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Estimation of Plasma IC_{50} of Donepezil Hydrochloride for Brain Acetylcholinesterase Inhibition in Monkey Using *N*-[^{11}C]methylpiperidin-4-yl Acetate ([^{11}C]MP4A) and PET

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Donepezil hydrochloride is a potent and selective inhibitor for brain acetylcholinesterase (AChE) and is currently used worldwide for the treatment of Alzheimer's disease. Until now, there is no *in vivo* study on the relation between the plasma concentration and the brain AChE inhibition. The purpose of this study was to estimate *in vivo* plasma IC_{50} of donepezil in living monkeys by measuring plasma donepezil concentration (LC/MS/MS) and brain AChE activity with positron emission tomography (PET) and *N*-[^{11}C]methylpiperidin-4-yl acetate, which is an acetylcholine analog recently developed by us for quantifying *in vivo* brain AChE activity. PET scans with donepezil at two doses, 100 μg/kg (donepezil-1; $N=5$) or 250 μg/kg (donepezil-2; $N=5$), were performed using the same monkeys at 4-week intervals. Before each PET scan, baseline PET scans ($N=10$ in total) were performed without donepezil. The plasma donepezil concentrations 14 min after intravenous injection were proportional to the doses, 17.2 ± 2.9 ng/ml (donepezil-1) and 44.0 ± 5.0 ng/ml (donepezil-2), and the mean AChE inhibitions in four neocortical regions as evaluated by PET were also dose-dependent, 27% (donepezil-1) and 53% (donepezil-2). In IC_{50} estimation, measured plasma donepezil concentrations were corrected for the change during PET scan. The IC_{50} values (estimate $\pm$ SE) were 42 ± 9.0 (ng/ml; donepezil-1), 34 ± 3.2 (donepezil-2), and 37 ± 4.1 (combined data). The present method may be useful for *in vivo* evaluation of other AChE inhibitors and novel drugs.
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INTRODUCTION

Degeneration of cholinergic basal forebrain neurons innervating the cerebral cortex is believed to contribute substantially to cognitive deficits seen in Alzheimer's disease (AD) (Bartus *et al*, 1982). This discovery triggered development of cholinesterase inhibitors such as donepezil with the aim to raise acetylcholine levels in the brain by blocking acetylcholinesterase (AChE). Donepezil is a reversible inhibitor that exhibits high specificity for centrally active AChE (Yamanishi, 1990; Rho and Lipson, 1997; Rogers *et al*, 1998).

In recent years, *N*-[^{11}C]methylpiperidin-4-yl acetate ([^{11}C]MP4A) (Irie *et al*, 1994) and *N*-[^{11}C]methylpiperidin-4-yl propionate ([^{11}C]MP4P) (Irie *et al*, 1994; Kilbourn *et al*, 1996) have been developed as radiotracers for brain AChE mapping and applied to quantification of neocortical AChE activity in healthy subjects (Namba *et al*, 1999; Koeppe *et al*, 1999) and in patients with AD (Iyo *et al*, 1997; Kuhl *et al*, 1999). Both tracers were also applied to evaluating the inhibitory effect of donepezil on brain AChE activity in AD patients (Kuhl *et al*, 2000; Shinotoh *et al*, 2001; Kaasinen *et al*, 2002) and in monkeys (Tsukada *et al*, 2004). As for donepezil, however, there has been no report on the quantitative relation between plasma concentration and brain AChE inhibition in the living subjects.

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In the case of reversible cholinesterase inhibitors such as tacrine and donepezil, inhibition of enzyme activity can be partially reversed by dilution, because of dissociation of the inhibitor from the enzyme with time (Hunter *et al*, 1989; Dawson, 1990; Kosasa *et al*, 2000). Furthermore, using microdialysis, it is reported that donepezil dose-dependently increases the extracellular acetylcholine concentration in rats (Kawashima *et al*, 1994; Kosasa *et al*, 1999) and in monkey (Tsukada *et al*, 2004), which may cause additional AChE inhibition (substrate inhibition) (Reiner and Radic, 2000). With positron emission tomography (PET), not only can we assess brain AChE inhibition by a reversible inhibitor without tissue-dilution effect, but also we can evaluate the inhibitory effects of increased synaptic acetylcholine levels on AChE *in vivo*.

In this study, we estimated *in vivo* plasma IC_{50} of donepezil for brain AChE inhibition in monkeys from measurement of plasma donepezil concentration and cerebral cortical AChE inhibition as evaluated with [^{11}C]MP4A-PET. We also discussed the difference between *in vivo* IC_{50} and *in vitro* IC_{50} for donepezil.

