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Manipulation of Tissue Contrast Using Contrast Agents for Enhanced MR Microscopy in *ex vivo* Mouse Brain

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Abstract

Detailed 3D mouse brain images may promote better understanding of phenotypical differences between normal and transgenic/mutant mouse models. Previously, a number of magnetic resonance microscopy (MRM) studies have successfully established brain atlases, revealing genotypic traits of several commonly used mouse strains. In such studies, MR contrast agents, mainly gadolinium (Gd) based, were often used to reduce acquisition time and improve signal-to-noise ratio (SNR). In this paper, we intended to extend the utility of contrast agents for MRM applications. Using Gd-DTPA and MnCl₂, we exploited the potential use of MR contrast agents to manipulate image contrast by drawing upon the multiple relaxation mechanisms and tissue-dependent staining properties characteristic of each contrast agent. We quantified r_1 and r_2 of Gd-DTPA and MnCl₂ in both aqueous solution and brain tissue and demonstrated the presence of divergent relaxation mechanisms between solution and tissue for each contrast agent. Further analyses using nuclear magnetic resonance dispersion (NMRD) of Mn²⁺ in *ex vivo* tissue strongly suggested macromolecule binding of Mn²⁺, leading to increased T₁ relaxation. Moreover, inductively coupled plasma (ICP) mass spectroscopy revealed that MnCl₂ had higher tissue affinity than Gd-DTPA. As a result, multiple regions of the brain stained by the two agents exhibited different image contrasts. Our results show that differential MRM staining can be achieved using multiple MR contrast agents, revealing detailed cytoarchitecture, and may ultimately offer a window for investigating new techniques by which to understand biophysical MR relaxation mechanisms and perhaps to visualize tissue anomalies even at the molecular level.

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Introduction

Magnetic resonance microscopy (MRM) is a specialized magnetic resonance imaging (MRI) technique for imaging *ex vivo* specimens at microscopic resolution in tens of microns (or $< \sim 10^4 \mu\text{m}^3$). Since the first MRM images were obtained in the 1980s (Aguayo et al., 1986, Johnson GA, 1986), MRM has been extensively used in various anatomical studies of normal and transgenic/mutant mice (Benveniste et al., 2000, Johnson et al., 2002, Natt et al., 2002, Cyr et al., 2005, van der Landen et al., 2006, Badea et al., 2007b), and developing mouse embryos (Schneider et al., 2003, Zhang et al., 2003). Unlike conventional histology, which requires tissue dissection, MRM provides capabilities to capture large amounts of anatomical information without destroying 3D tissue structures, and thus allows geometrically accurate visualization of anatomical structures. As greater numbers of transgenic or mutant mice have become available for translational studies of human diseases, MRM has become a highly important tool for anatomical phenotyping and morphologic screening. For instance, several groups have successfully completed comprehensive mouse brain atlasing of C57BL/6J and 129S1/SvImJ strains (MacKenzie-Graham et al., 2004, Ali et al., 2005, Ma et al., 2005) based on MRM images. MRM has also demonstrated success for phenotyping of several mutant mice strains such as the Reeler mouse (Badea et al., 2007b) and the CACNA1A knock-in migraine mouse (van der Landen et al., 2006).

Despite the great success of MRM for anatomical studies of various mouse strains and transgenic/mutant mice, MRM imaging that relies on intrinsic tissue contrast lacks the flexibility and target specificity offered by conventional histological staining. Although many of the MRM studies mentioned above have used MRI contrast agents (mainly gadolinium chelates), the main purpose of their use to date has been limited to reducing tissue T_1 relaxation time, so as to achieve improved overall signal-to-noise ratio (SNR) and shorter image acquisition time. These studies have therefore not explored the full capabilities of these agents for MRM; MR contrast agents can change not only T_1 , but also to alter tissue T_2 and T_2^* relaxation times. Moreover, multiple relaxation mechanisms and their field dependency can lead to change in relaxation enhancement effects of different contrast agents at different field strengths. For example, the MnCl_2 contrast agent has a scalar coupling relaxation mechanism, which exerts strong effect on T_2 relaxation especially at high field (Solomon, 1955, Bloembergen, 1957). In addition, when applied to *ex vivo* tissue specimens, commonly used MR contrast agents may exert distribution and tissue binding effects as a result of intrinsic chemical characteristics, and therefore produce differentiable MRI contrast between heterogeneous biological structures. Moreover, the differences in microscopic distribution of various contrast agents may illustrate different selective enhancement on T_2^* relaxation due to this contrast's sensitivity to microscopic tissue distribution (Fisel et al., 1991, Weisskoff et al., 1994). Therefore, it is quite possible that we can achieve better tissue differentiation and henceforth develop detailed tissue/cell specific MRM staining methods by employing various MR contrast agents.

