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REAL-TIME ANALYSIS OF MICROGLIAL ACTIVATION AND MOTILITY IN HEPATIC AND HYPERAMMONEMIC ENCEPHALOPATHY

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Abstract

Hepatic encephalopathy (HE) is a potentially fatal complication of acute liver failure, associated with severe neurological dysfunction and coma. The brain's innate immune cells, microglia, have recently been implicated in the pathophysiology of HE. To date, however, only *ex vivo* studies have been used to characterize microglial involvement. Our study uses *in vivo* two-photon imaging of awake-behaving mice expressing enhanced green fluorescent protein (eGFP) under the *Cx3cr1* promoter to examine microglial involvement in two different models of encephalopathy – a slower, fatal model of azoxymethane-induced HE and a rapid, reversible acute hyperammonemic encephalopathy (AHE) induced by an ammonia load. To investigate the potential contribution of microglia to the neurological deterioration seen in these two models, we developed a software to analyze microglial activation and motility *in vivo*. In HE, we found that microglia do not become activated prior to the onset of neurological dysfunction, but undergo activation with mildly impaired motility during the terminal stage IV. We demonstrate that this microglial activation coincides with blood–brain barrier (BBB) opening and brain edema. Conversely, both microglial activation and motility are unchanged during AHE, despite the mice developing pathologically increased plasma ammonia and severe neurological dysfunction. Our study indicates that microglial activation does not contribute to the early neurological deterioration observed in either HE or AHE. The late microglial activation in HE may therefore be associated with terminal BBB opening and brain edema, thus exacerbating the progression to coma and increasing mortality.

Keywords

azoxymethane; ammonia; microglia; two-photon; blood–brain barrier

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INTRODUCTION

HE is an important complication of acute liver failure that is primarily characterized by neurological dysfunction, accompanied with elevated blood ammonia (hyperammonemia) (Butterworth, 2011). Astrocytes are thought to be the main target of HE pathology as they possess the only enzyme in brain capable of removing ammonia (Martinez-Hernandez et al., 1977). Though astrocytes have been the main emphasis of study in HE, a cell type that has recently come into focus is microglia (Rodrigo et al., 2010; Butterworth, 2011; Zemtsova et al., 2011). Microglia have a unique role in the central nervous system (CNS); being resident immune cells that have both beneficial and harmful effects (Fuhrmann et al., 2010; Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Vinet et al., 2012). When activated, microglia take on an ameboid appearance that is thought to facilitate phagocytosis of both damaged and healthy cells. Activated microglia also secrete various cytokines to further amplify the inflammatory response in the CNS (Ransohoff and Cardona, 2010; Kettenmann et al., 2011; Prinz et al., 2011). Microglial activation and signaling are emerging topics that have been shown to affect disease progression in several conditions, such as Alzheimer's and Parkinson's disease, multiple sclerosis, sepsis, ischemia and neuropathic pain (Jack et al., 2005; Liu, 2006; Schwartz et al., 2006; Mount et al., 2007; Biber et al., 2011; Hayashi et al., 2011). Additionally, recent studies have shown that through the release of various mediators (such as ATP and brain-derived neurotrophic factor), activated microglia can alter synaptic transmission (Coull et al., 2005; Pascual et al., 2012). They may therefore constitute new players in brain disorders involving neuro-inflammation and/or cognitive impairment.

Previous studies have shown that a systemic and local inflammatory response and the release of multiple inflammatory cytokines worsen prognosis in patients and experimental HE (Bemeur et al., 2010a; Rodrigo et al., 2010; Butterworth, 2011; Zemtsova et al., 2011). A question that remains unexplored, however, is whether microglial dysfunction precedes and contributes to the initial neurological impairment seen in HE. In the late stages of HE, microglial activation has been demonstrated using ex vivo methods and several animal models (bile duct ligation, hepatic devascularization, portocaval shunts and chronic hyperammonemia), as well as in post mortem tissue from HE patients (Jiang et al., 2009b; Rodrigo et al., 2010; Agusti et al., 2011; Zemtsova et al., 2011). Additionally, the anti-inflammatory agents minocycline, N-acetylcysteine and ibuprofen have also been shown to improve outcome in HE (Jiang et al., 2009b; Bemeur et al., 2010b; Rodrigo et al., 2010). However, HE induced by portal vein ligation is not associated with microglial activation, despite this model causing significant hyperammonemia and neurological impairment (Bruck et al., 2011). Thus, another question that remains unanswered is to what extent isolated hyperammonemia contributes to any activation of microglia that might be present in HE.