MATERIALS AND METHODS

Principle of the [^{11}C]MP4A Method

[^{11}C]MP4A is a lipophilic acetylcholine analog with high AChE specificity (95% in monkey brain; unpublished data). Figure 1 represents a three-compartment model for [^{11}C]MP4A, consisting of an arterial blood compartment and two tissue compartments. The analog readily enters the brain by diffusion, and then a portion of incorporated [^{11}C]MP4A diffuses back into blood, whereas the remainder is specifically hydrolyzed by AChE into the hydrophilic metabolite, [^{11}C]methylpiperidinol ([^{11}C]MP4OH), which is trapped at the site of metabolic reaction within the brain. The entire process is described by three functional parameters: K_1, representing the rate constant for transport from blood to brain, k_2, representing the rate constant for transport from brain to blood, and k_3, representing the rate constant for hydrolysis by AChE. The k_3 value is used as an index of regional AChE activity.

[^{11}C]MP4A was synthesized by the reaction of its demethyl precursor with [^{11}C]methyl iodide as described previously (Namba *et al*, 1999).

Animals

Five male rhesus monkeys (Macacca mulatta, body weight 4–6.5 kg) were used for the study with PET. Monkeys were maintained and handled in accordance with the recommendations of the US National Institute of Health and the guidelines of the Central Research Laboratory, Hamamatsu Photonics. They were trained to sit on a chair twice a week for more than 3 months. Magnetic resonance images (MRI) of all monkeys were obtained with a Toshiba MRT-50A/II (0.5 T) under anesthesia with pentobarbital. The stereotactic coordinates of PET and MRI were adjusted based on the orbitomeatal (OM) line with a specially designed head holder. At least 1 month before the PET study, an acrylic plate, with which the monkey was fixed to the monkey chair, was attached to the head under pentobarbital anesthesia as described previously (Onoe *et al*, 1994).

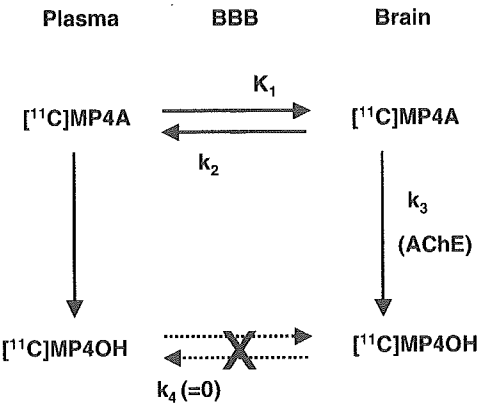

Figure 1 Schematic representation of the three-compartment model of [^{11}C]MP4A in the brain.

Measurement of Time Courses of Donepezil in Plasma

In preliminary studies using another three monkeys, to estimate the time when distribution volumes of donepezil between plasma and tissues reach the steady state, time courses of plasma donepezil concentration were measured after intravenous injection of donepezil at a dose of 250 μg/kg. A cannula was implanted into the posterior tibial vein for administration of donepezil. Another cannula was put into the femoral artery of the other leg to obtain arterial blood samples. Donepezil was intravenously administered to awake monkey by single bolus injection in a volume of 0.5 ml/kg. Arterial blood samples were collected (2 ml/tube) at 5, 10, 15, 30, 60, and 90 min after injection and the concentration was analyzed by liquid chromatography/two-mass spectrometry (LC/MS/MS) as described below. Donepezil hydrochloride was kindly supplied by Eisai Chemicals (Ibaraki, Japan).

Protocols for Donepezil Administration and PET Scan

PET scans with donepezil were performed at a dose of 100 μg/kg (donepezil-1; $N=5$) and 250 μg/kg (donepezil-2; $N=5$) using the same five monkeys at 4-week intervals. Before each PET scan with donepezil, baseline PET scans (Baseline-1 or Baseline-2; $N=5$ for each) were performed. Donepezil was intravenously administered by single bolus injection 15 min prior to the start of PET scanning. The experimental design is illustrated in Figure 2.

The PET scan was carried out with a high-resolution PET scanner (SHR-7000, Hamamatsu Photonics K.K., Hamamatsu, Japan) with transaxial resolution of 2.6 mm full-width at half-maximum (FWHM) and a center-to-center distance of 3.6 mm. PET images were reconstructed by the filtered-back projection with a Hunning 4.5 mm filter.

