood flow in the affected brain regions. Using *in vivo* two-photon laser scanning microscopy (2PLSM) of enhanced green fluorescent protein (EGFP)-expressing microglia in intact ischemic mouse neocortex we show that early spatiotemporal changes in microglial dynamics are dramatically affected by the remaining capillary BF around the microglial somata.

Materials and Methods

Transgenic mice

All procedures follow National Institutes of Health guidelines for the humane care and use of laboratory animals and underwent yearly review by the Animal Care and Use Committee at Georgia Health Sciences University. All efforts were made to minimize animal discomfort and reduce the number of mice used. The founding mice of the B6.129P-*Cx3cr1^{tm1Litt}/J* colony [CX3CR1-EGFP] and B6.Cg-Tg(Thy1-YFPH)2Jrs/J colony [YFP-H] were purchased from Jackson Laboratories. Mice of the CX3CR1-EGFP strain express EGFP in microglia under control of the endogenous *Cx3cr1* locus encoding the chemokine (C-X3-C) receptor 1 (CX3CR1, also known as fractalkine receptor) (Jung et al., 2000). In these mice microglia cells are clearly labeled with EGFP providing high contrast, thus facilitating the imaging of microglial somata and fine processes. YFP-H mice display bright fluorescence of YFP expressed in a fraction of pyramidal neurons of the neocortex aiding dendritic imaging (Feng et al., 2000). We used only heterozygous [CX3CR1-EGFP] mice in all experiments to preserve CX3CR1 signaling that affects microglia-neuron interactions (Cardona et al., 2006; Ransohoff and Cardona, 2010). Hybrid mice, with a small proportion of both fluorescent neocortical pyramidal neurons and fluorescent microglia, were generated by crossing [YFP-

H] and [CX3CR1-EGFP] strains. In total, 52 heterozygous [CX3CR1-EGFP], 12 hybrid [YFP-H]/[CX3CR1-EGFP] and 4 wild type male and female mice between 6 and 16 weeks of age were used in this study.

Preparation of mice for in vivo imaging

Microglia were imaged through a thinned skull cranial window centered at stereotaxic coordinates -1.8 mm from bregma, 2.8 mm lateral over the somatosensory cortex. Surgical procedures followed a protocol adapted from Grutzendler and Gan (2005). Mice were anesthetized with an intraperitoneal injection of urethane (1.5 mg/g body weight). Body temperature was maintained at 37°C with a heating pad (Sunbeam). A short ~ 1 cm L-shaped glass capillary (1.2 mm diameter) was inserted into the trachea and secured with sutures to minimize potential breathing problems. The scalp was gently separated from the cranium. A plastic ring (13 mm diameter) was glued to the skull with dental acrylic cement (Co-Oral-Ite Dental) to stabilize the head during craniotomy and imaging using a mouse headholder attached to a baseplate. A small aluminum bar with two tapped screw holes was embedded into the acrylic of the mice used in the bilateral common carotid artery occlusion (BCCAO) experiments. The heads of these mice were stabilized in the same position during consecutive imaging sessions by 2 screws tightened to a custom-made L-shaped adjustable metal arm fixed to the baseplate. A circular area of the skull (~ 1 mm diameter) was thinned under the Zeiss Stemi SV6 stereo zoom microscope with a high-speed dental drill (Midwest Stylus mini 540S) by $\frac{1}{4}$ bit. A final thickness of the skull (~ 30 μm) was achieved by gently scraping the bone with a microsurgical blade (Surgistar, #38-6900). A cortex buffer was applied to keep the thinned region moist during experiments.

The baseplate containing the headholder with the mouse resting on a heating pad was affixed to the Luigs & Neumann microscope stage for imaging. Rectal temperature was monitored continuously and maintained at 37°C . A sufficient level of anesthesia was confirmed by the lack of a toe-pinch reflex and heart rate (450 – 650 beats/min) monitoring using DAM-60 amplifier (WPI) and maintained with only minimal supplementation if necessary ($<10\%$ of the initial urethane dose) for up to 6 hours. Blood oxygen saturation was measured in a subset of mice ($n=11$) in photothrombotic stroke model using the MouseOx® pulse oximeter mounted on the left thigh. The oxygen saturation level remained above 90% for the duration of the experiment. Hydration was maintained by intraperitoneal injection of 0.9% NaCl (200 – 300 μl) with 20 mM glucose at 1 – 2 h intervals. A 0.1 ml bolus of 5% (w/v) Texas Red dextran (70 kDa) (Invitrogen) in 0.9% NaCl was injected into the tail vein for blood flow visualization. All chemicals were from Sigma Chemical unless indicated otherwise.