A possible explanation for the inconsistent results regarding microglial involvement in HE is the extensive use of ex vivo methods to quantify microglial activation. There is a clear need for *in vivo* imaging to complement previous studies and further explore the role of microglia in HE and their contribution to the onset of symptoms. However, anesthetics that are commonly used for *in vivo* imaging pose a major difficulty in studying HE, as they induce a similar CNS depression as HE (Marchetti et al., 2011). To test our hypothesis that microglial dysfunction precedes neurological impairment in HE, we therefore combined *in vivo* imaging of awake-behaving mice with two different models of encephalopathy – a rapid, reversible acute hyperammonemic encephalopathy (AHE) and a slower, fatal azoxymethane (AOM)-induced HE (Matkowskyj et al., 1999; Belanger et al., 2006). This allowed us to

determine whether microglia are activated prior to or after neurological deterioration, and whether hyperammonemia alone is sufficient to cause any microglial changes.

In our study, we find that microglia are only activated in the terminal stage IV of HE and not at all during isolated hyperammonemia or early stages of HE. Our data therefore indicate that microglial activation does not directly contribute to the initial neurological dysfunction seen in these two models. However, we confirm previous studies showing that microglia are involved in the terminal stage of HE, which is also characterized by blood–brain barrier (BBB) opening and brain edema (Belanger et al., 2006; Nguyen et al., 2006; Bemeur et al., 2010a). It is therefore conceivable that microglial activation may exacerbate these terminal changes, thereby contributing to mortality in HE.

EXPERIMENTAL PROCEDURES

Animal preparation for *in vivo* imaging in awake-behaving mice

C57BL/6J and heterozygous *Cx3cr1-eGFP* male mice 8–12 weeks were obtained from Jackson Laboratories. The mouse preparation was modified from published protocols (Dombeck et al., 2007; Greenberg et al., 2008). Briefly, mice were anesthetized using isoflurane (1.5% mixed with 1.5 L min⁻¹ O₂) and a custom-made steel mini-frame was fastened to their skull using cyanoacrylate-based glue allowing the animals to be head-restrained. Additionally, as hypothermia has been shown to improve outcome in AOM-induced HE, all the AOM-injected mice were also kept normothermic (36.5–37.5 °C) between and during experiments using heating pads. Blood glucose concentrations were also monitored and kept within normal limits using sterile 10% subcutaneous dextrose when necessary. The mice were then habituated to the head restraint and the setup over 2 days, during which they were briefly anesthetized with isoflurane to aid placement on the set-up. On the day of imaging, a 1.5 mm craniotomy was opened over the cortex carefully leaving the dura intact, and applying a coverslip. The mice were imaged on average 1 h after the craniotomy was performed. The AOM-injected mice were in addition re-imaged on the set-up at stage I and stage IV of hepatic encephalopathy. Both the AOM (100 µg/g from Sigma, St. Louis, CO, dissolved in 100 µL saline) and NH₄Cl (7.5 mmol kg⁻¹, 0.64 M dissolved in 0.2–0.3 ml of saline and pH adjusted to between 7.35–7.45) were injected intra-peritoneally (i.p.) (Belanger et al., 2006). Control animals received equivalent volumes of isotonic saline. All animal experiments were approved by the Animal Care and Use Committee of the University of Rochester.

In vivo two-photon laser scanning microscopy (2PLSM)

A Mai Tai laser (SpectraPhysics) attached to a confocal scanning system (Fluoview 300, Olympus) and an upright microscope (IX51 W) were used. For activation index (AI), we used a 20× objective (0.95 NA) with 3× additional optical zoom, laser power <25 mW to avoid photodamage and collected images 75–125 µm below the pial surface. Enhanced green fluorescent protein (eGFP) was excited at 890 nm and emission collected at 575–645 nm. Photomultiplier tube sensitivity and laser power were kept at similar levels to ensure comparable signal-to-noise levels in the images. Imaging sessions lasted no longer than 1.5 h. The analysis for microglial AI was performed on automatically thresholded xyz image stacks (10–15 frames, 2 µm z-resolution), with pre-defined region interest encompassing a single microglia, 512 × 512 pixels per frame (0.27 µm/pixel), and analyzed on a frame-to-frame basis (not on a collapsed image to avoid inaccuracy). The program scans each line of the frame horizontally and vertically to count the number of times the pixels change from black (gaps) to white (processes or soma). The horizontal and vertical transition counts are then averaged, and normalized to the mean at time 0 to obtain a percentage change in activation. Turnover-rate (TOR) was quantified using time-lapse xyz image stacks (2 µm z-

resolution; Δt : 5 min) with 512×512 pixels per image frame. The analysis was performed as described previously (Fuhrmann et al., 2010). Briefly, we calculate the sum of pixels lost and gained over time for single microglia within a thresholded region of interest, and divide this sum by the total area occupied by the microglia. Though this method has many advantages, a limitation of *in vivo* 2PLSM studies of microglia is that only cortex can be imaged, and not deeper structures.

