Statistical Parametric Mapping for Effects of Verapamil on Olfactory Connections of Rat Brain in Vivo using Manganese-enhanced MR Imaging

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(Received June 10, 2010; Accepted February 2, 2011)

Purpose: We investigated the effect of verapamil on the transport of manganese in the olfactory connections of rat brains in vivo using statistical parametric mapping and manganese-enhanced magnetic resonance (MR) imaging.

Methods: We divided 12 7-week-old male Sprague-Dawley rats into 2 groups of six and injected 10 μL of saline into the right nasal cavities of the first group and 10 μL of verapamil (2.5 mg/mL) into the other group. Twenty minutes after the initial injection, we injected 10 μL of MnCl₂ (1 mol/L) into the right nasal cavities of both groups. We obtained serial T₁-weighted MR images before administering the verapamil or saline and at 0.5, one, 24, 48, and 72 hours and 7 days after administering the MnCl₂, spatially normalized the MR images on the rat brain atlas, and analyzed the data using voxel-based statistical comparison.

Results: Statistical parametric maps demonstrated the transport of manganese. Manganese ions created significant enhancement (t-score = 36.6) 24 hours after MnCl₂ administration in the group administered saline but not at the same time point in the group receiving verapamil. The extent of significantly enhanced regions peaked at 72 hours in both groups and both sides of the brain. The peak of extent in the right side brain in the group injected with saline was 70.2 mm³ and in the group with verapamil, 92.4 mm³. The extents in the left side were 64.0 mm³ for the group with saline and 53.2 mm³ for the group with verapamil.

Conclusion: We applied statistical parametric mapping using manganese-enhanced MR imaging to demonstrate in vivo the transport of manganese in the olfactory connections of rat brains with and without verapamil and found that verapamil did affect this transport.

Keywords: manganese, MR image, rat, statistical parametric mapping, verapamil
cent studies have reported stereotaxic statistical mapping techniques for rat brain research using autoradiography\textsuperscript{11,12} and MR imaging.\textsuperscript{13–18} With these techniques, spatial normalization of the brain permits voxel-based analysis of the entire brain and more objective results than those of conventional ROI/VOI analysis. The results are less susceptible to changes in subject shape and size, and position, shape, and size of the ROI/VOI do not vary between observers. Furthermore, the new techniques allow group-wise statistical analysis and determination of common characteristics among subjects. These techniques of statistical mapping analysis were first developed and used for human brain research using positron-emission tomography (PET)\textsuperscript{19–22} and functional MR imaging (fMRI).\textsuperscript{23,24} Rat brain research uses a rat brain atlas\textsuperscript{25} like the atlas of Talairach and Tournoux\textsuperscript{26} used for human brain mapping.

Cross and associates investigated rat brain function using MEMRI and statistical mapping analysis with NEUROSTAT software.\textsuperscript{13–15} These methods using MEMRI are expected to become a basic and important method for rat brain research, so their applicability for such investigation requires validation. We applied Statistical Parametric Mapping 8 software (SPM8; Wellcome Department of Cognitive Neurology, London, UK [http://www.fil.ion.ucl.ac.uk/spm/]) for stereotaxic statistical mapping, which allows \textit{in vivo} imaging of olfactory connections in the rat brain using MEMRI, and used this mapping method to investigate the effect of a calcium-channel blocker (verapamil) on the transport of Mn in these connections.

Materials and Methods

\textbf{Subjects and MR imaging procedures}

We used 12 7-week-old male Sprague-Dawley rats weighing 246 ± 6 g (mean ± standard deviation [SD]) purchased from Charles River Japan (Yokohama, Japan) and housed in clean plastic cages in a temperature- and humidity-controlled facility with a constant 12-hour cycle of light and dark. The Animal Ethics Committee of the Osaka University School of Medicine approved the use of animals and the experimental protocol.

We divided the rats into 2 groups of six and injected 10 µL of saline into the right nasal cavities of one group (Group A) and 10 µL of the calcium-channel blocker verapamil (2.5 mg/mL) into the other group (Group B). Twenty minutes after the verapamil or saline injection, we injected 10 µL of manganese chloride (MnCl\textsubscript{2}) (1 mol/L) into the right nasal cavities of all rats.

We performed serial T\textsubscript{1}-weighted MR scans on each rat of the 2 groups before the administration of verapamil or saline and at 0.5, one, 24, 48, and 72 hours and 7 days after MnCl\textsubscript{2} administration (total of 7 scans). Except for the scans performed at 0.5 and one hour after MnCl\textsubscript{2} administration, all scans were performed after the rats were anesthetized with an intraperitoneal injection of 4% chloral hydrate solution (Sigma Aldrich, St. Louis, MO, USA; 400 mg/kg body weight). Because the effect of anesthesia lasts approximately an hour, we considered that the anesthetic administered before the administration of verapamil or saline would be sufficient for the next 2 scans and confirmed this by checking whether the rats awakened before the scans. Figure 1 illustrates the protocol for drug administration and MR imaging scans.

We acquired MR image data using an MR imaging system for animal experiments that was equipped with a 1.5-tesla permanent magnet (MRmini, DS Pharma Biomedical Co., Ltd., Osaka, Japan). T\textsubscript{1}-weighted images were acquired using a spoiled gradient-echo pulse sequence with parameters: echo time (TE)/repetition time (TR), 4.15/50 ms; matrix size, 256 × 128; 32 slices; pixel size, 0.246 × 0.246 mm; 0.98-mm slice thickness; and flip angle, 36°. Prior to acquiring the T\textsubscript{1}-weighted images used for study, we obtained trial images in the sagittal and transaxial planes and adjusted the position of each rat on the scanner to minimize image distortion.

