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Manganese-Enhanced MRI of Layer-specific Activity in the Visual Cortex from Awake and Free-moving Rats

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Abstract

Cortical responses to visual stimulation have been studied extensively in the rodent, but often require post-stimulation *ex vivo* examination of the tissue. Here, we test the hypothesis that visual stimulus-dependent cortical activity from awake and free-moving rats can be encoded following systemically administered MnCl₂, and activity subsequently readout using manganese-enhanced MRI (MEMRI), a technique that can be performed without sacrificing the animal. Unanaesthetized Sprague–Dawley rats, with or without systemic injection of MnCl₂, were maintained for eight hours in either a visually stimulating environment or darkness. To identify vision-dependent changes in cortical activity, animals were anesthetized and cortices were examined by 3D RARE MEMRI. Mean signal intensities in sub-cortical regions (e.g., superior colliculus and the lateral geniculate), and cortical regions (primary and accessory visual cortices) were compared. Cortex linearization was performed to aid in layer-specific signal intensity comparisons. Manganese administration alone globally increased signal intensity in the brain ($P < 0.0001$). In visually stimulated and unstimulated rats, layer-specific analysis revealed that stimulated rats had on average significantly ($P < 0.05$) higher signal intensities in layers IV and V of the primary visual cortex, as well as in deeper portions of the superficial superior colliculus, relative to dark adapted rats. Such differences went undetected without layer-specific analysis. We demonstrate, for the first time, the feasibility of layer-specific stimulus-dependant non-invasive MEMRI readout after encoding activity in awake and free moving rats. Future MEMRI studies are envisioned that measure the effects on cortical activity of sensory stimulation, as well as normal development, disease, plasticity, and therapy in longitudinal studies.

Visual processing, an important function of the central nervous system, starts in the retina and continues in several sub-cortical regions and the visual cortex. In the rat brain, visual information is first processed at the level of the lateral geniculate nucleus, which then provides thalamic input to visual cortical layers IV and deep layer III, (Paxinos, 1985). Pathways to other brain regions implicated in vision also originate in the primary visual cortex, including those to the superior colliculus (from layer V) and the accessory visual cortex (from layers II through VI).

Brain activity from awake and free-moving animals is assessed using either immunocytochemical staining techniques (Montero and Jian, 1995) or encoding activity based on the accumulation of an injected metabolic marker like 2-¹⁴C deoxyglucose (Cooper and

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Thurlow, 1991; Toga and Collins, 1981). In either case, readout is performed from histological slices and so requires sacrificing the animal. Non-invasive techniques are available, such as blood oxygenation-level dependent (BOLD) MRI or perfusion imaging that allow for multiple measurements from the same animal over time, although such methods have relatively lower spatial resolution and provide only indirect (i.e., hemodynamic) mapping of the location of brain activity. In addition, assessing animal brain activity inside a scanner requires anesthesia, which can modulate the results (Masamoto et al., 2007). There is a need for a high resolution imaging method that more directly measures encoded brain activity from awake and free-moving subjects.

Manganese-enhanced MRI (MEMRI) with systemically administered MnCl_2 has been used to examine neuronal activity in vivo (Berkowitz et al., 2006; Yu et al., 2005). Also, uptake of manganese following direct injection into neuronal structures been used for *in vivo* tract-tracing studies (Lindsey et al., 2007; Watanabe et al., 2001). The paramagnetic Mn^{2+} ion can enter active neurons through voltage-gated calcium channels, among others channels (Lee et al., 2005). Since Mn^{2+} efflux from cells is slow, its stimulation-dependent accumulation in active brain regions can be measured as a decrease in tissue T_1 at high spatial resolution hours later (Aoki et al., 2004; Alvestad et al. 2007; Sun et al., 2006). In this manner, MEMRI has measured sound-evoked activity in the midbrain from awake and free moving rodents, with spatial resolution surpassing that available in BOLD (Yu et al., 2005). However, in that study stimulation-dependent differences in whole cortical manganese accumulation were not found. It is not yet known if more spatially selective analysis of manganese accumulation in the cortex would be more sensitive to activity differences.

In this study, we used MEMRI to examine manganese accumulation from awake and free moving rats exposed to visually stimulating environment. We reasoned that preservation of layer-specific information from the cortex might be a more sensitive approach to analysis of activity-dependent changes, since some layers of the primary visual cortex (e.g., layer IV) are particularly responsive to stimulation (Cooper and Thurlow, 1991; Toga and Collins, 1981), and therefore would demonstrate activity-dependent changes in manganese accumulation.

Methods

The animals were treated in accordance with the NIH Guide for the Care and Use of Laboratory Animals and the ARVO Statement on Animals in Vision research.

Manganese injection and stimulation procedure

In all cases, adult female Sprague-Dawley rats ($n = 15$, 195 – 292 g) were housed and maintained in normal 12 hr light/12 hr dark cycling prior to the onset of the experiment. At the beginning of the experiment, rats were placed and maintained in darkness for 8–12 hours prior to additional procedures. All procedures (e.g., weighing rat, injecting MnCl_2 , anesthesia for MRI, and MRI exam) were done in the presence of dim red light or darkness. MnCl_2 was administered as an intraperitoneal injection (66 mg $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}/\text{kg}$, 0.1M) to awake rats. Rats were then either housed individually in darkness (“unstimulated”, $n = 5$), or placed in the stimulation apparatus (“stimulated”, $n = 5$) for eight hours. Only one animal occupied the stimulation apparatus at a time. An additional set of animals were unstimulated (i.e., dark adapted) and not treated with manganese (“noMn”, $n = 5$).