Modified encephalopathy score

A modified encephalopathy score adapted from previous publications was used to quantify the onset of the various stages of encephalopathy (Matkowskyj et al., 1999). This encephalopathy score relies on a behavioral scoring of the mice, where reduced spontaneous movement (passive) signified the prodromal stage, lethargy and a loss of the scatter reflex stage I, the onset of ataxia stage II, the loss of righting reflex stage III, and the loss of corneal reflex and coma stage IV. Animal movements were assessed using automated (continuous) video analysis (ANY-maze, Stoelting), and this was validated manually. *Cx3cr1-eGFP* mice were compared to WT mice using the modified encephalopathy score and ANY-maze, with no significant differences found following NH_4Cl or AOM injection.

Ammonia assay

50- μL heparinized tubes were used to collect blood from the femoral artery at baseline, 15, 30 and 60 min following ammonia injection and at baseline, stage I and stage IV of HE. A L-glutamate dehydrogenase (GDH)-based kit from Sigma was used for the quantification of plasma ammonia. Ammonia reacts with α -ketoglutaric acid (α -KGA) and nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of GDH to form L-glutamic acid and NADP^+ . The reduced light absorbance at 340 nm can then be measured in a spectrophotometer and is proportionate to the amount of ammonia available for oxidizing NADPH.

Electroencephalogram (EEG) and electromyogram (EMG) recordings

Wireless EEG and EMG electrodes were used (DSI Physiotel®). EEG electrodes were implanted epidurally, into a 0.5 mm craniotomy created using a dental drill, and secured with dental cement. EMG electrodes were implanted in the sternocleidomastoid muscles of the neck, and secured using nylon sutures. Recordings were analyzed offline using NeuroScore (DSI). Myoclonic seizures were defined on the EEG as single or multiple 3–9 Hz polyspike and wave discharges of 0.2–2 s duration, associated with myoclonic jerks determined by video recording, EMG and direct observation. EEG changes characteristic of later stages of HE were detected as triphasic high-amplitude slow-frequency discharge typical of metabolic coma states, and a reduction in EMG discharge reflecting the cessation of spontaneous movement.

^1H -NMR spectroscopy

Mice were anesthetized using 1.5% isoflurane then decapitated. A quick extraction of the forebrains (minus the cerebellum and brainstem) was performed. The brains were then frozen in liquid nitrogen and homogenized in 7.5 mL 12% PCA at 0 °C using a micro-sonicator. The homogenates were then centrifuged (15 min at 25000G), after which the supernatants were adjusted to pH 7.0 using KOH over an ice bath. They were then centrifuged once again, which separated the supernatant from KClO_4 . The supernatant was lyophilized and then reconstituted with 0.65 mL D_2O . ^1H -NMR spectra were acquired using a 600 MHz Varian UnityINOVA spectrometer equipped with a triple-axis gradient HCN probe (standard room temperature). Signals were acquired following a 90° pulse with a spectral width of 7200 Hz and 32 K data points. The time between pulses was 15 s and 64

signals were averaged for each spectrum. The sample temperature was 25 °C. Integrals of the relevant peaks were converted to $\mu\text{mol g}^{-1}$ wet weight and normalized NAA (methyl) to increase inter-sample consistency.

Histology of both cerebral hemispheres

After imaging *in vivo* at 0, 6 and 16 h, mice were anesthetized with isoflurane (1.5%) and immediately perfused transcardially with phosphate-buffered saline followed by 4% paraformaldehyde in phosphate-buffered saline (pH 7.4). The brains were dissected out and *Cx3cr1*-eGFP microglia in both the hemispheres with and without a craniotomy were immediately imaged using the 2PLSM set-up described above. This method was used to 'freeze' microglial morphology in the 'naïve' contralateral hemisphere that had not previously been subjected to craniotomy surgery or 2PLSM imaging. Thus, we could examine whether the craniotomy or imaging by themselves generated changes in microglial AI.

BBB permeability

Evans blue-albumin extravasation was used to assess BBB permeability. 2% Evans blue in phosphate-buffered saline pre-tagged to 4% albumin was used. 4 ml kg^{-1} mouse body weight was injected intravenously via a femoral vein catheter, and was allowed to circulate for totally 1 h. Care was taken to ensure that circulation time was accurate between animals. 5 min after the Evans blue-albumin injection, 7.5 mmol kg^{-1} NH_4Cl was injected i.p., alternatively saline (controls) or mannitol (positive control, data not shown). For the AOM experiments, the drug was injected the day before such that BBB permeability could be assessed at stage IV HE. The mice were then anesthetized with ketamine (0.12 mg g^{-1})/xylazine (0.01 mg g^{-1}), transcardially perfused and the brains extracted (minus the olfactory bulbs, cerebellum and brainstem) and weighed for later normalization. The brains were subsequently homogenized equally and centrifuged at 4 °C (15000 rpm for 15 min). Optical density was measured using spectrophotometry at 610 nm and the results compared to a previously made standard optical density curve.