Two-photon Laser Scanning Microscopy

Images were collected with IR optimized $40\times/0.8$ NA water immersion objective (Carl Zeiss), using the Zeiss LSM 510 NLO META multiphoton system mounted on the motorized upright Axioscope 2FS microscope (Zeiss). The scan module was directly coupled with the Spectra-Physics Ti:sapphire broadband mode-locked laser (Mai-Tai) tuned to 910 nm for 2-photon excitation. Emitted light was detected by internal photomultiplier tubes (PMTs) of the scan module with the pinhole entirely opened. The imaged microglia was typically within 100 μm of the pial surface and therefore in layer I. Three-dimensional (3D) time-lapse images were acquired at 1 μm increments using $3\times$ optical zoom, resulting in a nominal spatial resolution of 13.65 pixels/ μm (12 bits/pixel, 1.26 μs pixel time) across a 75×75 μm imaging field. Individual image stacks were taken at 19 – 21 minute intervals. If shifting of the focal plane occurred, the field of focus was adjusted and re-centered prior to acquiring the next image stacks (Risher et al., 2009; 2010). The blood flow was imaged in a repetitive line scan mode (1000 lines per scan) along the central axis of a capillary (1.15 ms/

line, 0.2 $\mu\text{m}/\text{pixel}$, 8 bits/pixel, 4.57 μs pixel time). Data acquisition was controlled by the Zeiss LSM 510 software.

Image analysis

Fiji distribution package (http://pacific.mpi-cbg.de/wiki/index.php/Main_Page) of NIH ImageJ (<http://rsb.info.nih.gov/ij/>) was used together with Bitplane AutoAligner for 2PLSM image analyses and processing. A median filter (radius=2) was applied to images in figures to reduce photon and PMT noise. Given the relatively poor axial resolution of 2PLSM (~2 μm), we used two-dimensional Maximum Intensity Projections (MIPs) of image stacks containing 31 sections through a microglial cell. The length of microglial processes and the speed of extension/retraction were analyzed with Simple Neurite Tracer plug-in for ImageJ (<http://homepages.inf.ed.ac.uk/s9808248/imagej/tracer/>) bundled with Fiji software. The number of new/lost processes and the average speed of process extension/retraction were calculated by comparing MIP images acquired at 19–21 minute intervals. To determine the average speed of extension/retraction for a particular microglial cell, we measured the length of extension/retraction of individual processes and then divided the total change in length for all extending/retracting processes by the time interval between two image stacks. Although the number of new/lost processes and the average speed of process extension/retraction were evaluated from MIPs of image stacks taken at a relatively slow sampling rate, these measurements were adequate to record structural changes, which underestimated actual values. Yet, faster morphological changes completed within 20 minutes were not recorded. Dendritic beading was recognized as the appearance of rounded regions extending beyond the diameter of the parent dendrite separated by “interbead” segments. Blood vessel diameter was estimated from xy planar images by measuring the distance between the edges of the vessel demarcated by the pixels with above the background level of intensity. Microvessels with a diameter <6 μm were defined as capillaries (Tsai et al., 2009). The velocity of red blood cells (RBCs) was measured from a space-time image formed by a stack of line scans along the central axis of a capillary where moving RBCs are seen as dark bands (Kleinfeld et al., 1998). The shortest 3D linear distance between the edge of a soma profile and capillary surface was calculated using the 3D measurement tool of the LSM 510 Image Examiner.

Photothrombotic stroke model

We utilized a variation of a ‘ring’ model of photochemically initiated thrombosis (Jiang et al., 2006; Risher et al., 2010; Wester et al., 2005) (Fig. 1A). In this model, the central area at risk of ischemic injury is surrounded by a zone of severely hypoperfused tissue. This penumbra-like area-at-risk is highly reproducible, undergoes progressive hypoperfusion and is easily definable in real-time thus facilitating time-lapse imaging. Briefly, using LSM 510 software a square-shaped region of interest (ROI; 1270×1270 μm) was positioned over the middle cerebral artery (MCA) territory in the somatosensory cortex (Fig. 1A). Green Ar-Kr laser (514 nm) of the LSM 510 system was used to repetitively irradiate only the perimeter (100 μm wide) of this ROI through a 10×/0.3 NA water immersion objective. Average power through the objective was ~3 mW. The square in the middle (1070×1070 μm) was not irradiated by the laser beam. A bolus of Rose Bengal (RB) was injected through the tail vein (0.03 mg/g body weight, diluted to 10 mg/ml in 0.9% NaCl) over 60 sec with a syringe pump (WPI) and activated by the green laser for 10 min. During this time RB mainly clears from the circulation (Risher et al., 2010; Zhang and Murphy, 2007), thus decreasing the possibility of the direct effect on microglia in case of the occurrence of leaking vessels in the ischemic zone. Vascular coagulation in the perimeter (Risher et al., 2010) was confirmed by visual inspection. Loss of BF was also verified by the laser speckle contrast imaging (Fig. 1B,C,D).

