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Improved Visualization of Neuronal Injury Following Glial Activation by Manganese Enhanced MRI

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Abstract

Research directed at anatomical, integrative and functional activities of the central nervous system (CNS) can be realized through bioimaging. A wealth of data now demonstrates the utility of magnetic resonance imaging (MRI) towards unraveling complex neural connectivity operative in health and disease. A means to improve MRI sensitivity is through contrast agents and notably manganese (Mn^{2+}). The Mn^{2+} ions enter neurons through voltage-gated calcium channels and unlike other contrast agents such as gadolinium, iron oxide, iron platinum and imaging proteins,

provide unique insights into brain physiology. Nonetheless, a critical question that remains is the brain target cells serving as sources for the signal of Mn^{2+} enhanced MRI (MEMRI). To this end, we investigated MEMRI's abilities to detect glial (astrocyte and microglia) and neuronal activation signals following treatment with known inflammatory inducing agents. The idea is to distinguish between gliosis (glial activation) and neuronal injury for the MEMRI signal and as such use the agent as a marker for neural activity in inflammatory and degenerative disease. We now demonstrate that glial inflammation facilitates Mn^{2+} neuronal ion uptake. Glial Mn^{2+} content was not linked to its activation. MEMRI performed on mice injected intracranially with lipopolysaccharide was associated with increased neuronal activity. These results support the notion that MEMRI reflects neuronal excitotoxicity and impairment that can occur through a range of insults including neuroinflammation. We conclude that the MEMRI signal enhancement is induced by inflammation stimulating neuronal Mn^{2+} uptake.

Keywords

Magnetic resonance imaging (MRI); Manganese enhanced MRI (MEMRI); Bioimaging; Glial activation; Microglia; Astrocyte; Glial-neuronal interactions; Inflammation

Introduction

Manganese (Mn^{2+}) is a potent magnetic resonance imaging (MRI) contrast agent used to improve anatomical visibility, most notably, neural structures and their connections. Unlike other agents, such as gadolinium, iron oxide, iron platinum and protein-based compounds, Mn^{2+} remains at a very early stage in clinical development, based in large measure to its inherent neurotoxicity (Dobson et al. 2004; Reaney et al. 2002; Silva et al. 2004). Nonetheless, a number of recent reports demonstrate that Mn^{2+} -enhanced MRI (MEMRI) in normal animal brains provide novel information relevant to anatomical, integrative, and functional assessments of neural connectivity. These findings are linked to the abilities of Mn^{2+} ions to efficiently enter neurons through voltage-gated calcium channels (Koretsky and Silva 2004; Pautler 2006; Silva and Bock 2008; Van der Linden et al. 2007).

A major drawback for the use of Mn^{2+} as a contrast agent in studies of human disease models rests in understanding its cellular mechanism and profiles (Immonen et al. 2008; Saito et al. 2012). Despite such potential limitations, significant attempts have been made, in recent years, to use MEMRI in studies of the pathobiology of neurodegenerative diseases utilizing relevant animal models (Bertrand et al. 2013; Perez et al. 2013; Drobyshvsky et al. 2012; Haenold et al. 2012; Morken et al. 2013; Saito et al. 2012; Wideroe et al. 2012; Boullier et al. 2011; Chan et al. 2011; Kim et al. 2011; Smith et al. 2011; Soria et al. 2011; Tang et al. 2011; Wideroe et al. 2011; Inoue et al. 2010; Kawai et al. 2010; van Meer et al. 2010). Nonetheless and paramount to the successful application of MEMRI is not simply the ability to deliver Mn^{2+} to the site of interest or of disease but in determining the cell types and cellular mechanisms that engage the ion and produce the signal enhancement observed. Based on our long standing interest in the links between neuroimmunity and neurodegenerative diseases we reasoned that pathological activation of the immune-competent glial cells could represent an obligatory event for any MEMRI signal enhancements. In support of this idea is a wealth of prior studies demonstrating MEMRI signal enhancements were co-localized with reactive glia (Haapanen et al. 2007; Wideroe et al. 2009; Wideroe et al. 2011; Morken et al. 2013). However it was never clear if such signal enhancements resulted from Mn^{2+} accumulation in the glial cells directly, or from elevated manganese uptake by neuronal cells stimulated by glial reaction.