Gd-DTPA and MnCl_2 are two MR contrast agents commonly used for *in vivo* studies. These agents are also known to have distinct *in vivo* bio-distributions when exogenously administered. *In vivo*, Gd-DTPA, an extracellular agent, is incapable of entering viable cells

(Quirk et al., 2003), whereas MnCl_2 can move across the neuronal cell membrane through calcium channels (Narita et al., 1990, Aschner and Aschner, 1991) or across synapses (Takeda et al., 1998). Gd-DTPA typically exerts its influence on tissue MR relaxation through dipole-dipole interaction (Lauffer, 1987). Mn^{2+} , besides having dipolar relaxation capabilities, has an additional scalar coupling relaxation effect, which especially affects T_2 relaxation at high field, (Bertini et al., 1996). Although relaxation mechanisms are well known in solution and to some extent *in vivo*, the *ex vivo* distribution of these compounds and their effect on MR relaxation rates in fixed specimens have not been precisely documented. Based on the different tissue distribution and biophysical properties demonstrated in various *in vivo* studies, we hypothesized that Gd-DTPA and MnCl_2 will also manifest different relaxation rates and distribution properties in *ex vivo* brain tissue, leading to spatially distinct image contrasts, and thus providing potential staining flexibility in MRM. To test our hypothesis, we administered these two contrast agents to widely available C57BL/6 mouse brains and acquired *ex vivo* images using 14T MRI system. We first characterized the tissue relaxation properties of each contrast agent in different brain regions; then, examined the dependence of tissue relaxation rates on staining duration and determined contrast agent concentration and imaging parameters to achieve desired image contrast for each MR contrast agent. Our specific goals in this study were (1) to determine staining and imaging parameters for high-resolution *ex vivo* magnetic resonance microscopy, (2) to examine the relaxation mechanisms, biophysical properties, and tissue affinities of these two MR contrast agents in mouse brains. In particular, we demonstrated the potential use of these two staining agents to improve *ex vivo* image contrast in the brain, furthermore the possibility of achieving tissue- or cell-specific contrast using such MRM staining methods.

Materials and Methods

A total of thirty adult male C57BL/6 mice (8 to 12 weeks old, Taconic Farm, Germantown, NY) were used in this study. Extracted brains were divided into 4 groups: (1) five animals were used as controls and received no contrast agent; (2) eight were used to optimize contrast agent concentration and measure relaxivity; (3) four were used for a contrast agent penetration study; and (4) thirteen (four for Gd-DTPA and nine for MnCl_2) were used to optimize MRM image acquisition.

Mouse brain preparation

All brains were fixed through transcardial perfusion. Specifically, after they were anesthetized with 1.5% isoflurane in 30% O_2 balanced by N_2O , control mice were transcardially perfused with 30-cc heparinized saline at room temperature, then with 10-cc 4% paraformaldehyde (PFA) in phosphate-buffered solution (PB). The brain was removed from the skull, post-fixed in 4% PFA for 12 hours, and transferred to 5 ml phosphate-buffered saline (PBS) solution afterwards. For those animals used for the MRI staining procedure, we prepared the brains as described above except for that we mixed contrast agents in 4% PFA used in transcardial perfusion and also in PBS buffer for extensive staining after fixation. In order to see whether the presence of Mn^{2+} in transcardial perfusion

buffer makes any difference on image contrast, three mice in the Mn^{2+} group were fixed without Mn^{2+} in 4% PFA during transcardial perfusion.

In the time course study of contrast agent penetration, mouse brains were incubated in a mixture of PBS and contrast agent for one, three, five, and seven days before T_1 measurement. For all other experiments, the brains were stored in the PBS buffer containing contrast agent of desired concentration for five to seven days before MRI acquisition.

Magnetic Resonance Imaging and Microcopy

All images were acquired with a 14T magnet (Magnex Scientific, England) connected to a ParaVision console (Bruker, Biospin, MA) with a gradient of 100 Gauss/cm (Bruker Biospin, MA). The brains prepared for *ex vivo* imaging were placed inside a glass tube filled with a proton-free susceptibility-matching agent (Perfluoro-compound FC-40, Fisher Scientific) to eliminate background signal. We used a Bruker 10 mm volume coil for all image acquisitions.

For two-dimensional T_1 , T_2 , and T_2^* measurements, we used an inversion-recovery prepared RARE (IR_RARE) sequence, a multi-slice multi-echo (MSME) sequence, and a multi-echo spoiled gradient echo (MGE) sequence. All images were acquired with an FOV of 0.95×0.95 cm, matrix size of 128×128 (in plane resolution of $74 \times 74 \mu\text{m}$), and slice thickness of 0.5 mm. Imaging parameters for MSME were: TR = 1200 ms, 8 echoes, 8.5 17.1 25.6 34.1 42.6 51.1 59.7 68.2 ms; for MGE were: TR=1200 ms, 8 echoes, first TE = 3.4 ms, echo spacing 2.45 ms; for IR_RARE were: TR/TE = 5000/7.9 ms, TI = 5.62, 55.62, 105.62, 205.62, 505.62, 1005.62, and 2005.62 ms (for brain stained with high concentration contrast agent), and TR/TE = 10000/7.9 ms, TI = 5.62, 105.62, 305.62, 505.62, 1005.62, 2005.62, and 3005.62 ms (for brains without contrast agent or in low concentration contrast agent).

