Mn Enhancement and Respiratory Gating for In Utero MRI of the Embryonic Mouse Central Nervous System

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The mouse is the preferred model organism for genetic studies of mammalian brain development. MRI has potential for in utero studies of mouse brain development, but has been limited previously by challenges of maximizing image resolution and contrast while minimizing artifacts due to physiological motion. Manganese (Mn)-enhanced MRI (MEMRI) studies have demonstrated central nervous system (CNS) contrast enhancement in mice from the earliest postnatal stages. The purpose of this study was to expand MEMRI to in utero studies of the embryonic CNS in combination with respiratory gating to decrease motion artifacts. We investigated MEMRI-facilitated CNS segmentation and three-dimensional (3D) analysis in wild-type mouse embryos from midgestation, and explored effects of Mn on embryonic survival and image contrast. Motivated by observations that MEMRI provided an effective method for visualization and volumetric analysis of embryonic CNS structures, especially in ventral regions, we used MEMRI to examine Nkx2.1 mutant mice that were previously reported to have ventral forebrain defects. Quantitative MEMRI analysis of Nkx2.1 knockout mice demonstrated volumetric changes in septum (SE) and basal ganglia (BG), as well as alterations in hypothalamic structures. This method may provide an effective means for in utero analysis of CNS phenotypes in a variety of mouse mutants. Magn Reson Med 59:1320–1328, 2008. © 2008 Wiley-Liss, Inc.

Key words: neuroimaging; fetal; MEMRI; contrast; motion correction

In recent years, the mouse has been established as the primary system for studying the genetic basis of mammalian development, and for modeling many human diseases. In particular, the mouse central nervous system (CNS) has been a major focus of developmental genetics research. Most mammalian brain development occurs prior to birth, and a continuing challenge has been the establishment of an in vivo and longitudinal methods of analysis of the mouse embryonic CNS within the maternal uterus.

Previous In Utero Brain Imaging Studies in Mice

As in the clinical setting, ultrasound imaging is currently the primary method for visualization of living mouse embryos (reviewed in Ref. 1). High frequency (40–50 MHz) ultrasound imaging, known as ultrasound biomicroscopy (UBM), enables real-time visualization of anatomy including the cardiovascular system and developing neural tube in the early to mid-gestational embryo. UBM is not well-suited for imaging late embryonic brain development, because of limited penetration of high-frequency ultrasound through the skull. In addition, current techniques for selective UBM contrast modification are limited. Micron-sized “microbubble” contrast agents are currently in use for imaging of the vascular space, but the size of these agents prevents extravasation and differential labeling within brain tissue.

Compared to ultrasound, magnetic resonance imaging (MRI) has excellent contrast versatility through combinations of sequence design and administration of diverse targeted and physiologically relevant contrast agents. High-field MRI has improved signal-to-noise ratio (SNR) and resolution to a scale relevant for mouse anatomy (reviewed in Ref. 2). The primary challenges to in vivo MRI of embryos include the relatively long acquisition times required to achieve adequate SNR, making susceptibility to motion a major consideration. With application of rapid acquisition sequences, in vivo embryo MRI has shown some promise. Notably, studies have demonstrated gross visualization of the mouse embryo CNS including the brain and brain ventricles (3–5). Increased anatomical detail was observed in larger rat embryos including appreciation of the forebrain, midbrain, hindbrain, and spinal cord (6). However, visualization of brain substructures such as cortex, striatum, or deep gray matter was not possible, in part because resolution in these studies has been limited to more than 195 μm isotropic or 156 μm in-plane with ≥800-μm slice thickness. Despite 10 years of progress, in utero MRI-based anatomical phenotyping of mutant mouse embryos has not yet been reported. To date, all mutant phenotype analyses of brain defects have used MRI on fixed embryos (7,8).

Challenges for Effective In Utero MRI of Living Mouse Embryos

MRI is a high-resolution, noninvasive imaging method that may provide a powerful approach for in vivo analysis of the mouse embryonic brain. Current achievable spatial resolution for in vivo MRI in the mouse is close to 100 μm isotropic, which should be adequate for analyzing many brain structures in late gestational mouse embryos (2). Two remaining challenges must be addressed: image artifacts due to physiological motion, and low intrinsic MR contrast in immature embryonic neural tissues. The goal of this study was to address these two challenges using re-
sporatory gating to minimize motion artifact and manganese (Mn)-enhancement to increase brain signal and contrast to achieve more meaningful in utero MRI of the embryonic mouse CNS.