For the stimulated group, rats were placed in a 32.5 cm $\times$ 32.5 cm $\times$ 17 cm tall housing constructed from four fourteen-inch computer monitors as walls, with a frame, floor, and ceiling of wood protected by steel and aluminum mesh. Other than the screen faces, all internal surfaces were painted flat black. During the eight hr stimulation period, a moving black and white square-wave grating was simultaneously displayed on each screen (VisionEgg, v.1.0,

Straw et al., 2006). A moving square wave grating was chosen both to avoid habituation over the course of the stimulation period, and to stimulate the primary visual cortex because simpler stimuli, like constant diffuse light, can have little to no effect (Cooper and Allen, 1995). Here, the rate of rotation (-20 to 20 degrees per second) and procession (0 to 5 cycle-widths per second), as well as the cycle width of the square-wave grating (0.50 to 0.17 cycles per degree visual angle, measured from one corner of the apparatus to the opposite corner) were variable. Each parameter would change every one to five seconds, independently of one another, to a random value within the aforementioned ranges. This range of cycle widths was chosen because adult Sprague-Dawley rats are capable of seeing sine wave gratings with a width of 0.5 cycles per degree visual angle, (Prusky et al., 2002). Because the primary visual cortex is more responsive to dim, rather than bright, square wave gratings (Cooper and Allen, 1995), the brightness of our display was set to eight lux, measured at the center of the container. The refresh rate for each screen was 60 Hz, well above the rat critical flicker frequency threshold of 20 Hz (Wells et al. 1999).

MEMRI procedure

The MRI scans began about 9 hours after manganese administration, (mean = $8\text{hr}52\text{min}$, SD = 17min). This duration was chosen to limit the effects of axonal transport of manganese on the observed pattern of accumulation – that mode of transport being rapid enough to carry manganese from the rat eye to the superior colliculus in 24 hours (Watanabe et al., 2001). Immediately prior to the MRI scan, rats were anaesthetized with 3.9 ml/kg urethane solution (36% solution, intraperitoneally, prepared fresh daily; Aldrich, Milwaukee, WI). Significantly less urethane (2.8 ± 0.4 ml/kg, mean $\pm$ SD, $p < 0.01$, two tailed t-test), was needed to yield the same depth of anesthesia in animals given manganese. To maintain appropriate core temperature during the scan, animals were placed on a heated recirculating water blanket. Rectal temperatures were continuously monitored and maintained throughout each experiment. MRI data were acquired on a 4.7 T Bruker Advance system using a whole body transmit-only coil and a 3 cm internal diameter receive-only surface coil placed dorsal to the head. Images were acquired using a RARE sequence (repetition time TR 330 ms, echo time TE 16.6 ms, RARE factor 8 , number of acquisitions NA 2 , matrix size $256 \times 256 \times 173$, field of view 3.84×3.84 cm², slice thickness 150 μm , 80 min/image). For each scan, a small vial serving as an external standard (containing a 10 to 1 mixture of water to 0.67 mM MnCl_2 in saline) was placed on the surface coil, always in the same location. All animals were alive at the end of the scan, and were subsequently sacrificed while still under anesthesia. An additional scan was performed on a phantom – a vial roughly the size of a rat's head containing the same solution found in the external standard – for later use in modeling the signal intensity change as a function of distance from the surface coil.

Data Processing

Using R (v.2.4.0, <http://www.r-project.org/>) scripts developed in-house as well as the scan of the aforementioned phantom, the signal intensity changes present due to signal reception with a surface coil were modeled, and corrected for in each image. Next, using MRIcro (v.1.40, Rorden and Brett, 2000), we manually defined several regions of interest (ROIs) in each brain. Selection of cortical and non-cortical ROIs (Figure 1) was based on literature values of cytoarchitectural and functional regions of the rat brain (Kreig, 1946; Pellegrino et al., 1979; Zilles, 1985), with Zilles (1985) being the primary source for selection of cortical ROIs.

Initially, a whole-region analysis was performed: The average signal intensity for each ROI was found (SI_{ROI}), and then normalized prior to group comparisons. In the comparison of the “noMn” and “unstimulated” groups, which was conducted to verify the presence of manganese enhancement, values were normalized to the mean signal intensity of the external standard ($SI_{Standard}$), i.e. $[SI_{ROI} / SI_{Standard}] \times 100$. Normalization to adjacent soft tissue – used in all

other comparisons – was not used here because some degree of enhancement was expected in all soft tissues following manganese administration.

In all comparisons of the “stimulated” and “unstimulated” groups, cortical tissue adjacent to each ROI was used to normalize signal intensities. For this purpose, we used the agranular retrosplenial cortex and granular retrosplenial cortex (“RSA” and “RSG”, respectively) as well as the posterior agranular insular cortex and the perirhinal area (“AIP” and “PRh”, respectively). These regions were chosen because activity differences between experimental groups were expected to be negligible (Toga and Collins, 1981), and because their large rostro-caudal expanse ensured that normalization tissue would be available near to each ROI. Although this approach, henceforth “tissue normalization”, requires a commitment to the fitness of tissue used for normalization (i.e. “Is it unaffected by visual stimulation?”), it has been used successfully in the past, albeit to different brain regions, (e.g. Angenstein et al., 2007). “Tissue normalization” affords two substantial benefits: First, any effects of systemic inter-individual differences in manganese handling (e.g. liver sequestration) are reduced. Second, the effect of signal intensity gradients due to surface coil use, which persist to some degree in spite of the phantom-based normalization, can be further minimized at all regions of interest. Without this spatial component of normalization, variance in visual ROIs could be reduced more so than in the more rostral non-visual ROIs, preventing their use as fair negative control regions. In whole-region comparisons between “stimulated” and “unstimulated” groups, ROI signal intensities from each hemisphere were normalized to adjacent regions of the RSA/RSG and AIP/PRh (i.e. $[SI_{ROI} / [SI_{RSA/RSG} + SI_{AIP/PRh}] / 2] \times 100$), then averaged to produce a single value for each ROI in each animal.