For high-resolution magnetic resonance microscopy, we used a 3D spoiled gradient echo sequence (3D SPGR). Two sets of high-resolution images were acquired with TR/TE = 35/10 ms, FOV: $1.3 \times 1.3 \times 0.95$ cm, Matrix: $512 \times 512 \times 256$ (resolution: $25 \times 25 \times 37 \mu\text{m}^3$), 8 averages (about 11 hours), and TR/TE = 35/8 ms, FOV: $1.45 \times 0.95 \times 0.95$ cm, Matrix: $400 \times 256 \times 256$ (resolution: $37 \times 37 \times 37 \mu\text{m}^3$), 16 averages (about 11 hours), respectively. The flip angle was chosen to be the Ernst angle to maximize the signal.

Inductively coupled plasma (ICP) mass spectrometry

For each brain used for relaxivity calculation, one hemisphere was used for ICP analysis, and the other was used for T_1 and T_2 measurement to calculate contrast agent relaxivities at 0.47T, 1.4T and 14T. Each brain sample was first dissolved in 2 ml 70% HNO_3 at 37°C overnight. Samples were then centrifuged at 5000 rpm and 4°C for 15 min. For brains stained with MnCl_2 , we diluted 0.75ml of the completely dissolved brain sample with 2 ml diluents (each 2L diluents containing 1.857 L water, 0.143 L 70% HNO_3 , 2 ml Triton, 20 ppb final concentration Strontium, 20 ppb final concentration Lutetium, and 40 ppb final concentration gold). For the corresponding Mn^{2+} staining buffer, we mixed 0.5 ml staining buffer with the 1.5 ml diluent. For those brains stained in Gd-DTPA, 50 μl of the dissolved

brain sample was diluted with 2 ml diluents. For the corresponding Gd-DTPA staining buffer, we mixed 50 μ l staining buffer with the 5 ml diluent. At each step, samples were carefully weighed for calculating the final Gd and Mn concentrations. We used Agilent 7500a ICP-MS to measure the amount of Gd and Mn in brain samples and the corresponding staining buffers.

Relaxivity measurement, choice of concentration and imaging parameters—

We used four different concentrations to calculate the relaxivities of each contrast agent: 0 mM, 2.5 mM, 5 mM, and 7.5 mM for Gd-DTPA, and 0 mM, 0.12 mM, 0.24 mM and 0.36 mM for MnCl₂. For aqueous solution and brain samples, T₁, T₂, and T₂* were measured using 2D IR_RARE, MSME, and MGE sequences described above. In addition, we performed inductively coupled plasma (ICP) mass spectrometry to determine accurate contrast agent concentrations in the brain. We also measured the *ex vivo* relaxivities of Mn²⁺ at two other field strengths (i.e. 0.47T and 1.4T) using the same brain samples that were analyzed via ICP. The relaxivity of each contrast agent was then obtained using linear regression analysis.

To determine the concentration and imaging parameters for high-resolution MRM, we first quickly scanned the brains stained with contrast agent of different concentrations using the 2D MGE sequence described previously. We then calculated the SNR and contrast-to-noise ratio (CNR) for multiple regions from brains stained with different concentrations of Gd-DTPA or MnCl₂, scanned at different TE's. Those brains that exhibited the best CNR were imaged again using a 3D gradient echo sequence (resolution: 50×74×74 μ m³) involving different flip angles (10°, 25°, 55°, 70°, and 90°) and TR/TE of 35/4 and 8 ms. The concentration and imaging parameters were selected based on the combined results from 2D and 3D measurements.

Time course of contrast agent penetration—Mouse brains stained with 5 mM Gd-DTPA and 0.24 mM MnCl₂ were imaged at one day, three days, five days, and one week after soaking in PBS buffer with contrast agents of desired concentrations. We measured T₁ using IR_RARE sequences at each time point. We then calculated T₁ at each time point in order to characterize tissue T₁ change over the time.

Data Analysis—Region of interest (ROI) analysis was used to investigate changes of tissue T₁, T₂, and T₂* after MRI contrast agent staining, and the relaxivities of Gd-DTPA and MnCl₂ in *ex vivo* mouse brain. ROIs pertaining signal from the whole brain, cerebral cortex, striatum, thalamus, hippocampus, and cerebellum were manually outlined based on Paxinos mouse brain atlas (Franklin and Paxinos, 2007) using AFNI (Cox, 1996). We used Matlab (Mathworks, Natick, MA) to analyze MRM data. T₁, T₂, and T₂* in each ROI were fitted using non-linear least square or first-order polynomial algorithms based on averaged MR signal intensity. In tissue relaxivity calculations, concentrations measured from ICP analysis were used. We used Mouse BIRN Atlasing Toolkit (MBAT) (Boline JK et al., 2006) for brain segmentation and structure labeling. All the structures were identified and labeled based on the Allen Brain Atlas (Lein et al., 2007) and Paxinos mouse brain atlas. ImageJ software package (Abramoff et al., 2004) was used for image visualization. Unless otherwise specified, all statistical analyses in the study were performed using a two-tailed t-

test. Statistical significance was accepted at a confidence level of 0.95. All of the numerical data were presented as averages $\pm$ standard deviation.