Thus, we sought to better elucidate the cellular basis of the MEMRI signal in studies of neurodegenerative diseases. We reasoned that if the cell association of the signal enhancement is determined, MEMRI can be developed as a potential imaging tool to monitor real-time glial-neuronal interactions. To this end, we investigated relationships between microglial and astrocytic activation linked to Mn^{2+} uptake. These studies were both in vitro as performed in glial and neuronal cells and in vivo using MRI following lipopolysaccharide (LPS) treatments in mice. The results showed that astrocytic reactions result in MEMRI signal enhancement by stimulating neuronal Mn^{2+} ion uptake.

Materials and methods

PC-12 differentiation

A rat adrenal pheochromocytoma-derived cell line—PC-12 Tet-Off, was used to study neuronal Mn^{2+} uptake. PC-12 cells were utilized here because they serve as a relevant in vitro model system for primary neuronal cells (Greene and Tischler 1976; Vignali et al. 1996). They have been widely used to study voltage-gated Ca^{2+} channels (Singh et al. 2013; Zhang et al. 2012) and effects of Mn^{2+} exposure on these cells (Kwik-Urbe et al. 2003; Reaney et al. 2002; Zheng and Zhao 2001). Following withdrawal of doxycycline (Dox) from the medium, PC-12 cells were differentiated with nerve growth factor (NGF, R&D Systems 1156-NG/CF).

Cell co-culture and activation

Primary cultured mouse astrocytes and microglia were prepared from *NOD-scid IL2R_g^{null} (NSG)* newborn pups as described in (Yamamoto et al. 2007; Kiyota et al. 2009). Differentiated PC-12 cells were de-attached and seeded in multi-well plates. Primary astrocytes and microglia were placed on inserts and co-cultured with PC-12 cells respectively at the cell ratio of 1:1 (10^5 cells). The co-cultured cells were then treated with a combination of cytokines including interferon gamma (IFN- γ) and tumor necrosis factor alpha (TNF- α) at the following concentrations: 25 (IFN- γ) and 2.5 (TNF- α), 50 and 5.0, or 100 and 10.0 ng/ml to induce glial activation. After 9 h of treatment, MnCl_2 solution was administered in the medium at concentrations of 80, 160 and 320 μM . An untreated cell group was used for control measurements. Cells were washed and resuspended at 2 and 15 h after MnCl_2 treatment. Inductively coupled plasma mass spectrometry (ICP/MS) was used to measure the Mn^{2+} concentration. Experiments were performed with triplicate samples.

Mouse model of acute neuroinflammation

All animal procedures performed in this study were approved by the University of Nebraska Medical Center Institutional Animal Care and Use Committee. LPS was intracranially (i.c.) injected to induce focal inflammation in male wild-type *C57BL/6* mice ($n=13$, 6–8 weeks old). Using a stereotactic injection device, 10 μg (LPS) in 2 μl PBS was administrated into the left hemispheric caudoputamen (coordinates from bregma: 3.0 mm lateral, 0.5 mm anterior, and –3.5 mm ventral). The LPS solution was delivered with a 27 g needle at the flow rate of 0.2 μl per minute. A sham-operated control group ($n=11$) was injected with 2 μl PBS through the i.c. route with the same parameters as the LPS group.

MEMRI

MnCl_2 (50 mM) was administrated i.p. with the dose of 60 mg/kg consecutively four times at 24 h intervals before MRI. Another group of mice ($n=10$) with i.c. LPS injection was added to study the effect of LPS alone on MRI signal enhancement. This group was administrated with saline through the i.p. route with the volume of 6 ml/kg at the same times as the MnCl_2 injection. The LPS mice injected with MnCl_2 are designated as LPS + MnCl_2 .

hereafter, and the LPS mice injected with saline are designated as LPS + saline. The sham-operated mice with i.c. PBS injection are designated as PBS + MnCl₂. One day after LPS or PBS injection, six LPS + MnCl₂ mice, five LPS + saline mice and five PBS + MnCl₂ mice were scanned using MRI. The remaining mice were scanned 7 days after LPS or PBS injection. After MRI, the mice were euthanized and brains were removed for histology.