Global ischemia models

Two different types of global ischemia models were employed. Complete global ischemia with a sudden loss of BF was accomplished by cardiac arrest (CA) induced by an injection of 1 ml of air into the tail vein. Transient global ischemia was achieved by temporary BCCAO with subsequent reperfusion (Murphy et al., 2008). Both common carotid arteries (CCAs) were carefully separated from the muscles and vagus nerves through a ventral cervical incision under the stereo zoom microscope. Occlusion was accomplished by ligation with 5-0 silk sutures (Ethicon) and reperfusion was achieved by untying knots. 2PLSM images were acquired in three intermittent sessions (before BCCAO for 30 min, during BCCAO for 30 min and after reperfusion for 60–120 min) because the mouse was moved between 2-photon microscope stage and the stereo zoom microscope. This transfer was necessary to ensure proper ligation of CCAs and subsequent full reperfusion. Changes in BF were first confirmed visually by observing changes of surface BF in the stereo zoom microscope and then by the laser speckle imaging and 2PLSM imaging of BF. Laser Doppler recordings were also used in a subset of experiments.

Cerebral blood flow (CBF) measurements

Two-dimensional maps of CBF with high spatiotemporal resolution were acquired by laser speckle imaging as described elsewhere (Dunn et al., 2001; Risher et al., 2010; Sigler et al., 2008). Briefly, the cortical surface was illuminated through Edmund Optics anamorphic beam expander by a 785 nm StockerYale laser at an angle of $\sim 30^\circ$ and imaged with a 4x/0.075 NA objective. The Zeiss AxioCam MRm CCD camera controlled by AxioVision software (Zeiss) was used to capture 300 images at 13 Hz with 20ms of exposure time. Individual images of variance were created from raw speckle images by using two-dimensional variance filtering (3×3 pixel kernel size, $3.23 \mu\text{m}/\text{pixel}$) function of ImageJ. A single 32-bit image of the standard deviation was calculated by taking the square root of the averaged variance image. An image of laser speckle contrast (k) was obtained by dividing the standard deviation image by the mean of all raw images.

We quantified relative changes in CBF velocity using speckle correlation time values (τ_c) as described elsewhere (Dunn et al., 2001; Risher et al., 2010; Tom et al., 2008). Briefly, k images were converted to relative (τ_c) images with the asymptote algorithm method using the equation $\tau_c \approx 2Tk^2$, where T is the exposure duration of the camera (Tom et al., 2008). Since the velocity of blood flow is assumed to be inversely proportional to speckle correlation time (Dunn et al., 2001), inverse speckle correlation time images ($1/\tau_c$) were calculated in control, after photothrombotic occlusion, and at the end of experiments after cardiac arrest induced by 1 ml of air embolization into the tail vein. The post-cardiac arrest ($1/\tau_c$) image (corresponding to biological zero flow) was subtracted from the ($1/\tau_c$) control and experimental image to create relative CBF images (percentage of baseline CBF, Fig. 1D). To quantify relative changes in CBF velocity, 28–31 ROIs covering most of the area-at-risk but omitting major vessels were drawn. A mean control (baseline) value of ($1/\tau_c$) and mean ($1/\tau_c$) postocclusion values were calculated using these ROIs, and the percentage of baseline CBF was computed.

The laser Doppler recordings of CBF were acquired by PeriFlux 5000 system (Perimed PF5010 laser Doppler perfusion monitoring unit) equipped with a small Probe 407-1 (Perimed) and PeriSoft analysis software.