In practice, a monkey was fixed on the monkey chair with stereotactic coordinate aligned parallel to the OM line in the gantry of PET scanner after overnight fasting. The monkeys were fully awake during the whole PET procedures. A cannula was implanted into the posterior tibial vein for administration of donepezil and [^{11}C]MP4A. Another

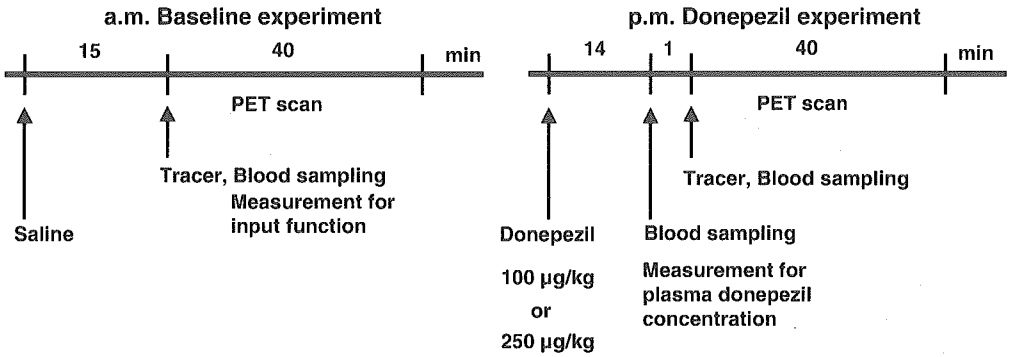

Figure 2 Illustrating of a time schedule for baseline experiment and donepezil experiment.

cannula was put into the femoral artery of the other leg to obtain arterial blood samples. PET scan was performed under dim light. After 30 min of transmission scanning using ^{68}Ge–^{68}Ga external standard, a monkey received an intravenous infusion of [^{11}C]MP4A (100–350 MBq/kg) in 3 ml of saline for 30 s. PET scan started simultaneously and 16 sequential frames were acquired dynamically over a period of 40 min. The image data were reconstructed, and regions of interest (ROIs) were placed in the frontal, temporal, parietal and occipital cortices on the PET image of the brain with reference to the corresponding MRI of each monkey brain and then the time–activity curve in ROIs were obtained.

Measurement of Arterial Input Function for [^{11}C]MP4A

Seventeen arterial blood samples were collected (0.5 ml/tube) in a 1.5 ml microtube containing 0.1 mg of physostigmine, an inhibitor of cholinesterases, in 0.01 ml of heparinized saline. Blood sampling was started at PET scan start time and continued for 30 min at appropriate time intervals. The total radioactivity in the plasma and the ratio of authentic [^{11}C]MP4A to the metabolite [^{11}C]MP4OH were measured, and then the time–activity curve of authentic [^{11}C]MP4A in plasma (input function; Ca(t)) was calculated as reported previously (Namba *et al*, 1999).

Kinetic Analysis for [^{11}C]MP4A

The regional K_1–k_3 parameters of [^{11}C]MP4A were estimated from PET data based on a three-compartment model as described below (Namba *et al*, 1999), where $C_S(t)$ represents the theoretical function for authentic tracer, $C_M(t)$ the metabolite, $C_T(t)$ the total radioactivity and $\otimes$ the mathematical operation of convolution.

$$C_S(t) = \int_0^t K_1 \mathrm{Ca}(\theta) \mathrm{e}^{-(k_2+k_3)(t-\theta)} \mathrm{d}\theta \quad (1)$$

$$C_M(t) = \int_0^t k_3 C_S(\theta) \mathrm{d}\theta \quad (2)$$

$$C_T(t) = C_S(t) + C_M(t) = \frac{K_1}{k_2+k_3}[k_3 + k_2 \mathrm{e}^{-(k_2+k_3)t}] \otimes \mathrm{Ca}(t) \quad (3)$$

The K_1–k_3 values were estimated by fitting $C_T(t_i)$ (i = 1–16) to the observed PET data using an iterative nonlinear least-squares optimization.

Measurement of Plasma Donepezil Concentration

An arterial blood sample was obtained 1 min before the start of PET scan (14 min after injection of donepezil) and analyzed for donepezil by LC/MS/MS as follows (Kosasa *et al*, 2000). The analysis was entrusted to ADME/TOX Research Institute, Daiichi Pure Chemicals, Ibaraki, Japan. Blood samples were centrifuged at 3000 rpm for 15 min, and obtained plasma samples were stored at −20°C. Electrospray ionization-MS/MS was carried out on an API 3000 (Applied Biosystems, Tokyo, Japan) mass spectrometer equipped with a LC system, Agilent 1100 (Agilent Technologies, Tokyo, Japan). The spectrometer was set to admit the protonated molecules $[M+H]^+$ at *m/z* 380 (donepezil) and *m/z* 394 (internal standard; (*R,S*)-1-benzyl-4-[2-[(5,6-dimethoxy-1-indanon)-2-yl]-ethyl]piperidine hydrochloride), with monitoring of the product ions at *m/z* 91 (donepezil) and *m/z* 91 (internal standard). The limit of quantification for donepezil was set at 5 ng/ml.