MRI was performed 24 h after the last MnCl₂ injection on a 7 T/21 cm horizontal bore scanner (Bruker, Billerica, MA) operating ParaVision 5.1 with a volume coil for RF transmission and a 4-channel phased-array coil for signal reception. Mice were scanned using T₁ mapping (fast spin echo with variable TR from 0.4 s to 10 s, 12 slices, slice thickness=0.5 mm, in-plane resolution=0.1×0.1 mm²) and T₁-wt MRI (gradient recalled echo, TR=20 ms, flip angle=20°, 3D isotropic resolution=0.1×0.1×0.1 mm³).

To reduce the influence of the inhomogeneous signal reception by the surface coil, N3 field inhomogeneity correction (Sled et al. 1998) was first performed on each image using MIPAV (CIT, NIH). The brain was then manually aligned to the LONI atlas (LONI, UCLA) in Analyze (AnalyzeDirect, KS). The alignment is necessary for accurate quantification of signal enhancement due to manganese uptake.

MRI signal enhancement was measured on a slice-by-slice basis in the axial direction. On each slice, the injection site was first identified. The mean value and standard deviation (SD) of the signal intensity about the corresponding location of the needle hole on the contralateral hemisphere was measured (in a larger ROI), then the intensity threshold was defined as the mean value plus 2 SDs. This threshold was applied on the ipsilateral hemisphere as the lower boundary to identify enhanced area about the needle hole. The enhanced volume was the summation of the enhanced areas multiplied by the slice thickness. The total enhanced volume was then normalized to the needle depth. The signal enhancement ratio was calculated by dividing the mean signal intensity in the enhanced volume by mean intensity on the contralateral side.

Immunohistology

Mice were euthanized immediately after MRI. Brains were collected and fixed in 4 % paraformaldehyde overnight and embedded in paraffin. The paraffin blocks were cut into 5 μm thick sections. These brain tissue sections were then labeled with mouse monoclonal antibodies for glial fibrillary acidic protein (GFAP) (1:1000, Dako). Microglia were stained with rabbit polyclonal antibodies to ionized calcium binding adaptor molecule 1 (Iba-1) (1:500; Wako Chemicals). The polymer-based HRP-conjugated anti-mouse and anti-rabbit Dako EnVision systems were used as secondary detection reagents and 3,3'-diaminobenzidine (DAB, Dako) used as a chromogen. All paraffin-embedded sections were counterstained with Mayer's hematoxylin. Deletion of primary antibodies served as controls. Images were captured with a 10× objective using Nuance EX multi-spectral imaging system (Cambridge Research Instruments), and Nuance EX image analysis software (Media Cybernetics) was used for quantification of GFAP and Iba-1 expression in the region of interest (in and around the injection line) as reflected by intensity/μm². Area-weighted average intensity was calculated for GFAP and Iba-1 expression by dividing the total signal intensity, for each partitioned area, by area (μm²).

For immunofluorescence labeling, brain sections about the injection line were treated with the paired combination of primary antibodies mouse synaptophysin (SYN, 1:1000), and rabbit microtubule-associated protein 2 (MAP2, 1:750). Primary antibodies were labeled with secondary anti-mouse and anti-rabbit antibodies conjugated to the fluorescent probes Alexa Fluor 488 and Alexa Fluor 594, and nuclei were labeled with DAPI (4,6-diamidino-2-phenylindole). Slides were coverslipped with ProLong Gold anti-fade reagent. Then slides

were stored at -20°C after drying for 24 h at room temperature. Images were captured at wavelengths encompassing the emission spectra of the probes, with a 10X objective by Nuance EX multispectral imaging system (Cambridge Research Instruments), and Nuance EX image analysis software (Media Cybernetics) used for quantification. For SYN and MAP2 expression, area-weighted average fluorescence intensity was calculated in the region of interest (in and around the injection line) by dividing the total signal intensity, for each partitioned area, by area (μm^2) as intensity/ μm^2 .