Statistics

SigmaStat (Systat) was used for statistical analyses. A two-tailed unpaired Student's t -test, one way ANOVA followed by Tukey post-hoc test and Kruskal-Wallis ANOVA on Ranks followed by Dunn's post-hoc test were used to compare group means for parametric and the

median values for nonparametric data, respectively. A two-tailed paired *t*-test, one-way RM ANOVA followed by Bonferroni post-hoc test and Friedman RM ANOVA on Ranks followed by Tukey post-hoc test were used to compare means and the median values over different time points, respectively. The linear regression analysis was applied to quantify the strength of the relationship between two variables. A two-tailed Fisher exact test was used to analyze data arranged in contingency tables. The significance criterion was set at $P < 0.05$. Data are presented as mean $\pm$ s.e.m.

RESULTS

Microglial process dynamics in the hypoperfused area-at-risk

We have created a square-shaped photothrombotic occlusion surrounding an ischemic penumbra-like hypoperfused area-at-risk as has been described by us recently (Risher et al., 2010). Laser speckle imaging revealed a drop in CBF immediately after the photothrombotic occlusion (Fig. 1B–D). CBF in the area-at-risk dropped to $62.9 \pm 7.2\%$ of control at ~ 2 min after photothrombosis ($n=5$ mice and declined to $39.1 \pm 4.2\%$ at 1 h ($n=4$ animals). 2PLSM confirmed that microglial processes were continuously undergoing cycles of extension and retraction under control conditions (Nimmerjahn et al., 2005) (Fig. 1E–G). Immediately after photothrombotic occlusion some microglia in the area-at-risk retained their process activity (Fig. 1H), whereas the processes of other microglia cells became less dynamic (Fig. 1I). Several microglia cells with active processes (7 cells in 4 mice) were imaged up to 6 hours after photothrombosis, and loss of process activity eventually was observed in all cases.

Because the area-at-risk in this model inevitably undergoes progressive hypoperfusion (Jiang et al., 2006; Risher et al., 2010; Wester et al., 1995) we investigated whether the motility of microglia processes depended on local BF. In the mouse neocortex the average 3D linear distance from the center of the cell nuclei to the nearest microvessel center-line is $\sim 15 \mu\text{m}$ (Tsai et al., 2009). When mean distance was calculated, all distances shorter than $5 \mu\text{m}$ were excluded. This correction in the automated method used to collect data was necessary because no intervals could possibly be shorter than one cell soma radius plus one microvessel radius (i.e. $5 \mu\text{m}$), as was estimated in the study by Tsai et al. (2009). In our experiments we have measured the shortest 3D distance from a surface of the microvessel to a surface of the microglial somata, therefore no correction was necessary. Hence, it could be expected that the average 3D linear distance of microglia cells somata is about $10 \mu\text{m}$ from a surface of the nearest microvessel. We have selected microglia cells with a capillary located within $10 \mu\text{m}$ of soma (mean $3.1 \pm 0.6 \mu\text{m}$, 20 cells in 12 animals) and monitored RBC velocity by repetitive line scans every 20 minutes (Fig. 2A,B). The velocity of RBC before photothrombosis was $623 \pm 40 \mu\text{m/sec}$ (20 capillaries in 12 animals). Capillary diameter was not affected by photothrombosis (before $3.6 \pm 0.1 \mu\text{m}$ and after $3.6 \pm 0.1 \mu\text{m}$, $P=0.1$, paired *t*-test). As expected for this model (Risher, et al., 2010) the RBC capillary velocity was continuously decreasing with fluctuations during the next 6 hours (Fig. 2C). When measured immediately after photothrombotic occlusion, the RBC capillary velocity decreased to $59 \pm 11\%$ of control values, as recorded around 10 microglia cells in 7 mice, while increased BF was observed around the somata of another 10 cells in 5 animals. Therefore we classified microglia cells into three groups based on changes in capillary BF around somata, i.e., cells with *severe* BF loss ($84 \pm 6\%$ loss, $n=6$ cells), *moderate* BF loss ($22 \pm 5\%$ loss, $n=4$) and *increased* BF ($87 \pm 10\%$ increase, $n=10$). We did not observe targeted outgrowth of large processes from microglia cells towards capillaries with reduced BF. However, in one case we detected fine microglia processes that started to wrap around a capillary during two imaging sessions separated by 20 min. Microglia processes continue to cover this capillary during the next 95 min of imaging, possibly shielding a leaky part. Microglia had high process activity in all groups before stroke (Fig. 2D,F,H) and severe BF loss resulted in a

visible decrease of process activity (Fig. 2E), while moderate loss or increase in BF had no visible effect (Fig. 2G,I, respectively). To quantify these observations, we measured the total length of processes (Total length), the number of newly formed processes (New process), the number of lost processes (Lost process), the total length of extension per minute (Extension), the total length of retraction per minute (Retraction), and the Extension/Retraction ratio (Ext/Ret). The mean values of all these parameters (except for Lost process) were significantly decreased as compared to control for microglia cells experiencing severe loss of BF around their somata, while the differences in the mean values for cells with moderate or increased BF were not significant when compared to pre-stroke values (Fig. 2J–O). Yet, the rate of Extension and Retraction had a strong linear correlation with BF around microglial somata (Fig. 2P,Q). We conclude that a severe BF reduction near the somata coincided with a decrease of process activity reflecting the beginning of morphological transformation.

