Water permeability of capillaries in the subfornical organ of rats determined by Gd-DTPA$^2$ enhanced $^1$H magnetic resonance imaging

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The water permeability of capillaries in the subfornical organ (SFO) of rat was measured by a $^1$H nuclear magnetic resonance method in combination with a venous injection of a relaxation reagent, gadolinium-diethylene triamine-$N,N,N',N''$-N$^-$-pentaacetic acid (Gd-DTPA$^-$), which could not pass through the blood–brain barrier (BBB). Judging from results of Gd-DTPA$^-$ dose dependency in the intact brain and the BBB-permeabilized brain, Gd-DTPA$^-$ could not have leaked out from the capillaries in the cortex, thalamus or SFO, but it could have been extravasated in the posterior lobe of the pituitary gland. The longitudinal ($T_1$) relaxation time of water in the SFO region was measured by inversion-recovery magnetic resonance imaging at 4.7 T. The $T_1$ relaxation rates ($1/T_1$) before and after Gd-DTPA$^-$ infusion were $0.70 ± 0.02$ s$^{-1}$ (mean ± S.E.M., $n = 9$) and $1.53 ± 0.11$ s$^{-1}$ ($n = 9$), respectively. The rate constant for water influx to the capillaries was estimated to be $0.84 ± 0.11$ s$^{-1}$ ($n = 9$) which corresponds with a diffusive membrane permeability ($P_d$) of $3.7 \times 10^{-3}$ cm s$^{-1}$. Compared with values found in the literature available on this subject, this $P_d$ value for the capillaries in the SFO was the same order of magnitude as that for transmembrane permeability of water for the vasa recta, and it may be 10–100 times larger than that of the blood–brain barrier in the cortex. Areas of the cortex and thalamus showed minimal changes in the $T_1$ relaxation rate (ca 0.09 s$^{-1}$), but these values were not statistically significant and they corresponded to $P_d$ values much smaller than those found in the SFO. From these results, we conclude that the capillaries in the SFO have one of the highest water permeability values among all of the capillaries in the brain. It is also suggested that this magnetic resonance imaging, based on $T_1$ relaxation rate, is a useful method to detect local water permeability in situ.

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The circumventricular organs (CVOs) are structures bordering the third and fourth ventricles and are considered to be important sites for communicating between neural cells, cerebrospinal fluid and blood. CVOs have been characterized as regions of the brain with permeable capillaries, which can pass marker dyes from blood (Davson et al. 1987). This high tracer transportability of vessels in the CVOs was explained by the histological findings of the capillary endothelium, which has cytoplasmic vesicles, caveolae, pits, fenestrations and a few tight junctions (Bouchaud & Bosler, 1986; Gross, 1992). Several substances such as horseradish peroxidase and $\alpha$-aminoisobutyric acid (AIB) have been used as markers, and it has been revealed that the various parts of the CVOs have different permeability values. The order of permeability of AIB from highest to lowest was pineal gland > the posterior lobe of the pituitary gland > the subfornical organ (SFO), area postrema (AO), and median eminence (ME) > subcommissural organ (SCO) (Brodie et al. 1960a,b; Broadwell & Brightman, 1976; Bouchaud & Bosler, 1986; Gross et al. 1987; Gross, 1992). The SFO might be a part of the sensory system involved in body fluid homeostasis, and it might transfer information on the osmolarity of serum to neurons. However, no direct observations on water permeability in the SFO have been reported.

In order to measure water permeability in the SFO in situ, we applied $^1$H magnetic resonance imaging (MRI). The principle of the method is based on that reported by Fabry & Eisenstadt (1975). Most of the $^1$H nuclear magnetic resonance (NMR) signal from biological tissue derives
from water. The intrinsic longitudinal relaxation time ($T_1$) of water in the intravascular, interstitial and intracellular water is in the range of 1.5–2 s. This occurs because the water exchange between these compartments averages the differences in $T_1$ relaxation times of the water in the compartments. In the brain, almost all capillaries are impermeable, forming the blood–brain barrier (BBB). It is possible to distinguish between intravascular and extravascular water molecules by adding an impermeant relaxation reagent to the intravascular fluid (Fig. 1). Gadolinium-diethylene triamine-$N,N,N',N'',N''-$pentaacetic acid (Gd-DTPA$^{2-}$) is a well-known $T_1$ relaxation reagent which cannot cross the intact blood–brain barrier (Abbott et al. 1999). The reagent accelerates the relaxation of the intravascular water protons by two orders of magnitude. In the absence of diffuse water exchange across the capillary membrane, the extravascular water protons relax at the normal rate. If the transmural water exchange rate is in the order of the $T_1$ relaxation rate or higher, the extravascular water protons can relax more rapidly by diffusing out of the extravascular space and into the presence of the relaxation reagent. In this case, the rate constant of the diffusive water efflux can be estimated from the relaxation rate of the extravascular water protons. This method was previously used to measure the water permeability of cellular membranes, such as Necturus gallbladder epithelial membrane and salivary acinar cells in vitro (Steward & Garson, 1985; Steward et al. 1990), and the values obtained were in good agreement with those based on optical measurements of the changes in cellular volume (Persson & Spring, 1982). In this study, we detected water permeable sites in CVOs using $T_1$-weighted MRI. Then we confirmed the capillary impermeability of Gd-DTPA$^{2-}$ in the SFO. Finally, we estimated the water permeability of capillaries in the SFO from the changes in the $T_1$ relaxation rate of the water.