\textbf{Data analysis}

Data analyses were performed using MRIcro Version 1.37 developed by Rorden (http://www.sph.sc.edu/comd/rod/), SPM8, MATLAB version R2007a (The MathWorks Inc., Natick, MA, USA).
USA), and SPM for Windows (SPMwin) Version 1.01 (coded by Sergey Pakhomov and Nick Tsyganov of the Institute of the Human Brain of the Russian Academy of Sciences in collaboration with the Wellcome Department of Cognitive Neurology, University College, London, UK). We used MATLAB to execute SPM8, calculate a statistical value of each voxel on images pre-processed by SPM8, and view statistical maps of results.

We based our study analyses methods on the mapping techniques for rat brains developed by Cross and associates,13 which were essentially the same as the techniques used for statistical image analysis in research on human brain mapping.27–31 The steps in data analysis were: (1) transfer of the data to a personal computer; (2) conversion of the data to ANALYZE data format; (3) extraction of brain regions; (4) intra-subject realignment; (5) spatial normalization; (6) smoothing; and (7) voxel-intensity normalization. Further details of each step follow.

**Extraction of brain regions**

Eliminating non-brain regions from MR images allows implementation of the intra-subject realignment and spatial normalization steps. Using MRcro, we manually drew a binary mask for the brain on each MR image (which was transferred to a personal computer and converted to ANALYZE data format) and then removed the non-brain regions from the images.

**Intra-subject realignment**

For a single rat, 12 sets of 7 images, each obtained at different time points, were realigned in the same direction using the “coregister” function of SPM8. We used the image obtained before administration of verapamil or saline as a reference image and set the images obtained after administration (6 images for each subject) as target images. We used rigid-body transformation with 6 parameters (x, y, and z translation and rotation), normalized mutual information32 as a cost function, and applied Powell’s optimization method33 with the “coregister” function of SPM8. We averaged each set of 7 realigned images for the same subject and used this averaged image to estimate the parameters of spatial normalization for the corresponding set of 7 realigned images, as described below.

**Template**

Voxel-based statistical image analysis across different subjects requires spatial normalization of individual images to the coordinates of a standard atlas, which necessitates a template image. We generated a T1-weighted MR image template as the rat brain atlas in stereotaxic coordinates by first performing T1-weighted MR scans on 2 rats that had not been treated with saline, verapamil, or MnCl2 (one scan/one rat), extracting the brain images as described above and individually transforming them to the rat brain atlas using the “display” function of SPM8, iteratively translating, rotating, and scaling the images in 3 dimensions to match the rat brain atlas, and generating a mean image from the matched images as the seminal template. We then used the “normalization” function of SPMwin to individually normalize the 2 pre-transformed images (obtained after brain extraction) to this seminal template by 9-parameter affine transformation (x, y, and z translation, rotation, and scaling). The final template image (Fig. 2A) was generated by averaging these 2 normalized images and reformatted to a matrix sized 91 × 71 × 141 (voxel size, 0.2 × 0.2 × 0.2 mm³).

**Spatial normalization**

We spatially normalized the 84 images in which the brain was extracted and realigned by 12 affine parameters (x, y, and z translation, rotation, scaling, and shear) using the “normalization” function of SPM8. We estimated the parameters for normalization of each image by normalizing the above corresponding averaged image (for the same subject) to the above template image. All images were then normalized to the template in the stereotaxic rat brain atlas space. These processes allowed voxel-based comparison between different subjects.

**Spatial smoothing and voxel-intensity normalization**

Spatial smoothing is performed in SPM analysis for human brain-mapping studies, mainly to improve the signal-to-noise ratio of images, reduce the impact of misregistration of data into the space of the template, allow for anatomical variability among rat brains, and facilitate multiple comparison correction using Gaussian random fields.11,21 Smoothing also increases statistical power as a secondary effect.24,34–38 All spatially normalized images were smoothed using a 3-dimensional isotropic Gaussian kernel with a full-width at half-maximum (FWHM) of 0.8 mm. We determined the FWHM value from the results of “increasing statistical power by smoothing” described below.

Normalization of voxel intensity is necessary to compare images obtained at different time points and those obtained for different subjects because an MR image has a relative intensity. We normalized voxel intensity in this study by dividing voxel values...
Fig. 2. (A): Rat atlas template for coronal slices generated from the T1-weighted magnetic resonance (MR) images obtained for 2 rats not treated with manganese chloride (MnCl₂). The number in millimeters below each image represents the axial distance from the bregma according to the rat brain atlas. (B): Right- and left-side volumes of interest (VOIs) used to determine the smoothing parameter suitable for increasing the statistical power of voxel-based comparison. The number in millimeters below each image represents the axial distance from the bregma according to the rat brain atlas.

by the estimated average whole brain value in each normalized and smoothed image. We estimated the average whole brain value as a mean value of those voxels that survived after we discounted voxels outside the brain in the “extraction of brain regions” step described above; based on a zero voxel value outside the brain, surviving voxels were those with values greater than zero.

