Magnetic Transfer Contrast Accurately Localizes Substantia Nigra Confirmed by Histology

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Background: Magnetic resonance imaging (MRI) has multiple contrast mechanisms. Like various staining techniques in histology, each contrast type reveals different information about the structure of the brain. However, it is not always clear how structures visible in MRI correspond to structures previously identified by histology. The purpose of this study was to determine if magnetic transfer contrast (MTC) or T2 contrast MRI was better at delineating the substantia nigra (SN).

Methods: MRI scans were acquired in vivo from two nonhuman primates (NHPs). The NHPs were subsequently euthanized, perfused, and their brains sectioned for histologic analyses. Each slice was photographed before sectioning. Each brain was sectioned into approximately 500 sections, 40 μm each, encompassing most of the cortex, midbrain, and dorsal parts of the hindbrain. Levels corresponding to anatomic MRI images were selected. From these, adjacent sections were stained using Kluver-Barrera (myelin and cell bodies) or tyrosine hydroxylase (dopaminergic neurons) immunohistochemistry. The resulting images were coregistered to the block-face images using a moving least squares algorithm with similarity transformations. MR images were similarly coregistered to the block-face images, allowing the structures on MRI to be identified with structures on the histologic images.

Results: We found that hyperintense (light) areas in MTC images were coextensive with the SN as delineated histologically. The hypointense (dark) areas in T2-weighted images were not coextensive with the SN but extended partially into the SN and partially into the cerebral peduncles.

Conclusions: MTC is more accurate than T2-weighting for localizing the SN in vivo.

Key Words: Dopamine, MRI, magnetic transfer contrast, nonhuman primate, substantia nigra, T2

There has been growing interest in visualizing in vivo a critical region of the midbrain, the substantia nigra (SN) because of its role in neurodegenerative and psychiatric disorders, such as Parkinson’s, Huntington’s, and schizophrenia (1). Yet until recently, the SN has defied depiction with magnetic resonance imaging (MRI) techniques. A reliable MR technique could serve as a potential marker for determining more timely diagnoses or monitoring of disease progression during treatment.

Methods and Materials

The studies were performed on two male macaques. The first, a Macaca fascicularis, was approximately 6 years old and weighed 7.6 kg. The second, a Macaca mulatta, was approximately 7 years old and weighed 8.4 kg. These animals had been used in previous studies involving microelectrode recording from occipital visual
cortical areas V1, V2, and V4, but no recordings were made in or near the structures studied in this article nor were there any interventions likely to alter their anatomy (8–10).

For the present experiments, it was necessary to attach a head-fixation system on the monkeys’ skulls to stabilize them during MRI imaging. Thus, under isoflurane general anesthesia, high-strength plastic (PEEK—polyether ketone) strips were bolted to the skull with ceramic screws (Thomas Recording, Giessen, Germany) and connected to a head-fixation system (11). Before MRI imaging, the animals were anesthetized with a mixture of ketamine 5 to 25 mg/kg and acepromazine .1 to .5 mg/kg, followed by atropine .05 mg/kg subcutaneously. During the entire imaging procedure, the animals were kept anesthetized, and heart rate and arterial hemoglobin oxygen saturation were monitored via an optical sensor taped to one finger (Invivo Millennia 3155MVS and 3150 M; Invivo, Gainesville, Florida).

Animals were euthanized, consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association, by deep sodium pentobarbital anesthesia (≥100 mg/kg IV), followed by exsanguinations by cardiac perfusion first with a solution of .9% NaCl and 1% sodium nitrite, then with 4% paraformaldehyde in 1 mol/L phosphate buffer (PB). This method of euthanasia is consistent with the recommendations of the American Veterinary Medical Association Guidelines on Euthanasia.

All experimental procedures and care of the animals were carried out in compliance with guidelines established by the National Institute of Health and were approved by the University of Alabama at Birmingham Animal Care and Use Committee.