**METHODS**

**Preparation of animals**

Twenty-six Wistar-Hamamatsu rats (300–350 g, 7–9 weeks old) were used in the study. The actual number of rats used in each experiment was as follows: anatomical imaging, 3; angiography, 4; $T_1$ relaxation in intact brains, 11; dose dependency of Gd-DTPA$^{2-}$, 3; permeabilization of the BBB, 3 (the details for each experiment are described in the following sections). Rats were anesthetized with pentobarbital (50 mg (kg body wt)$^{-1}$ I.P.). After inserting the tracheal cannula, anesthesia was changed to 1 % enflurane in a gas mixture of 36% O$_2$–2% CO$_2$–62% NO delivered through the tracheal cannula by an artificial ventilator (tidal volume 4 ml, ventilation rate 50 min$^{-1}$; Harvard Apparatus, MA, USA) (Takamata et al. 2001). An intravenous cannula was inserted in either the right or left femoral vein and polyethylene tubing was connected with a 5 ml syringe for injection. Body temperature was maintained using a warm-water circuit. Arterial blood was taken from the right femoral artery through an indwelling catheter. After the experiments, the rats were killed by an overdose of anaesthesia (pentobarbital, 500 mg (kg body wt)$^{-1}$ I.V.). All of the animal experiments conducted in this study were carried out under the rules and regulations of the Animal Care and Use Committee, National Institute for Physiological Sciences, Japan, which are based on the Guiding Principles for the Care and Use of Animals, as approved by the Council of the Physiological Society of Japan.

**Acquisition of MR images**

Rats were placed in the prone position on a custom-built Plexiglas sledge, and the position of the head was fixed with a pair of earplugs and a bite bar. A home-built $^1$H radio frequency (RF) surface coil (18 or 23 mm in diameter) was placed on the surface of the head, and the centre of the coil was positioned on the bregma. $^1$H MR images were obtained by a BioSpec spectrometer built for animal imaging (ABX-4.7/40, Bruker, Karlsruhe, Germany). ParaVision operating software (version 2.1, Bruker) was employed to obtain the images. A 4.7 T horizontal-bore magnet with a free bore size of 40 cm was equipped with an active shielded gradient (B-GA12, Bruker). The RF powers of the RF pulses were adjusted by employing a spherical water sample (8 mm in diameter) set in the centre of the RF coil.

The imaging parameters used for $T_1$-weighted sagittal gradient-echo imaging were as follows: 2.5 cm × 2.5 cm field of view (FOV), 128 × 128 data matrix, 1 mm slice thickness, 100 ms relaxation delay ($T_B$), 4.2 ms echo-time ($T_E$), 7 slices and 2 or 4 accumulations. A sinc-shaped pulse (duration 2 ms, with a bandwidth 2.5 kHz) was used for excitation. The RF power was adjusted for 180 deg pulse in the centre of the RF coil, which roughly corresponded to the 90 deg pulse in the depth of the lateral ventricles of the rats. The total time required for an image was 28 or 56 s. A high resolution $T_1$-weighted image was obtained for depicting anatomical structures. The imaging parameters used were as follows: 2.5 cm × 2.5 cm FOV, 256 × 256 data matrix, 1 mm slice thickness, 100 ms $T_B$, 6.76 ms $T_E$, 5 slices and 8 accumulations.

The $T_1$ relaxation times were measured using an inversion-recovery fast-imaging sequence (fast-$T_1$ MRI) employing a single 180 deg pulse prior to the acquisition of a series of low flip angle detection pulses (FLASH) (Haase et al. 1989). The parameters used were as follows: 2.5 cm × 2.5 cm FOV, 128 × 128 data matrix, 1 mm slice thickness, 1 slice and 2 accumulations. An adiabatic pulse (4 ms duration, 2.5 kHz bandwidth) was used for inversion (Silver et al. 1984). The RF power was adjusted so that it was two times that required for a 180 deg pulse at the centre of the RF coil. This produced a 180 deg pulse at the depth of the lateral ventricles of the rats. A series of 16 Gaussian-shaped RF pulses (90 deg flip angle in the centre of the RF coil, 1 ms duration and 3 kHz bandwidth) with a short $T_E$ (3.1 ms) was used for detection. Eight or ten inversion recovery delays ($T_i$, 38–7000 ms) were used, and were optimized for the $T_1$ measurements in a range of $T_i$ relaxation times from 500 ms to 1.5 s. The total time required for the fast-$T_1$ measurement was 9.5 or 12.75 min. In order to test the accuracy and reproducibility of the fast-$T_1$ measurements, a cylindrical sample, with a diameter and length of 12.5 mm and 3 cm, respectively (containing 0.2 mM MnCl$_2$ solution) was measured under the same conditions employed for the rat brain measurements. As a reference, the $T_1$ relaxation rate of the 0.2 mM MnCl$_2$ solution was measured by the conventional inversion-recovery pulse sequence (180–VD–90–acquire) using a bird-cage RF coil (52 mm in diameter) which can produce a uniform excitation RF pulse over the whole sample range, where 180 and...
mannitol solution was injected into the internal carotid artery (4.25 μmol (kg body wt)_1) within 30 s, followed by a continuous injection of Gd-DTPA²⁻ (0.14–1.4 mmol (kg body wt)_1) to keep the plasma concentration of Gd-DTPA²⁻ at around 10 mM (7–13 mM). The infusion of Gd-DTPA²⁻ was monitored by a T₁-weighted gradient-echo fast imaging method in order to confirm that it reached a steady state. After 30 min of the Gd-DTPA²⁻ infusion, the T₁ relaxation times were measured again. This pair of T₁ relaxation rates was used to obtain an estimation of the diffusive water permeability of capillaries in the SFO. In separate experiments, T₁ relaxation rate constants were measured for a range of Gd-DTPA²⁻ doses (0.38–4.5 mmol (kg body wt)_1) and a suitable dosage of Gd-DTPA²⁻ was determined. At each dosage, Gd-DTPA²⁻ was applied intravenously over 1–2 min. The stability of the Gd-DTPA²⁻ concentration was confirmed by the image intensity of the T₁-weighted MRI. Fifteen minutes after the injection, the T₁ relaxation rate constant was measured by the fast-T₁ MRI method.