Voxel-based comparison
We performed 12 kinds of paired t-test between the baseline images obtained prior to MnCl₂ administration and those images obtained after, for each of the 6 rats, at each time point, and for each group. T-scores of the paired t-tests were calculated voxel by voxel. The statistical threshold was set to \( P<0.05 \) and corrected for multiple comparisons using the false discovery rate (FDR). To increase the statistical power of the voxel-based analysis, we restricted the voxels to be analyzed to brain regions within coronal slices obtained for the region from the olfactory bulb to a distance \(-4.6\) mm from the bregma, defined according to the standard rat brain atlas. Regions of significant enhancement (voxels with t-score exceeding the statistical threshold) were identified and localized in the standard atlas.

Region of interest (ROI) analysis
To verify results from the above voxel-based comparison, we analyzed ROIs that we selected referring to the significantly enhanced regions in voxel-based comparison (Table 1) and the stereotaxic coordinates. Concretely, we set 12 ROIs at: both sides of the olfactory bulb on the coronal slice \(+5.4\) mm from the bregma, both sides of the lateral olfactory tract on the coronal slice \(+3.2\) mm from the bregma, both sides of the anterior commissure anterior part of the coronal slice \(+3.2\) mm from the bregma, both sides of the caudate putamen (striatum) on the coronal slice \(+2.4\) mm from the bregma, both sides of the olfactory tubercle on the coronal slice \(+1.0\) mm from the bregma, and both sides of the piriform cortex on the coronal slice \(-2.0\) mm from the bregma.

ROI analysis was performed as follows. We calculated the intensity of each ROI by averaging intensities in each ROI for spatially and voxel-intensity normalized rat brain data (without smoothing after spatial normalization). We calculated the percentage of change at a given time point for each rat and calculated ROI using:

\[
C_t = \frac{R_t - R_{pre}}{R_{pre}} \times 100, \tag{1}
\]

where \( C_t \) represents the percentage of change at the time point, \( R_t \) is ROI intensity at the time point, and \( R_{pre} \) is the intensity of the ROI prior to administration of MnCl₂. We obtained each mean percentage of change by averaging the percentages of change of the 6 rats in the corresponding group, ROI, and time point.