Motion is often a concern in abdominal MRI studies. Two approaches have been used to deal with this challenge in human MRI: 1) rapid acquisition sequences often in combination with breathhold; and 2) respiratory and/or cardiac gating methods. Previous in utero rodent embryo imaging efforts applied both fast imaging sequences (3,4,6), and respiratory gating (5) to minimize motion artifacts. Imaging of other abdominal organs such as the liver have also utilized physiological gating methods. These have included prospective approaches in which data acquisition is triggered to respiratory and/or cardiac signals, as well as retrospective approaches in which physiologically similar data are reconstructed with exclusion of dissimilar data during postprocessing (9,10).

Mn-enhanced MRI (MEMRI) methods have been used with considerable success for MR contrast enhancement in the rodent CNS from neonatal to adult stages (11–14). Mn is a paramagnetic ion that acts as a calcium analog, accumulating in a number of CNS cell types including active neurons, and providing positive contrast on T2-weighted MRI. When administered generally, for example via intraperitoneal (IP) injection, Mn first crosses the blood brain barrier (BBB) primarily at the choroid plexus (15) or more widely if the BBB is disrupted (16,17). MEMRI studies of developing cerebellum in the early postnatal period correlated Mn-enhancement anatomically with elaboration and differentiation of the internal granular layer (11). The utility of MEMRI methods extends well beyond observation of brain anatomy, with recent studies demonstrating preferential activation (12) and connectivity (18,19) of stimulated sensory systems.

MEMRI methods have not previously been applied to visualization of the embryonic CNS, in which both BBB and neuronal function and connectivity are immature (20). During this period, MEMRI as a marker of neuronal function might be expected to show correlation with expression of molecular markers of neuronal maturation, such as neuron specific tubulin (Tuj1; Ref. 21), or to reflect BBB integrity (20) (see Discussion for further exploration in light of the results of this study). Although Mn-containing compounds have been found to be relatively nontoxic to adult animals in previous MEMRI studies (22); in toxicology studies, embryonic exposure was associated with significant toxicity (23,24). Therefore, we expected that the desirable Mn-enhancing MRI effects may have to be balanced against the toxic effects of Mn on fetal mice.

Objectives of the Current Study
The overall goal of the current study was to develop an MRI approach for in vivo microimaging of the mouse CNS inside the maternal uterus. The specific objectives were four-fold. First, we aimed to provide 3D brain images over a range of developmental stages with isotropic resolution of 100 μm. Second, we sought to develop a method that combined respiratory gating and MEMRI to provide CNS-specific contrast enhancement with minimal motion artifact. Third, knowing the potential toxic effects of Mn administration on mouse embryos, we sought to investigate whether a compromise could be struck between Mn-enhancement and toxicity for fetal neuroimaging. Finally, having analyzed the enhancement characteristics in the embryonic mouse CNS, we aimed to perform imaging and analysis of an appropriate mouse model with brain defects to determine the utility of our approach for in utero phenotype analysis.

MATERIALS AND METHODS

Animals
All procedures used in these studies were approved by the Institutional Animal Care and Use Committee at New York University School of Medicine. Timed-pregnant ICR strain (Taconic Farms, Germantown, NY) mice were used for studies of wild-type mice, where the stage was denoted as the day following identification of a vaginal plug (embryonic day 0.5 [E0.5] is defined as noon of the plug day).

For investigation of toxicity, embryo survival was assessed at two points following Mn administration to assess for survival at MRI, and survival to near-birth. Short-term survival in E12.5 and E14.5 embryos was assessed 24 h after high-dose Mn administration using UBM visualization of heart contraction (procedure described below). Survival was documented as percent of living embryos in the litter (number living/total × 100). Five pregnant mice were evaluated in each group. Mice often cannibalize dead fetuses at birth, making it difficult to assess survival at that time. Therefore, longer-term survival was assessed on the day prior to birth, E18.5, to be able to accurately assess the total number of embryos, living and dead, in each litter. Following administration of Mn at 20, 40, and 80 mg/kg at E11.5 and E13.5, and 80 mg/kg at E16.5, we assessed survival (at E18.5) by euthanizing the pregnant mother by cervical dislocation and performing rapid laparotomy to identify likely viable (responsive to gentle probe touch) and likely nonviable (unresponsive to probe touch) or resorbing (degraded) embryos. For longer-term survival data, each group was represented by at least four litters. The specific number of litters for each group is reported in the Results section.