Statistical analysis

T-test was used to compare the cell manganese concentrations in the in vitro study. Correlation between manganese uptake by PC-12/glia cells and cytokine level were performed using Pearson's correlation coefficient calculation. In the in vivo MEMRI study, *t*-test was used to compare all the variables in between LPS and PBS injected groups, whereas Pearson's correlation coefficient was used to test for correlations between signal enhancement and glial reactivity.

Results

Mn²⁺ uptake by PC-12 cells

The manganese concentration in PC-12 cells co-cultured with astrocytes and microglia was shown in Fig. 1a and b, respectively. Mn²⁺ concentration in PC-12 cells was plotted against cytokine level (IFN- γ /TNF- α , ng/ml) at 2 and 15 h of MnCl₂ incubation time (left and right plots, respectively). In this figure, several properties of the baseline manganese uptake by PC-12 cells (when IFN- γ /TNF- α =0/0) are manifest. First of all, the measurements at MnCl₂ concentration=0 shown in Fig. 1 by dash-dot lines demonstrated that PC-12 cells endogenous manganese was low, suggesting the manganese in PC-12 must be taken from the extrinsic source—the MnCl₂ solution. Secondly, the manganese uptake by PC-12 cells depends on the availability of the extrinsic manganese (i.e., the concentration of MnCl₂ solution). It can be seen that the PC-12 manganese concentration is positively correlated with MnCl₂ concentration in all the plots in Fig. 1 (Pearson's correlation, $r = 0.900$, $p < 0.05$). The incubation time of MnCl₂ apparently plays a role in PC-12 manganese uptake as well. The manganese concentration in PC-12 cells at 15 h was much higher than at 2 h, evidenced by comparing the left and right columns in Fig. 1 ($p < 0.05$ at MnCl₂=160 and 320 μM). These observations were in agreement with previous findings (Silva et al. 2004).

The role of glial reaction on PC-12 manganese uptake involves a number of factors including the glial cell type, and the level and time of glial reaction. Figure 1a illustrates that the change in PC-12 manganese uptake resulted from astrocytic activation. At short inflammatory cytokine treatment time (2 h), PC-12 manganese uptake was almost linearly increased with cytokine levels at 160 μM (Pearson's correlation, $r = 0.990$, $p < 0.01$) and 320 μM (Pearson's correlation, $r = 0.998$, $p < 0.01$). Prolonged treatment (15 h) seemed to suppress the manganese uptake by PC-12 cells with cytokine levels at 160 and 320 μM MnCl₂. However the negative correlation between the manganese concentration and cytokine level was not significant ($p = 0.064$ at 160 μM , $p = 0.052$ at 320 μM).

On the other hand, microglial reaction had no significant impact on PC-12 manganese uptake. At the high MnCl₂ concentration (320 μM) and short treatment time (2 h), it showed the trend of negative impact (Pearson's correlation, $r = -0.935$, $p = 0.065$). The PC-12 manganese uptake remained constant at all other MnCl₂ concentrations at both 2 and 15 h.

It was interesting to see that at 15 h Mn²⁺ uptake by PC-12 cells co-cultured with astrocytes at baseline (IFN- γ /TNF- α =0/0) was twice the concentration of Mn²⁺ as PC-12 cells co-cultured with microglia. This result could be due to increase in Mn²⁺ efflux from astrocytes

in response to high extracellular Mn^{2+} (Wedler et al. 1989), which could cause increase in extracellular Mn^{2+} concentration, and thus lead to more Mn^{2+} uptake by PC-12 cells.

Manganese uptake by glial cells

No significant exogenous manganese uptake by glial cells was observed in this study at any extracellular MnCl_2 concentration, glial activation level, or times of exposure (data not shown). The ICP/MS measurements showed that the intrinsic manganese content in astrocytes and microglia was 50–70 ppb, which is about 30–50 times higher than that of PC-12 cells at baseline (measured at 0 μM MnCl_2). The intrinsic manganese concentrations of glial cells measured in this work are in agreement with previous studies (Takeda 2003; Tholey et al. 1987). The high intrinsic Mn^{2+} concentration in glial cells indicates that Mn^{2+} is an important element for the function of glial cells.