Increasing statistical power by smoothing
To determine the optimal FWHM parameter for smoothing in this study, we investigated the parameter of the kernel of a 3-dimensional isotropic Gaussian filter that increased t-score in the statistical images. First, we generated the statistical
| T   | t-score | Group A (with saline) | Group B (with verapamil) |
|-----|---------|------------------------|--------------------------|
|     | X(mm)   | Y(mm)                  | Z(mm)                    | structure | X(mm)   | Y(mm) | Z(mm) | structure |
| 24 h| 10.1    | -1.2                   | 2.0                      | 7.4        | r.OB    | 10.3  | -0.4  | 4.6      | 7.8  | r.OB    |
|     | 36.6    | -0.6                   | 4.4                      | 6.2        | r.OB    | 5.7   | -2.0  | 2.0      | 6.8  | r.OB    |
|     | 17.0    | -1.2                   | 2.0                      | 5.4        | r.OB    | 8.9   | -2.0  | 2.6      | 5.2  | r.OB    |
|     | 11.2    | -0.2                   | 3.4                      | 4.6        | r.OB    | 19.1  | -0.6  | 5.0      | 5.0  | r.OB    |
|     | 14.2    | 2.6                    | 6.0                      | 3.6        | r.aca   | 9.0   | -2.0  | 3.8      | 5.0  | r.OB    |
|     | 7.6     | 2.2                    | 7.4                      | 3.0        | l.aca   | 24.8  | -1.4  | 6.0      | 4.4  | r.OB    |
|     | 5.4     | 2.4                    | 8.4                      | 2.2        | l.Tu    | 12.3  | 2.2   | 4.4      | 1.2  | r.Pir   |
| 48 h| 9.5     | -1.0                   | 3.6                      | 6.8        | r.OB    | 10.3  | -0.4  | 4.6      | 7.8  | r.OB    |
|     | 9.7     | -0.6                   | 4.8                      | 6.4        | r.OB    | 7.7   | -2.0  | 2.0      | 6.8  | r.OB    |
|     | 9.4     | -1.0                   | 5.0                      | 5.6        | r.OB    | 8.9   | -2.0  | 2.6      | 5.2  | r.OB    |
|     | 9.0     | -2.0                   | 2.6                      | 5.4        | r.OB    | 19.1  | -0.6  | 5.0      | 5.0  | r.OB    |
|     | 9.1     | -2.2                   | 3.2                      | 4.6        | r.OB    | 18.0  | -2.0  | 3.8      | 5.0  | r.OB    |
|     | 10.3    | 0.4                    | 4.6                      | 4.2        | r.OB    | 24.8  | -1.4  | 6.0      | 4.4  | r.OB    |
|     | 9.9     | -2.4                   | 5.4                      | 3.2        | r.LO    | 15.9  | -3.4  | 7.8      | 2.0  | r.Pir   |
|     | 9.5     | -1.8                   | 6.8                      | 3.2        | r.aca   | 25.6  | -1.8  | 6.6      | 3.4  | r.aca   |
|     | 12.9    | -1.4                   | 6.2                      | 2.4        | r.aca   | 26.0  | -2.4  | 7.0      | 2.8  | r.aca   |
|     | 7.3     | 2.6                    | 8.4                      | 2.2        | l.Tu    | 8.7   | 2.6   | 5.4      | 2.2  | l.Cpu   |
|     | 13.3    | -0.8                   | 7.6                      | 1.0        | l.OB    | 15.9  | -3.4  | 7.8      | 2.0  | r.Pir   |
|     | 6.7     | 2.2                    | 7.0                      | 0.2        | l.aca   | 17.0  | -3.0  | 8.6      | 1.4  | r.Tu    |
|     | 4.9     | 1.8                    | 4.6                      | 0.2        | l.Cpu   | 12.2  | -4.4  | 7.6      | 1.4  | r.Pir   |
|     | 5.8     | -4.4                   | 9.4                      | -1.4       | r.Pir   | 13.0  | 2.0   | 6.6      | 1.2  | l.aca   |
|     | 5.1     | 6.0                    | 8.8                      | -2.0       | l.Pir   | 12.6  | -2.4  | 8.8      | 0.4  | r.Tu    |
|     | 7.1     | 5.8                    | 9.2                      | -3.6       | l.Pir   | 10.9  | -2.6  | 8.8      | -0.2 | r.Tu    |
|     | 9.8     | -6.2                   | 9.0                      | -4.4       | r.Pir   | 9.2   | 5.0   | 8.2      | -0.8 | l.Pir   |
|     |         |                        |                          |            |         |       |       |          |      |         |
| 72 h| 6.5     | 1.0                    | 2.6                      | 5.2        | l.OB    | 7.4   | -1.0  | 5.0      | 7.2  | r.OB    |
|     | 8.0     | 1.4                    | 4.0                      | 5.0        | l.OB    | 6.9   | -1.6  | 2.6      | 6.8  | r.OB    |
|     | 13.1    | 2.8                    | 4.0                      | 3.8        | l.LO    | 11.6  | -1.8  | 4.6      | 5.4  | r.OB    |
|     | 17.2    | 2.8                    | 5.6                      | 3.2        | l.LO    | 10.8  | -2.0  | 4.0      | 5.2  | r.OB    |
|     | 36.0    | 0.8                    | 5.4                      | 2.6        | l.CPu   | 12.3  | 2.2   | 4.4      | 3.6  | r.LO    |
|     | 13.2    | 3.8                    | 6.6                      | 2.0        | l.Pir   | 17.8  | -2.8  | 6.4      | 3.2  | r.LO    |
|     | 12.1    | 3.8                    | 7.4                      | 1.4        | l.Pir   | 11.3  | 2.2   | 5.4      | 3.2  | l.LO    |
|     | 11.4    | -2.0                   | 8.8                      | 0.8        | r.Tu    | 25.1  | 1.8   | 6.0      | 1.8  | l.Cpu   |
|     | 10.3    | 2.0                    | 6.6                      | 0.6        | l.aca   | 16.3  | -5.0  | 6.8      | 1.6  | r.Pir   |
|     | 9.4     | -2.2                   | 5.2                      | 0.2        | r.CPu   | 77.5  | -2.6  | 8.6      | 1.4  | r.Tu    |
|     | 26.2    | -1.4                   | 8.2                      | -0.2       | r.LPO   | 25.3  | -4.6  | 7.6      | 1.0  | r.Pir   |
|     | 11.1    | 3.0                    | 7.4                      | -0.8       | l.CPu   | 26.5  | -2.8  | 9.0      | 0.2  | r.Tu    |
|     | 7.2     | 0.0                    | 5.2                      | -0.8       | vhc     | 23.7  | 1.0   | 6.8      | 0.0  | r.Lac   |
|     | 12.0    | -3.0                   | 9.4                      | -1.0       | r.Pir   | 26.9  | -2.8  | 8.8      | -0.6 | r.VP    |
|     | 8.1     | -1.4                   | 7.2                      | -1.6       | r.Rt    | 4.0   | 3.4   | 5.2      | -1.4 | l.ic    |
|     | 8.4     | -3.6                   | 8.2                      | -2.0       | r.Pir   | 28.0  | 6.2   | 8.2      | -2.2 | l.Pir   |
|     | 10.4    | -4.6                   | 10.0                     | -2.2       | r.Pir   | 9.3   | -3.4  | 10.0     | -2.8 | r.Pir   |
|     | 9.5     | -4.6                   | 10.0                     | -3.0       | r.Pir   | 12.9  | -6.2  | 9.0      | -3.8 | r.Pir   |
|     | 7.1     | -6.0                   | 8.6                      | -3.0       | r.Pir   |       |       |          |      |         |
|     | 31.5    | -0.8                   | 8.8                      | -4.2       | r.Pir   |       |       |          |      |         |
Table 1. (Continue)