In addition to whole-region analysis, all ROIs but the medial geniculate were analyzed to preserve layer-specific information. For the superficial superior colliculus (sSC) and dorsal lateral geniculate nucleus (dLGN), images were imported into ImageJ (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, <http://rsb.info.nih.gov/ij/>, 1997–2008) and signal intensities as a function of depth were measured by drawing lines (8–12 per coronal slice) perpendicular to the structures’ surfaces (Silva et al., 2008). This approach works well for the smaller regions of interest in the highly contoured diencephalon and mesencephalon, but becomes impractical when assessing the relatively expansive regions of the cortex. Therefore, cortices were instead processed with in-house software (see below) that linearized the cortex without data loss due to interpolation, facilitating layer-specific analysis of manganese accumulation. Mean signal intensities as a function of depth into the cortical regions were determined in a fashion similar to that used in retinal studies (see Berkowitz et al., 2007) or structural MEMRI studies, (Angenstein et al., 2007; Silva et al., 2008).

In preparation for cortex linearization, brains were digitally extracted using a semi-automated process. Briefly, an R script developed in-house advanced a provisional brain/no brain border out from the center (which had been marked manually) of each coronal slice, locally stopping the advance when a large change in signal intensity was encountered. Once the voxels occupied by brain were provisionally identified, this script “filled in” abrupt surface contours of the brain (e.g. the longitudinal fissure), producing a silhouette of the brain. In early testing, this “filling in”, which did not take place within 1mm of our ROIs, improved the quality of cortex linearization (described next). All results of this step were inspected, and any errors in identifying the brain/no-brain border were manually corrected. Finally, the brain silhouette was slightly dilated, leaving the brain surrounded with a small, consistent, halo of non-brain, (see top of Figure 2).

Cortex linearization was accomplished with an R script developed in-house. A high order ($\geq 10^{\text{th}}$) polynomial was fit to the halo of non-brain in the top and bottom half of each coronal slice. Every 30 μm along each polynomial, a 2250 μm -long perpendicular to the polynomial

was drawn (see top of Figure 2). For each voxel hosting the origin of a perpendicular, the rostro-caudal change in the brain's surface within a $450\ \mu\text{m} \times 450\ \mu\text{m} \times 1050\ \mu\text{m}$ (x, y, z) area centered on that voxel was assessed, then simplified using a linear best-fit. Each perpendicular from that voxel was tilted caudally or rostrally according to that best-fit. Along each of these lines, now normal to the brain surface, the signal intensity was sampled every $30\ \mu\text{m}$. The result of this processing was a linearized image arranged as a function of distance from the host polynomial (see middle of Figure 2). At this stage, the linearized images show slight inconsistencies in the location of the brain surface, attributable both to imperfections in the digital extraction of the brain, and in the fit of each polynomial. To control for this, a moving-average was used to align the brain surface near the top of each linearized image: Within a narrow window centered at each column of values recovered from a single line normal to the brain, the average signal intensity as a function of distance from the polynomial was found. The window's width, $930\ \mu\text{m}$, was chosen after pilot testing with several widths: Much larger windows were less sensitive to local changes in brain surface position, while narrower windows (330 and $660\ \mu\text{m}$) provided relatively less stable signal intensity averages for the subsequent image adjustment: The location of the largest increase in signal intensity (i.e. $[\text{SI}_{\text{point } N} - \text{SI}_{\text{point } (N-1)}]$, moving away from the polynomial) was used for alignment, (see bottom of Figure 2). Provisionally, this marks the location of the brain/no-brain border. A more definitive border was identified post-hoc: Signal intensity profiles of the Oc1 and Oc2L were compared to the high-resolution structural study of Aoki et al. (2004), wherein a manganese-enhanced signal intensity peak occurred at layer II in the rat occipital lobe. Signal intensity profiles of Oc1 and Oc2L were shifted superficially (in Figure 4, to the left) by $90 \pm 30\ \mu\text{m}$ to center the high-intensity peak at depths corresponding to the superficial half of layer II/III. The same adjustment ($90 \pm 30\ \mu\text{m}$) was made to Par1 and Temp1 profiles.

The quality of this linearization approach will impact any subsequent layer-specific analysis: Distortions will arise unless lines drawn into the brain are, within a small margin of error, normal to brain surface. In addition to visually inspecting the results of the linearization program for each subject (including outputs like at the top of Figure 2, generated for every coronal slice from each animal), we measured its accuracy in several slices sampled from two brains. From each of the two brains, a coronal section was selected at caudal limit of the pineal body, the rostral limit of the hippocampus – those two limits marking the broad range within which all ROIs were located – and from halfway between those limits. From each of the two brains, a parasagittal slice passing through the left superior colliculus and a horizontal slice positioned midway through the brain were also selected. In each slice, 14 to 18 evenly-spaced lines perpendicular to the brain surface were manually drawn in the spans of cortex that include and surround our cortical regions of interest; within the aforementioned rostral and caudal borders, and dorsal to the rhinal fissure, but outside of a 2.4mm -wide buffer centered at the longitudinal fissure. The angles of these manually drawn perpendiculars (θ_{manual}) were compared to the in-plane angles of automatically drawn lines (θ_{auto}) from the same locations ($|\theta_{\text{manual}} - \theta_{\text{auto}}|$). Difference values from coronal and non-coronal slices were averaged separately to provide an overall assessment of the error introduced by the linearization program in the coronal plane, and in the rostro-caudal tilt of perpendiculars. Using these values, error in automatically drawing lines normal to the brain was then estimated, $(\tan^{-1} [\sqrt{[\tan \theta_{\text{Coronal Error}}]^2 + [\tan \theta_{\text{Non-Coronal Error}}]^2}])$. The size of this error is likely dependent on factors like image resolution, and is therefore specific to the data linearized as well as the linearization method itself. As such, the error size is best understood in comparison to the error from another method applied to this data set: Lines were manually drawn a second time, a day after the first, from the same points. The angles from the second day (θ_{manual2}) were then compared to those from the first ($|\theta_{\text{manual}} - \theta_{\text{manual2}}|$).