For mutant mouse analyses, Nkx2.1 heterozygous mutant mice were bred to produce homozygous null offspring (Nkx2.1−/−) as well as heterozygous (Nkx2.1+/−) and wild-type (Nkx2.1+/+) littermate controls. Polymerase chain reaction (PCR) of adult or embryo tail DNA was used to identify genotypes, using primers for Neo and Nkx2.1 (25).

Administration of Mn Chloride
A 100 mM aqueous solution of Mn chloride (MnCl2) was made by dissolving MnCl2 tetrahydrate (FW = 197.9; Sigma-Aldrich, St. Louis, MO, USA) in ultrapure water. This solution was diluted in sterile phosphate buffered saline (PBS) to a concentration of 30 mM, which was administered via IP injection at a dose per weight of 20, 40, or 80 mg/kg (0.1, 0.2, or 0.4 mmol Mn/kg) to the pregnant mouse 24 h prior to imaging. To avoid directly injuring embryos with the IP injection, the mouse was held in a supine position with the head tilted down during the injection to allow for abdominal organs to fall away from
the base of the abdomen. The needle tip was inserted in the midline midway between the umbilicus and the pubis to avoid the bladder and embryos inferiorly, and the liver superiorly. For survival studies, MnCl₂ solution was administered mid-day on the noted embryonic day.

MRI

For imaging, mice were anesthetized with isoflurane gas delivered via vaporizer/anesthesia machine (VMC Matrix, Orchard County, NY, USA); 5% isoflurane in air for induction followed by 0.5–1.5% isoflurane in air via nosecone for maintenance. Following anesthetic induction, the mouse was placed side-down in the mouse holder with the lateraled uterine horns in the abdomen positioned within the surface coil, a half-cylinder shaped home-built coil (30 mm length and 14 mm diameter) originally designed for supine spine imaging (Fig. 1a). The coil was used for both transmit and receive modes. Images were acquired using a SMIS console (Surrey Medical Imaging Systems, Guildford, UK) and 7T, 200-mm horizontal bore magnet (Magnex Scientific, Abingdon, UK) with actively shielded gradients (Magnex; gradient strength = 250 mT/m, rise time = 200 μs). A T₁-weighted three-dimensional (3D) gradient echo sequence was used achieving 100-μm isotropic resolution: repetition time (TR) = 40 ms; echo time (TE) = 5 ms; flip angle = 35° (gated sequence) or 65° (nongated); field-of-view (FOV) = 24 mm × 24 mm × 48 mm; matrix size = 240 × 240 × 480. Respiratory motion was monitored using a pneumatic pillow (BioPAC Systems, Goleta, CA, USA) fixed to the abdomen of the pregnant mouse with a Velcro™ strap (Fig. 1a).

For gating, the analog respiratory signal was band-pass filtered to decrease baseline drift (Fig. 1b), and converted to transistor-transistor logic (TTL) using thresholding to identify the active part of the breathing cycle (Fig. 1c) (Acqknowledge Software; BioPAC Systems). The 3D excitation pulse was applied every TR regardless of breathing motion to maintain relative steady state magnetization (Fig. 1d). Data acquisition was gated such that the same phase encoding step was reiterated until gating conditions were met, i.e., until the mouse was not actively breathing (Fig. 1e). Total gated imaging time per mouse was 70–90 min (one average). Ungated imaging time was 120 min (two averages).

UBM

Mouse preparation and imaging with UBM has been described in detail previously (26). Briefly, pregnant mice were anesthetized with sodium pentobarbital (6 mg/100 g body weight, IP) with added MgSO₄·H₂O (14 mg/100 g body weight) to reduce spontaneous uterine contractions. After sedation, the maternal abdomen was incised and the entire uterus was gently pulled out of the abdomen to count the total number of embryos. The embryos/uterus were replaced in the abdomen, and the pregnant mouse placed supine in the bottom of a two-level holding stage. A 100-mm Petri dish, modified by cutting a 25-mm central hole and stretching a thin 35-mm rubber membrane over the hole, was mounted over the abdomen. Part of the uterus containing one or two embryos was carefully pulled through a 15-mm central slit in the rubber membrane into sterile PBS filling the Petri dish. Each embryo was imaged with 40-MHz UBM (Vevo 770; VisualSonics, Toronto, Canada) to determine whether regular embryonic contractions of the heart were occurring. After assessing the exposed embryos, they were gently placed back in the uterus and the next one or two embryos pulled out into the PBS for UBM. In this way, we could image the entire litter of embryos (typically about 12 embryos) in each mouse in less than 1 h.