Assessment of capillary permeability of Gd-DTPA²⁻
We employed T₁-weighted gradient-echo MRI and the T₁ relaxation reagent Gd-DTPA²⁻ to detect the water-permeable regions. In the T₁-weighted gradient-echo imaging with a 90 deg excitation pulse, the observed signal intensity (I) of the image reached a steady value:

\[
I = I_0(1 - \exp(-T_0/T_1))\exp(-T_0/T_2),
\]

where T₂* is the apparent transverse relaxation time, I₀ is the signal intensity corresponding to the fully relaxed magnetization of the sample, T₀ is the repetition time of the excitation pulse and T₁ is the echo-time after the excitation pulse. The T₁ and T₂ relaxation rate constants are mainly determined by the concentration of the reagent Gd-DTPA²⁻. Judging from the relaxivity of Gd-DTPA²⁻ (K = 1/[T₁ × [Gd-DTPA²⁻]] = 4 mM s⁻¹) for T₁; and the T₁/T₂ ratio = k = 1–3; Muller, 1996; Caravan et al. 1999) and eqn (1), the Gd-DTPA²⁻ infusion induces shorter T₁ and T₂ relaxation times. In cases where a low concentration of Gd-DTPA²⁻ (~10 mM) is employed, the T₁/T₂ and T₁/T₂* ratios are relatively small, so the image intensity will depend on the T₁ relaxation rate, and produce a broad peak at around 5–10 mM of Gd-DTPA²⁻. Therefore, the T₁ relaxation rate of the higher signal intensity region could be estimated as being higher than that of the surrounding lower signal intensity areas. Thus, it was easy to detect high signal intensity regions due to either high Gd-DTPA²⁻ permeability or high water exchanges between the intravascular and the extravascular spaces.

In order to confirm the capillary permeability of Gd-DTPA²⁻, we measured changes in the signal intensity of the T₁-weighted images and the T₁ relaxation rates of rat brain when the BBB was permeabilized by hyperosmotic stress (Nagy et al. 1979). In addition to a cannula in the left femoral vein for the injection of AMI-227 (Advanced Magnetics Inc., MA, USA). AMI-227 is a dextran-coated ultraslim superparamagnetic iron oxide (USPIO) particle (400–900 kDa), which will stay in the extravascular space and be detected as a high signal intensity in the extravascular components reflected water efflux from the extravascular space (Fig. 1) were calculated: (iii) the rate constant of diffusive water efflux from the extravascular space (kₑ) given by:

\[
kₑ = Rₑ - Rₙ₂,
\]

where Rₑ is the relaxation rate constant for the extravascular water; (iv) the rate constant of diffusive water efflux from the extravascular space (kₑ) given by:

\[
kₑ = Rₑ - Rₐₑ,
\]

where Rₐₑ is the relaxation rate constant for the intravascular water containing Gd-DTPA²⁻; (v) kₑ is also represented by:

\[
kₑ = k_V/Vₑ,
\]

where Vₑ and Vₑ are the volumes of extravascular water and intravascular water, respectively; and (vi) diffusive water permeability (Pₑₑ) is calculated by:

\[
Pₑₑ = k_V/Vₑ
\]

where Aₑ is the surface area of vessels.
When we applied this analysis to the data obtained by MRI, we optimized the fast-\(T_1\) MRI pulse sequence to measure the slow component in order to obtain a single exponential relaxation (\(R_s\)), because the fast-\(T_1\) MRI is not suitable for measuring the faster relaxation component (\(T_1 < \sim 25\) ms), and the conventional inversion-recovery MRI is prohibitively time consuming. As shown in eqn (4), it is also true that the \(R_s\) and \(R_e\) should give the same results (Steward et al. 1990). Moreover, as shown in eqns (2) and (3), \(R_i\) depends only on the exchange rate of water at a high intravascular concentration of Gd-DTPA\(^{2-}\), but \(R_e\) depends on the intravascular relaxation rate (\(R_{ld}\)), which was much higher than the water exchange rate (\(k_i\)). Therefore, as long as we can measure the slow component, we do not need to control the Gd-DTPA\(^{2-}\) concentration strictly. This makes such experiments much easier to perform and facilitates reliability. The intrinsic \(T_1\) relaxation rate constant for the extravascular water (\(R_e\)) was assumed to be that obtained before the Gd-DTPA\(^{2-}\) injection, because the fraction of vascular space in the SFO is approximately 2.5% (Sposito & Gross, 1987), and accordingly, almost all of the observed water molecules are in the extravascular space.

**Statistics**

All numerical values are expressed as means ± S.E.M. or means ± S.D. Student’s \(t\) test was employed for the statistical significance tests, and it was considered that \(P\) values of less than 0.05 indicated significance.

**RESULTS**

Detection of water permeable sites using \(T_1\)-weighted MRI

We employed \(T_1\)-weighted gradient-echo MRI and the \(T_1\)-relaxation reagent Gd-DTPA\(^{2-}\) to detect the water-permeable regions. A typical sagittal \(T_1\)-weighted MR image under steady-state Gd-DTPA\(^{2-}\) intravascular infusion is shown in Fig. 2A. The SFO area was clearly imaged as a high signal intensity (arrowhead). Signal intensities in the pituitary gland area were also maintained at a high level during the Gd-DTPA\(^{2-}\) infusion. This suggested either a high water permeability or extravasation of the Gd-DTPA\(^{2-}\) in the SFO and the pituitary gland. The pituitary gland was positioned at the edge of the zone detectable by the RF coil, and we could not detect any differences between the posterior, intermediate and anterior lobes of the pituitary gland during the Gd-DTPA\(^{2-}\) perfusion. Among the circumventricular organs, this method made it possible to detect changes in signal intensity in the SFO and posterior lobe of pituitary gland. It was difficult to discriminate the organum vasculosum laminae terminalis (OVLT), subcommissural organ (SCO) and pineal gland areas from the surrounding cerebrospinal fluid (CSF) and/or surrounding cerebrospinal fluid (CSF) and/or

![Figure 1. A kinetic model for water exchange in the subfornical organ based on Gd-DTPA\(^{2-}\)-impermeable capillaries](image1)