MRI Acquisition

All MR images were acquired on a 4.7-T vertical bore primate-research-dedicated scanner (Varian, Palo Alto, California). The scanning protocol for both subjects included PD-weighted images, MTC images, and T2-weighted images. Identical image geometry was used with each contrast. Four slices through the brain stem parallel to the anterior commissure–posterior commissure line were acquired. The MTC images were acquired using the same PD sequence with the addition of a 4-W, 20 msec, constant magnitude MTC preparation pulse at 10 kHz below the resonance frequency of water. For the T2-weighted images, a simple spin echo sequence (repetition time/echo time 5000/50 msec) was used.

Histology

So that the MRI slices would match the histological sections, the brains were blocked using a stereotactic frame to produce sections that were axial and anterior commissure–posterior commissure aligned. Each brain was then removed from the skull and placed in a solution of 25% sucrose in PB for several days until it sank to the bottom of the vessel. The sucrose solution was replaced with fresh solution each day. Each brain was then removed from the sucrose bath, and the dorsal surface was trimmed flat so that the brain could be positioned ventral side up. The brains were then placed on the stage of a Model 860 American Optical sliding microtome (American Optical, Buffalo, New York) and frozen with dry ice. After removing an initial few thick sections, the brain was sectioned at a thickness of 40 μm and sections stored in individual wells in PB solution containing sodium azide (1%), and stored at 4°C.

Approximately 500 sections were obtained from each animal encompassing most of the cortex, the midbrain, and the dorsal parts of the hindbrain. The block face was photographed at 3024 x 1998 resolution using a Nikon D70 SLR digital camera (Nikon Corporation, Tokyo, Japan) before sectioning each slice. Illumination was provided by two 250-W photoflood lamps (Osmar Sylvania, Danvers, Massachusetts). Image acquisition was triggered automatically by a custom trigger mounted on the microtome. Images were automatically downloaded to a computer for storage using remote camera operation software (Sofortbild, http://www.sofortbildapp.com).

Adjacent sections from two series per brain were stained with either KB stain to identify axons and cells or processed for TH immunohistochemistry to identify dopamine cells and fibers.

Kluver-Barrera

Sections were prepared by washing in PB (4 x 30 min) and then placed on charged slides and allowed to dry overnight. Slides were then incubated in dH2O for 5 min, followed by dehydration in 50% ethanol (EtOH) and 70% EtOH for 5 min each. Slides were then placed in .1% luxol fast blue MBS solution and placed in a 65°C water bath with a slight agitation for 24 hours. Once removed, the tissue was rehydrated in 95% EtOH, 70% EtOH, 50% EtOH, and dH2O for 5 min each. Tissue was placed in .05% lithium carbonate solution, aqueous for 12 min with constant agitation, followed by 70% EtOH (2 min, constant agitation), 70% EtOH (1 min, constant agitation), and 50% EtOH (1 min). Tissue was checked for differentiation under the light microscope and then placed in dH2O (2 x 5 min). The sections were counterstained in .1% cresyl violet acetate solution, aqueous for 10 min, followed by dehydration in 50% EtOH (2 min), 70% EtOH (2 min), 95% EtOH (2 x 2 min), and 100% EtOH (2 x 2 min). The slides were placed in xylene (2 x 2 min) and then coverslipped using Eukitt.

TH Immunohistochemistry

Free-floating sections were washed in PB (1 x 5 min), incubated in 1% sodium borohydride solution made in PB for 15 min, washed in PB (4 x 5 min), incubated in 1.5% hydrogen peroxide solution made in PB for 30 min, and then washed in phosphate buffered saline (3 x 5 min). The tissue was then incubated in 10% normal horse serum in PB for 30 min, washed in PB (4 x 5 min), followed by the primary antibody (Anti-Tyrosine Hydroxylase, MAB318, EMD Millipore Corporation, Billerica, Massachusetts) at a dilution of 1:1000 in 3% normal horse serum in PB for 72 hours at 4°C at a constant agitation. The tissue was then washed in PB (4 x 5 min) and incubated in biotinylated horse antimmunoglobulin G 1:200 in a 1.5% normal horse serum solution made in PB for 1 hour. The tissue was washed in PB (4 x 5 min) before being treated with reagents from the avidin-biotin peroxidase kit (ABC standard kit, Vectastain; Vector Laboratories, Burlingame, California) using the recommended dilutions of 1:100 for both solution A and solution B in PB for 45 min. The sections were then washed in PB (4 x 5 min) and then incubated in 3,3’-diaminobenzidine (10 mg/15 mL PB) containing 0.3% hydrogen peroxide for 2 min to visualize the reaction product. Control samples were processed in an identical
fashion except for the exclusion of the primary antibody. These control sections did not exhibit any specific staining. The stained sections were mounted on slides, dehydrated, and cover slipped as detailed earlier.