In the layer-specific analysis of stimulated and unstimulated groups, linearized data were used to acquire an averaged profile of signal intensity as a function of depth for each cortical ROI,

similar to the procedures in Angenstein et al. (2006) and Silva et al. (2008). As in the whole-region analysis, “tissue normalization” was applied to the signal intensity profiles, (i.e. $[SI_{ROI} / [SI_{RSA/RSG} + SI_{AIP/PRh}] / 2] \times 100$). Because a layer-specific response could also occur in both the superficial SC and the dLGN, the same layer-specific analysis was performed in those regions. The cortical depths from the MEMRI data were associated with histologic layers using averaged literature values of cortical thicknesses (Kreig, 1946; Zilles, 1985; Gabbot and Stewart, 1987). The same was done for layering in the superficial SC (Ortega et al., 1995, Pellegrino et al., 1979; Toga & Collins, 1981), and to find the overall depth of the dLGN (Montero and Guillery 1968; Pellegrino et al., 1979; Toga & Collins, 1981). Literature values were adjusted using a shrinkage factor of 15% (Gabbot and Stewart, 1987) before applying them to the MRI data. Finally, error in drawing lines normal to the brain surface will artificially displace data from each cortical layer in averaged profiles; the more oblique a line, the more of its length passes through each cortical layer. To correct for this, profile depths were adjusted according to the estimated error in automatically drawing lines normal to the brain surface, (adjusted depth = initial depth $\times$ cos[error]).

To further explore the spatial relationship of manganese uptake and brain region, we also utilized the linearized data in a voxel-wise analysis of the cortex. In this proof-of-concept analysis, we examined a broad sheet of cortex between the caudal limit of the pineal body and the rostral limit of the hippocampus, dorsal to the rhinal fissure, and outside of a 2.4mm-wide buffer centered at the longitudinal fissure. First, images were subjected to a variant of “tissue-normalization” wherein values from a given slice, N, were normalized to the mean value in the RSA/RSG and AIP/PRh for the N–1 through N+1 slice. The flattened cortical sheet from each animal was then manually aligned using non-rotational rigid body transformations. Large co-localization errors were not expected here, as the subject-to-subject size variation in the cortical sheet was minimal, and common alignment of the brain surface was accomplished earlier in processing. Lastly, the two hemispheres of the brain were then averaged together.

Finally, we performed the following voxel-wise analysis on non-linearized data, which is commonly used on pre-clinical imaging data: After signal intensity normalization, which proceeded as in the voxel-wise analysis of the linearized data (see above), images from “stimulated” and “unstimulated” animals were registered to one another (rigid-body) and averaged to form a single template image in SPM5 (<http://www.fil.ion.ucl.ac.uk/spm/>). SPM5 was then used to spatially normalize each animal’s data to the template prior to statistical analysis, which was confined to the same broad portions of the brain as the voxel-wise analysis of linearized data (see above). Although this approach is relatively easy to implement, it carries the risk of degrading layer-specific information through interpolation. We sought to explore the utility of this approach in the context of layer-specific analysis of MEMRI data.

Statistics

For the whole-region comparisons, normalized signal intensities for all regions of interest were compared using a two-way mixed ANOVA (Region $\times$ Condition). For layer specific ROI analysis, the signal intensity profiles were compared with a two-way (Depth $\times$ Condition) mixed ANOVA, then further analyzed by performing a series of planned t-tests (see e.g. Angenstein et al., 2007) at each depth. One-tailed t-tests were used, as manganese accumulation was expected to be elevated in the visual regions of stimulated animals, (Cooper and Allen, 1995; Cooper and Thurlow, 1991; Toga and Collins, 1981). Analysis was restricted to cortical depths corresponding to layers II through VI of each ROI, with layer I being excluded from analysis due to concerns about partial-volume averaging with non-brain. Concerning the layer-specific analysis of the sSC and dLGN, the superficial-most 125 μ m of these structures were excluded from analysis, analogous to the exclusion of layer I from cortical analysis. In the

aforementioned cases, $P < 0.05$ was considered statistically significant. These data are presented as mean $\pm$ SEM unless otherwise noted.

In the voxel-wise analysis of linearized data, one-tailed t-tests were used to compare the “stimulated” and “unstimulated” groups. Three thresholds were used to visualize the group differences; $P < 0.05$, $P < 0.005$, and $P < 0.0008$. The least stringent ($P < 0.05$), visually links the voxel-wise analysis and the analysis of signal intensity profiles, while the most stringent threshold would be the Bonferroni-corrected threshold if analysis were restricted to space occupied by layer IV of Oc1 (using the original, rather than the resampled, voxel size). Under this arrangement, locations where below-threshold voxels from each comparison (“stimulated” $>$ “unstimulated” and “unstimulated” $>$ “stimulated”) are closely intertwined reveal the false positive rate at each threshold.