Loss and recovery of microglial process activity in occlusion and reperfusion

To further verify observations that microglial process activity depends on BF around somata, we examined if microglia would react similarly to a complete BF loss. As shown in Fig. 3A–C, a complete loss of BF with sudden onset, as during cardiac arrest (CA), resulted in the total loss of microglial process activity immediately after CA with no recovery 1 hour later. No obvious morphological changes were observed in the first 60 minutes after CA with fine microglial morphology retained for 3 hours after CA. Quantitative analysis revealed no New processes formed and no Lost processes after CA. There was a significant decrease in the Extension (before CA: 7.8 ± 1.2 $\mu\text{m}/\text{min}$, after CA: 0.5 ± 0.1 $\mu\text{m}/\text{min}$, $n=7$ cells, $p<0.001$, paired t-test), and in the Retraction (before CA: 7.8 ± 1.2 $\mu\text{m}/\text{min}$, after CA: 0.5 ± 0.1 $\mu\text{m}/\text{min}$, $n=7$ cells, $p<0.005$, paired t-test). However CA did not induce change in the Total length ($p=0.9$, paired t-test) (Fig. 3D) and in the Ext/Ret ratio (before CA: 1.2 ± 0.1 , after CA: 1.3 ± 0.4 , $n=7$ cells, $p=0.96$, paired t-test) suggesting that some residual BF is necessary to begin a microglial morphological transformation.

Having confirmed that complete and sudden BF loss during CA immediately hindered microglial process activity, we used 2PLSM to determine the extent of microglial recovery from a nearly complete BF loss after reperfusion in the BCCAO model (Fig. 3E–G). Capillary BF measurements by repetitive line scans confirmed a nearly complete loss of BF during BCCAO (RBC velocity before BCCAO 706.3 ± 113.5 $\mu\text{m}/\text{sec}$, during 0.8 ± 0.4 $\mu\text{m}/\text{sec}$, $n=9$ capillaries, $p<0.05$, one way RM ANOVA) with full recovery after reperfusion (1146.0 ± 1.1 $\mu\text{m}/\text{sec}$) (Fig. 3H). Microglia clearly lost baseline process activity during BCCAO and recovered activity within the first 60 minutes after reperfusion (Fig. 3I–K). Quantification confirmed that there were no New processes formed during BCCAO (Fig. 3M). A significant decrease in the number of the Lost processes and in the rate of the Extension and the Retraction was also observed during BCCAO (Fig. 3N–P). All parameters returned to the pre-occlusion values after 60 minutes of reperfusion. However, the Total length (Fig. 3L) and the Ext/Ret ratio were not significantly affected during BCCAO and reperfusion. All these data taken together imply that morphological microglial activation requires some residual BF because complete perfusion loss as during BCCAO and CA did not support microglial deramification.

Loss of microglial process activity does not depend on the microenvironment around distal processes

Microglial process dynamics in the cells sampled in the area-at-risk depended on the capillary BF measured within 10 μm of the somata. Therefore, we evaluated whether the capillary BF around peripheral processes (>30 μm from somata) influenced their activity. Thirty seven areas randomly selected in the area-at-risk in 11 mice were monitored

immediately after photothrombosis. In the presence of a stalled capillary, distal processes were active in 15 areas (Fig. 4A,B), while in 5 areas peripheral processes were less active. In areas where capillaries were flowing at the site of distal processes these processes were active in 11 cases, while they were less active in 6 areas (Fig. 4C,D). The Fisher exact probability test revealed that frequency of observations of active peripheral processes was not dependent on the presence of a flowing capillary around these processes ($p=0.72$).