| T  | t-score | Group A (with saline) | Group B (with verapamil) |
|----|---------|-----------------------|--------------------------|
|    |         | X(mm) | Y(mm) | Z(mm) | structure | X(mm) | Y(mm) | Z(mm) | structure |
| 7 d | 8.7     | 1.8  | 5.6  | 4.8  | 1.OB      | 8.3   | 1.0  | 4.8  | 7.6  | 1.OB |
|     | 8.2     | 3.4  | 6.8  | 2.6  | 1.Pir     | 14.2  | 0.8  | 4.2  | 5.6  | 1.OB |
|     | 7.4     | 1.6  | 6.2  | 1.8  | 1.Cpu     | 9.6   | 1.0  | 5.2  | 5.6  | 1.OB |
|     | 7.2     | -2.8 | 6.2  | 1.0  | r.Cpu     | 7.5   | -1.8 | 6.6  | 3.6  | r.aca |
|     | 12.1    | 1.8  | 7.4  | 0.6  | l.aca     | 19.5  | 2.4  | 7.6  | 2.0  | l.aca |
|     | 9.1     | 3.2  | 8.6  | 0.6  | 1.Tu      | 19.5  | 1.8  | 8.6  | 1.4  | 1.Tu  |
|     | 10.2    | 3.4  | 5.2  | -0.2 | 1.CPu     | 11.5  | 1.2  | 6.8  | 1.2  | 1.Cpu |
|     | 9.5     | -3.2 | 4.2  | -0.8 | r.CPu     | 12.2  | 3.2  | 8.4  | 0.8  | 1.Tu  |
|     | 20.0    | -3.8 | 3.2  | -1.0 | r.CPu     | 21.7  | 3.6  | 8.2  | -0.8 | 1.CPu |
|     | 8.4     | 0.4  | 2.6  | -1.4 | 1.Cg2     | 10.5  | -5.2 | 9.0  | -0.8 | r.Pir |
|     | 24.6    | 4.2  | 8.8  | -1.8 | 1.Pir     | 14.7  | 2.4  | 7.8  | -1.6 | l.ic  |
|     | 11.7    | -3.2 | 4.4  | -1.8 | r.ic      | 18.4  | 4.8  | 9.8  | -2.8 | l.Pir |
|     | 5.9     | -4.0 | 5.4  | -2.0 | r.ic      |        |      |      |      |      |
|     | 14.7    | 3.4  | 10.0 | -3.4 | l.PLCo1   |        |      |      |      |      |
|     | 8.1     | 1.0  | 6.0  | -3.4 | l.MDL     |        |      |      |      |      |
|     | 12.9    | -2.0 | 9.4  | -3.8 | r.ic      |        |      |      |      |      |
|     | 10.5    | 4.8  | 4.2  | -4.2 | l.Hf     |        |      |      |      |      |
|     | 15.6    | 4.4  | 3.6  | -4.4 | l.CA2    |        |      |      |      |      |
|     | 11.1    | -6.2 | 9.0  | -4.6 | r.Pir     |        |      |      |      |      |
|     | 6.4     | 1.8  | 10.0 | -4.6 | l.ic      |        |      |      |      |      |

T indicates time of post-administration of manganese chloride (MnCl₂).
X, Y, and Z indicate the coordinates in millimeters on the rat brain atlas.
X shows the coordinate on the horizontal axis and Y, on the vertical axis on a coronal slice.
Z shows distance from the bregma on a coronal slice.
The structure indicates pertinent regions with reference to the atlas and coordinates of X, Y, and Z.
Each list is sorted by Z column.
Numbers of t-score peak points are up to 20.
Structure abbreviations: aca, anterior commissure, anterior part; CA2, field CA2 of the hippocampus; Cg2, cingulate cortex, area 2; CPu, caudate putamen (striatum); d, day; fi, fimbria of the hippocampus; h, hour; ic, internal capsule; l., left side; LO, lateral orbital cortex; LPO, lateral preoptic area; MDL, mediodorsal thalamic nucleus, lateral part; mt, mammillothalamic tract; OB, olfactory bulb; Pir, piriform cortex; PLCo1, posterolateral cortical amygdaloid nucleus, layer 1; r., right side; Rt, reticular thalamic nucleus; T, time; Tu, olfactory tubercle; vhc, ventral hippocampal commissure; VP, ventral pallidum.

Results

Voxel-based comparison

Figure 3A shows the t-score maps of Group A (treated with saline). Significant enhancement of the olfactory bulb (after signal enhancement from Mn²⁺) was first detected in the t-score map comparing the images obtained 24 hours after MnCl₂ administration with the baseline images obtained prior to MnCl₂ administration, and the enhancement showed bilateral asymmetry (Fig. 3A, arrow 1). Significant enhancement was also detected at the anterior commissure anterior part of the right side of the brain (Fig. 3A, arrow 2). Moreover, significant enhancement in the right side was detected at the piriform cortex, olfactory tubercle, and lateral preoptic area (Fig. 3A, arrows 3 and 4) on the t-score map comparing the images obtained 72 hours after MnCl₂ administration and at baseline. Significant signal enhancement in the left side of the brain occurred 24 hours later than that in the right side of the brain. Significant enhancement in the left side of the brain was detected at the prelimbic cortex (Fig. 3A, arrow 5), anterior commissure anterior part, ventral pallidium, lateral orbital cortex, and piriform cortex (Fig. 3A, arrow 6) when comparing the images obtained 48 hours after MnCl₂ administration.
Fig. 3. The t-score maps in Group A (A) and Group B (B) at 30 min, one, 24, 48, and 72 hours and 7 days after administration of manganese chloride (MnCl₂). The columns represent the different coronal slices at the same time point, and the rows, the same coronal slices at different time points. The numbers in millimeters in the left column represent axial distances from the bregma according to the rat brain atlas. Arrows in (A) indicate the following: 1, the first appearance of significant enhancement on the right-side bulb; 2, lateral olfactory tract and anterior commissure anterior part in the right-side brain; 3, piriform cortex, olfactory tubercle, and lateral preoptic area in the right-side brain; 4, piriform cortex in the right-side brain; 5, prelimbic cortex; 6, anterior commissure anterior part, ventral pallidum, lateral orbital cortex, and piriform cortex in the left-side brain; 7, anterior commissure anterior part, olfactory tubercle, and piriform cortex in the left-side brain; 8, lateral preoptic area, ventral pallidum, olfactory tubercle, and piriform cortex in the left-side brain; 9, anterior commissure anterior part, ventral pallidum, and olfactory tubercle in the left-side brain. Arrow in (B) indicates the first appearance of significant enhancement on the right-side bulb. The numbers shown in millimeters on the left-side column represent axial distances from bregma.