Results

Relaxivity measurement and microscopic tissue distribution

We first determined contrast agent concentrations in *ex vivo* brain using ICP analysis. Table 1 shows contrast agent concentrations in the PBS staining buffer (5 ml) before and after brain staining and in the corresponding *ex vivo* brains. Buffer Gd-DTPA concentration was not significantly affected by brain staining over time. There was only slight change in Gd-DTPA concentration, going from 5 mM to 4.9 mM and from 2.5 mM to 2.4 mM after brain incubation (Table 1). On the other hand, Mn^{2+} concentrations in the buffer dropped significantly after brain staining; while, its concentration in the brain was much higher than that of the buffer. In addition, there was no obvious linear relationship between resultant brain Mn^{2+} concentration and initial buffer concentration (see Table 1).

Table 2 summarizes the relaxivities of the two contrast agents in both aqueous solution and *ex vivo* mouse brain at 14T. The molar relaxivities (r_1 and r_2) of Gd-DTPA and MnCl_2 were analyzed using linear regression based on R_1 and R_2 measured at five different concentrations in both solution and tissue (whole brain). The r_1 of Gd-DTPA in aqueous solution and *ex vivo* brain were measured to be $3.88 \pm 0.12 \text{ mM}^{-1} \cdot \text{s}^{-1}$ and $3.58 \pm 0.01 \text{ mM}^{-1} \cdot \text{s}^{-1}$, respectively. Meanwhile, the r_2 of Gd-DTPA in *ex vivo* brain ($13.84 \pm 0.79 \text{ mM}^{-1} \cdot \text{s}^{-1}$) was significantly higher than in aqueous solution (5.434 ± 0.11) (see Table 2). The MR relaxivity of MnCl_2 trended differently than Gd-DTPA. The r_1 of MnCl_2 in solution was $6.3 \pm 0.1 \text{ mM}^{-1} \cdot \text{s}^{-1}$, lower than the r_1 of $8.57 \pm 0.55 \text{ mM}^{-1} \cdot \text{s}^{-1}$ in *ex vivo* tissue. MnCl_2 also exhibited a relatively high transverse relaxation enhancement effect, with greater r_2 in aqueous solution ($162.44 \pm 3.74 \text{ mM}^{-1} \cdot \text{s}^{-1}$) than in *ex vivo* brain ($124.9 \pm 15.44 \text{ mM}^{-1} \cdot \text{s}^{-1}$).

Table 3 shows the relaxivities of Mn^{2+} in *ex vivo* brain tissues at different field strengths, with r_1 the highest at 0.47T ($69.89 \pm 6.67 \text{ mM}^{-1} \cdot \text{s}^{-1}$). The r_1 drops dramatically as the field strength increases, whereas r_2 increases with magnetic field increases.

We further evaluated the Gd-DTPA tissue distribution based on the relaxivities and ICP results. Gd-DTPA has a similar r_1 in *ex vivo* tissue to the r_1 value in aqueous solutions (Table 2), indicating that the T_1 relaxation enhancement of Gd-DTPA in *ex vivo* tissue is also caused by the same dipole relaxation mechanism that governs paramagnetic proton relaxation in solution. The r_1 values of Gd-DTPA in tissue and solution also imply that there is no protein or macromolecule binding of Gd-DTPA in brain tissue. We, therefore, can use the concentrations of Gd-DTPA in tissue and staining buffer to estimate the tissue space that is occupied by Gd-DTPA after brain staining. From the ICP results (Table 1), we found that the final brain Gd-DTPA concentration was 1.97 mM if 2.5 mM staining buffer was used or 3.81 mM if 5 mM staining buffer was used. Assuming that the total tissue space could be accessible by Gd-DTPA, the final Gd-DTPA tissue concentration would be equal to that of staining buffer since we are using large volume of buffer to stain the brain. By taking the ratio of Gd-DTPA concentration in tissue to that in staining buffer, we estimated that Gd-

DTPA occupied about 77% of the total tissue space. This result indicates that Gd-DTPA is not uniformly distributed in *ex vivo* brain tissue. In addition, as Fisel and Weisskoff pointed out, the microscopic distribution of contrast agent can generate local magnetic gradient that influences proton relaxation over a longer range than the dipole relaxation (Fisel et al., 1991, Weisskoff et al., 1994). This together with electron-proton dipole-dipole interaction can cause higher T_2 relaxation enhancement in tissue than in solution. Indeed, this is what we observed in our study: higher r_2 in *ex vivo* brain tissue than in aqueous solution (Table 2: $13.84 \pm 0.79 \text{ mM}^{-1} \cdot \text{s}^{-1}$ in tissue vs. $3.58 \pm 0.01 \text{ mM}^{-1} \cdot \text{s}^{-1}$ in solution). This further suggests that Gd-DTPA is not uniformly distributed in tissue, and is probably confined in local microscopic space.