MR Image Analysis

Contrast index (CI) was defined as the difference in mean MRI signal intensity (SI) between CNS tissue (in superior spinal cord or posterior brain stem) and non-CNS embryo tissue (face or neck) divided by the average of these values, in percent:

\[ CI = \frac{(SI_{\text{CNS}} - SI_{\text{emb}}) - (SI_{\text{CNS}} + SI_{\text{emb}})/2}{100}. \tag{1} \]

Image analysis and semiautomated segmentation were performed using Analyze (AnalyzeDirect, Overland Park, KS, USA) and Amira (Mercury Computer Systems, Berlin, DE, USA) software packages. The semiautomated segmentation scheme was modeled after previously reported segmentation methods (27). As an initial step, the dataset was manually reoriented using the Oblique Section function in Analyze to allow for visualization of standard coronal, sagittal, and horizontal planes through the brain. The following steps were then taken for each dataset: 1) regions were identified based on comparison with sections in the Kaufman mouse embryo atlas (28) and the Altman embryonic rat brain atlas (29); 2) the regions were segmented by seeding and expanding the regions in 3D using the magic wand feature of the Amira Segmentation Editor; 3) segmentations were refined by manual exclusion of extension.

![FIG. 1. Imaging setup with prospective respiratory gating.](Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.)
into adjacent structures (based on correlation with the atlas); and 4) segmented regions were further filtered by application of the Smooth function (modified Gaussian filter). When boundaries were difficult to appreciate they were defined based on 3D morphology (on adjacent or orthogonal sections), position of visualized adjacent structures, and comparison with the atlases (as described in Ref. 27). Note that the Altman rat brain atlas (29) was used for coronal and sagittal correlation of brain regions that are similar in mice and rats, and was not used for any scale measurements. For analysis of the mutant embryos, because of the structural abnormalities, there is not expected to be complete agreement with the atlases. In that case, the extensive histological images from previous analyses of the Nkx2.1 model (25) were referenced. The volume of the segmented region (in voxels) was measured using the Amira Tissue Statistics function and converted to cubic millimeters (mm$^3$). Statistical comparisons of volumes of whole brain (WB), basal ganglia (BG), and septum (SE) were made from measurements on wild-type ($N = 3$; Nkx2.1$^{+/+}$ or Nkx2.1$^{+/−}$), and mutant ($N = 3$; Nkx2.1$^{−/−}$) embryos, where the BG volume represents one side. The two-tailed Student’s $t$-test was used to test for statistical significance.

Phenotyping embryos as either wild-type or Nkx2.1 mutant was also assessed based on identification in the thalamus of the ventral extension of the third ventricle and mamillary body in a coronal section (Fig. 6e and f). For this study, six observers blinded to the genotype of the embryos were educated about these features with histological images for reference (25), and were then asked to label 6 (2D) MEMRI images as either wild-type or mutant based on identification of the midline ventricle and mamillary body. The results were reported as the percentage of correct identification (number correctly identified/total × 100).

Histology

For comparison with MRI, histological samples were prepared in the following manner. The pregnant mouse was euthanized with IP sodium pentobarbital (0.1 ml = 10 mg/100 g body weight) and cervical dislocation, and the embryos were removed, cardioperfused (vascular perfusion of Maternal respiration was not significantly restricted by the holder based on observations of regular spontaneous breathing throughout the acquisition and pink skin color upon removal from the coil following MRI acquisition. Figure 2 demonstrates the application of MEMRI and respiratory gating to MRI of the E14.5 CNS. With gating, in the absence of Mn-enhancement (Fig. 2a), the outline of an E14.5 embryo was barely visible, with no intraembryonic contrast. With Mn-enhancement and no gating (Fig. 2b), the contrast between CNS and non-CNS tissues was increased; however, the image quality was degraded due to motion artifacts. The combination of MEMRI and respiratory gating (Fig. 2c) increased signal and decreased motion artifact for improved visualization of the embryonic CNS.