Below-threshold voxels were expected to be somewhat sparse. As such, 210 μm thick sections, centered at depths corresponding to layers II/III, IV, V, and VI of the primary visual cortex, were selected from the flattened brain and the most significant voxels at each coordinate projected to the sections’ surfaces. The locations of our ROIs were superimposed, as well as a partial map of cortical regions adapted from Kreig, (1946). The latter being useful to visualize the broader functional regions from which our ROIs were selected: The borders of the primary visual cortex correspond well to those of area 17, and the lateral and medial accessory visual cortex correspond well to respective portions of area 18. Caudal portions of area 7 also belong to the accessory visual cortex, (Paxinos, 1985).

Statistical analysis of the standard voxel-wise approach proceeded as in the voxel-wise analysis of linearized data, but without averaging the brain hemispheres. Selection of cortical depths (for display purposes) was approximate here. Depths were matched as closely as permitted by the (non-resampled) voxel size to those used in the voxel-wise analysis of linearized data.

Results

Concerning the Precision and Accuracy of the Linearization Program

In coronal sections, the mean absolute value difference between manually drawn lines and automatically drawn lines was 5.40° in coronal slices ($SD = 4.01$) and 5.13° in non-coronal slices ($SD = 3.93$). In the comparison of manually drawn lines on two different days revealed a mean absolute value difference of 4.20° in coronal slices ($SD = 3.09$) and 3.23° in non-coronal slices ($SD = 2.90$). Estimation of the error in drawing lines normal to the brain surface (see methods) yields 7.42° for automatically drawn lines, and 5.29° for the day-to-day manual error.

MEMRI vs. MRI in Visually Unstimulated Rats

Comparison of MEMRI data of unstimulated animals with MRI of animals not given manganese revealed a significant main effect of condition ($F[1,6] = 111.02$, $P < 0.0001$), of ROI ($F[1,6] = 19.89$, $P < 0.0001$), and a condition by ROI interaction ($F[1,6] = 32.60$, $P < 0.0001$). A series of t-tests (one-tailed, post-hoc) confirmed that manganese administration increased signal intensity in each ROI (all $P < 0.0005$).

MEMRI of Visually Stimulated vs. Unstimulated Rats

Whole-region comparisons—Comparison of data from stimulated and unstimulated animals revealed a significant main effect of region of interest (ROI) ($F[1,6] = 44.63$, $P < 0.0001$), but no main effect of condition, and no interaction, ($P > 0.05$) were observed (Figure 3).

Layer-specific comparisons—Comparison of data from stimulated and unstimulated animals revealed a main effect of depth in all regions (Oc1 – $F[1,40] = 20.87$; Oc2L – $F[1,36] = 24.46$; Par1 – $F[1,52] = 11.85$; Temp1 – $F[1,38] = 12.37$; dLGN – $F[1,15] = 41.07$; SC – $F[1,18] = 55.22$, $P < 0.0001$ for all). No region showed a main effect of condition (all $P > 0.10$). A Depth $\times$ Condition interaction was only detected in the primary visual cortex ($F[1,40] = 1.79$, $P < 0.005$). No such interaction arose in the primary somatosensory cortex, primary auditory cortex, accessory visual cortex (all $P = 1$), dLGN ($P > 0.60$), or sSC ($P = 0.06$). Planned one-tailed t-tests at each depth revealed that stimulated animals had significantly higher signal intensities in the primary visual cortex at depths corresponding to almost all of layer IV, and the superficial third of layer V ($P < 0.05$), as well as in the superficial superior colliculus, at depths corresponding to the optic nerve layer of the superior colliculus. No differences ($P > 0.05$) were found between stimulated and unstimulated animals in other regions. These results are summarized in Figure 4.

Proof-of-Concept Voxel-wise Analysis of Linearized Data—Visual inspection of Figure 5 (left) suggested that, of our four cortical ROIs, Oc1 showed the highest density of below-threshold voxels. The broader primary visual cortex, especially near some of its borders, displayed a noticeably higher density of below-threshold voxels than in the Oc1 ROI. Density was relatively lower in the Oc2L ROI. However, other portions of the accessory visual cortex, including parts of the rostral half of area 7 and the medial division of 18 showed stronger responses to visual stimulation.

Standard Voxel-wise Analysis—No voxels reached the most stringent threshold ($P < 0.0008$) in the entire primary visual cortex. Very few voxels reached even the intermediate threshold ($P < 0.005$), totaling 25 in the primary visual cortex (occupying $<2\%$ of the region's volume), with none appearing in the dLGN or sSC. The density of these voxels was lower in the primary auditory cortex and primary somatosensory cortex, but no other patterns were apparent at that threshold. Results for the cortex are presented in Figure 5 (right).

Discussion

The major result of this study is that systemic administration of a relatively low dose of manganese can be used to encode layer-specific visual cortex activity in awake and free moving rats, as readout with MEMRI. To-date, the use of MEMRI to study cortical activity has largely been restricted to protocols which involve some combination of the following; blood brain barrier opening with mannitol at the time of Mn^{2+} administration, anesthesia while the brain activity of interest is taking place (e.g. Aoki et al., 2004; Lu et al. 2007), or scanning after brain extraction (e.g. Sun et al., 2006; Brozoski et al., 2007). In contrast, using systemically administered manganese, and without compromising the BBB or scanning *ex vivo*, Yu et al. (2005) measured activity in the rodent midbrain in response to auditory stimulation. Also, cortical activity has been investigated in a model of epilepsy using MEMRI (Alvestad et al., 2007) at 24 hours post systemic manganese injection. The present results support, in general, the findings of Yu et al. and Alvestad et al. and extend them, for the first time, to study normal visual cortex *in vivo*.