MRI signal enhancement and enhanced volume measurements

At Day 1 after LPS/PBS injections, signal enhancement was found only in 2 mice in each group. Signal enhancement at Day 1 was negligible as compared to Day 7, and was not significantly different between the LPS + MnCl_2 and PBS + MnCl_2 groups (Data not shown). At Day 7 after LPS injection, T_1 -wt images of the LPS + MnCl_2 group showed strong signal enhancement around the injection line compared to both the surrounding tissue and the corresponding region in the contralateral hemisphere. The T_1 -wt image of a Day 7 LPS + MnCl_2 mouse was shown in Fig. 2a in three orthogonal planes: coronal (upper left), sagittal (upper right) and axial (bottom left). The areas around the injection line were encased by red boxes and shown in magnified windows in Fig. 2a. The signal enhancement in this area was robust. The T_1 -wt image of a PBS + MnCl_2 mouse was shown in Fig. 2b. The PBS injected mice showed minimal enhancement compared to the LPS injected mice. The enhancement ratios calculated in the PBS + MnCl_2 and LPS + MnCl_2 groups are shown in Fig. 2c. Statistical analysis showed significantly higher signal enhancement ratio in the LPS + MnCl_2 group as compared to the PBS + MnCl_2 group, $p < 0.01$. Similarly as shown in Fig. 2d, enhanced tissue volumes (after normalization by injection depths) were significantly larger in the LPS + MnCl_2 group than in the PBS + MnCl_2 group, $p < 0.001$. There was no obvious signal enhancement in the mice injected with only LPS but not MnCl_2 (the LPS + saline group) as shown in Fig. 2e.

Immunohistology

At Day 1 after LPS/PBS injection, no reactive astrocytes were found, and only microglia detected by Iba-1 were observed in LPS injected mice around the injection line. A brain slice of a LPS + MnCl_2 mouse at Day 1 is shown in Fig. 3a. The Iba-1 staining on a region around the injection line (red box) is shown in a magnified (20X) window. As described in the preceding paragraph, no significant MEMRI signal enhancement was observed in these mice (data not shown). Both astrocytic and microglial reactivity were detected at Day 7 in LPS injected mice. The top row in Fig. 3b shows a brain slice of a LPS + MnCl_2 mouse at Day 7. The GFAP and Iba-1 staining on a region around the injection line are shown in magnified (20 $\times$) windows. It can be seen that a large number of activated astrocytes were detected by GFAP, and activated microglia by Iba-1, in this region. A brain slice of a PBS + MnCl_2 mouse is shown in the 2nd row in Fig. 3b. The areas stained by GFAP and Iba-1 in the region about the injection line (red box) were much smaller compared to the LPS + MnCl_2 mouse. In quantitative analysis, at Day 7, the astrocytic reactivity represented by GFAP expression ($p < 0.01$) and microglial reactivity by Iba-1 expression ($p < 0.05$) were significantly higher in the LPS + MnCl_2 group than in the PBS + MnCl_2 group (Fig. 3c). Immunofluorescence labeling for neuronal markers (SYN and MAP2) showed no significant difference in neuronal loss between LPS + MnCl_2 and PBS + MnCl_2 groups around the injection lines (data not shown).

Figure 4a shows that the correlation between astrocyte and microglial reactivity is significant (Pearson's correlation coefficient, $r=0.62$, $p<0.05$) in the LPS + MnCl₂ group at Day 7. More interesting is that, at Day 7 in the LPS + MnCl₂ group, there was a significant correlation between astrocytic reactivity and enhanced tissue volume calculated from MEMRI data (Pearson's correlation coefficient, $r=0.66$, $p<0.05$) as shown in Fig. 4b. No correlation was found between microglial reactivity and enhanced tissue volume.

Discussion

Intrinsic manganese content in neurons is low compared to glial cells, and neuronal manganese uptake is partly dependent on the availability of extrinsic manganese. Here we investigate the effect of glial reaction on glial and neuronal manganese uptake. The results suggest that inflammatory cytokine induced astrocytic activation (Yenari and Giffard 2006) stimulates Mn²⁺ uptake of neurons, and that this uptake is proportional to the level of activation. On the other hand, microglial reaction has no direct effect in stimulating neuronal Mn²⁺ uptake, and intensive microglial reaction may even suppress uptake.