The injected Gd-DTPA\(^{2-}\) remains inside the vessels, so the relaxation rate of blood (\(R_{ld}\)) is accelerated. The diffusive water permeability of capillaries (\(P_{ac}\)) is described by either \(k_iV/A_\nu\) or \(k_eV_e/A_c\), where \(k_i\) and \(k_e\) are the rate constants of water efllux from and influx to capillaries, respectively, \(V_i\) and \(V_e\) are the volumes of the intravascular and extravascular spaces, respectively, and \(A_c\) is the surface area of the capillaries. The extravascular water can be treated as a single compartment because there is a faster water exchange between the interstitial and the intracellular spaces (\(P_{ac}\)). Accordingly, the intrinsic \(R_i\) can be presented as 

\[
(R_iV_i + R_cV_c)/(V_i + V_c)
\]

The abbreviations used are as follows: \(R_i\), the relaxation rate constant of the extravascular water; \(R_c\), the relaxation rate constant of the intracellular water; \(V_i\), the volume of the intravascular space; \(V_c\), the relaxation rate constant of the intracellular water; \(V_{ac}\), the volume of the intracellular space; \(k_i\) and \(k_e\), the rate constants of water efllux from and influx to the cells, respectively; \(A_\nu\), the surface area of the cells; \(P_{ac}\), the diffusive water permeability of the cell membrane.

![Figure 2. T1-weighted 1H MRI and 3D-angiography of the rat brain](image2)

A, a mid-sagittal high-resolution \(T_1\)-weighted MRI during intravenous injection of Gd-DTPA\(^{2-}\). The area of subfornical organ (SFO) is indicated by an arrowhead. B, a mid-sagittal section of a 3D-angiography of the brain with the vascular contrast reagent AMI-227. The abbreviations used are as follows: pca, pericallosal artery; aca, anterior cerebral artery; ACA, anterior communicating artery; icv, internal cerebral vein; gcv, great cerebral vein; sss, superior sagittal sinus.
vasculature. The area postrema (AP) was out of the area detectable by the RF coil. There were minimal changes in the cortex and thalamus areas. In order to confirm the positions of the arteries and veins, a 3D-angiography of the brain was obtained with a vascular contrast reagent, AMI-227. As shown in Fig. 2B, in the mid-sagittal section with a voxel resolution of 143 μm x 143 μm x 137 μm, arteries and veins were clearly depicted by a high signal intensity, but no vessels were detected in the SFO area. These results suggested that the capillaries in the SFO have higher permeability than the blood–brain barrier in the cortex and thalamus. Increases in the signal intensity of the CSF in ventricles were also detected in most of the rats (but not in all cases). This was likely to have been caused by leakage of Gd-DTPA2− from the choroid plexus due to the high osmotic stress of the bolus infusion of 500 mM Gd-DTPA2−.

$T_1$ relaxation rate constants in the intact rat brain

We measured the $T_1$ relaxation rate using an inversion-recovery fast-imaging sequence (fast-$T_1$ MRI). In this pulse sequence, we used an adiabatic pulse for the inversion of the magnetization. This adiabatic pulse produces a 180 deg inversion over a broad range of RF power, so it was possible to apply a uniform 180 deg pulse over the rat brain. In order to confirm accuracy, we measured a phantom region of interest (ROI) containing a 0.2 mM MnCl₂ solution, and the resulting $T_1$ relaxation rate was 1.77 s⁻¹ using a conventional inversion-recovery pulse sequence with uniform block RF pulses. The dependencies on vertical and horizontal distances that were demonstrated are shown in Fig. 3A and B, respectively. Figure 3C shows a typical position of the rat brain and the RF coil, along with ROIs detected in Fig. 3A and B. Almost the same $T_1$ relaxation rates were obtained with approximately 4% of standard deviation in areas with a radius within 10 mm (with a horizontal distance < 7 mm at a depth of 7 mm, or with a vertical distance from the centre of the RF coil < 10 mm). There was a tendency for increased scattering of values in the area distant from the RF coil (with a

![Figure 3. The accuracy and reproducibility of the fast-T1 measurements using a surface coil](image-url)

A cylindrical sample (12.5 mm in diameter with a length of 3 cm) containing 0.2 mM MnCl₂ solution ($1/T_1 = 1.77$ s⁻¹) was measured by the fast-$T_1$ MRI using a surface coil (23 mm in diameter) under the same conditions employed for the rat brain. Relative $1/T_1$ of ROIs was presented. A, dependency on a vertical distance of 2 to 14 mm from the centre of the RF coil. B, dependency on a horizontal distance of −12 (nose side) to 11 mm (tail side) from the centre of the RF coil at a depth of 7 mm. The effects of variations in the flip angle of the sampling pulse (+6%) on the accuracy of the $T_1$ measurement are also shown. The mean and standard deviation are presented in parentheses. C, the positions of the RF coil, the centre of the RF coil and the range of measurement of vertical and horizontal dependencies are superimposed on a typical sagittal section of the rat brain. The positions of the ROIs in the SFO, cortex and thalamus are also shown.
horizontal distance > 7 mm at a depth of 7 mm, or with a vertical distance from the centre of the RF coil > 10 mm), which might be caused by a low signal-to-noise ratio for images in these areas. No influence on the accuracy of the $T_1$ measurement was shown with variations of the flip angle of the sampling pulse (±6%). The average positions of the SFO (7.26 ± 0.1 mm in depth, −2.80 ± 0.23 mm horizontal, $n = 9$), cortex (4.96 ± 0.11 mm in depth, −2.32 ± 0.21 mm horizontal, $n = 9$) and thalamus (7.78 ± 0.09 mm in depth, −1.88 ± 0.23 mm horizontal, $n = 4$) are also presented in Fig. 3C. From these results, we can conclude that it is possible to obtain accurate measurements of the $T_1$ values in the SFO, thalamus and cortex areas using the fast-$T_1$ MRI method.