Brain water content

Brain edema was assessed using wet-to-dry ratios of brain weight as described previously (Haj-Yasein et al., 2011). Briefly, mice were anesthetized using ketamine (0.12 mg g^{-1})/xylazine (0.01 mg g^{-1}), decapitated and their forebrains rapidly extracted. The brains were then placed on small pre-weighed petri dishes, and immediately weighed to obtain an estimate of wet brain weight. The brains were then completely dried in an oven (72 h at 70 °C), before being re-weighed to obtain dry weight. WDR was calculated by taking wet brain weight – dry brain weight/wet brain weight.

Statistics

All analysis was performed using IBM SPSS Statistics 19 and all tests were two-tailed where significance was achieved at $\alpha = 0.05$ level. Where $n \geq 10$ for normally distributed data, the unpaired *t* test was used for independent samples, and paired *t* test for paired samples. For independent samples where $n < 10$ and non-parametric the Mann–Whitney *U* test was used.

RESULTS

AHE is characterized by a rapid progression of neurological deterioration and myoclonic seizures

We chose a rapidly progressing, reversible model of AHE to determine whether microglia contribute to the onset of ammonia-related neurological symptoms, including myoclonic seizures. This allowed us to test whether microglia respond to hyperammonemia directly, as prolonged elevations of ammonia might result in activation due to secondary cell injury or death (Zemtsova et al., 2011). We first characterized the model using a modified encephalopathy score to record onset of symptoms (Matkowskyj et al., 1999). To record the myoclonic seizures, both extracellular field potential, wireless epidural EEG and EMG electrodes were used (Fig. 1a). We induced AHE by injecting $7.5 \text{ mmol kg}^{-1} \text{ NH}_4\text{Cl}$ i.p. Following the injection the mice exhibited an acute progression of symptoms; becoming passive, lethargic and developing ataxia (prodromal to stage II) accompanied by myoclonic seizures. The neurological impairment was reversible and the mice recovered completely by $17.94 \pm 1.8 \text{ min}$ (Fig. 1a, Table 1).

We next measured the extent of plasma ammonia ($[\text{NH}_4^+]$) increase, and changes in several brain amino acid concentrations using an enzymatic ammonia assay and $^1\text{H-NMR}$, respectively. AHE was associated with a rapid increase in plasma $[\text{NH}_4^+]$ from 0.084 ± 0.013 to $1.69 \pm 0.20 \text{ mM}$ (Fig. 1b). Consistent with the literature, AHE induced a significant increase in glutamine, decrease in glutamate and had no effect on GABA (Table 2) (Ratnakumari et al., 1994; Belanger et al., 2006). As changes in microglial morphology have previously been linked to BBB opening and brain edema, we also measured these two parameters in our model (Lynch et al., 2004; Wu et al., 2010). Using the Evans blue-albumin extravasation technique and wet-to-dry brain weight measurements, we found no change in BBB permeability (3.54 ± 0.19 compared to $3.82 \pm 0.14 \text{ } \mu\text{g Evans blue-albumin/g brain}$ during control and AHE, respectively) or brain edema ($77.6 \pm 0.0017\%$ and $77.5 \pm 0.0013\%$ brain water during control and AHE, respectively) (Fig. 1c, d). Taken together, our AHE model displayed a rapid deterioration in neurological function with the onset of myoclonic seizures and increased plasma $[\text{NH}_4^+]$. These features, however, were reversible and not associated with any BBB opening or brain edema.

Isolated hyperammonemia is not associated with microglial activation or dysmotility

We proceeded to determine whether AHE affects microglia prior to or after the onset of neurological symptoms. We used 2PLSM to image microglia in mice expressing eGFP under the *Cx3cr1* promoter (Fig. 2a). *CX3CR1* is a G-protein-coupled receptor involved in adhesion and migration of microglia and other immune cells (Lee et al., 2010).