The intrinsic manganese content in astrocytes and microglia is much higher than in PC-12 cells as shown in this study and others (Takeda 2003; Tholey et al. 1987). The high intrinsic Mn²⁺ concentration in glial cells indicates that Mn²⁺ is an important element for the function of these cells. Studies have found that activity of several enzymes in the central nervous system is Mn²⁺ dependent, such as superoxide dismutase, ATPase, and glutamine synthase (Wedler and Toms 1986; Stallings 1991; Bentle and Lardy 1976; Doherty et al 1983; Hazell 2002). No significant exogenous Mn²⁺ uptake by glial cells was observed in this study at any extracellular MnCl₂ concentration or glial activation level or time.

The in vivo imaging study was designed to reveal the effects of microglial and astrocytic reactions on MEMRI signal enhancement. The imaging times of Day 1 and Day 7 were chosen because, after 1 day of LPS injection, microglial reaction is well defined, and astrocytic reaction generally takes place after 3–4 days, and becomes well established around 7 days (Jana et al. 2008).

The in vivo study has clearly demonstrated the co-localization and strong correlation between reactive astrocytes and MEMRI signal enhancement. By incorporating the in vitro finding that reactive astrocytes stimulate neuronal manganese uptake rather than absorb manganese themselves, we are confident to conclude that the MEMRI signal enhancement resulted from the elevated manganese uptake in neurons stimulated by astrocytic reaction.

In GFAP stained brain slices, we also found activated astrocytes in regions remote from the injection line in the LPS + Mn²⁺ mice. However the number of the activated astrocytes in these regions was small. The neuronal Mn²⁺ uptake caused by these astrocytes was not detected in MRI due to the limited sensitivity.

Several groups have studied the relationship between MEMRI signal enhancement and glial reaction in a range of animal models of human disease, including but not limited to, cathepsin D deficiency (Haapanen et al. 2007), epilepsy (Immonen et al. 2008), ischemia (Wideroe et al. 2009; Wideroe et al. 2011; Morken et al. 2013) and prenatal X-ray exposure (Saito et al. 2012). However, results have been varied. Some studies showed elevated MEMRI signal enhancement co-localized with activated microglia (Haapanen et al. 2007) or both activated microglia and astrocytes (Wideroe et al. 2009; Wideroe et al. 2011; Morken et al. 2013). However the study of prenatal X-ray exposure (Saito et al. 2012) found negative correlation between brain tissue longitudinal relaxivity (R₁) and reactive astrocytes. Because R₁ determines MEMRI signal intensity, this study suggested that astrocytic reaction suppressed signal enhancement. Another study of epilepsy (Immonen et al. 2008) found no

correlation between MEMRI signal change and astrocytic reaction. The inconsistent results from these studies could be due to the variety of disease models used. In these diseases, a variety of pathobiological events occur in addition to glial reaction, such as cell swelling and necrosis (Wideroe et al. 2009; Wideroe et al. 2011; Morken et al. 2013), apoptosis (Haapanen et al. 2007), and hippocampal mossy fiber spouting (Immonen et al. 2008). These events can impair neurons that could either stimulate or suppress neuronal Mn^{2+} uptake, causing inconsistent MEMRI signal enhancement. One particular study (Kawai et al. 2010) using rats, found strong positive correlation between MEMRI signal enhancement and astrocytic reaction in a stroke model, and proposed that the signal enhancement is caused by Mn^{2+} accumulation in reactive astrocytes through voltage-gated Ca^{2+} channels. However this study did not investigate the neuronal reaction to glial activation, and thus cannot exclude the contribution of elevated Mn^{2+} uptake by reactive neurons to signal enhancement.

Astrocytes react rapidly to various neurodegenerative insults. Reactive astrocytes protect neurons by secluding the injury site from the rest of the CNS area, and secreting multiple neurotrophic factors to aid neuronal survival. However the astrocytic processes have been implicated in the pathogenesis of a variety of neurodegenerative diseases, including but not limited to, Alzheimer's disease, Parkinson's disease, HIV-associated neurocognitive disorders, acute traumatic brain injury, and inflammatory demyelinating diseases. It is believed that rapid and severe astrocytic reaction initiates or augments inflammatory response by secreting various pro-inflammatory molecules leading to neuronal death and brain injury (Gendelman and Masliah 2008); (Yenari and Giffard 2006).