Fig. 4. Maximum intensity projection (MIP) of t-score maps in a transverse view at 30 min, one, 24, 48, and 72 hours and 7 days after administration of manganese chloride (MnCl₂). Upper row shows results in Group A and lower row, in Groups B.

Statistical Mapping for Effect of Verapamil

V o l .1 0 N o .2 , 2 0 1 1

administration with the baseline images. Furthermore, 24 hours later (72 hours after administration of MnCl₂), significant enhancement was detected at the anterior commissure anterior part, olfactory tubercle, piriform cortex, lateral preoptic area, and ventral pallidum (Fig. 3A, arrows 7 and 8). Significant enhancement remained even after that in the right side of the brain disappeared (Fig. 3A, arrow 9).

Figure 3 shows the t-score maps of Group B (treated with verapamil). As in Group A (treated with saline), significant enhancement of the olfactory bulb (Fig. 3B, arrow) was first detected in the right side of the brain. The t-score map demonstrated enhancement 48 hours after MnCl₂ administration, which was 24 hours later than its demonstration in Group A.

In addition to visual representation of the results by t-score maps, Table 1 lists coordinates and structures of t-score peak points in paired t-test maps. Except for the olfactory bulb, characteristic regions common to Groups A and B that were significantly enhanced were the right side of the anterior commissure anterior part at 48 hours (Group A: t-score = 9.5, X = -1.8 mm, Y = 6.8 mm, Z = 3.2 mm; Group B: t-score = 9.5, X = -1.8 mm, Y = 6.8 mm, Z = 3.2 mm); right side of the piriform cortex at 48 hours (Group A: t-score = 5.8, X = -4.4 mm, Y = 9.4 mm, Z = -1.4 mm; Group B: t-score = 8.8, X = -4.0 mm, Y = 9.4 mm, Z = -0.8 mm); and the right side of the piriform cortex at 72 hours (Group A: t-score = 7.1, X = -6.0 mm, Y = 8.6 mm, Z = -3.0 mm; Group B: t-score = 9.3, X = -5.4 mm, Y = 10.0 mm, Z = -2.8 mm).

Figure 4 demonstrates rostral to caudal progressions of Mn²⁺ over time in Groups A and B by
maximum intensity projection (MIP) of the t-score maps in a transverse view (Fig. 4). In addition to visual representation, the extents of significantly enhanced regions in Groups A and B are shown (Fig. 5). The extents in the right side brain in Groups A and B were 46.2 mm³ and 40.4 mm³ at 48 hours; 70.2 mm³ and 92.4 mm³ at 72 hours; and 7.2 mm³ and 0.3 mm³ at 7 days. The extents in the left side brain in Groups A and B were 18.0 mm³ and 14.9 mm³ at 48 hours; 64.0 mm³ and 53.2 mm³ at 72 hours; and 31.9 mm³ and 11.0 mm³ at 7 days. The extents peaked at 72 hours in both groups and both sides of the brain. Although the extents were larger in the right side of brain than the left side at 24, 48, and 72 hours in both groups, they were smaller than those in the left side at 7 days in both Groups A and B.

**ROI analysis**

Figure 6 demonstrates the averaged percentages of change in enhancement on selected ROIs. We also performed Wilcoxon rank-sum tests of the percentages of change (Table 2) at 24 hours in the ROI of the right side of the olfactory bulb, a region with differing t-maps between Groups A and B (Figs. 3, 4), and at 24, 48, and 72 hours in the ROI of the right lateral olfactory tract, which also differed between Groups A and B (Fig. 6B). The mean percentage of change in the ROI of the right olfactory bulb at 24 hours in Group A was 35.1% and in Group B, 33.8%. The corresponding standard deviation in Group A was 4.4% and in Group B, 12.1%. None of the Wilcoxon rank-sum tests showed significant difference.

**Increasing statistical power by smoothing**

For the VOI set on the right side of the rat brain template, the average t-score at 24 hours in Group A was maximized with the use of a 3-dimensional isotropic Gaussian filter with an FWHM of 0.8 mm (Fig. 7A). The maximum t-score was 10.4. For the same VOI, the average t-score was maximized at FWHM of 1.0 mm at 48 hours in Group A (maximum t-score 7.7); 1.6 mm at 48 hours in Group B (maximum t-score 9.8); and 1.2 mm at 72 hours in Group B (maximum t-score 7.7). For the left side VOI, the average t-score at 7 days in Group B was maximized at an FWHM of 0.8 mm, and the maximum t-score was 5.2 (Fig. 7). The other average t-scores did not vary significantly.