In functional MEMRI studies, the cortex is typically analyzed as a homogenous tissue, and little to no activity-dependent differences in cortical manganese accumulation have been reported without disrupting the blood brain barrier. In this study, a similar analysis pointed to the same conclusion (Figure 3). Since layers of a given cortical region respond differently to sensory stimulation, we reasoned that a layer-specific analysis might be able to detect subtle stimulation-dependent changes in cortical activity with greater sensitivity. Our results strongly support this approach, with visually-stimulated animals exhibiting increased manganese

accumulation only at depths of 500 to 750 μm in the Oc1 ROI within primary visual cortex, roughly corresponding to layer IV and the superficial third of layer V (Figure 4).

Methods for assessing brain activity at a high spatial resolution (e.g., $2\text{-}^{14}\text{C}$ deoxyglucose) permit the observation of cortical layer-specific activity, while lower-resolution methods like BOLD and PET often do not (e.g. Van Camp et al. (2006), but see Logothetis et al., (2002) and Silva and Koretsky (2002)). In BOLD and PET, image registration and spatial normalization to a template image prior to statistical analysis are common practices. In both steps, interpolation can combine information from adjacent cortical layers, reducing sensitivity to lamina-specific changes beyond what might be expected at a given image resolution. This conclusion is supported by visual comparison of a standard voxel-wise analysis (Figure 5 right panels), which involved interpolation of signal intensity values, and the proof-of-concept voxel-wise analysis of linearized data (Figure 5 left panels), which did not. The overall lower sensitivity of the standard approach is apparent. Note that the differential sensitivity in Figure 5 was not due to averaging of brain hemispheres. A separate analysis of the linearized data, in which hemispheres were not first averaged (not shown), also revealed a larger volume of voxels reaching each threshold than in the standard voxel-wise analysis. These considerations support the use of linearized data, which involves fewer interpolative steps and thus maintains higher layer-specific sensitivity.

In this study, there were some limitations regarding how the linearized data were generated: First, the use of polynomials here may not be ideal. Though much of the rat brain's dorsal surface can be linearized well using polynomials, these functions cannot easily accommodate the most abrupt contours of the cortical surface (e.g. the longitudinal fissure), limiting the cortical space available for study. In such places, a more refined technique (e.g. Schleicher et al., 2005) might be adapted. Second, linearization entails sampling the original data along lines normal to the brain surface, but proper positioning is non-trivial. Our approach appeared to work well over the expanse of the cortex analyzed, with an estimated error of 7.42° from normal, compared to the estimated error in manually drawn lines of 5.29° from normal. However, if errors differ substantially from line to line, distortion will arise in linearized data: Cortical layer borders will be displaced in the set of signal intensities derived from each line, more so in one than the other. Small line-to-line differences are nevertheless of little consequence: For instance, to displace data from layer VI's deep border in Oc1 by 30 microns – the smallest voxel width in the resampled linearized images – the error must exceed 11.5° . In the present study, no noticeable distortion arose in the analyzed portions of the brain. Finally, unlike the ROI-based analyses, our proof-of-concept voxel-wise analysis utilized rigid-body transformation to co-align the data from each subject. Future co-alignment improvements will greatly enhance the quality of this voxel-wise technique, and may be achieved by applying a two-dimensional (rostral-caudal and mediolateral) spatial normalization algorithm to the linearized data.

Beyond providing a test case for MEMRI analysis techniques, the present results support previous findings in which visually stimulated rodents demonstrate increased activity in layer IV of the primary visual cortex, relative to darkness (Cooper and Allen, 1995). The increased activity in layer V observed here is also consistent with results in the literature: In comparisons of cortical activity following visual stimulation or enucleation, layer IV, and a combination of layers V and VI of the primary visual cortex, showed a greater difference than all other layers of the cortical regions analyzed (Toga and Collins, 1981).

Substantial activity has also been observed in the accessory visual cortex with visual stimulation, and more so in the lateral division than in the medial (Toga and Collins, 1981; Cooper and Thurlow, 1991). That pattern was not observed here, and this difference between the present data and the literature is appreciated when comparing the voxel-wise analysis of

linearized data (Figure 5, left) with the 2-¹⁴C deoxyglucose-based activity map in Cooper and Thurlow (1991): Whereas the patterns in the primary visual cortex are similar – including the somewhat stronger response in the medial half of area 17 – the pattern in the accessory visual cortex (area 18) is reversed, with an appreciable response occurring in portions of the medial division but not the lateral division in the present study (Figure 5). Because neither of the aforementioned studies utilized binocular visual stimulation, this conflict may arise from differences in stimulation. In the rat, the visual pathway is highly lateralized, with the overwhelming majority of information arriving at the primary visual cortex coming from the contralateral eye. Although direct inter-hemispheric connections between primary visual cortices are negligible, this is not the case for the accessory visual cortex, (Paxinos, 1985). The opportunity exists, therefore, for direct modulation of accessory visual cortex activity based on the information received by the contralateral cortex. However, the degree of inter-hemispheric coordination in this region of the rat brain is unknown.

Concerning the superior colliculus, stimulation-dependent activity has been detected in the stratum griseum superficiale of the sSC (sgsSC), as well as in the layer just deep to it, the optic nerve layer, with immunocytochemical staining (Montero and Jian, 1995). The same pattern of activity has also been observed using 2-¹⁴C deoxyglucose, but more robustly in response to flashing patterned light, rather than non-flashing patterned light as used here (compare Cooper and Allen, 1995 with Cooper and Thurlow, 1991). Although we found activity-dependent increases in manganese uptake in the sSC, they appeared to reside in the optic nerve layer, rather than in the sgsSC.