Choice of concentration, staining time, and imaging parameters

To determine the contrast agent concentration to use, we characterized image contrast between three brain regions - cortex, thalamus, and striatum - using a 2D MGE sequence. Figure 1 shows the CNR (cortex vs. thalamus and cortex vs. striatum) as a function of TE for brains immersed in the two contrast agents at different concentration. For brains stained with Gd-DTPA, we found that the best CNR for the selected regions (cortex vs. thalamus and cortex vs. striatum) was achieved with a concentration of 5 mM (Figures 1C and 1D). Brains stained with Mn^{2+} showed different optimal concentrations in the same selected regions (Figures 1A and 1B). The CNR between cortex and thalamus (Figure 1A) peaked around TE=8 ms, at a concentration of 0.24 mM. However, the CNR between cortex and striatum decreased as TE increased, and reached its maximum at a concentration of 0.36 mM. These results suggest that the optimal concentration for these experiments for Gd-DTPA is ~5 mM, and for Mn^{2+} , 0.24 ~ 0.36 mM. Based on these data, we chose to use 5 mM Gd-DTPA and 0.24 or 0.36 mM MnCl_2 in our subsequent MRM studies.

We determined image parameters using a low resolution 3D FLASH (i.e. 3D SPRG) sequence with different flip angles and TE's at the TR of 35 ms. Images in Figure 3 show how contrast changes in mouse brains stained with 5 mM Gd-DTPA (Figure 3A) and 0.24 mM MnCl_2 (Figure 3B) at different flip angles (10° , 25° , 55° , 70° , and 90°) and with TE's of 4 ms and 8 ms. As shown in the top panel of Figures 3A and 3B, we observed no significant change in image contrast with increased T_1 weighting, i.e., increased flip angle, which indicated a loss of T_1 contrast in fixed brain tissue (Schumann et al., 2001). However, the overall image contrast was greatly enhanced as the echo time was increased from 4 ms to 8 ms. Accordingly, we chose to image the brain samples with TE of 8 ms at the Ernst angle, to optimize image contrast and SNR.

To understand the dependency of tissue relaxation times on the duration of contrast agent staining, T_1 of mouse brains stained with 5 mM Gd-DTPA or 0.24 mM MnCl_2 were measured at one day, three days, five days, and seven days after incubation in the PBS buffers doped with corresponding contrast agent. As shown in Figure 2, we plotted the changes in T_1 in different brain regions over time, which clearly demonstrated that after a five-day incubation period, the contrast agent concentration reaches equilibrium across the whole brain. To ensure full penetration and equilibrium of contrast agent, all of the brains

used for subsequent MRM studies were incubated for at least five to seven days before imaging.

Structure and Image Contrast of *ex vivo* Brain in Gd-DTPA and MnCl₂

Figure 4 shows coronal, sagittal, and axial views of mouse brains in 0.24 mM MnCl₂ (Figure 4A) and 5 mM Gd-DTPA (Figure 4B). Gd-DTPA and MnCl₂ greatly enhanced overall image contrast to allow visualization of detailed anatomical structures of mouse brain. We further labeled different brain structures (Figure 5B) using MBAT software based on the *ex vivo* brains stained with either 5 mM Gd-DTPA (Figure 5A) or 0.36 mM MnCl₂ (Figure 5C). Different brain structures such as hippocampus, caudate putamen, thalamus (including thalamus nuclei), globus pallidus, habenula, and corpus callosum can be clearly identified from these high-resolution images. Furthermore, our data also demonstrate that the two contrast agents stain the brains differently, in multiple brain regions, as shown in Figure 4 and 5. For example, in the cerebellar cortex, the image contrast between granular layer and molecular layer of brains stained by these two different agents is reversed. The granular layer was stained dark by MnCl₂ but appeared bright for brains in Gd-DTPA. In addition, the distinct layer (possibly Purkinje layer in the cerebellar cortex) between granular and molecular layers that is stained dark in Gd-DTPA brains is not apparent in the MnCl₂-stained brain (Figure 4). In the cerebral cortex, Mn²⁺ seems to stain a particular cortical layer dark (Figure 4A sagittal view and 5A axial view). On the other hand, Gd-DTPA appears to stain the cortex uniformly. The lamina structure of hippocampus is more obvious in the Mn²⁺-stained brains than in those stained by Gd-DTPA (Figure 4 and 5). Interestingly, both contrast agents stained the dentate gyrus bright, but pyramidal layer in CA region appears better resolved by Gd-DTPA staining. These subtle differences in image contrast offered by these two MRI staining agents not only allow better structure delineation and segmentation but also demonstrate the ability to achieve MR differentiation staining by using different MR contrast agents.

Discussion

This study clearly demonstrates that Gd-DTPA and MnCl₂ affect T₁, T₂, and T₂* relaxation times through different relaxation mechanisms in solution and *ex vivo* brain samples. Moreover, these contrast agents exhibited different biological properties and tissue affinities. As a result, MR images of *ex vivo* mouse brains immersed in Gd-DTPA or Mn²⁺ solutions revealed different local image contrasts. The distinct image contrast indicated independent tissue differentiation abilities of each contrast agent, suggesting that tissue differentiation can be achieved through the use of different MRI contrast agents, providing flexibility analogous to that inherent in conventional histology.