The dose dependency of the $T_1$ relaxation rate constant is shown in Fig. 4 for a range of doses of Gd-DTPA$^{2-}$ from 0.38 to 4.5 mmol (kg body wt)$^{-1}$. In the SFO, the $T_1$ relaxation rate was increased by the Gd-DTPA$^{2-}$ injection, and then reached a steady level (approximately 1.6 s$^{-1}$) with a dosage of more than 1.5 mmol (kg body wt)$^{-1}$. This plateau was expected from eqn (2). The observed $T_1$ relaxation rate depends on the water exchange rate, and is not dependent on the intravascular relaxation rate, which was accelerated by the high dosage of Gd-DTPA$^{2-}$. In the thalamus and cortex, the $T_1$ relaxation rate constants were maintained at the original values (approximately 0.7–0.8 s$^{-1}$), within the range of errors of $T_1$ measurement (Fig. 4B). In the skin, veins, and the posterior and anterior lobes of the pituitary gland, $T_1$ relaxation rate constants were increased proportionally when the dosage of Gd-DTPA$^{2-}$ was increased (Fig. 4A; data for the anterior lobe of the pituitary gland and veins are not shown). Due to their fast $T_1$ relaxation, we could not determine $T_1$ relaxation rates at the higher dosage of Gd-DTPA$^{2-}$. From these results, we determined the dosage of Gd-DTPA$^{2-}$ (1.5–2.5 mmol (kg body wt)$^{-1}$) employed for the following $T_1$ relaxation measurement of the SFO.

Typical sets of $T_1$ relaxation of water in the SFO and cortex are shown in Fig. 5. The relaxation represented a single exponential decay, not a bi-exponential decay, within the error of measurement. In this experiment, the plasma concentration of Gd-DTPA$^{2-}$ was maintained at approximately 10 mM, which corresponds to the plasma $T_1$ relaxation rate of approximately 40 s$^{-1}$. Therefore, almost all of the magnetization of the intravenous compartment relaxed before the inversion-recovery delay ($T_1$) of 100 ms. In other words, contamination of the blood compartment in the SFO, cortex and thalamus could be neglected, not

![Figure 4. Dose dependency of $T_1$ relaxation rate constants over the Gd-DTPA$^{2-}$ dosage range](image)

**Figure 4. Dose dependency of $T_1$ relaxation rate constants over the Gd-DTPA$^{2-}$ dosage range**

*Figure 4.* A, a typical result obtained from a rat with a Gd-DTPA$^{2-}$ dosage range from 0.38 to 4.3 mmol (kg body wt)$^{-1}$. $T_1$ relaxation rate constants and standard errors of the regression are presented. For each dosage, Gd-DTPA$^{2-}$ was applied intravenously within 1–2 min. $T_1$ relaxation rate constants were measured 15 min after the injection using the fast-$T_1$ MRI method. B, Gd-DTPA$^{2-}$ dose dependency obtained from nine rats. Filled symbols present the same data shown in A obtained from a sequential experiment on a single rat. Open symbols present data from eight rats, and so each symbol represents data obtained from a different rat. The line is a polynomial fit plotted to guide the eye of the reader. The positions of the ROIs are the same as shown superimposed on a sagittal image of the brain in Fig. 7D.
only due to its small volume fraction (2–3%), but also because of this faster relaxation. The $T_1$ relaxation rate values in the SFO, cortex and thalamus were measured before and after the Gd-DTPA$^{2-}$ infusion, and the results are summarized in Table 1. Only the SFO region demonstrated a statistically significant increase ($P < 0.05$) in $T_1$ relaxation rate. Regions of the cortex and thalamus showed minimal increase, but the differences were not statistically significant. The $T_1$ relaxation rate of the pituitary gland could not be determined precisely because of the low signal-to-noise ratio and also the fast relaxation.

### Capillary permeability of Gd-DTPA$^{2-}$

The anatomical definition of the Gd-DTPA$^{2-}$ compartment is essential for a definition of the water permeability in the SFO. It is known that Gd-DTPA$^{2-}$ does not cross the intact blood–brain barrier (Abbott et al. 1999). However, there are no reports on the Gd-DTPA$^{2-}$ permeability of the capillaries in the SFO. Accordingly, we conducted tests to determine whether or not Gd-DTPA$^{2-}$ could cross the capillaries in the SFO. The intensity of the $T_1$-weighted MR images of the SFO, cortex (motor area), thalamus and the great cerebral vein were measured every 30 s after the Gd-DTPA$^{2-}$ injection.

### Table 1. $T_1$ relaxation rate constants (s$^{-1}$) of SFO, cortex and thalamus, and differences ($\Delta R$) due to Gd-DTPA$^{2-}$ injection

<table>
<thead>
<tr>
<th></th>
<th>Control $n$</th>
<th>After Gd-DTPA$^{2-}$ $n$</th>
<th>$\Delta R$ $n$</th>
</tr>
</thead>
<tbody>
<tr>
<td>SFO</td>
<td>0.695 ± 0.016 9</td>
<td>1.532 ± 0.114 9</td>
<td>0.836 ± 0.109 9</td>
</tr>
<tr>
<td>Cortex</td>
<td>0.663 ± 0.013 9</td>
<td>0.749 ± 0.017 9</td>
<td>0.086 ± 0.023 9</td>
</tr>
<tr>
<td>Thalamus</td>
<td>0.699 ± 0.027 4</td>
<td>0.792 ± 0.060 4</td>
<td>0.093 ± 0.040 4</td>
</tr>
</tbody>
</table>

$n$, number of animals. *Statistically significant difference between the control value and values after the Gd-DTPA$^{2-}$ injection ($P < 0.05$).