Studies of the visual system using 2-¹⁴C deoxyglucose (Cooper and Thurlow, 1991; Cooper and Allen, 1995; Toga and Collins, 1981) and immunocytochemical staining (Montero and Jian, 1995) have readily found stimulation-dependent increases in dLGN activity. The same was expected here given recent successes at detecting changes in sub-cortical activity with MEMRI (Alvestad et al. 2007; Brozoski et al., 2007; Yu et al., 2005). Nevertheless, in this study, no difference between stimulated and unstimulated animals was observed using whole-region analysis of the dLGN. This result persisted with layer-specific analysis, in contrast to the results for the sSC and primary visual cortex.

There are several possible explanations for the unexpected results in the sSC and dLGN. Comparisons of MEMRI data with known histology are non-trivial – even when both sources of information are from the same animal, precise and straightforward structural comparisons are still complicated by factors like tissue shrinkage (see Silva et al., 2008). In the case of the dLGN, partial inclusion of adjacent structures that respond less avidly to patterned light, like the ventral lateral geniculate nucleus (Toga and Collins, 1981) could have occurred during analysis. However, this cannot fully explain the lack of a difference between groups reported here. In the sSC, slight misalignment of MEMRI data with histological labels could partially explain the differences between our results and previous literature. Indeed, partial-volume averaging of structures within the sSC with non-brain could create the illusion of a lower-intensity extension of the zonal layer lying outside of the true superficial border of the sSC, thereby shifting signal intensities deep to (in Figure 4, to the right of) their corresponding structures. Scanning animals at a higher spatial resolution would address this possibility.

Although the dose of manganese used in this study was likely sufficient for detecting sub-cortical activity (in Alvestad et al. 2007, 80mg/kg MnCl₂; in Brozoski et al., 2007, 20mg/kg MnCl₂), in the current study, the timing between manganese administration and scanning may not have been ideal for sub-cortical structures: A span between the two events of 24 hours or more is used in other studies (Alvestad et al. 2007; Brozoski et al., 2007; Yu et al., 2005). Our data from animals given no manganese showed that ample manganese accumulation occurred in the dLGN and sgsSC, making this explanation unlikely.

It may be that the patterns of dLGN and sSC activity described in studies using monocular visual stimulation differ substantially from the patterns of activity that take place during binocular stimulation, but imaging literature addressing this matter is sparse. In a qualitative 2-¹⁴C deoxyglucose study by Biral et al. (1982), wherein patterned stimuli were presented binocularly, the superior colliculi appeared “strongly darker” than the lateral geniculate nuclei in visually stimulated animals, but the two regions had similar optical densities in control animals. These results contrast with those of Cooper and Allen (1995), wherein the lateral geniculate trended towards a greater response to monocular visual stimulation than did the superior colliculus. Increased activity in the sSC during binocular, rather than monocular, stimulation (Van Camp et al. 2006) partially accounts for this conflict, as well as the sub-cortical findings of this study. It may also be that dLGN activity in rats is greater during monocular, instead of binocular, stimulation. However, we are aware of only one imaging study lending insight into this possibility: Studying the canine visual system with BOLD, Willis et al. (2001) found that a smaller area of the dLGN was active during binocular stimulation, compared to monocular. However, dLGN-wide differences in BOLD signal change were non-significant.

Regardless of the findings in sub-cortical structures, MEMRI non-invasively detected mean differences in layer-specific cortical activity of awake and free-moving animals. Such a capability would be advantageous whenever anesthetic use could influence results, as in studies of learning, the motor system, or top-down modulation of sensory processing. Although the advantages of this technique would be diminished if MnCl₂ itself disrupts normal cortical function, the dose of MnCl₂ used in the present study (66 mg/kg) is not expected to adversely affect cortical activity in rodents. Previously, we established that a 33% lower dose of MnCl₂ (44 mg/kg) was not associated with changes in neuronal function at either 4 hrs or 7 days post injection, relative to control rats (Berkowitz et al., 2007). In addition, at 30 days post-injection, there was a lack of anatomical and physiological changes in control rats (Berkowitz et al., 2006). Although a preliminary study using 44 mg/kg dose of MnCl₂ produced reliable signal intensity changes compared to animals not given manganese (data not shown), we found empirically that a somewhat higher dose of manganese (66 mg/kg) produced more robust contrast changes, likely increasing the chances of detecting changes in stimulated from unstimulated cortex. This dose is similar to, or lower than, that used in other rodent MEMRI studies, and is well below that needed to induce neurotoxicity (Silva et al., 2004; Yu et al., 2005). Thus, it is reasonable to consider the 66 mg/kg dose as non-toxic and the uptake of manganese as a quantitative biomarker of ion activity regulation *in vivo*.

The methods employed in this study should be applicable to investigations in other species. Although our approach to cortex linearization might be difficult to implement in species with highly convoluted brain surfaces, other techniques are available. In some species, especially primates, the possibility of manganese toxicity needs to be seriously considered (Chen et al., 2006). Various approaches are being developed to address potential toxicity issues, such as the use of fractionated manganese administration (Bock et al., 2008) or co-administration of N-acetylcysteine (Hazell et al., 2006). In addition, Teslascan is an FDA-approved manganese-based contrast agent for human use which may prove to be particularly useful if sensitivity gains can be achieved which offset the lower amounts of injected manganese contained within Teslascan, relative to the present study.

Prior to the present study, non-invasive monitoring of stimulation-dependent layer-specific changes in cortical ion activity *in vivo* had not been reported. Future studies will examine the possibility of measuring, in the same animal, both retinal and cortical activity longitudinally. Such an approach would allow one to uniquely measure structural and functional changes that occur in normal development and in disease – through its natural course or with an intervention. Based on the present data, MEMRI is likely to be a widely applicable method for measuring

activity in the visual cortex, and layer-specific analysis may enhance the sensitivity of MEMRI to activity in other brain regions as well.