Relaxation mechanisms and tissue affinities of Gd-DTPA and MnCl₂

Paramagnetic contrast agents affect tissue relaxation primarily through electron-proton dipole-dipole interaction (Lauffer, 1987). In addition, for paramagnetic ions like Mn²⁺, which have long electron spin relaxation times, the contribution of scalar coupling to transverse relaxation becomes prominent, especially at high magnetic field (Bertini et al., 1996). This may lead to the strong T₂ relaxation enhancement effect of Mn²⁺, both in

solution and in tissue, as observed in our study at 14T. However, this scalar coupling relaxation has negligible effect on T_1 relaxation at relatively high magnetic fields (Bloembergen, 1957), since T_1 relaxation enhancement for Mn^{2+} ion occurs mainly through dipole-dipole interaction. Dipole relaxation can be significantly affected by a change in the molecular tumbling rate, a reflection of binding to macromolecules, and has strong field dependency. Previously, the manganese ion has been shown to provide highly enhanced T_1 relaxation effect ($r_1 = 56 \text{ mM}^{-1} \cdot \text{s}^{-1}$ at 20 MHz) in the rat myocardium due to intracellular protein binding (Nordhoy et al., 2004). In our study, the relaxivity of Mn^{2+} in *ex vivo* mouse brains, measured at the same field strength, agrees with results published in the literature, implying protein or macromolecule binding of Mn^{2+} ion. This potential binding to proteins or macromolecules is further supported by field-dependent relaxivity measurements (NMRD) of Mn^{2+} in *ex vivo* brain (Table 3). Moreover, ratios of r_2/r_1 at relatively high field strengths further demonstrate the significant contribution of scalar relaxation mechanism to transverse relaxation at high field. Perhaps more important, ICP analysis has revealed that manganese ion concentration is much higher in the brain than in the corresponding staining buffer. Indeed, the buffer Mn^{2+} concentration dropped significantly after brain incubation, which suggests that the process of Mn^{2+} tissue staining involves mechanism other than passive diffusion in excised tissue. These findings together would seem to suggest tissue Mn^{2+} binding mechanisms that are independent of active cellular uptake described in MEMRI experiments (Lin and Koretsky, 1997, Pautler and Koretsky, 2002).

Gd-DTPA, unlike Mn^{2+} , influences proton relaxation mainly through dipole-dipole interaction. The relaxivity values in aqueous solution agree well with literature results. Using the tissue Gd-DTPA concentration obtained from ICP analysis, the r_1 of Gd-DTPA was found to be $3.58 \pm 0.01 \text{ mM}^{-1} \cdot \text{s}^{-1}$. This value is close to that in aqueous solution, indicating the same T_1 paramagnetic relaxation mechanism in *ex vivo* tissue. In addition, the lower Gd-DTPA concentration (Table 1) in *ex vivo* samples as revealed by ICP analysis suggests that there is tissue space inaccessible to Gd-DTPA. This result further indicates that Gd-DTPA most likely stains tissue through passive diffusion. Based on the ratio of Gd-DTPA concentration in tissue to that in solution, we estimated that, on average, Gd-DTPA was distributed to a tissue space occupying approximately 77% of the total brain volume. Given the fact that Paraformaldehyde fixation can crosslink proteins and increase the overall membrane permeability, this surprisingly high percentage suggests at least parts of the original intracellular space becomes accessible to Gd-DTPA following the tissue fixation. In addition, the higher r_2/r_1 ratio in *ex vivo* brain compared to that measured in solution (Table 2) suggests that Gd-DTPA is not uniformly distributed in excised brain tissue. This microscopic Gd-DTPA tissue distribution creates microscopic local magnetic field gradients, which contribute to transverse relaxation enhancement (Weisskoff et al., 1994, Kovacevic et al., 2005) leading to increased r_2 in *ex vivo* tissue. Furthermore, the similar r_1 in aqueous solution and *ex vivo* tissue indicates that there is little or perhaps no tissue binding or active staining process for Gd-DTPA in excised brain tissue.

Bio-distribution and its effect on image contrast

Gd-DTPA, as mention earlier, distributed into the *ex vivo* brain tissue through passive diffusion and demonstrated a selective T_2 and T_2^* relaxation enhancement in *ex vivo* brain