Typical results shown in Fig. 6 are based on a sagittal image of the midline ROIs (390 μm × 390 μm). In the SFO, the image intensity was decreased transiently by the i.v. injection of Gd-DTPA$^{2-}$, and then increased by approximately 100% compared with the control level. This transient decrease in the image intensity might be due to a bolus infusion of a high concentration of the Gd-DTPA$^{2-}$ (500 mM), which could have caused a large magnetic susceptibility difference between vessels and the extravascular space. As a result, the $T_1^*$ relaxation time in the tissue was decreased, which forced a decrease in the image intensity. When the Gd-DTPA$^{2-}$ was distributed in the extracellular space, it usually took 10–15 min to reach a steady state, with a venous Gd-DTPA$^{2-}$ concentration of approximately 10 mM. Therefore, these transient changes could not be explained by a simple $T_1$ effect due to the Gd-DTPA$^{2-}$. However, the righthand part of Fig. 6

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**Figure 5.** $T_1$ relaxation of water protons in the SFO and the cortex with and without intravenous infusion of Gd-DTPA$^{2-}$

Data are plotted as the natural logarithm of the difference of the image intensity ($I_t$), after the inversion recovery time ($\tau$), from the fully relaxed magnetization ($I_0$). The lines indicate the results of linear fitting data. The relaxation rate constants and standard errors of the regression for the SFO and cortex before the Gd-DTPA$^{2-}$ injection (SFO and cortex) were $0.627 \pm 0.017$ s$^{-1}$ ($r^2 = 0.996$) and $0.646 \pm 0.007$ s$^{-1}$ ($r^2 = 0.999$), respectively. The relaxation rate constants for the SFO and cortex after the Gd-DTPA$^{2-}$ injection (SFO (Gd-DTPA) and cortex (Gd-DTPA)) were $1.695 \pm 0.101$ s$^{-1}$ ($r^2 = 0.995$) and $0.744 \pm 0.006$ s$^{-1}$ ($r^2 = 0.999$), respectively.

**Figure 6.** The changes in signal intensity of $T_1$-weighted 1H MRI induced by the intravenous injection of Gd-DTPA$^{2-}$

Data are typical results induced by the intravenous injection of Gd-DTPA$^{2-}$ (1.4 mmol (kg body wt)$^{-1}$ within 30 s followed by 0.7 mmol (kg body wt)$^{-1}$ h$^{-1}$). The image intensities in the SFO, cortex, thalamus and the great cerebral vein (vein) were normalized by those obtained before the Gd-DTPA$^{2-}$ injection. The inset image is a $T_1$-weighted sagittal gradient-echo image of a rat brain at the midline showing the position of the ROIs. The actual size of ROIs were 2 pixels × 2 pixels (0.153 mm$^2$).
contains the important findings. We did not observe any delayed increase in the signal intensity in the SFO, which would indicate leakage of the Gd-DTPA\(^2-\) from the vessels to the interstitial space. The area of the high signal intensity in the SFO did not change over a period of 2 h. If extravasation of Gd-DTPA\(^2-\) occurs, the area and signal intensity of the high signal intensity region should expand and increase time-dependently.

In order to confirm the low Gd-DTPA\(^2-\) permeability in the SFO, we measured changes in the signal intensity of the \(T_1\)-weighted images and \(T_1\) relaxation rates when the BBB was permeabilized by hyperosmotic shock. The changes seen in the SFO and nine ROIs are summarized in Fig. 7. In this experiment, we reduced the initial infusion rate of Gd-DTPA\(^2-\) in order to reduce the effects of the magnetic susceptibility of the high concentration of Gd-DTPA\(^2-\). Therefore, the image intensities in the SFO and the blood in the anterior communicating artery (ACA) increased faster and without the transient dip. However, the dilution process induced by the distribution of Gd-DTPA\(^2-\) in the extravascular extracellular space caused a late decrease in the image signal intensity. After these transient changes, the extracellular Gd-DTPA\(^2-\) concentration was approximately 11 mM, and the signal intensities in all of the ROIs showed steady levels. When the BBB was broken by the bolus injection of 25% (w/v) mannitol, the image intensity in the SFO increased again, and this increase was continued after the transient changes induced by the mannitol injection. Image intensities in the cortex and thalamus were maintained at their original signal intensities before the mannitol injection, but they were also increased very sharply by the mannitol injection, with a gradual increase maintained thereafter. These results suggest that the mannitol injection caused a breakdown of the BBB such that the Gd-DTPA\(^2-\) leaked out into the extravascular space.

Figure 7. Transient changes in the signal intensity of the \(T_1\)-weighted images and the \(T_1\) relaxation rates of rat brain when the BBB was permeabilized due to hyperosmotic stress

After 5 min under control conditions, Gd-DTPA\(^2-\) was injected over 4 min at a rate of 0.57 mmol (kg body wt\(^{-1}\) min\(^{-1}\) (black box), then maintained at a constant infusion rate of 0.14 mmol (kg body wt\(^{-1}\) h\(^{-1}\) (bold line). After 35 min of the Gd-DTPA\(^2-\) infusion, 25% (w/v) mannitol solution (5 ml (kg body wt\(^{-1}\))) was injected from the internal carotid artery (open arrow), which made capillaries in the right hemisphere permeable, including the mid-sagittal part of the thalamus, hypothalamus and the midbrain. A–C, changes in \(T_1\)-weighted image intensity of a single voxel (195 \(\mu\)m x 195 \(\mu\)m x 1000 \(\mu\)m) were plotted every 56 s. D, the positions of the 10 ROIs are shown superimposed on a sagittal image of the brain.
space in the cortex, thalamus and SFO. These results are consistent with the changes in the $T_1$ relaxation rates in the SFO, cortex and thalamus, where values increased from 0.678, 0.681 and 0.661 $s^{-1}$ to 1.90, 1.88 and 1.67 $s^{-1}$, respectively, after the mannitol injection.