The Correction of Plasma Donepezil Concentration

Even after reaching the steady state following intravenous bolus injection of donepezil in monkeys, plasma donepezil concentration decreases gradually, and consequently brain AChE activity may be changed during 40-min PET scan. We actually measure brain AChE activity as an average value during the PET scan, more correctly, during the residence time of authentic [^{11}C]MP4A in the brain. By this reason, in the estimation of plasma IC_{50}, we used the corrected value of plasma donepezil concentration, that is, the concentration at the mean residence time (MRT) of authentic [^{11}C]MP4A in the brain. The correction of plasma donepezil concentration was made as follows.

First, based on the results of measurements of plasma donepezil concentration in three monkeys after intravenous injection of donepezil at a dose of 250 μg/kg, the standard curve (Cp(t) in Figure 3) was obtained by averaging the results from three monkeys, followed by curve fitting with bi-exponential function as $\mathrm{Cp}(t) = 84.9\mathrm{e}^{-0.058t} + 46.6\mathrm{e}^{-0.011t}$. Of each monkey in donepezil-1 and donepezil-2 studies, we measured the plasma concentration only once, 14 min after

injection of donepezil (open triangle in Figure 3). The correction curve, $Cp'(t)$, was defined as the mono-exponential curve which has the same slope as the second term of $Cp(t)$ and pass through the measured point. Second, based on the result of nonlinear least-square analysis of [^{11}C]MP4A data in the occipital cortex, MRT (min) for authentic [^{11}C]MP4A in the brain was calculated for each monkey as follows:

$$\mathrm{MRT} = \frac{\int_0^{40} t\mathrm{Cs}(t)\mathrm{d}t}{\int_0^{40} \mathrm{Cs}(t)\mathrm{d}t}$$

where $Cs(t)$ represents a theoretical curve for authentic [^{11}C]MP4A in the brain. Finally, because PET scan was performed after 15 min donepezil injection, the corrected value for plasma donepezil concentration was obtained as $Cp'(15 + \mathrm{MRT})$ from the correction curve, $Cp'(t)$, at the time $15 + \mathrm{MRT}$ min (open square in Figure 3).

Estimation of Plasma IC_{50}

The value of plasma IC_{50} of donepezil, that is, the concentration of donepezil in plasma that inhibits brain AChE activity by 50% in the steady-state conditions of distribution volumes between plasma and brain, was calculated using a simple one-parameter model as follows:

$$\mathrm{AChE\ inhibition} = \frac{[\mathrm{I}]}{\mathrm{plasma}\ IC_{50} + [\mathrm{I}]}$$

where AChE inhibition represents mean k_3 reduction in the four regions and [I] represents the corrected donepezil concentration in plasma. The plasma IC_{50} values were estimated in three ways, that is, for each dose experiment, separately, and from the combined data. The standard errors of plasma IC_{50} estimates were calculated with the variance–covariance matrix (Veng, 1977; Carson, 1986).

Statistically, the following null hypothesis was used to compare the plasma IC_{50} values between each dose experiment. The null hypothesis was that the plasma IC_{50} values obtained from the two-doses experiments would be sampled from the same normal distribution and the null hypothesis was to be rejected at the 0.05 level probability.

RESULTS

Time Courses of Donepezil Concentration in Plasma

After intravenous injection of donepezil at a dose of 250 μg/kg in three monkeys, the shape of concentration curve plotted in semilogarithmic scale, $Cp(t)$, showed a biphasic pattern (Figure 3). Nonlinear regression analysis was used to determine the standard concentration curve as $Cp(t) = 84.9e^{-0.058t} + 46.6e^{-0.011t}$, which is composed of the early phase (half-life; 12 min) and the later phase (63 min). From the shape of this curve, it was assumed that the steady-state conditions of distribution volumes between plasma and brain had been established 15 min after intravenous injection of donepezil in monkeys.