'Activated' or 'reactive' microglia take on an amoeboid appearance where their processes thicken and become fewer (less complex), thought to facilitate phagocytosis (Zemtsova et al., 2011). This change has previously been used in histological studies of HE to get a quantitative, but static 2-dimensional measure of microglial activation (Rodrigo et al., 2010; Agusti et al., 2011). We developed a MatLab-based program to measure the degree of microglial activation in real-time. The software carries out the analysis on a frame-by-frame basis using a 3-dimensional xyz-stack with a pre-defined region of interest encompassing individual microglia, and is automatically thresholded. We avoided the use of a collapsed z-stack to increase accuracy. The program counts the number of black gaps, or transitions, between processes by scanning each line of the image horizontally and vertically to find the number of times the pixels change from black to white. The horizontal and vertical counts are then averaged. The count from each cell was normalized to the mean count at time frame 0 (Fig. 2b). Using this analysis, a resting microglia with complex processes would have

higher mean transition counts than an activated amoeboid cell. To convey these results more intuitively, a decrease in transition count was represented as a percentage increase in AI relative to time 0. Using this approach we were able to track the progression of individual microglia from resting to activated over time *in vivo*.

We found that AHE caused no change in AI during the entire 1 h recording session after ammonia administration (Fig. 2c, d). As an additional control, we tested whether the cranial window by itself increased AI, and found no evidence of this as long as the cranial window was carefully prepared with intact dura mater and of a small diameter (1.5 mm) (Fig. 2h, i) (Takata and Hirase, 2008).

We next wanted to investigate whether dynamic microglial function is altered in AHE. Previous *in vivo* studies of microglia found that these cells have highly motile processes that constantly survey their microenvironment (Nimmerjahn et al., 2005; Fuhrmann et al., 2010). The turnover rate (TOR) of microglial processes is a measure of motility. TOR is thought to reflect microglial capability to survey the brain microenvironment for potential pathogens (Fuhrmann et al., 2010). Using a custom-made MatLab software, we measured TOR by overlapping consecutive 2PLSM image z-stacks collected every 5 min to determine the gain and loss of pixels. The sum of these pixels was divided by the total number of pixels occupied by the microglial cell (Fig. 2f). We found no significant change in TOR per cell during the 1 h recording of AHE (Fig. 2g). We additionally quantified microglial density to explore whether AHE causes any net loss or gain of microglia. There was, however, no change in cell density indicating that there is no infiltration of peripheral leukocytes or loss of microglia during this disease process (Fig. 2h). Taken together, these *in vivo* data indicate that microglia do not contribute significantly to the neurological symptoms and myoclonic seizures seen in AHE.

HE induces a slow but fatal decline in neurological function, with late onset BBB opening and brain edema

We proceeded to investigate whether microglia are involved in the slower deterioration of neurological function seen in HE. We induced HE by injecting the liver toxin AOM (100 $\mu\text{g/g}$ i.p.). This mouse model of HE has previously been well characterized and is highly reproducible (Matkowskyj et al., 1999; Belanger et al., 2006). Additionally, AOM-induced HE is known to be associated with a significant release of pro-inflammatory cytokines during stage IV and is therefore a relevant model for studying microglial involvement in HE (Bemeur et al., 2010a,b). To characterize the timescale of the neurological dysfunction in HE, we used the same modified encephalopathy score and electrode recordings described above. HE caused a slower decline in neurological function, with the mice passing through the prodromal stage (passive) of encephalopathy and all four subsequent stages (lethargy, ataxia, loss of righting reflex and coma, Fig. 3a, Table 1). During the terminal coma stage IV, the mice displayed a triphasic EEG pattern typical of metabolic encephalopathies. If no intervention was given the coma would worsen, leading to death at 17.14 ± 1.3 h. We subsequently quantified the plasma $[\text{NH}_4^+]$ increase. HE was associated with a progressive increase in plasma $[\text{NH}_4^+]$ from baseline (0.074 ± 0.0098 mM), to stage I (0.17 ± 0.024 mM) and finally to stage IV (0.63 ± 0.067 mM) (Fig. 3b). We then measured the concentrations of brain glutamine, glutamate and GABA, and found a similar pattern to that of AHE (Table 2) (Ratnakumari et al., 1994; Belanger et al., 2006).

Various models of HE, including AOM, have previously been shown to cause BBB opening and brain edema (Butterworth, 2002; Belanger et al., 2006; Nguyen et al., 2006; Butterworth et al., 2009; Bemeur et al., 2010a). Consistent with these results, we detected an increased extravasation of Evans blue-albumin during stage IV (5.16 ± 0.39 $\mu\text{g g}^{-1}$ brain), indicating an opening of the BBB, but found no change during stage I (3.49 ± 0.21 vs control $3.48 \pm$

0.16 μg Evans blue-albumin g^{-1} brain) (Fig. 3c). Additionally, we found that only stage IV HE was associated with an increase in brain water content ($78.3 \pm 0.0014\%$ brain water at stage IV compared to $77.5 \pm 0.00095\%$ in controls) (Fig. 3d). In conclusion, HE induced a slow but irreversible decline in neurological dysfunction, with BBB opening and brain edema during the terminal stage IV of the disease.