Dose-Dependent MEMRI Contrast and Toxicity

At E12.5 and E14.5 (images not shown), doses of 20 mg/kg (Fig. 3a), 40 mg/kg (Fig. 3b) and 80 mg/kg (Fig. 3c) were analyzed to examine the relationship between Mn dose and CNS contrast enhancement. The mean CI was measured at E12.5 and E14.5 MEMRI following Mn administration of 20 mg/kg, and 40 mg/kg, and at E12.5, E14.5, and E17.5 following the 80 mg/kg dose (Fig. 3d; $N = 4$ in each group, error bars represent SD). In each age group, the CI increased with increasing Mn dose. A dose-dependent improvement in embryo survival with decreased dose was also observed at both earlier stages (Fig. 3e, $N$ for each group as noted below the bar; E11.5/low (dose) $N = 5$, E11.5/medium $N = 4$, E11.5/high $N = 4$, E13.5/low $N = 4$, E13.5/medium $N = 4$, E14.5/high, and E16.5/high $N = 15$). The dose of 80 mg/kg demonstrated decreased survival to

Results

Mn-Enhancement and Respiratory Gating for In Utero MRI

The two main challenges for in utero MRI of the embryonic CNS are low contrast between CNS and other embryonic tissues, and embryo motion. In this study, respiratory gating was used primarily at E12.5 and E14.5. At E17.5, respiratory motion was usually not apparent on MRI probably because the larger embryos fit more snugly within the shaped surface coil permitting less motion of the embryos with respiration. In addition, the setup tended to isolate the embryos from other potential sources of physiological motion, including cardiac and intestinal motion. Maternal respiration was not significantly restricted by the holder based on observations of regular spontaneous breathing throughout the acquisition and pink skin color upon removal from the coil following MRI acquisition. Figure 2 demonstrates the application of MEMRI and respiratory gating to MRI of the E14.5 CNS. With gating, in the absence of Mn-enhancement (Fig. 2a), the outline of an E14.5 embryo was barely visible, with no intraembryonic contrast. With Mn-enhancement and no gating (Fig. 2b), the contrast between CNS and non-CNS tissues was increased; however, the image quality was degraded due to motion artifacts. The combination of MEMRI and respiratory gating (Fig. 2c) increased signal and decreased motion artifact for improved visualization of the embryonic CNS.

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![FIG. 2. Mn-enhancement and respiratory gating work in combination to improve in utero MRI. Images at E14.5 with gating and without Mn (a), with Mn (80 mg/kg) and without gating (b), and with Mn and gating demonstrated significant improvement in image quality with both Mn-enhancement and respiratory gating (scale bar is 1 mm).](image-url)
E18.5, which was most pronounced and significant ($P < 0.0001$) at E12.5, and was also observed at E14.5 ($P < 0.06$).

The survival of late-stage (E17.5) embryos injected with 80 mg/kg was not significantly decreased from control litters ($P = 0.23$), so lower doses were not used in this group.

Survival to E18.5 was low with the highest doses of Mn, especially at E12.5 (Fig. 3e). However, previous literature (23) and our observations of the size of nonviable or resorbing embryos indicated that most survived for a few days following Mn administration. To assess survival 24 h after 80 mg/kg Mn administration, UBM was used to assess cardiac activity in litters of mice at E12.5 (after E11.5 administration) and at E14.5 (after E13.5 administration). Short-term survival of embryos was 98.3% at E12.5 (SD = 3.7%, $N = 5$) and 96.9% at E14.5 (SD = 4.2%, $N = 5$), not significantly decreased from the survival of control embryos to E18.5 (Fig. 3e). From these studies, we conclude that toxicity is primarily a long-term, chronic effect that is less pronounced with increasing developmental stage. Higher doses of Mn are relatively safe for later-stage embryos, and can also be used for short-term studies of earlier stage embryos, provided postnatal survival is not critical to the experiment.

Interestingly, on MRI we occasionally observed embryos with global enhancement throughout the body, markedly different than the normal CNS-specific enhancement (Fig. 4). Following assessment of survival, embryos with this pattern of generalized enhancement were found to be small and resorbing, indicative of early embryonic demise. This suggests that well-localized Mn-enhancement within the CNS occurs only in living, metabolically intact embryos, and may be a useful marker to gauge embryonic viability.