**Discussion**

**Voxel-based comparison and ROI analysis**

We used using voxel-based comparison and ROI analysis to investigate the olfactory connections in the groups treated with saline (Group A) and with verapamil (Group B). Voxel-based comparison demonstrated: (1) more prominent significant enhancement in the statistical images of the 2 groups on the right side, which can be explained by the administration of MnCl₂ into the right nasal cavity in both groups (Fig. 3A, arrow 1; Fig. 3B, arrow); (2) significantly enhanced regions and their order of detection on the t-maps of Group A (Fig. 3A, Table 1) that support the findings of Cross and associates; (3) similar regions of significant enhancement and extent of enhancement in Groups A and B at 48 and 72 hours and 7 days (Figs. 3–5, Table
Fig. 6. Percentages of change in region of interest (ROI) intensity for the 2 groups versus time after administration of manganese chloride (MnCl$_2$). The vertical axis indicates the mean percentage of change in ROI intensity averaged over 6 rats. The horizontal axis is time in hours after administration of MnCl$_2$. The graphs represent the regions of interest of: (A) the right (r.OB) and left olfactory bulb (l.OB) with saline or verapamil; (B) the right (r.lo) and left lateral olfactory tract (l.lo); (C) the right (r.aca) and left anterior commissure anterior part (l.aca); (D) the right (r.CPu) and left caudate putamen (l.CPu); (E) the right (r.Tu) and left olfactory tubercle (l.Tu); and (F) the right (r.Pir) and left piriform cortex (l.Pir).

Table 2. Results of Wilcoxon rank-sum tests for percentage of change in region of interest analysis between the 2 groups

| Time  | VOI    | Group A | Group B | Wilcoxon rank-sum test |
|-------|--------|---------|---------|------------------------|
| 24 h  | r.OB   | n 6     | 6       | $P=0.9372$             |
|       | Mean   | 35.1%   | 33.8%   |                        |
|       | SD     | 4.4%    | 12.1%   |                        |
|       | Median | 35.7%   | 36.1%   |                        |
| 24 h  | r.lo   | n 6     | 6       | $P=0.3939$             |
|       | Mean   | 23.3%   | 38.2%   |                        |
|       | SD     | 12.8%   | 28.9%   |                        |
|       | Median | 24.3%   | 31.4%   |                        |
| 48 h  | r.lo   | n 6     | 6       | $P=0.9372$             |
|       | Mean   | 26.4%   | 38.5%   |                        |
|       | SD     | 12.0%   | 27.3%   |                        |
|       | Median | 31.5%   | 28.3%   |                        |
| 72 h  | r.lo   | n 6     | 6       | $P=0.3095$             |
|       | Mean   | 15.3%   | 35.9%   |                        |
|       | SD     | 16.1%   | 30.6%   |                        |
|       | Median | 19.2%   | 28.9%   |                        |

Time indicates the time of post manganese chloride (MnCl$_2$) administration. n, number of samples; r.OB, region of interest (ROI) of the right-side olfactory bulb; r.lo, ROI of right-side lateral olfactory tract; SD, standard deviation; VOI, volume of interest.

1), which show that verapamil did not affect the pathway to transport Mn; and (4) initial detection of significant enhancement from Mn$^{2+}$ 48 hours after MnCl$_2$ administration in Group B (Fig. 3B) and 24 hours after in Group A (Fig. 3A), which reflects some differences in Mn$^{2+}$ accumulation in the right side of the olfactory bulb between the 2 groups. However, the results of voxel-based comparison and ROI analysis are contradictory 24 hours after administration of MnCl$_2$ (Fig. 6, Table 2) because
Fig. 7. Average t-scores in the volumes of interest (VOIs) as a function of the smoothing parameter, full-width at half-maximum (FWHM), in mm for 30 min, one, 24, 48, and 72 hours and 7 days after administration of manganese chloride (MnCl₂). The 4 graphs represent the results of (A) the right-side VOI for Group A, (B) the right-side VOI for Group B, (C) the left-side VOI for Group A, and (D) the left-side VOI for Group B.

of the larger variance (standard deviation) of changes in enhancement on the right side of the olfactory bulb in Group B than Group A. The null hypothesis (that there was no difference between data of pre- and 24-hour post-administration of MnCl₂ within Group B) could not be rejected in the paired t-test with the statistical threshold corrected for multiple comparison problem, that is caused by multiple testing for many voxels in the brain. It is satisfactory to consider that voxel-based comparison and ROI analysis showed no effect of verapamil on the transport of Mn in the olfactory connections.

Cross’s group have reported investigations of functional transport of Mn in the olfactory system of normal rats,13 alterations in function transport after injury,14 and age-related decrease in axonal transport15 with the statistical mapping method using MEMRI. In this study, we investigated the effect of a calcium-channel blocker (verapamil) on the transport of Mn in olfactory connections using the statistical method with MEMRI. Cross’s team used a similar injection protocol to ours and showed that verapamil inhibits Mn²⁺ accumulation in the olfactory bulb. They injected 3 µL of the calcium-channel blocker verapamil (5 mg/2 mL) into the right nasal cavity 20 min before the administration of MnCl₂,42 and we injected 10 µL of verapamil (2.5 mg/mL). Pautler and Koretsky also showed that the calcium-channel blocker diltiazem interfered with Mn²⁺ accumulation in the olfactory bulb.43

Contrary to the above findings, our results showed no effect of verapamil on the accumulation of Mn²⁺ in the olfactory bulb. It is unclear whether the different results are attributable to the injection protocol for verapamil or other unknown factors. Divalent metal transporter 1 (DMT1) has recently been reported to play an important role in the uptake of Mn²⁺ into the brain from the nasal cavity,44–46 and transporter proteins ZIP8 and ZIP14 have been characterized as various divalent cations.46–49 As well, in case of rats under certain conditions, there may be other pathways to transport Mn from the nasal cavity to the olfactory bulb than voltage-gated calcium channels. Further investigation is required.