Baseline Study

In baseline studies, the regional k_3 values of [^{11}C]MP4A were measured using the same monkeys ($N = 5$) before the two doses of donepezil studies at 4-week intervals, once before (Baseline-1) and once (Baseline-2) after donepezil administration (100 μg/kg). The baseline k_3 values (mean ± SD; min^{-1}) in the four cerebral cortical regions were as follows: (Baseline-1) temporal, 0.174 ± 0.014; frontal, 0.173 ± 0.016; occipital, 0.145 ± 0.015; parietal, 0.142 ± 0.036. (Baseline-2) temporal, 0.186 ± 0.040; frontal, 0.171 ± 0.026; occipital, 0.154 ± 0.032; parietal, 0.144 ± 0.031. Figure 4 shows the change in cerebral cortical mean k_3 for five monkeys between the two baseline studies. The mean and SD values of the cerebral cortical mean k_3 were 0.158 ± 0.019 in Baseline-1 (○) and 0.164 ± 0.030 in Baseline-2 (□). Though the mean value in Baseline-2 was higher by 3.5% compared with Baseline-1, the difference was statistically not significant ($P = 0.55$ using a paired t-test). Actually, two of the five monkeys showed reductions (−8.7 and −7.0%) and three showed increases (2.2, 6.4, and 23.8%). As for the reproducibility of baseline k_3 values, mean absolute difference in cerebral cortical k_3 values between two experiments was 9.2%.

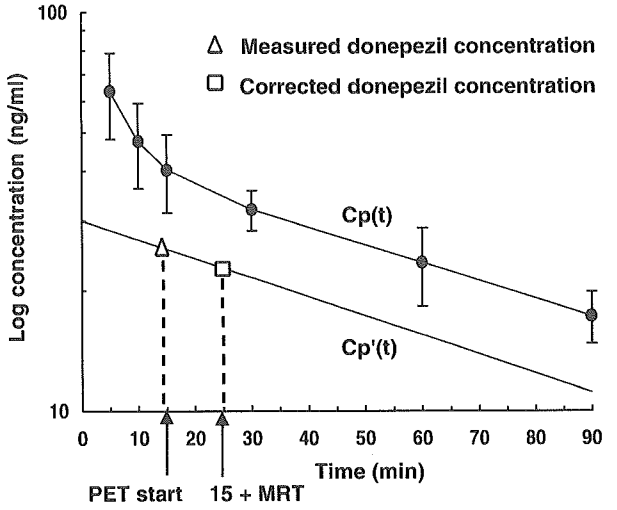

Figure 3 (Upper curve) A time course of plasma donepezil concentration after intravenous injection of 250 μg/kg donepezil in three monkeys: The curve showed a biphasic pattern which was fitted with bi-exponential function as $Cp(t) = 84.9e^{-0.058t} + 46.6e^{-0.011t}$. (Lower curve) The $Cp'(t)$ represents a correction curve, derived from the slower component of $Cp(t)$ and the measured value (Δ) in each monkey. The corrected value (□) was calculated as the $Cp'(15 + \mathrm{MRT})$ value, where MRT represents the estimated value of mean residence time of authentic [^{11}C]MP4A in the brain for each monkey.

Donepezil Study

Figure 5 shows the dose dependency of regional AChE inhibition as calculated by k_3 change between baseline experiments and donepezil experiments. In both doses of donepezil, the AChE reductions were almost the same across the four regions examined, 27 ± 0.6% in donepezil-1 (100 μg/kg) and 53 ± 0.7% in donepezil-2 (250 μg/kg). In the estimation of plasma IC_{50}, therefore, the mean value for the four regions was used as AChE inhibition for each monkey.

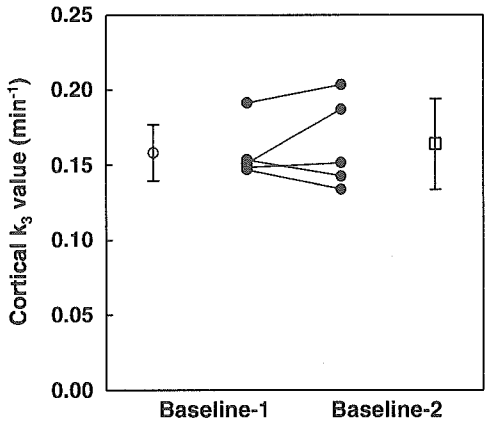

Figure 4 Reproducibility of cerebral cortical k_3 values between two baseline experiments performed at 4-week intervals after single intravenous injection of donepezil in five monkeys at a dose of 100 μg/kg. The open circle and open square represent the mean of cortical k_3 values in five monkeys before and after donepezil injection, respectively. The closed circles represent cerebral cortical k_3 values for repeated scans of each monkey.