Developmental Stage-Dependent Mn-Enhancement in the CNS

At the 80 mg/kg dose, Mn was observed to effectively accumulate in the entire spinal cord and ventral brain of the developing CNS from E12.5 (Fig. 5a and d) with increased forebrain enhancement becoming more visible at later time points (Fig. 5b, c, e, and f). Although Mn-enhancement of the spinal cord was robust at all time points observed (Fig. 5a–c), enhancement of the forebrain, particularly dorsally, was minimal at the earliest time point.

Neural differentiation occurs during this period, and might be expected to correlate with Mn-enhancement if it functions in embryos as it does in adults, as a surrogate of neuronal function. We assessed stage-dependent neuronal differentiation using immunohistochemical staining for a marker of neuronal differentiation, neuron-specific tubulin or Tuj1 (21). Expression of Tuj1 in sections of E12.5 (Fig. 5g), E14.5 (Fig. 5h), and E17.5 (Fig. 5i) brains showed good qualitative correlation with Mn-enhancement, with immature ventricular zone tissue showing both low Tuj1
FIG. 5. Developmental stage–dependent changes in Mn-enhancement (Mn dose = 80 mg/kg). Sagittal (a–c) and coronal (d–f), orientation of image plane indicated by diagonal solid lines on corresponding sagittal images) MEMRI images of E12.5 (a,d), E14.5 (b,e), and E17.5 (c,f) embryos showed increasing forebrain enhancement with age (scale bars in all panels are 0.5 mm). CNS development during this period includes neuronal differentiation, which can be observed with immunohistological staining for Tuj1. At all stages, Mn enhancement correlated qualitatively with Tuj-1 expression (marker of differentiated neurons) (g–i; red dashed box in d–f represents the region shown in histology), with decreased Mn-enhancement in the ventricular zone (arrowheads in d,e,g,h) and subventricular zone (arrowheads in f,i). At E17.5, layering in the dorsal aspect (f) correlates with high Tuj1 expression in the CP and lower Tuj1 expression in the subplate (SP) (i). The enhancing structure (*, f) deep to the SP correlates with the position of the choroid plexus in the lateral ventricle.

FIG. 6. Mn enhancement enabled 3D analysis of the developing CNS from in vivo MEMRI images (Mn dose = 80 mg/kg). The spinal cord (SC) and ventral brain enhanced at all time points (a–c). There was a progressive enhancement from posterior to anterior and ventral to dorsal with increasing age allowing segmentation of the SC and ventral hindbrain, midbrain, and diencephalon at E12.5 (a); the SC and entire hindbrain, midbrain, diencephalon, and ventral telencephalon at E14.5 (b); and the SC and WB at E17.5 (c) (scale bar is 1 mm). At E17.5, the boundaries of brain subregions including the cortex (aqua), thalamus (magenta), BG (green), and pyriform cortex (red) could be appreciated (d), enabling semiautomated segmentation and 3D analysis of these structures as well as the olfactory bulbs (orange) and SE (blue) (e). Of note, although there was some image noise that contributed to variation in signal within the segmented regions; however, other variation was likely due to tissue structures themselves. For example, the BG (d, green) contains two subregions at this level that were differentiable based on signal intensity: the ventromedial portion represents the globus pallidus (bright), and the dorsolateral portion represents the striatum (intermediate intensity). Similarly, the dark spot in the center of the thalamus (magenta) was likely a portion of the fourth ventricle that extends into that region.
3D Segmentation and Analysis From MEMRI

The increase in contrast between CNS and non-CNS tissue facilitated semiautomated segmentation of the enhanced region using Amira image analysis software. At E12.5, the enhanced region consisted of the spinal cord and ventral brain (Fig. 6a). At E14.5, more of the brain became enhanced enabling more complete segmentation of the midbrain and ventral forebrain (Fig. 6b). At E17.5, the neocortex had adequate enhancement to allow complete CNS segmentation (Fig. 6c). Additionally, at E17.5 several substructures were visible within the forebrain, including the cortex, thalamus, and hypothalamus, SE, BG, amygdala, and pyriform cortex (Fig. 6d). MEMRI contrast was sufficient to enable several subregions to be segmented from the dataset using semiautomated methods for 3D visualization (Fig. 6e). Of note, within some of the outlined regions in Fig. 6d, subregions of different signal intensity can be identified. Although some global variation results from image noise, local regions often correlated with meaningful substructures. Within the BG, for example, the ventromedial region (bright) corresponded with the globus pallidus, and the dorsolateral region (intermediate intensity) corresponded with the striatum. Within the thalamus, the central dark spot corresponded to a portion of the fourth ventricle.