Increasing statistical power by smoothing

Cross and colleagues13 used ‘‘NEUROSTAT’’ software to develop a statistical method for mapping the rat brain, whereas we adapted the SPM package, an open source program for research on human brain mapping. We also performed spatial smoothing after spatial normalization (anatomical standardization), but Cross’s group13 did not. Advantages of spatial smoothing include improved signal-to-noise ratio34 and Gaussian random fields for multiple comparison correction,35 but the disappearance of small signals reduces spatial resolution.24,36–38 Nevertheless, spatial smoothing is a pre-processing step in the SPM analysis for human brain research, and we included this pre-processing step.

We used the 3-dimensional isotropic Gaussian filter with an FWHM of 0.8 mm for the above-mentioned voxel-based comparison, and it was difficult to determine optimal smoothing for the statistical maps. An FWHM value that is too large for smoothing prevents detection of small regions enhanced by Mn²⁺, and one that is too small reduces the statistical power, as described. Because recent studies made no reference to the smoothing parameter for statistical mapping of the rat brain using MEMRI,11–18 we set the parameter such that the t-score was increased. Figure 7 shows that smoothing increased the t-score. We selected a value of 0.8 mm for FWHM in this study because we obtained the maximum t-score at this value
when we compared the images acquired at 24 hours after MnCl₂ administration and at baseline (Fig. 7A), and this value was the smallest of all the other corresponding values (Fig. 7). However, further investigation is needed of the optimal parameter value for smoothing and should take into account the other pre-processing parameters (e.g., resampled voxel size at the spatial normalization), size of enhanced regions in the rat brain, equipment used to acquire data, and specifications concerning the acquisition parameters. Some of these parameters have been investigated in the field of human brain mapping, but the rat brain is considerably smaller than the human brain, and the scanner used in this study was intended for use with small animals.

Improvement and limitations of this analysis

The statistical mapping method adopted by Cross and associates and used in this study is considered more objective than conventional ROI/VOI analysis because its analytic steps, except for the process of extracting the brain, are automated. We performed the brain extraction manually and observed some differences in the extracted brain regions among operators. Therefore, automating the brain extraction process would render the statistical mapping method in this study more objective.

The statistical mapping method also permits group-wise analysis that is independent of individual results, but it cannot estimate individual Mn-enhanced MR imaging intensities. However, the spatial normalization (anatomical standardization) technique used in this mapping method enables an objective ROI/VOI analysis that can measure the intensities. The 3-dimensional stereotaxic rat brain regions (ROIs/VOIs) predefined in the rat brain atlas allow the ROI/VOI analysis. The analysis with the 3-dimensional stereotaxic ROI/VOI has been used in the field of the human clinical assessment for quantitative cerebral blood flow by using single-photon emission computed tomography (SPECT) or positron emission tomography (PET). Predefined stereotaxic VOIs for the entire brain allow analysis of the whole rat brain by this method. Our ROI analysis employed such a stereotaxic ROI/VOI template method with only 12 ROIs.

Our analysis is limited regarding the accuracy of the coordinates of significant enhanced (peak t-score) voxels and regarding temporal resolution.

Although anatomical standardization can be performed to identify the structures of significantly enhanced voxels by comparing the coordinates of the voxels against the standard brain atlas, this was difficult in this study for several reasons. The spatial resolution of the MR scanner was not sufficient to visualize the small structures of the rat brain, and the T₁-weighted MR imaging template that we prepared of the rat brain was not identical to the standard rat brain atlas. In the atlas, the significantly enhanced regions (not voxels) were identified as neighboring structures. Increased spatial resolution of the scanner and use of a template better resembling the atlas should improve structure identification. Thus, this limitation can be certainly attributed to the inaccuracy in spatial normalization to the atlas. Although we must note the algorithm of spatial normalization, we performed only affine transformations (without non-linear warping) because the rat brains in this study did not vary significantly in shape. We visually confirmed that the spatially normalized images were well matched in shape to the rat brain template image. We also performed spatial smoothing to reduce the impact of misregistration of the data into the space of the template to allow for anatomical variability among the rat brains. In one side, another problem might be the movement of peak points by spatial smoothing.

With regard to temporal resolution, we performed 7 scans, one prior to MnCl₂ administration and 6 others, at 0.5, one, 24, 48, and 72 hours and 7 days after. Increasing the number of scans performed and reducing the intervals between scans can improve temporal resolution. However, in this study, increasing the number of scans performed would have increased the loads on the rats by their being anesthetized before each scan.

Conclusion

We applied SPM using MEMRI to demonstrate in vivo the transport of Mn in the olfactory connections of the rat brain with/without verapamil as statistical maps and found that verapamil affected Mn transport in the olfactory connections. This method appears useful both for analyzing the transport of Mn in olfactory connections and investigating the effect of a calcium-channel blocker on Mn transport.

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