Microglial activation and dysmotility is only present in the terminal stage of HE

To explore whether microglia contribute to the neurological dysfunction seen during HE, we used the same 2PLSM-imaging protocol of *Cx3cr1*-eGFP mice as described above (Fig. 4a). We imaged these mice at baseline and during stage I and the terminal stage IV of HE. Equivalent control imaging experiments were performed on mice receiving i.p. injections of isotonic saline. HE caused a non-significant decrease in AI from $0.00 \pm 1.78\%$ at baseline to $-1.46 \pm 1.39\%$ at stage I. This was followed by a significantly increased AI at stage IV to $6.77 \pm 2.42\%$ compared to control (Fig. 4b, c).

We then investigated whether HE was associated with altered microglial motility, quantified using TOR. We found that HE caused a slight but non-significant increase in TOR per cell during stage I ($6.01 \pm 0.93\%$ compared to the 6 h control of $4.67 \pm 0.71\%$). However, during stage IV HE, TOR per cell decreased significantly to $2.83 \pm 0.5\%$ compared to the 16 h control of $4.92 \pm 0.86\%$ (Fig. 4d). HE also caused no change in microglial density (Fig. 4e).

Taken together, our *in vivo* data indicate that HE induces microglial activation in the terminal stage IV but we did not detect any changes prior to the onset of neurological deterioration. Additionally, the microglial activation we observed cannot be attributed solely to hyperammonemia, as AHE induced no microglial changes. Finally, we found a slight decrease in the surveying capacity of microglia during stage IV in HE, which likely represents cellular dysfunction.

DISCUSSION

This is the first study to investigate microglial activation and motility in HE and AHE *in vivo* using 2PLSM in awake mice. Microglial activation was only present in stage IV of HE, coinciding with BBB opening and brain edema. The involvement of microglia late in the disease would imply that these cells are not responsible for the neurological dysfunction seen in AHE or the earlier stages of HE. It is possible, however, that the late microglial changes in HE may exacerbate potentially fatal brain edema and therefore increase mortality by setting up a vicious cycle of inflammation and BBB leakage. Intervention aimed at inhibiting the microglial activation and response could therefore prevent a fatal outcome in HE (Jiang et al., 2009a; Rodrigo et al., 2010; Agusti et al., 2011; Butterworth, 2011).

Several *ex vivo* and *post mortem* studies have shown that HE is strongly associated with the release of numerous cytokines, causing both a systemic and local (CNS) inflammatory response (Jiang et al., 2009a; Rodrigo et al., 2010; Zemtsova et al., 2011). It is possible that the systemic inflammatory response is responsible for the initial opening of the BBB and the activation of microglia shown in ours and previous studies (Rodrigo et al., 2010; Zemtsova et al., 2011). On the other hand, a recent *ex vivo* study in patients with HE due to liver cirrhosis has shown that microglia can be activated prior to any increase in pro-inflammatory markers (Zemtsova et al., 2011). Hence during HE, microglia might also be responding to local signals within the brain and may initiate the sequence of neuro-inflammation and BBB opening as a result. Once activated, microglia would be expected to amplify the inflammatory response in the brain, increasing vascular permeability and worsening edema (Bemeur et al., 2010a; Kettenmann et al., 2011). Our results also indicate that microglial motility and consequently surveying capacity may be reduced during stage

IV of HE. Consistent with this observation, cultured microglia exposed to ammonia for longer periods also display retracted processes and defective phagocytosis (Zemtsova et al., 2011). Microglial dysfunction might thus add further insult to the injury in HE, and the recently described microglial modulation of synaptic transmission may also be altered (Coull et al., 2005; Pascual et al., 2012). Microglial activation and dysfunction may therefore be critical changes contributing to the fatal final pathway of HE.

One of the aims in our study was to examine whether isolated acute hyperammonemia is sufficient to activate microglia. Several *ex vivo* studies have recently shown that chronically elevated ammonia (>6 h) causes microglial activation and neuro-inflammation (Rodrigo et al., 2010; Zemtsova et al., 2011). However, the late occurrence of activation implies that the microglial changes might be secondary to cell injury or death, and not a direct response to ammonia. Our AHE model resulted in an acute, reversible hyperammonemic encephalopathy with a moderate increase in plasma ammonia to ~1.7 mM. We did not detect any microglial activation or change in motility, despite the mice experiencing severe symptoms including seizures. However, a limitation of the AI and TOR analysis is that they may not be able to detect small changes in microglial activation and motility. Additionally, in this study we use *in vivo* imaging to detect dynamic changes in microglial morphology in HE and AHE, and thus we cannot exclude a subtle early involvement of microglia in these two conditions (for example changes in gene expression). However, we provide evidence that in the acute setting ammonia alone is not sufficient to activate microglia, and acute ammonia neurotoxicity is not dependent on microglial changes, BBB opening or brain edema to provoke symptoms.