due to nonuniform microscopic tissue distribution. MnCl_2 , on the other hand, has a more complicated bio-distribution than Gd-DTPA. Brains stained with MnCl_2 showed distinct tissue- or region-specific relaxation time differences. For example, in cerebellar cortex, the change of R_1 is significantly higher in granular layer than in molecular layer (Figure 6A). Mn^{2+} , a calcium analog, is known to be able to enter neurons via calcium channels (Narita et al., 1990, Aschner and Aschner, 1991), and has high affinity to membrane and intracellular proteins (Nordhoy et al., 2003). Unlike the molecular layer that is consisted of neuronal dendrites, fiber tracks, and small interneurons, the granular layer is a densely populated neuronal cell layer. It is therefore very likely that relatively more Mn^{2+} is drawn to this layer and accumulates in the neuronal cell bodies that reside there. This Mn^{2+} tissue microscopic distribution further creates a local microscopic gradient that leads to the small but significant increase in R_2^* relaxation observed in the granular layer (Figure 6B). Enhanced T_1 and T_2^* relaxation associated with accumulation of Mn^{2+} in the granular layer, in turn, leads to the unique contrast we observed in the T_2^* -weighted image (Figures 8B and 8C): darker granular layer and brighter molecular layer. The differentiation of the cerebellar lamina that we observed using Mn^{2+} in *ex vivo* mouse brain is also consistent with previous *in vivo* MRI studies in rats and mice using MnCl_2 (Aoki et al., 2004, Silva et al., 2008). Moreover, the presence of Mn^{2+} in the buffer used for transcardial perfusion was not a factor for the overall image contrast enhancement. The image contrast remains similar between Mn^{2+} stained brains that were initially fixed with or without Mn^{2+} in the transcardial perfusion buffer (Figure 7). Together, these results suggest that the presence of activity-independent Mn^{2+} tissue binding or uptake mechanisms, which do not require cells or tissue to be alive. Such biochemical mechanisms appear to dominate the tissue contrast observed in selective brain regions in our *ex vivo* study, and further suggest that some of the observed manganese enhanced MRI (MEMRI) contrast (e.g. the cerebellar lamina) may be from activity-independent uptake or binding.

Although both contrast agents can significantly shorten T_1 , T_2 , and T_2^* relaxation times in *ex vivo* brains imaged at 14T, images (Figures 3A and 3B, top panel) acquired with the T_1 -weighted sequence revealed relatively poor tissue contrast compared to those acquired with the T_2^* -weighted sequence (Figures 3A and 3B, bottom panel). This difference suggests that image contrast in *ex vivo* mouse brain originates mainly from T_2 or T_2^* contrast, possibly due to the loss of T_1 contrast in fixed tissue (Schumann et al., 2001). We, therefore, used T_2^* -weighted images in all our *ex vivo* MRM studies. This is different from the imaging protocols used in Johnson's group, in which T_1 -weighted images were used primarily for high resolution MRM (Badea et al., 2007a). Difference in specimen preparation may account for the different choice of imaging protocols. In the active staining method developed by Johnson's (Johnson et al., 2002) group, brains were perfuse-fixed with 50 mM ProHance and subsequently fixed in 10% formalin without contrast agent. However, in our protocol, 5 mM Gd-DTPA 4% PFA solution was used for both transcardial perfusion and subsequent incubation. As a result, contrast agent distribution and final concentration in the brain are possibly different, leading to different tissue T_1 , T_2 and T_2^* relaxation times and differently optimized MR imaging parameters.

Images of mouse brains stained with Gd-DTPA or MnCl_2 revealed different staining patterns and varying contrast in multiple regions of the brain (Figure 4). This tissue differentiation and potentially flexible staining capability offered by MR contrast agents suggests a path toward future studies that might detail the principles behind the biophysical and biochemical distributions of these agents that may facilitate the development of new agents with more selective staining properties. When looking at the gene expression maps from the Allen Brain Atlas (<http://www.brain-map.org>), we found that the staining patterns of both MnCl_2 and Gd-DTPA showed positive correlation with certain gene expression profiles. For example, the spatial expression of calretinin or calbindin 2 (calb2, the main intracellular Ca^{2+} binding protein expressed in cerebella granular cells) showed striking correspondence with the staining patterns of MnCl_2 and Gd-DTPA (Figures 8A, 8B, and 8C). Moreover, the image contrast observed between Gd-DTPA and MnCl_2 stains was reversed in these regions. In another example, the distinctive dark line between the two layers in the cerebellar cortex of our Gd-DTA-stained brains shows remarkable correlation with lecithin cholesterol acyltransferase (Lcat, an important protein in lipid metabolism expressed in cerebellum) expression (Figures 8C and 8D). However, it is important to note that the correlations observed here are not specific to Calb2 or Lcat. For example, other genes such as Zinc1 (zinc finger protein of the cerebellum 1) and CAMKK2 (calcium/calmodulin-dependent protein kinase kinase 2) also showed correlation pattern similar to that observed for Calb2. Although correlations between the staining patterns (using Mn^{2+} or Gd-DTPA) and the gene expression maps from the Allen Brain Atlas are not specific to particular genes, these interesting correlations suggest that MRM staining may have a great potential in high-resolution molecular imaging with the development of highly selective or target specific “smart” MR contrast agents. As a result, MRM is not only an important tool for anatomical screening and phenotyping, but it may also play a significant role in studies of gene expression (Liu et al., 2007) and pathological diagnosis (Blackwell et al., 2008).