If the capillaries in the SFO were permeable by the Gd-DTPA$^{2-}$, we should not observe this additional increase in the image intensity in the SFO. This is also the case in the skin and the anterior lobe of the pituitary gland, as both of these regions have ordinary capillaries that are permeable to Gd-DTPA$^{2-}$. The image intensities in both of these regions were increased by the infusion of Gd-DTPA$^{2-}$, and minimal changes were demonstrated by the mannitol injection. Therefore, the Gd-DTPA$^{2-}$ was distributed throughout the extracellular space in these regions before the mannitol injection. The changes in the image intensity in the posterior lobe of the pituitary gland, one of the CVOs, were almost the same as those in the skin and the anterior lobe of the pituitary gland, suggesting that capillaries in the posterior lobe of the pituitary gland might be permeable to Gd-DTPA$^{2-}$. A region in the hypothalamus was measured as a reference because this region also receives blood perfusion from the superior hypophyseal artery, a branch from the internal carotid artery, which perfuses the posterior lobe of the pituitary gland. The hypothalamus showed results similar to those obtained in the cortex and thalamus. Therefore, the mannitol infusion might have affected these regions so that the capillaries were permeable to Gd-DTPA$^{2-}$. An ROI in the pons was employed as a negative control, in order to demonstrate any changes shown in this experiment, because the pons is perfused by the basilar artery, and accordingly, the mannitol could not reach this region. Changes in the signal intensity of the cerebrospinal fluid in the fourth ventricle were small and increased slowly before the mannitol injection, and then increased significantly after the injection. In preliminary experiments using an isotonic Gd-DTPA$^{2-}$ solution (100 mM), it was difficult to detect any Gd-DTPA$^{2-}$ leakage into the CSF. These results suggested that the choroid plexus might have a low Gd-DTPA$^{2-}$ permeability under the control conditions, and they also suggested that the hypertonic stress might have significantly permeabilized the choroid plexus. Quantitative handling of data might be difficult because the ventricular system is an open system. The CSF is secreted by the choroid plexuses in the lateral and fourth ventricle, and it is then transported to the subarachnoidal space and drained off via the arachnoid granulations into the dural sinus. Therefore, Gd-DTPA$^{2-}$, leaked from the choroid plexuses, is also transported to the subarachnoidal space. Since pores of about 1 $\mu m$ in diameter are present in the apical surface of the granulations, the Gd-DTPA$^{2-}$ may be drained off into the venous system (Takamata et al. 2001).

### DISCUSSION

**Capillary permeability of Gd-DTPA$^{2-}$**

It is believed that the blood–brain barrier is impermeable to Gd-DTPA$^{2-}$ (742 Da) (Abbott et al. 1999). The question arises: is this true of the capillaries in the SFO? As summarized by Abbott et al. (1999), almost all of the reports published have treated abnormal BBB that suffered from pathological conditions, such as ischaemia. We found only a limited number of reports on the capillary permeability of Gd-DTPA$^{2-}$ in the intact brain. Whole-body autoradiographic studies in rats after intravenous administration of $^{153}$Gd-DTPA$^{2-}$ and Gd-$^{[14]}$DTPA$^{2-}$ (Täuber et al. 1986) showed that Gd-DTPA$^{2-}$ was very rapidly distributed throughout the body except for the central nervous system and fetus. Since the distributions of both of the labelled Gd-DTPA$^{2-}$ compounds were similar, there was no indication of a dissociation of the Gd-DTPA$^{2-}$ complex. The pharmacokinetics and tissue distribution of a similar substance, the gadobenate ion (Gd-BOTPA$^{2-}$; 862 Da), have also been reported (Lorusso et al. 1999). Gd-BOTPA$^{2-}$ was also distributed over the whole body, except for the central nervous system. There was no mention of the CVOs in the reports on these autoradiography studies, and there was no staining in the SFO region of the sagittal section of rat brain. Thus, Täuber’s study (Täuber et al. 1986) may actually be the first showing Gd-DTPA$^{2-}$ capillary permeability in the CVOs. As shown in Fig.7, the $T_1$-weighted MR image intensity of the SFO was increased by the injection of Gd-DTPA$^{2-}$, then maintained at a stable level, but it was increased again by the injection of hypertonic mannitol solution. The $T_1$ relaxation rate was also accelerated from 0.68 to 1.9 $s^{-1}$. After the BBB breakdown, image intensities in the SFO, cortex and thalamus were almost the same, and they increased continuously. These results indicate that Gd-DTPA$^{2-}$ can enter the interstitial spaces of the SFO, cortex and thalamus after the BBB breakdown, and the concentration of Gd-DTPA$^{2-}$ may be the same in those...

#### Table 2. Transepithelial diffusive water permeability

<table>
<thead>
<tr>
<th>Tissues</th>
<th>$P_4$ (cm $s^{-1}$)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subfornical organ (rat)</td>
<td>$3.7 \times 10^{-3}$</td>
<td>Morgan &amp; Berliner, 1968</td>
</tr>
<tr>
<td>Vasa recta (rat)</td>
<td>$2 \times 10^{-3}$</td>
<td>House, 1974</td>
</tr>
<tr>
<td>Muscle capillary (dog)</td>
<td>$2.8 \times 10^{-4}$</td>
<td>Kuwahara &amp; Verkman, 1988</td>
</tr>
<tr>
<td>Outer medullary collecting tubule (rabbit, 37 °C)</td>
<td>$5.8 \times 10^{-4}$</td>
<td></td>
</tr>
<tr>
<td>MDCK monolayer (type I, 20 °C)</td>
<td>$2.6 \times 10^{-4}$</td>
<td>Lavelle et al. 1997</td>
</tr>
<tr>
<td>Bladder epithelium (guinea-pig, 37°C)</td>
<td>$4.6-8.6 \times 10^{-5}$</td>
<td>Lavelle et al. 1998</td>
</tr>
</tbody>
</table>
regions that show similar $T_1$ relaxation rates (1.7–1.9 s⁻¹). Therefore, it must be difficult for the Gd-DTPA²⁻ to cross the capillaries in an intact SFO. The Gd-DTPA²⁻-induced shortening of the $T_1$ relaxation time was mainly caused by water exchange between the intravascular and the extravascular fluid. In contrast, capillaries in the posterior lobe of the pituitary gland could pass Gd-DTPA²⁻ freely because there was no change following mannitol administration. The faster $T_1$ relaxation, which could not be measured precisely, also supports the concept of extravasation of Gd-DTPA²⁻ in the pituitary gland.