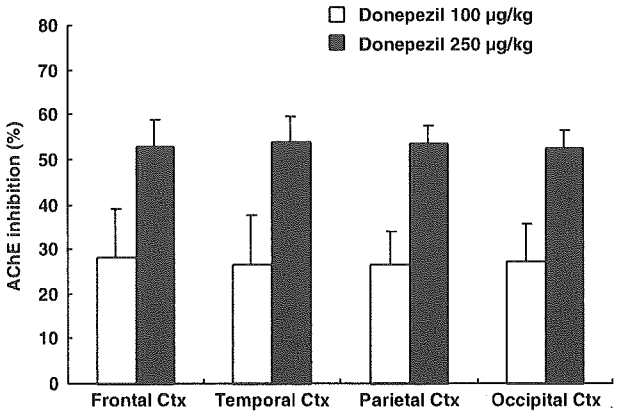

Figure 5 Dose effects of donepezil on AChE activity as measured by [^{11}C]MP4A in the cerebral cortical regions of monkeys. Data are expressed as a percent inhibition for five monkeys per treatment condition. The difference in AChE inhibition between the two doses was statistically significant in all regions ($P<0.05$ using a paired *t*-test).

Donepezil Concentration in Plasma

The plasma donepezil concentrations 14 min after intravenous injection were 17.2 ± 2.9 ng/ml (mean ± SD) in donepezil-1, and 44.0 ± 5.0 ng/ml in donepezil-2 experiment. The ratio of the measured donepezil concentration in plasma between donepezil-1 and donepezil-2 experiment (2.6-fold) was almost the same as the ratio of administered dosage of donepezil (2.5-fold).

Since the concentration of donepezil in plasma gradually decreased during PET scan, in the estimation of plasma IC_{50} of donepezil, we used the corrected value of plasma donepezil concentration, which was calculated as follows. Representative time–radioactivity curve of authentic tracer in the brain calculated by nonlinear least-squares analysis is shown in Figure 6. From the time–activity curve of authentic tracer in the brain, MRT (min) of authentic tracer was calculated in each dose experiment, which was 7.7 ± 1.5 (mean ± SD) in donepezil-1, and 10.1 ± 1.2 in donepezil-2 experiment. Using the methods as described (Figure 3), the corrected donepezil concentration (ng/ml) at 15 + MRT (open square in Figure 3) after injection of donepezil was estimated to be 15.8 ± 2.5 (mean ± SD) in donepezil-1 and 39.3 ± 4.0 in donepezil-2 experiment.

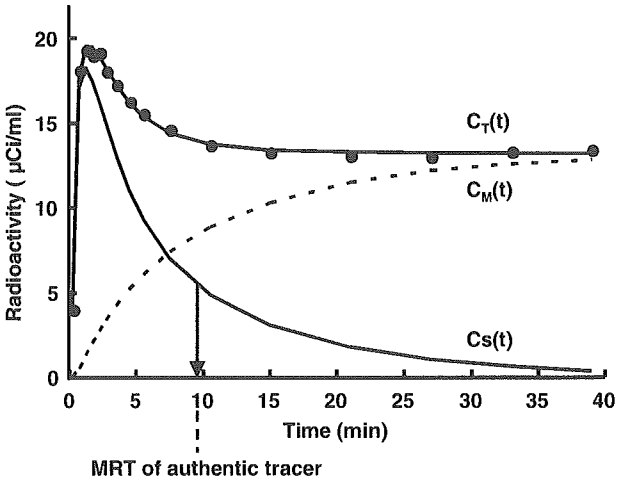

Figure 6 Time–radioactivity data in the occipital cortex of one subject given with donepezil at a dose of 250 μg/kg and the fitted curves by nonlinear least-squares analysis: $C_T(t)$, representing the theoretical curve for the total radioactivity; $C_M(t)$, the metabolite; Cs(*t*), the authentic tracer. MRT represents the mean residence time of the authentic tracer in the brain.

Plasma IC_{50}

The plasma IC_{50} values were estimated from the values of corrected plasma donepezil concentration and cerebral cortical mean AChE inhibition as measured by PET. The plasma IC_{50} values (ng/ml) were 42 ± 9.0 (estimate ± SE) and 34 ± 3.2 in donepezil-1 and donepezil-2 experiments, respectively. Though the IC_{50} in donepezil-2 experiment was about 20% lower than that in donepezil-1 experiment, the difference was not statistically significant ($P = 0.35$). Therefore, we have estimated plasma IC_{50} from combined data, which was 37 ± 4.1 ng/ml. Figure 7 shows the concentration-inhibition curve for the case of IC_{50} of 37 ng/ml.