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Figure 1.

An overhead view showing cortical regions of interest (ROIs), with – for visualization purposes – a superimposed drawing showing the relevant surface topography, adapted from Kreig (1946). Also, (A–D) four coronal sections of the same brain, (position indicated on overhead view) showing all regions of interest. Cortical regions of interest are named for the broader functional region of which they are part. ROI abbreviations: “Oc1”-primary visual cortex (primarily within the binocular division), “Oc2L”-accessory visual cortex (lateral division), “sgsSC”-stratum griseum superficiale of the superior colliculus, “dLGN”-dorsal lateral geniculate nucleus, “Par1”-primary somatosensory cortex, “Temp1”-primary auditory cortex, “MG”-medial geniculate, “RSA/RSG”-agranular retrosplenial cortex and granular

retrosplenial cortex, “AIP/PRh”-posterior agranular insular cortex and perirhinal area. Note that layer-specific analysis of the superficial superior colliculus (“sSC”) includes the stratum griseum superficiale, as well as tissue immediately superficial to it, the zonal layer, and tissue immediately deep to it, the optic nerve layer.

Figure 2.

Top: A coronal section through a brain in its native position, (i.e. oriented not in stereotaxic space, but as it was under the surface coil during scanning). Blue pixels show locations where the brain was “filled in”, and the resulting silhouette of the brain dilated, before fitting polynomials for the top (dorsal) and bottom halves of the brain. Thick red lines show these high order polynomials. Thin red lines show every tenth perpendicular line, upon which signal intensities were sampled to construct the flattened out image. As part of the linearization procedure, perpendiculars are tilted in the rostro-caudal plane according to the location of the brain surface within a $450 \times 450 \times 1050 \mu\text{m}$ area, the coronal profile of which is illustrated by the yellow box. Middle: Linearized data corresponding to the polynomials for the top and bottom halves of the coronal section. Due to resampling, each pixel of flattened image represents $30 \times 30 \times 150 \mu\text{m}$ of space. To ensure values are aligned at the provisional brain surface, signal intensity profiles are analyzed within a $930 \mu\text{m}$ moving window. The green box – positioned at an unanalyzed zone near the longitudinal fissure – illustrates the extent of this window. Bottom: The flattened data from the top half of the brain, after alignment to the provisional brain/no-brain border. The green arrow illustrates the correction made at the boxed area in the middle of the figure.

Figure 3.

The mean signal intensities in visual and negative control regions of interest for animals given manganese, normalized to mean signal intensities in the adjacent RSA/RSG and AIP/PRh regions of the cortex. White bars represent stimulated animals, grey bars represent unstimulated animals. Abbreviations: “Oc1”-primary visual cortex, “Oc2L”-accessory visual cortex (lateral division), “sgsSC”-stratum griseum superficiale of the superior colliculus, “dLGN”-dorsal lateral geniculate nucleus, “Par1”-primary somatosensory cortex, Temp1-primary auditory cortex, “MGN”-medial geniculate. A two-way mixed ANOVA (Region $\times$ Condition) indicated a main effect of region ($P < 0.0001$), but no main effect of condition (stimulated vs. unstimulated) and no interaction, ($P > 0.05$). Error bars are $\pm$ SEM.

Figure 4.

Profiles of signal intensity (SI) as a function of depth into the cortex for the visual cortical ROIs (primary visual – “Oc1”, accessory visual – “Oc2L”) and negative control regions (primary somatosensory – “Par1”, primary auditory – “Temp1”), as well as signal intensity profiles for the superficial superior colliculus (“sSC”) and dorsal lateral geniculate nucleus (“dLGN”). White points represent stimulated animals, black points represent unstimulated animals. The rough correspondence between depth and histological layering is marked with roman numerals for cortical regions, and with letters in the sSC (zonal layer – “z”, stratum griseum superficiale – “sgs”, optic nerve layer – “op”). In Oc1 and sSC, asterisk indicates $P < 0.05$ one-tailed t-test for depths in the designated span. Error bars are $\pm$ SEM. Note that broad profile patterns shared by both groups may have several different influences, including Gibbs ringing, manganese accumulation from basal neuronal activity, or manganese accumulation in non-neuronal cells.

Figure 5.

A comparison of the proof-of-concept voxel-wise analysis of linearized data and the standard voxel-wise analysis of non-linearized data showing overhead statistical maps at different depths into the cortex. The top of each image is the rostral border of the analyzed cortex, with the left border indicating the position of the longitudinal fissure. Depths were chosen based on the layering in the primary visual cortex, with each image being centered in layers II/III, IV, V, and VI (brackets). For visualization purposes only, a cortical map adapted from Kreig (1946) is overlaid on the images to indicate approximate positions of our regions of interest (labeled with green text) relative to established surface topography. Broad functional regions – subsets of which were used for ROIs – correspond well to the overlaid map, with the primary visual cortex occupying area 17, and the lateral and medial accessory visual cortex respectively

occupying most of the lateral and medial sections of area 18. The rostral half of area 7 is also part of the accessory visual cortex (Paxinos, 1985). P-thresholds are indicated at the bottom. Left: Linearized data from both hemispheres were averaged before statistical analysis. The green shaded band shows (1.2 mm of) the 2.4 mm-wide buffer of unanalyzed space, centered at the longitudinal fissure. Right: 3D renderings of non-linearized data showing data only from the right hemisphere, which displayed a higher total number of below-threshold voxels from the “stimulated” vs. “unstimulated” comparison than did the left. Depths are approximate here. The narrowing of the images from top to bottom, as well as the accompanying manipulation of overlays, results from progressive removal of cortex to reveal deeper layers.