Since dendrites are terminally injured in some areas of hemodynamic penumbra (Zhang and Murphy, 2007; Risher et al., 2010) and since microglia may actively contribute to synapse elimination (Tremblay et al., 2010; Wake et al., 2009), we assessed whether microglial distal process sensing activity depends on the presence of damaged dendrites, identified by permanent dendritic beading (Andrew et al., 2007; Zhang et al., 2005). Simultaneous imaging of dendrites and microglia in 56 areas-at-risk in 12 hybrid [YFP-H]/[CX3CR1-EGFP] mice revealed active peripheral microglial processes in 21 areas with beaded dendrites (Fig. 4E,F) and in 19 areas with normal appearing dendrites. Coincidentally, distal microglial processes were less active in 9 areas with healthy dendrites (Fig. 4G,H) and in 7 areas with beaded dendrites. Statistically, the proportion of observations of active or halted distal microglial processes was not dependent on the presence of injured or healthy dendrites around these peripheral processes ($p=0.57$, the Fisher exact probability test). All data taken together imply that distal microglial processes retain their basal level of activity even in the pathological microenvironment if somata have sufficient BF.

DISCUSSION

We investigated real-time microglial morphology and process activity in ischemic conditions using *in vivo* 2PLSM. In the hypoperfused penumbra-like area-at-risk, microglial process dynamics were linearly correlated with local BF around microglial somata. During severe BF loss around the somata, microglia displayed a reduced total length of all processes, indicating the beginning of deramification. The microglial ramified phenotype was preserved in the case of complete BF loss as during CA or transient BCCAO. Under these ischemic conditions, microglial process activity was entirely stalled and recovered after reperfusion in transient BCCAO. There was no significant relationship between microglial process activity and the microenvironment (i.e. local BF in distal capillaries and dendritic beading) surrounding peripheral processes. We propose that while the withdrawal of microglial processes is generally considered a hallmark of microglial activation, morphological manifestation of such activation requires some level of residual BF around microglial somata.

Deramification is one of the most prominent cytoarchitectural changes of activated microglia (Kreutzberg, 1996; Stence et al., 2001), but the underlying mechanisms have not yet been completely elucidated. It has been proposed that two purinergic pathways differentially regulate microglial process dynamics (Gyoneva et al. 2009). Under normal conditions, chemoattractive responses of ramified microglia to the site of microinjury is controlled by G_i -coupled purinergic $P2Y_{12}$ receptors activated by ATP/ADP released from the damaged cells (Davalos et al., 2005; Haynes et al., 2006). Under pathological conditions, activated microglia deramification appears to be mediated by the G_s -coupled purinergic A_{2A} receptors activated by adenosine (Orr et al., 2009). Extracellular adenosine levels increase several fold in the early stages of cerebral ischemia, reflecting a mismatch between degradation and synthesis of cytoplasmic ATP (Latini and Pedata, 2001; Parkinson et al., 2000; Phillis et al., 1996). Both neurons and astrocytes can release adenosine under ischemic/hypoxic conditions (Martin et al., 2007; Parkinson et al., 2002). This correlates with rapid downregulation of $P2Y_{12}$ receptor expression on activated microglia (Haynes et

al., 2006) and rapid upregulation of the adenosine A_{2A} receptor expression (Orr et al., 2009), enhancing deramification.

Remodeling of the actin cytoskeleton is a critical factor in both cellular motility and morphological changes in many different cell types (Pollard and Borisy, 2003). Microglial protrusion extension and retraction is mediated by polymerization and depolymerization of the actin filament network (Hines et al., 2009; Orr et al., 2009), which is ATP-dependent (Atkinson et al., 2004). The cortical ATP level declines rapidly to near zero within several minutes of global ischemia but stays at 50–70% of normal in the penumbra during focal stroke (Lipton, 1999). Severe energy deprivation, such as during CA or BCCAO, may result in the net unregulated polymerization of monomeric G-actin to F-actin (Atkinson et al., 2004), disrupting the actin filament dynamics and leading to a complete arrest of microglial process motility. Apparently the residual BF in the area-at-risk remains sufficient for mitochondrial ATP generation at the level required to maintain the ATP-dependent actin polymerization/depolymerization. Possibly the severe reduction of BF around the microglial somata mediates deramification via the purinergic A_{2A} receptor pathway. It is unclear what molecular pathways are involved and future studies are necessary to reveal the underlying mechanisms. The data suggests that the morphological activation of microglia requires some residual BF around the somata to support oxidative metabolism.