MEMRI Phenotype Analysis of the Nkx2.1 Mutant Mouse Forebrain

2D and 3D MEMRI analysis should be quite helpful for analyzing anatomical abnormalities in mutant mouse models. Based on the observed Mn-enhancement and improved visualization of ventral forebrain structures from E14.5, we chose to study the Nkx2.1 knockout model (25), which was shown previously by histology to have several abnormalities within the ventral forebrain, both telencephalon and diencephalon (25,30). Many of these abnormalities were visible on 2D MR images of mutant vs. wild-type embryos, including in the anterior brain, a narrowing of the SE and enlargement of the BG (Fig. 7b vs. a); and more posteriorly, midline fusion of the hypothalamus (Fig. 7f vs. e), with loss of the ventral portion of the third ventricle. Matched sections of fixed brains stained with H&E, show qualitative correlation between MRI and histology (Fig. 7c, d, g, and h). When blinded observers (N = 6) were asked to identify a set of images from six embryos as wild-type (N = 3) or mutant (N = 3) based on identification in the thalamus of the midline third ventricle and mammillary body (Fig. 7e, f), they identified the phenotypes correctly in 100% of cases. Semiautomated segmentation and volumetric analysis of ventral structures in wild-type and mutant datasets allowed improved comparison between wild-type and mutant embryos than were previously possible based on 2D histological methods alone (Fig. 7i, j). Volumetric measurements of altered structures showed a significant decrease in the volume of the SE (P = 0.03), and a significant increase in the volume of the BG (P = 0.02) (Fig. 7k), whereas the WB volume was not significantly different between mutant and wild-type embryos (P = 0.6).

Our MEMRI analysis of the Nkx2.1 mutant phenotype is preliminary, using data from only three homozygous mutants. Segmentations by a second observer blinded to the genotypes of the mouse embryos demonstrated the same trend with larger BG and smaller SE in the mutants but the difference did not reach statistical significance (data not shown), possibly because this observer did not have the privilege of using the mutant histology for comparison. Future studies are required to fully determine the utility of this approach for in utero detection and longitudinal analysis of the Nkx2.1 mutant brain phenotypes. Nevertheless, these data are encouraging that this approach can provide a robust method for in vivo analysis of highly relevant forebrain phenotypes in mutant mouse models, including Nkx2.1 mice.

DISCUSSION

These results demonstrated that the combination of MEMRI and prospective respiratory gating provides an effective method for in vivo imaging of the developing CNS in mid- to late-gestational mouse embryos. With analysis at three different developmental stages, this study showed the potential for a significant expansion of MEMRI methodology to include in vivo embryonic imaging. Specifically, we showed that MEMRI enhancement in the developing CNS qualitatively correlates with the profile of neuronal differentiation, showing a general trend of increasing CNS enhancement from posterior/ventral to anterior/dorsal as a function of embryonic stage. In combination with respiratory gating to reduce motion artifacts, in utero MEMRI enabled robust 3D segmentations of the whole CNS, as well as morphometric analyses of a number of important subregions of the developing brain and spinal cord.

Although preliminary, our analysis of Nkx2.1−/− mice showed promise for application of this method for in utero phenotyping and characterization of mutant mouse brains during development. Indeed, this study represents the first example of mutant mouse phenotyping using in utero MR brain imaging. The main morphological features of the Nkx2.1 mutant phenotype were all detected with MEMRI, including a decrease of Nkx2.1-derived ventral structures (namely the globus pallidus, SE, some hypothalamic nuclei, and the pituitary) and a reciprocal expansion of adjacent structures (including the striatum), presumably due to respecification of the Nkx2.1 cells to adjacent fates (30). Previous analyses used 2D histological methods, and made no attempt to quantitatively evaluate the anatomical changes. Our preliminary quantitative results are promising that future in utero MEMRI approaches may help to enhance understanding of the 3D changes occurring in the...
Nkx2.1 mutant model, providing volumetric and morphometric analyses.