Monitoring glial-neuronal interactions dynamically using noninvasive imaging technologies is a unique and powerful method, which can be used to understand the pathobiology of neurodegenerative diseases, provide diagnosis and prognosis, and aid in the development of therapeutic methods. MRI is a noninvasive imaging technology providing high spatial resolution, excellent soft tissue contrast and real-time measurements. Because Mn^{2+} crosses the brain–blood barrier and enters neurons through voltage-gated calcium channels (Inoue et al. 2011), MEMRI has proven to be a powerful tool to study neuronal viability, activation and impairment. The results in this study show the potential to use MEMRI monitoring of glial-neuronal interactions in normal and abnormal conditions. Our immunohistological results showed no significant neuronal death caused by LPS injection at the times when MEMRI was performed. In the future we plan to extend the study to later stages at which neuronal death induced by glial reaction occurs. We expect to see decreased MEMRI signal enhancement due to the neuronal death as shown in several previous studies (Bertrand et al. 2013; Perez et al. 2013; Haenold et al. 2012). Therefore a longitudinal MEMRI study showing neuronal excitation by astrocytic reaction and neuronal death later provides valuable information of the progression of pathobiology.

In conclusion, we demonstrated that astrocytic reaction induces elevated neuronal Mn^{2+} uptake that results in MEMRI signal enhancement. This study demonstrates that MEMRI can be used not only to monitor neuronal vitality and activity but also to monitor astrocyte-neuronal interactions in animal model systems of neurodegenerative diseases.

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Fig. 1.

a Mn^{2+} concentration in PC-12 cells co-cultured with astrocytes plotted against cytokine treatment level (IFN- γ /TNF- α , ng/ml) at 2 h (*Left*) and 15 h (*Right*) of MnCl_2 incubation. **b** Mn^{2+} concentration in PC-12 cells co-cultured with microglia plotted against cytokine treatment level (IFN- γ /TNF- α , ng/ml) at 2 h and 15 h of MnCl_2 incubation

Fig. 2.
T₁-wt images and enhancement quantification. **a** T₁-wt image of a LPS + MnCl₂ mouse. The image was shown in sagittal, coronal and axial directions (*counter-clockwise starting*

from upper right). Areas around the injection line were encased by *red boxes* and shown in magnified windows. **b** T1-wt image of a PBS + MnCl₂ mouse. **c** Enhancement measurements. The LPS + MnCl₂ group showed significantly higher signal enhancement than the PBS + MnCl₂ group ($p<0.01$). **d** Enhanced volume. Enhanced volumes (normalized by dividing it by respective Injection depths). The LPS + MnCl₂ group showed significantly larger enhanced volumes ($p<0.01$). **e** T1-wt image of a LPS only (no MnCl₂ injection) mouse. No obvious signal enhancement was observed in the mice injected with only LPS but not MnCl₂

Fig. 3.

Immunohistology. **a** A brain section of a LPS + MnCl₂ mouse at Day 1 after LPS injection. The reactive microglia by Iba-1 in the regions encased in *red boxes* about the injection line are shown at 20×. **b** A brain section of a LPS + MnCl₂ mouse (*top row*) and of a PBS + MnCl₂ mouse (*2nd row*). The reactive astrocytes by GFAP and microglia by Iba-1 in the regions encased in *red boxes* around the injection line are shown at 20×. **c** Glial reactivity quantification. Astrocytic and microglial reactivity represented by GFAP and Iba-1 expressions (Intensity/μm²) were significantly higher in the LPS + MnCl₂ group compared to the PBS + MnCl₂ group ($p < 0.01$ and $p < 0.05$, respectively)

Fig. 4. Correlation comparisons. **a** Correlation between astrocytic and microglial reactivity ($r=0.62$, $p<0.05$). **b** Correlation between astrocytic reactivity and enhanced volume ($r=0.66$, $p<0.05$)