In summary, we characterized the relaxivities and tissue distributions of two commonly used contrast agents, Gd-DTPA and MnCl_2 , in *ex vivo* mouse brains. We demonstrated that their unique relaxation and tissue properties led to differentiated MR “staining” in *ex vivo* mouse brains, which greatly enhanced the capability of MRI to delineate tissue structures in addition to providing improved SNR. Our results also suggest that through the use of specific contrast agents, one may potentially perform tissue/cell-specific MRM, which in combination with histology can greatly enhance our ability to delineate brain cytoarchitecture and image molecular level changes in normal or transgenic mouse models.

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Figure 1.

CNR vs. TE of mouse brains stained with Gd-DTPA or MnCl₂ at different concentrations based on 2D MGE sequence. Top panel: brains in 0.12, 0.24, or 0.36 mM MnCl₂ showing image contrast between cortex and thalamus on the left, cortex and striatum on the right; bottom panel: brains in 1, 2.5, 5, or 7.5 mM Gd-DTPA, left: cortex v.s. thalamus, right: cortex v.s. striatum.

Figure 2.

Changes in tissue T₁ relaxation time over time for mouse brains stained with 5 mM Gd-DTPA and 0.24 mM MnCl₂. These data demonstrate that at least three days are needed for the contrast agent to fully penetrate brain tissue. To ensure good tissue staining, approximately five days are needed before imaging.

Figure 3. Coronal sections of mouse brains stained with 5 mM Gd-DTPA (3A) or 0.24 mM MnCl₂ (3B). Images were acquired using 3D FLASH sequence with different flip angles (from left to right, 10°, 25°, 55°, 70°, and 90°) and TEs (top: TE = 4 ms, bottom: TE = 8 ms).

Figure 4. high-resolution MRM of *ex vivo* mouse brain stained with 0.24 mM MnCl_2 (4A) or 5 mM Gd- DTPA (4B) reveals detailed anatomical structure with different image contrast in certain regions brain, e.g., cerebellum, cortex, and hippocampus.

Figure 5.

Cross-section images of an *ex vivo* mouse brains stained with 5 mM Gd-DTPA (5A) or 0.36 mM MnCl₂ (5C) and segmented brain structures (5B). These two contrast agents showed different tissue differentiation abilities. For example, Frimbria is better visualized in brain stained with Mn²⁺. Lamina structure of hippocampus is more pronounced, especially the axial view (8C), in brain stained with Mn²⁺. More interestingly, the image contrast of cerebellar cortical layers (granular and molecular layers) is reversed between the brains stained Gd-DTPA and Mn²⁺. By cross examination of MRM images obtained using these two staining agents, detailed anatomical structures can be better resolved.

Figure 6.

Changes in R_1 , and R_2^* values in cerebellar molecular and granular layers of mouse brain after stained with 0.36 mM $MnCl_2$. The higher R_1 and R_2^* in granular layer indicate that Mn^{2+} is possibly accumulated in neuronal cells in this layer.

Figure 7.
Images of *ex vivo* mouse brains fixed without (A) and with (B) Mn^{2+} in 4% PFA during transcatheter perfusion. Image contrast is not significantly affected by the different staining protocols.

Figure 8.

Regions in the cerebellum of brains stained with MnCl₂ or Gd-DTPA showed correlation with gene expression patterns of Ca²⁺ binding protein calretinin (calb2) in cerebellum and lectithin cholesterol acyltransferase (Lcat). Calb2 and Lcat expression images were obtained from online Allen Brain Atlas (<http://www.brain-map.org>).

Table 1

concentrations of Gd-DTPA and MnCl₂ staining buffer and ex vivo brain. Total buffer volume is 5 ml.

Mn ²⁺	Concentration in buffer (initial) (mM)	Concentration in buffer (after staining) (mM)	Concentration in the brain (mM)
	0	0.00014	0.002
	0.12	0.082	0.29
	0.24	0.19	0.59
	0.36	0.23	0.71
Gd-DTPA			
	2.5	2.36	1.97
	5.0	4.92	3.81

Table 2relaxivities of Gd-DTPA and MnCl₂ in water and ex vivo brain at 14T

	Gd-DTPA		MnCl ₂	
	In water	In tissue	In water	In tissue
r_1 (mM ⁻¹ ·s ⁻¹)	3.88 ± 0.12	3.58 ± 0.01	6.3 ± 0.1	8.57 ± 0.55
r_2 (mM ⁻¹ ·s ⁻¹)	5.434 ± 0.11	13.84 ± 0.79	162.4 ± 3.74	124.9 ± 15.44
Ratio: r_2/r_1	1.4	3.86	25.79	14.57

Table 3

relaxivities of MnCl_2 in ex vivo brain measured at different field strength: 0.47T, 1.4T, and 14T. The NMRD profile strongly suggests macromolecule binding of Mn^{2+} in exercised brain tissue.

B_0 (T)	Larmor freq (MHz)	r_1 ($\text{mM}^{-1} \text{S}^{-1}$)	r_2 ($\text{mM}^{-1} \text{S}^{-1}$)	Ratio: r_2/r_1
0.47	20	69.89 ± 6.67	98.01 ± 8.82	1.40
1.4	60	56.78 ± 4.35	105.92 ± 8.89	1.87
14	600	8.57 ± 0.55	124.9 ± 15.44	14.7