In conclusion, we demonstrate that microglia are not involved in the initial neurological deterioration of both AHE and HE, but HE is associated with both activation and reduced motility of microglia in stage IV of the disease. Using *in vivo* imaging, we were thus able to examine microglial changes in a clinically relevant setting, while the mice were awake and undergoing symptoms of encephalopathy. Further research is warranted to elucidate the proximal cause of the initial neurological dysfunction seen in the two models tested in this study. Additionally, the specific role of microglia in terminal disease pathway of HE needs to be explored, which may create more opportunities to develop targeted therapies for this potentially fatal disease (Jiang et al., 2009a; Agusti et al., 2011).

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Abbreviations

AHE	acute hyperammonemic encephalopathy
AI	activation index
AOM	azoxymethane
ATP	adenosine-5'-triphosphate
BBB	blood–brain barrier
CNS	central nervous system
EEG	Electroencephalogram

eGFP	enhanced green fluorescent protein
EMG	Electromyogram
GABA	gamma-aminobutyric acid
GDH	L-glutamate dehydrogenase
HE	hepatic encephalopathy
¹H-NMR	¹ H nuclear magnetic resonance
i.p.	intra-peritoneal
NADP	nicotinamide adenine dinucleotide phosphate
TOR	turn-over rate
α-KGA	α-ketoglutaric acid
2PLSM	two-photon laser scanning microscopy

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Fig. 1.

Characterization of the acute hyperammonemic encephalopathy (AHE) model. (a) AHE was induced by administering $7.5 \text{ mmol kg}^{-1} \text{ NH}_4\text{Cl}$ intraperitoneally (i.p.). A timescale for the onset of neurological symptoms classified using a modified encephalopathy score is shown: prodromal (lethargy), I (lethargy/loss of scatter reflex), II (ataxia), III (loss of righting reflex), IV (coma/loss of corneal reflex). Electroencephalogram (EEG, placed epidurally) and electromyogram (EMG, placed in neck musculature) electrodes were placed with wireless transmitters to allow for free movement of the mice. Representative EEG and EMG traces are shown from baseline and after NH_4Cl injection when the mice were experiencing symptoms. NH_4Cl results in myoclonic seizures with typical poly-spike and wave discharges seen on EEG. $n = 6$. (b) NH_4Cl -induced AHE causes a rapid and large increase in plasma $[\text{NH}_4^+]$. * $P < 0.0001$, $n = 10$, paired t test. (c) AHE causes no opening in blood–brain barrier (BBB), measured by extravasated Evans blue-albumin. $n = 5$ for both groups, ns = not significant ($P = 0.15$), Mann–Whitney U test. (d) AHE does not change brain water content, measured by the dry-to-wet weight ratio. $n = 10$ (control) and 6 (AHE), ns = not significant ($P = 0.59$), Mann–Whitney U test. Data are shown as mean $\pm$ SEM.

Fig. 2.

in vivo two-photon laser scanning microscopy (2PLSM) reveals no microglial activation or dysmotility in AHE. (a) Diagram illustrating the 2PLSM imaging setup used to examine microglial involvement in AHE. Representative microglial images illustrate the morphology of a normal resting microglia (top right) and an activated microglia (bottom right). Activated microglia take on an ameboid appearance where the process thicken and become fewer. Scale bars represent 10 μm . (b) Diagram illustrating the software analysis program used to measure the degree of microglial activation. The activation index (AI) is based on counting transitions from black (gaps) to white (processes) pixels by scanning each frame of a thresholded xyz-stack horizontally and vertically within a pre-defined region of interest encompassing a single microglia. This gives an indication of how ameboid the microglia become. High magnification images show that resting microglia (top right) have a higher mean count than activated ameboid microglia (bottom right), which is represented as a percentage increase in AI normalized to the time 0 frame. Scale bars represent 5 μm (left image) and 3 μm (right high magnification images). (c) Representative 2PLSM images during AHE at 0, 30 and 60 min after injection show no change in microglial activation (collapsed z-stacks). Scale bar represents 15 μm . (d) Quantification of AI during AHE shows no significant change. $P > 0.05$, $n = 30$ cells from 6 mice for each group, unpaired t test. (e) Microglial motility can be measured using TOR. Each z-stack is compared to the previous one with a time interval of 5 min. The sum of pixels lost (red) and gained (green) indicates microglial motility (TOR). Scale bar represents 5 μm . (f) Quantification of microglial TOR per cell during AHE reveals no significant change in motility. Each data point represents the mean TOR per cell over a 30-min period, time 0 represents a 30-min baseline prior to injection. $P = 0.78$ (time 0 vs 30 min), $P = 0.61$ (time 0 vs 60 min), $n = 30$ cells from 6 mice for each group, unpaired t test. (g) AHE causes no change in microglial density. $n = 6$ mice for each group. (h) 2PLSM images of perfusion fixed *Cx3cr1-eGFP* mouse cerebral cortex showing representative images of 'resting' microglia from the ipsilateral hemisphere where the cranial window was located (left two images) and the contralateral hemisphere (right two images). Scale bars represent 20 μm . (i) Cranial window preparation (ipsilateral hemisphere) does not cause microglial activation when compared to