During development, organisms are particularly sensitive to toxic insult. Of relevance for these investigations, toxicity of Mn-containing compounds has been studied extensively due to concerns about Mn exposure in the workplace of miners and metal-workers (recent review, Ref. 31). Mn is relatively nontoxic to adult animals except in the brain, where prolonged exposure to Mn through inhalation or ingestion has been shown to lead to Parkinson-like symptoms. However, previous studies showed that a high dose of MnCl₂ in the pregnant female mouse resulted in fetal toxicity evidenced by increased late resorptions and decreased weight of surviving embryos from doses of 4 mg/kg/day when administered from day 6 to day 15 of gestation (for a total dose of 40 mg/kg) (23). When administered as a single subcutaneous dose, 50 mg/kg of MnCl₂ resulted in increased late resorption that was most significant when Mn was administered at E9 or E10, with decreased effect at E11 or E12 (24).

Although toxicity was a more significant limiting factor for application of MEMRI to embryos compared to previous applications to postnatal animals, the dose-dependent contrast effects and survival data were encouraging that a balance could be struck between acceptable CNS contrast and survival for many applications. Short-term survival (to 24 h) following 80 mg/kg Mn exposure in E11.5 and E13.5 embryos was very high, similar to survival to E18.5 after 80 mg/kg Mn exposure at E16.5. These results indicate that for short-term analyses or for analyses in late-stage embryos, this dose is likely to be acceptable. For longitudinal imaging and mid-gestational studies where postnatal follow-up is critical, a lower dose may provide acceptable image quality with acceptable survival statistics. Evaluation of the long-term implications of embryonic Mn-exposure at any dose was beyond the scope of this study, and future investigation of the neurodevelopmental impact of Mn exposure will be required before this method finds widespread applications.

A number of questions remain regarding the mechanisms of Mn uptake in the brain and accumulation in specific cell types. Since the BBB is immature and largely nonfunctional prior to birth, it is likely that Mn permeability through the BBB is more effective at these early developmental stages compared to adults. Elucidation of the kinetics of Mn uptake in the brain will require further longitudinal imaging studies. At 24-h post-Mn injection, we observed good qualitative correlation between neuronal differentiation, as assessed by Tuj1 immunohistochemistry, and Mn enhancement on MEMRI. Although not

FIG. 7. In utero MEMRI was used for mutant phenotype analysis (Mn dose = 80 mg/kg). The ventral brain defects in Nkx2.1 knockout mice can be appreciated with MEMRI. A decrease in ventral midline structures such as the SE (blue outline) and expansion of the BG (green outline) was appreciated in the Nkx2.1⁻/⁻ brains (b) compared to wild-type littersmates (a). H&E-stained sections showed the matched region in the mutant (d) and wild-type (c) embryos. More posteriorly, in the Nkx2.1⁻/⁻ brain (f), MEMRI visualized a midline fusion of the hypothalamus with loss of the extension of the third ventricle (v3) to the floor of the diencephalon compared to wild-type (e) (mamillary body: M). Corresponding histological sections demonstrated the same midline defects (g,h). Semiautomated segmentation of the BG (green), SE (blue), and WB (yellow) from wild-type (i) and Nkx2.1⁻/⁻ (j) datasets showed significant changes in the volumes of the BG and SE (k, dark bars are wild-type, light bars are mutant, *P < 0.03), but no significant difference was observed in the volume of the WB (P = 0.6, scale for WB on right). The volume shown for BG is for one side, i.e., half the total volume.
explicitly tested here, these results may indicate that a broader requirement for Mn accumulation may include neuronal connectivity and electrical activity present only in mature cell types. Spontaneous electrical activity during mouse embryonic development has been observed in a number of regions of the CNS (32). Indeed, both early synchronous electrical activity, and related calcium (Ca) ion flux have been suggested to be necessary signals for cellular differentiation and establishment of mature neural circuitry throughout the brain (33,34). The mechanism for these developmental processes is not yet completely understood, and the extent to which Mn mimics Ca in vivo is still not clear. In future, it will be interesting to investigate whether in utero MEMRI can provide more insights into these important electrochemical developmental events.

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