From each of these stained series, 3 to 4 sections were selected that corresponded to each of the anatomic MRI images. The slides were then scanned at 20× on an Aperio ScanScope CS (Aperio, Vista, California) to create complete microscopic images of the midbrain area.

The resulting histologic images were then coregistered with the block face images. In turn the MR images were also coregistered to the block face images. After this preprocessing, the transformed histologic images corresponded to the MR images and could be directly compared.

Coregistration

All image processing was performed on a MacBook Pro (Apple Inc., Cupertino, California) using the open source software package FIJI (a version of the National Institutes of Health’s ImageJ; http://fiji.sc). The image processing algorithms described below were all tools available in the default FIJI installation.

Histology images were initially subsampled to approximately 1000 × 1000 pixels. The block-face photos were cropped to 1188 × 1184 pixels. The MR images were used at the native resolution of 256 × 256 or 128 × 128 pixels. Uneven illumination was removed from the histological images using a rolling ball background removal algorithm (14) assuming a light background. The histological, block-face, and MR images were then contrast enhanced using contrast-limited adaptive histogram equalization (15).

The MR images and histology images were then coregistered to the block-face images. To guide the coregistration, 10 features were marked on each image. The marked features consisted of the most anterior points of the crus cerebri, the most posterior point of the interpeduncular fossa, the most lateral points of the crus cerebri, the most anterior point of the cerebral aqueduct, and the most posterior point of the tectum. These features were selected because they are easily identified in all of the imaging modalities used in this study. These 10 features served as the control points for nonrigid image registration based on an “as rigid as possible” moving-least-squares algorithm (16,17).

The midbrain was then segmented out of the histologic images by hand tracing. The crus cerebri in the KB stained sections and the SN in the TH stained sections were delineated by hand. The coregistered images and the outlines of the crus cerebri and SN were then overlaid. After the images were coregistered and overlaid, figures for the article were assembled and labeled in Adobe Photoshop Elements 7 (Adobe Systems Inc., San Jose, California).

Results

Figure 1 illustrates the difference in contrast between PD- and MTC-weighted images. The MTC pulse introduces additional contrast that can be used to delineate structures in the brainstem. Figure 2 illustrates the alignment of the histological data with the block-face and MRI image data from one slice in subject two. All the histological and MR contrast images were successfully aligned to the block-face resulting in mutual coregistration of all of the imaging modalities.

Figure 3 illustrates the location of the cerebral peduncles and the SN as determined via postmortem histology at three levels of the midbrain. The cerebral peduncles were delineated using the KB stain (left column, Figure 3) and the SN was delineated using TH immunohistochemistry (right column, Figure 3). Cerebral

![Figure 1](image1.png)

**Figure 1.** (A) Proton density and (B) magnetization transfer contrast images from the same subject and same axial section at the level of the substantia nigra.
peduncles are outlined in blue, and SN is outlined in red. The top row shows sections through the caudal portion of the SN at the level of the cerebellar peduncles (black arrow, A) (C) KB and (D) TH axial sections at the level of cranial nerve III rootlets (black arrow, C). (E) KB and (F) TH axial sections through the rostral portion of the SN at the level of the optic tract (black arrow, E). Scale bar, 1 cm.