Intriguingly, the rate of process extension and retraction had a strong correlation with BF around the somata. Although, peripheral process motility appears independent of the capillary BF around those processes, these data should be interpreted more cautiously, because simultaneous recording of BF at the distal and proximal capillaries were not typically conducted. We do not feel this is a serious limitation since the key findings suggest that microglia with a sufficient level of BF around the somata can retain their function even in the nearby pathological microenvironment at distal sites. Our observations are consistent with recent reports identifying microglial somata and primary giant processes as sites of mitochondria location (Banati et al., 2004; de Gannes et al., 2000). From these sites energy could be supplied to drive the microglial peripheral processes that are advantageous for microglial tissue protection and surveillance. Accordingly, microglial process surveying dynamics were maintained at the sites of terminal dendritic beading, which is an early sign of acute neuronal injury and commitment to cell death (Enright et al., 2007; Hori and Carpenter, 1994; Risher et al., 2010). Microglia actively participate in the remodeling of synaptic circuitry disrupted by ischemia, possibly by tagging some synapses for elimination during prolonged direct contact after ischemic insult (Wake et al., 2009). Some distal microglial processes were less active in the presence of beaded dendrites while others retained their basal activity. Future experiments will be necessary to determine whether less active peripheral microglial processes were in direct contact with beaded dendrites, perhaps targeting their synapses for elimination.

Recovery of microglial process activity after transient BCCAO illustrates the functional and structural resiliency of microglia to ischemic conditions. This is the first study reporting the existence of the transient “dormant” state of microglia in the ischemic brain. Future long-term imaging studies are necessary to determine the fate of individual microglial cells after transient BCCAO and whether this dormant state could be the first event leading to the microglial transformation into an amoeboid state. Nevertheless, the surprising results of this study highlight an unexpected influence of local BF near the microglial somata on microglial process activity, signifying microglial activation early during ischemia as a much more dynamic and complex process than was previously anticipated.

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Figure 1.

Microglial cell dynamics vary in the hypoperfused area-at-risk in the photothrombotic model. **A**, Blood vessels visualized below the thinned skull imaging window in the mouse somatosensory cortex. Blood plasma was fluorescently labeled with Texas Red dextran (70 kDa). Drawing shows the perimeter area “a” to be illuminated with 514 nm laser beam during bolus injection of RB. Within the perimeter “a”, RB mediates photothrombotic occlusion of blood vessels. The area-at-risk within the central zone “b” ($1070 \times 1070 \mu\text{m}$) was not exposed to the laser light. 2PLSM imaging of microglia was performed within this area “b”. **B**, Control grayscale image of laser speckle contrast of cortical vasculature shown in (A) reveals flowing blood vessels that appear dark. **C**, Loss of blood flow immediately after RB photoactivation for 10 min. A decline of BF in the imaging field is clearly seen. Scale bar in B is for B–D. **D**, Pseudocolored image of relative CBF reveals that BF in the central area-at-risk remains above 50% immediately after photothrombosis. To facilitate comparison, grayscale laser speckle contrast image was overlaid with thresholded image where blood flow less than 50% of control appears as various shades of blue (see calibration bar). **E–G**, Control microglia morphology and dynamics before photothrombosis. MIPs of image stacks acquired during time-lapse recording at the beginning (E) and 20 minutes later (F) are overlaid in (G). Overlay image shows abundant extension (green) and retraction (red) of microglial processes. Scale bar in E is for E–G. **H–I**, Activity of individual microglial cells is altered in the area-at-risk in the first 20 minutes after photothrombosis. Overlays showing the merged images of microglia captured at 0 and 20 minutes following photothrombotic occlusion reveal extension (green) and retraction (red) of microglial processes. Example of microglia with retained process activity is presented in (H) and with lost process activity is shown in (I). Scale bar in I is for H–I.

Figure 2.