DISCUSSION

This is the first report on the estimation of *in vivo* plasma IC_{50} of donepezil using [^{11}C]MP4A-PET and monkeys. Based on the preliminary studies on pharmacokinetics of donepezil in plasma following intravenous injection in three monkeys, we have designed the experimental protocol based on single intravenous injection of donepezil. We have estimated the plasma IC_{50} at two different doses of donepezil, 100 and 250 μg/kg, to examine the possible inhibitory effects of enhanced synaptic acetylcholine levels

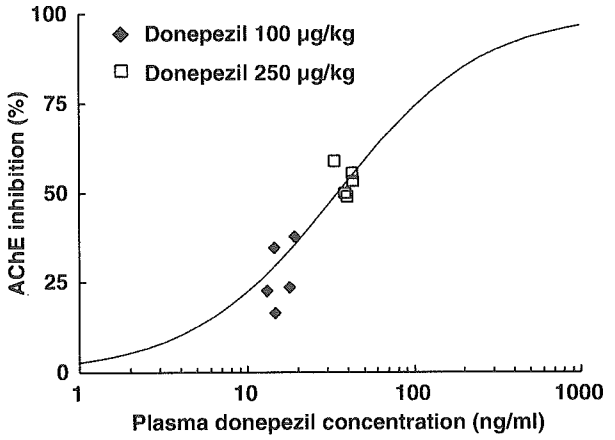

Figure 7 Relation between the plasma donepezil concentration and the percent inhibition of brain AChE activity. The curve represents the concentration–inhibition curve for the estimated IC_{50} value of 37 ± 4.1 ng/ml (estimate ± SE) from the combined data.

resulting from AChE inhibition by donepezil on the hydrolysis rate (k_3) of [^{11}C]MP4A.

In order to determine the experimental protocol such as the sampling time for measurement of plasma donepezil concentration and the start time of [^{11}C]MP4A-PET scan, we have performed preliminary studies on the time courses of donepezil concentration in plasma after intravenous bolus injection of donepezil at a dose of 250 μg/kg in three monkeys. With a bolus injection, since the plasma curve is simple in itself, that is, representing the response to an impulsive input, it would be much easier to determine the time of reaching the steady state with regard to the distribution volume of donepezil between plasma and brain by analysis of the shape of the curve, compared with infusion. Another merit of intravenous injection, compared with oral administration, is to achieve high drug concentration in the brain from minimal dose, which is important from ethical point of view. The plasma donepezil concentration showed a bi-exponential curve (Figure 3), composed of the rapid component ($T_{1/2}$; 12 min) representing the distribution of the drug from blood to tissues and the slow component ($T_{1/2}$; 63 min) corresponding to the redistribution and the elimination of the drug from body. The result suggests that the distribution of donepezil to all tissues was very rapid and that the steady state had been established within 15 min after intravenous injection. In mice experiments using [^{11}C]donepezil, it is reported that the ratio of donepezil concentration between blood and brain became constant within 5 min after intravenous injection (De Vos *et al*, 2000). Furthermore, in monkey experiments, the maximal increase in intracerebral acetylcholine levels resulting from AChE inhibition by donepezil occurred as early as 14 min after intravenous injection of donepezil as measured by microdialysis (Tsukada *et al*, 2004). These results support our assumption that distribution volume of donepezil between plasma and brain reaches the steady state rapidly enough, before the start of PET scan (15 min after intravenous injection of donepezil).

In general, nonlinear least-squares analysis using measured input function data is known to be the most reliable method for k_3 estimation, though arterial blood sampling and metabolite analysis are required. Even with this analysis, due to higher hydrolysis rate of [^{11}C]MP4A compared with [^{11}C]MP4P and also due to higher AChE activity in the cortical regions of monkey compared with human, [^{11}C]MP4A is limited to measurement of k_3 in brain regions with relatively low AChE activity such as cerebral cortical regions. By this reason, we have measured cerebral cortical k_3 change to evaluate the inhibitory effect of donepezil on brain AChE activity. In donepezil studies, the values of regional AChE inhibition showed a very small variability (1–2%, Figure 5) for both donepezil doses, which validates the reliability of the present method.

It is reported that when living brain slices from mice were exposed to AChE inhibitors such as physostigmine and pyridostigmine, AChE mRNA levels were markedly increased 30 min after treatment, followed by enhancement of AChE in cerebral cortical regions (Kaufer *et al*, 1998). By this reason, we have performed two baseline experiments, once (baseline-1) before and once (baseline-2) after the donepezil administration. The beseline-2 experiment (after exposure of 100 μg/kg donepezil) showed only a slightly higher k_3 (3.5%; statistically not significant) compared with baseline-1 experiment, indicating that the possibility of AChE induction by donepezil is low at least under the present condition, that is, 1 month after single intravenous injection of donepezil at a dose of 100 μg/kg.