the contralateral hemisphere after perfusion/fixation with paraformaldehyde, ns = not significant ($P = 0.39$), $n = 30$ cells from 6 mice for both groups, paired t test. Data are shown as mean $\pm$ SEM.

Fig. 3.

Characterization of azoxymethane (AOM)-induced hepatic encephalopathy (HE). (a) HE was induced by administering the liver toxin AOM ($100 \mu\text{g g}^{-1}$ i.p.). A timescale is shown for the comparatively slower onset of neurological symptoms in HE (classified using a modified encephalopathy score). Representative EEG and EMG recordings are shown from baseline, stage I and stage IV. AOM causes a triphasic pattern of EEG discharge during the coma stage IV, typically seen in severe metabolic encephalopathies. $n = 6$. (b) HE causes a slower and less pronounced increase in plasma ammonia ($[\text{NH}_4^+]$) from baseline to stage I (approx. 6 h after injection) and stage IV (approx. 16 h after injection). $*P = 0.0011$, $**P < 0.0001$, $n = 10$, paired t test. (c) HE is associated with increased BBB permeability (opening) during stage IV, but not stage I, seen by increased extravasation of Evans blue-albumin. ns = not significant ($P = 0.92$), $**P = 0.0079$, $n = 5$ (baseline), 6 (stage I HE), 5 (stage IV HE), Mann–Whitney U test. (d) Brain water content is increased in stage IV, but not stage I of HE. ns = not significant ($P = 0.86$), $**P = 0.00067$, $n = 10$ (control), 6 (stage I HE) and 6 (stage IV HE), Mann–Whitney U test. Data are shown as mean $\pm$ SEM.

Fig. 4.

The terminal coma stage of HE is associated with microglial activation and dysmotility, which is not present during the onset of symptoms. (a) Diagram illustrating the 2PLSM imaging setup following AOM administration. (b) Representative 2PLSM images from HE at 0 h, stage I and stage IV (collapsed z-stacks). During the latter stage the microglia appear more ameboid and activated (see yellow arrowheads). Scale bar represents 15 μm . (c) Overlay of representative images from HE at 0 h and stage IV (collapsed z-stacks) show microglial activation during the latter stage. Red represents microglia at 0 h, green represents microglia at stage IV and yellow represents the area of overlap (unchanged pixels). Scale bar represents 15 μm . (d) Quantification of AI during control and HE reveals an increase during stage IV. $*P = 0.023$, $n = 30$ cells from 6 mice for each group, unpaired t test. (e) Quantification of microglial TOR per cell show a slight decrease in motility during stage IV of HE. Each data point represents the mean TOR per cell over a 30-min period, time 0 represents a 30-min baseline prior to injection. $*P = 0.041$, $n = 30$ cells from 6 mice for each group, unpaired t test. (f) HE causes no change in microglial density. $n = 6$ mice for each group. Times 6 and 16 h are equivalent to stages I and IV, respectively. Data are shown as mean $\pm$ SEM.

Table 1

Symptom onset in HE and AHE

	Prodromal (passive)	Stage I (lethargy)	Stage II (ataxia)	Stage III (righting reflex loss)	Stage IV (corneal reflex loss, coma)	No. of seizures
HE	3.5±0.3 h	6.8±0.9 h	9.2±0.8 h	14.6±1.9 h	16.3±1.6 h	0
AHE	3.5±0.76 min	4.3±0.91 min	5.1±1.3 min			8.40±1.01

Table 2

Brain amino acids

	Glutamine ($\mu\text{mol/g}$ wet weight)	Glutamate ($\mu\text{mol/g}$ wet weight)	GABA ($\mu\text{mol/g}$ wet weight)
Control	5.21 $\pm$ 0.17	15.41 $\pm$ 0.24	2.62 $\pm$ 0.096
HE Stage IV	7.91 $\pm$ 0.36	13.60 $\pm$ 0.38	2.65 $\pm$ 0.071
Control	4.85 $\pm$ 0.47	14.29 $\pm$ 0.19	2.01 $\pm$ 0.14
AHE	8.69 $\pm$ 0.33	12.49 $\pm$ 0.15	2.19 $\pm$ 0.12