Microglia activity depends on local blood flow around cell body. **A**, MIP image showing microglia (green) and capillary (red; blood plasma labeled with Texas Red dextran) from which BF was recorded (white arrow). Striking within capillaries is a sign of a flowing blood vessel caused by scanning of moving non-fluorescent RBC. BF near ($<10\ \mu\text{m}$) microglial somata was recorded by repetitive line scans before and after photothrombosis. Arrow indicates the position and direction of the line scan. **B**, Stack of line scans from the capillary shown in (A) before (left) and after photothrombosis (PT) (right) with moving RBC represented by dark bands. The inverse of the slope of these bands is proportional to the instantaneous RBC velocity calculated as $\Delta x/\Delta t$ (inset). **C**, RBC velocity calculated from line scans in (B) is showing a BF decrease after photothrombosis. Arrows indicate the time points corresponding to line scans shown in (B). Red bar indicates timing of photothrombosis. Values are shown as percent of baseline RBC velocity before stroke. Under control conditions cardiac contributions primarily underlie fluctuations of RBC velocity, but post-occlusion fluctuations are likely to reflect fast redistribution of upstream BF after photothrombosis (Schaffer et al., 2006). **D–I**, Paired overlay images of microglia acquired before (D, F, H) and after (E, G, I) photothrombosis showing the effect of changes in capillary BF around microglial somata on extension (green) and retraction (red) of microglial processes. Overlay images were created by merging MIP images captured at 20 minute time intervals. Following severe loss of capillary BF (E) (61–100% loss of control RBC velocity) microglial processes were shorter and their activity was decreased when compared to control image (D). Moderate loss of capillary BF (G) around somata (12–32% loss of control RBC velocity) had no noticeable effect on process activity as compared to control (F). Increase in capillary BF (I) (32–136% increase of control RBC velocity) also produced no visible changes in the activity of microglial processes when compared to pre-stroke image (H). Scale bar in D is for D–I. **J–O**, Summary of 20 microglial cells from 12 animals showing a significant effect of severe loss of capillary BF around somata on microglial activity. When compared to control, a severe reduction in BF resulted in a

significant decrease of the Total length of processes (J), decline in New processes (K), slower average speed of process Extension (M) and Retraction (N), and a drop in the Ratio of the Extension to the Retraction speed (* $P < 0.05$, One way ANOVA; $^{\dagger}P < 0.05$, Kruskal-Wallis ANOVA on Ranks). **P, Q**, Motility of microglial processes strongly correlated with the local capillary BF around cell body ($r = 0.68$, $P < 0.01$ for the Extension and $r = 0.61$, $P < 0.01$ for the Retraction; regression lines shown).

Figure 3.

Microglia activity during global ischemia is determined by blood flow. **A–C**, CA immediately stalled all processes. Each overlay image of microglia was produced by merging MIP images acquired with 20 minute time intervals. Processes were highly active with abundant extension (green) and retraction (red) just prior to CA (**A**) and then virtually lost all activity during the first 0–20 minutes after CA (**B**), that is also evident at later time points (40–60 minutes after CA) (**C**). Scale bar in **A** is for **A–C**. **D**, Summary of 7 microglial cells from 5 mice showing no change in Total length of processes before and 20 minutes after CA. **E–G**, MIP image sequence of microglial cell and capillary before (**E**), during (**F**) and after (**G**) BCCAO showing complete loss of BF during occlusion (**F**) and recovery during reperfusion (**G**). Scale bar in **E** is for **E–G**. **H**, RBC velocity calculated from line scans along 9 capillaries in 5 mice demonstrating a nearly complete loss of BF during BCCAO as well as successful reperfusion (* $P < 0.05$, one way RM ANOVA). **I–K**, Overlay image sequence of microglial cell before (**I**), during (**J**) and after (**K**) BCCAO. Overlay images were created by merging MIP images captured with 20 minutes time interval. Process activity seen before BCCAO (**I**) is extremely decreased during BCCAO (**J**), and then recovers 40–60 minutes after BCCAO (**K**). Scale bar in **I** is for **I–K**. **L–P**, Summary from 7 microglial cells in 4 animals revealing loss and recovery of microglial processes activity during and after BCCAO. There was no significant change in Total length of processes (**L**) during the experiment, but there were significant changes in the number of New (**M**) and Lost (**N**) processes and average speed of process Extension (**O**) and Retraction (**P**) (* $P < 0.05$, one way RM ANOVA, $^{\dagger}P < 0.05$, Friedman RM ANOVA on Ranks).

Figure 4.

Microenvironment around distal microglial processes does not influence their activity. **A–B**, Single plane image sequence of peripheral microglial processes (green) and stalled capillary (red) in the area-at-risk imaged at 76 and 97 minutes after photothrombosis. Microglial processes are active despite complete loss of BF. Scale bar in A is for A–D. **C–D**, Another image sequence of distal process (arrowhead, green) and flowing capillary (red) in the area-at-risk following 36 and 49 minutes after photothrombosis. Process activity is seized even in the presence of a flowing capillary. **E–F**, Distal processes (arrowhead) are active around a beaded dendrite (arrow) at 156 and 161 minutes after photothrombosis. Scale bar in E is for E–H. **G–H**, Halted processes (arrowhead) seen in close apposition to the normal appearing dendrite (arrow) at 161 and 167 minutes after photothrombosis.