The reason why much bigger molecules, e.g. horseradish peroxidase (HRP; 40 kDa), penetrate capillaries in the SFO is still puzzling. HRP has been used as a marker of endocytosis (Gross, 1992). Histologically, most of the capillaries in the SFO are classified as Type I and Type III. Type I capillaries are relatively thick-walled capillaries, with a high density of cytoplasmic vesicles, caveolae and pits. Type I capillaries are found in almost all of the areas of the SFO, and they are predominant in the transitional sub-region between the rostral and central parts of the nucleus. Type III capillaries have a thin endothelium with numerous fenestrations, vesicular profiles and perivascular lacunae. Type III capillaries are present only in the transitional, central and caudal SFO sub-regions. There are loosely interconnected tight junctions in the endothelium in both types of capillaries, but there are no gap junctions (Bouchaud & Bosler, 1986; Gross, 1992). After intravenous administration, HRP was usually found in the cytoplasmic vesicles, which shuttle bidirectionally between plasma membranes. It is considered that endocytosis is the major transport pathway of HRP in the BBB, and not paracellular pathways (Gross, 1992; Lu et al. 1993; Banks & Broadwell, 1994). When wheat germ agglutinin is conjugated to HRP (WGA–HRP), it can enter the brain 10 times more rapidly than HRP alone despite its larger molecular size (77 kDa). It is likely that WGA–HRP undergoes adsorptive transcytosis through cerebral endothelia from blood to brain via specific subcellular compartments within the endothelium (Banks & Broadwell, 1994). Trypan blue (961 Da) is almost the same size as Gd-DTPA²⁻, but it forms a complex with plasmatic protein in blood (Bouchaud & Bosler, 1986). Therefore, it might be transported by endocytosis, as was observed with albumin–Gd-DTPA (92 kDa) and carboxymethyl dextran–Gd-DTPA (38.9 kDa) (Siauve et al. 1999). The capillaries in the subcommissural organ (SCO) have no fenestration, but HRP appears in the extravascular space (Bouchaud & Bosler, 1986). This also suggests the importance of the endocytosis of HRP. As far as we are aware, no reports have been published on the transcellular transport of Gd-DTPA²⁻. Therefore, this contradiction in the relationship between molecular size and permeability could be explained by the endocytosis of HRP.

Another tracer commonly employed for the detection of capillary permeability is α-aminoisobutyric acid (AIB). Because AIB is a small (103 Da) and neutral amino acid, it is able to cross the tight junctions not only in the CVOs but also in the normal BBB (Gross et al. 1987). The epithelial paracellular pathway shows cation selectivity. This selectivity is caused by negatively charged groups that might form a line in tight junctions, because the pH at which alterations in cation permeability (isoelectric pH) occur is between pH 3 and 5 (Powell, 1981). Therefore, it is not easy for Gd-DTPA²⁻ to pass through the tight junctions within the physiological range of pH. Our molecular knowledge of the tight junction has been advanced significantly by the identification of claudin (Furuse et al. 1998). The tight junction is not a simple static barrier, but rather combinations of claudin proteins that produce a specific pore. For example, in the thick ascending limb of Henle claudin-16 forms a pore as an Mg²⁺ channel (Tsukita et al. 2001). In the central nervous system, claudin-11 was found in the myelin sheath, and claudin-5 has been found in the endothelial cells in capillaries. It is likely that claudin-5 is involved in the low permeability of the BBB, but the details of the molecular mechanisms involved are still under investigation (Tsukita et al. 2001). Judging from the results of this study, we can say that it is difficult for Gd-DTPA²⁻, a moderate size molecule with a strong negative charge, to pass through the capillaries in the SFO, but it might cross the capillaries in the posterior lobe of the pituitary gland. The development of caludin-5 knockout mice may provide some insight into the capillary permeability of Gd-DTPA²⁻.

**Estimation of water permeability**

As far as we can determine, there are no reports on $P_d$ values for the SFO. On the assumption that the relaxation time course of the slow component ($R_s$) was mainly determined by the rate of the diffusive water exchange between the extravascular space and the intravascular space, we have calculated the rate constant ($k_w$) in the SFO to be $0.84 \pm 0.11$ s⁻¹ ($n=9$). As shown in eqn (5), in order to convert the $k_w$ to a diffusive permeability coefficient ($P_{db}$), the volume/area ratio of the volume of the extravascular space ($V_e$) and the surface area of the capillaries ($A_s$) is required. From several morphological studies of the SFO in rats (Sposito & Gross, 1987; Gross, 1992), we have taken the volume fraction of the capillaries as 2.3 ± 0.5% of the tissue, which corresponds to the $V_e/A_s$ ratio. Using these values, the apparent diffusive water permeability of the surface of capillaries in the SFO is estimated to be $3.7 \times 10^{-3}$ cm s⁻¹. This $P_{db}$ value is similar to values obtained for the vasa recta of rat kidney ($2 \times 10^{-3}$ cm s⁻¹, Morgan & Berlinger, 1968), and is 10 times larger than that of capillaries in dog skeletal muscle ($2.8 \times 10^{-4}$ cm s⁻¹, House, 1974). It is also much bigger than that in non-leaky epithelium such as the renal collecting duct and bladder epithelium (Table 2). Most of the water permeability studies on the brain
capillaries have determined hydraulic conductivity (Lp) values and some were obtained values of the permeability surface area product (PS). The Lp value for brain capillaries that form the blood–brain barrier (3 x 10⁻⁷ cm atom⁻¹ s⁻¹) is two orders smaller than that of muscle capillaries (2.5 x 10⁻⁵ cm atom⁻¹ s⁻¹) (House, 1974) and the vasa recta (3.1 x 10⁻⁵ cm atom⁻¹ s⁻¹) (Morgan & Berliner, 1968). Since Lp and Ps have different units, these Lp values did not compare directly with the diffusive water permeability (Ps), but Ps values of the SFO were similar to those of the vasa recta. Therefore, it is likely that the Ps value of the SFO might be 10–100 times higher than that of the brain capillaries that form the blood–brain barrier. The very small changes in the relaxation rates seen in the thalamus and cortex in this study also support this estimation. Therefore, we conclude that the SFO region has one of the highest water permeability values among all of the capillaries in the brain.

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