Figure 4 illustrates the correspondence of the histological results and the MTC contrast images. The levels of section and the outlines are identical to those in Figure 3. The hypointense regions of the T2 maps do not correspond well with the SN (red outline). In the rostral and caudal sections the hypointense regions fall within the densely myelinated dorsal region of the cerebral peduncles. In the intermediate section at the level of cranial nerve III, the hypointense regions extend into the cerebral peduncles and the SN but are not coextensive with either structure.

**Discussion**

In this study, we imaged the midbrain and the SN of two nonhuman primates in vivo with a PD-weighted gradient recalled echo sequence with a MTC preparation pulse as described by Wolff and Balaban (13). Previous studies have compared in vivo
including fast spin echo and turbo spin echo (18), which results from RF excitation of out-of-plane slices during image acquisition (21).

Early MRI studies of Parkinson’s disease used T2-weighted images to delineate the SN (22,23), and researchers interpreted the hypointense region to result from iron in the SN pars reticulata (24). However, Rutledge et al. (25) and Máng et al. (26) have observed that the correlation between this hypointense region and the SN was imperfect. Because T2-weighted images do not accurately depict the SN, others have used additional MRI techniques, including diffusion-weighted imaging (4), inversion recovery imaging (27,28), quantitative T1 mapping (3), and susceptibility weighted imaging (2).

Recently, Sasaki et al. (1,7) used a T1-weighted fast spin echo sequence to visualize the SN and locus ceruleus. They referred to this technique as neuromelanin-sensitive MRI, arguing that the contrast results from neuromelanin as a byproduct of dopamine and noradrenaline metabolisms. They applied this technique to distinguish patients with schizophrenia and depression from healthy control subjects (1). They found increased signal intensity in the SN in patients with schizophrenia, which is consistent with elevated levels of dopamine, and reduced signal intensity in the locus ceruleus in patients with depression, which is consistent with dysfunction of the noradrenergic system. To image the SN in patients with Parkinson’s disease, Schwarz et al. (29) recently used a T1-weighted fast spin echo sequence with additional magnetization transfer contrast pulses. They found smaller regions of hyperintensity with reduced contrast in the SN in patients compared with controls, consistent with the loss of dopamine neurons in the SN pars compacta. Because neuromelanin is a byproduct of dopamine and noradrenaline synthesis, alterations to these catecholamines or loss of dopaminergic neurons could have an effect on images sensitive to neuromelanin. The resulting contrast is likely due to a combination of paramagnetic T1-shortening effects of neuromelanin and MTC leading to a hyperintense signal on T1-weighted images (29). However, the role of neuromelanin has not been confirmed with postmortem studies and should be a focus of future research. Nevertheless, the results of our study, together with those of Shibata et al. (1) and Schwarz et al. (29) lend support to the accurate localization of the SN with magnetization transfer contrast as confirmed by us using histology in nonhuman primates. Our results indicate this imaging technique may be useful for studying or monitoring neurodegenerative and psychiatric disorders for which there is evidence of SN pathology, such as Parkinson’s disease and schizophrenia.

Although this study was intended to explore the use of MTC to delineate the SN (incorporating the pars compacta and the pars reticulata), delineating the ventral tegmental area (VTA) in the same way would be of great importance. However, because of the morphology of the VTA and the resolution limitations of our MRI hardware, there was insufficient in-plane resolution in the MR images to make a definitive statement about the suitability of using MTC to image the VTA.

There are other considerations when using the magnetization transfer contrast. Acquisition times are lengthened, requiring a reduction in the number of slices or in the image resolution. Longer acquisition times are an especially important consideration when studying patient populations. Furthermore, the additional MTC pulses result in increased power deposition, approaching specific absorption rate limits.

With advancing MRI technologies, there will be continued improvement in the visualization of the midbrain and other
subcortical structures. Future studies will benefit from additional techniques such as diffusion tensor imaging, which has already been applied to the segmentation of SN subregions (30), as well as state-of-the-art high resolution and high field strength imaging of the midbrain (31). However, although comparing MR contrasts with published atlas data is certainly a valid technique, the ultimate test of any new MR contrast method will be to compare the MR data with coregistered histology from the same subjects, which will likely involve the use of appropriate animal models.

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