We observed a good correlation between the dose of donepezil and the reduction of cerebral cortical k_3 value, about 27 and 53% reductions at 100 and 250 μg/kg, respectively (Figure 5). The values of k_3 reduction were almost the same among four cerebral cortical regions. Therefore, in the estimation of plasma IC_{50}, we used the cortical mean as the value of AChE inhibition for each monkey. As for donepezil plasma concentration at steady state in human, the value of 26.4 ± 3.9 ng/ml (mean ± SD) was reported from measurements in healthy subjects after oral doses of 5 mg donepezil for 28 days (Tiseo *et al*, 1998). Using this value of plasma donepezil concentration in human and the IC_{50} value (37 ng/ml) obtained in the present monkey study, brain AChE inhibition in human is calculated as 41%, which is compatible with the reported AChE inhibition (27–40%) as measured with PET in patients with AD under treatment by donepezil at a dose of 3–10 mg/day using [^{11}C]MP4A (Shinotoh *et al*, 2001; Kaasinen *et al*, 2002) and [^{11}C]MP4P (Kuhl *et al*, 1999), supporting the validity of the present method for estimation of plasma IC_{50} of donepezil in monkeys.

Donepezil is thought to exert its therapeutic effect by increasing the concentration of acetylcholine through reversible inhibition of its hydrolysis by AChE, thereby enhancing cholinergic functions. This mechanism of action has been experimentally confirmed in rats (Kawashima *et al*, 1994; Kosasa *et al*, 1999) and monkeys (Tsukada *et al*, 2004), as measured qualitatively by microdialysis. Since [^{11}C]MP4A is a substrate-type radiotracer, the increase in synaptic acetylcholine levels would affect k_3 of [^{11}C]MP4A through two mechanisms as follows. First, acetylcholine by itself acts as a competitive inhibitor for [^{11}C]MP4A. Second, acetylcholine in high concentration may cause substrate inhibition through binding to a regulatory site on AChE (Reiner and Radic, 2000). To examine whether such indirect

inhibitory effects of donepezil through the change in synaptic acetylcholine levels might be detectable using [^{11}C]MP4A-PET, we carried out donepezil-1 and donepezil-2 experiments and compared plasma IC_{50} values between different doses. We expected that the IC_{50} obtained at higher dose would become lower if such indirect inhibitory effects of donepezil are large. Though donepezil-2 experiment showed about 20% lower plasma IC_{50} (34 ng/ml) than donepezil-1 experiment (42 ng/ml), the difference was not significant statistically. It is unclear whether the change in synaptic acetylcholine levels can be detectable *in vivo* using [^{11}C]MP4A-PET. Further studies are needed in this respect. In the discussions below, we have used plasma IC_{50} estimated from the combined data, which was 37 ± 4.1 ng/ml.

In this study, the plasma IC_{50} of donepezil was obtained on a basis of the total concentration as 37 ng/ml (89 nM). Using the reported protein-bound fraction (92.6%) of donepezil in human plasma (Mihara *et al*, 1993), we obtain plasma IC_{50} of donepezil on a basis of free form as 6.6 nM (89 nM × (100−92.6) %). At the steady state, free donepezil concentration in brain is also 6.6 nM. This value is almost the same as *in vitro* IC_{50} of donepezil (6.7 nM), as measured using rat brain tissue homogenate at a highly diluted condition (600-fold), where protein binding is considered to be negligible (Ogura *et al*, 2000). A close relation between *in vivo* plasma IC_{50} on a basis of free donepezil (6.7 nM) obtained in this study and the reported *in vitro* IC_{50} (6.6 nM) implies that we can estimate the value of brain AChE inhibition from measurement of plasma total concentration of the drug in each subject based on the plasma IC_{50}, which is obtainable from *in vitro* IC_{50} and information on the plasma protein binding.

Based on the plasma IC_{50} estimated in this study (89 nM on a basis of total donepezil concentration) and distribution volume of donepezil in rats brain (6–8 ml/g) reported by Kosasa *et al* (2000), the total donepezil concentration in brain at 50% AChE inhibition (*in vivo* brain IC_{50}) is estimated to be in the range of 500–700 nM, which is almost two orders larger than *in vitro* IC_{50} (6.7 nM). Such a large difference in IC_{50} values between *in vivo* and *in vitro* experiments may be due to strong tissue binding of donepezil in the brain. Of the total concentration of donepezil in the brain, the free fraction may be less than 1%, which provides the inhibitory effect on brain AChE activity in clinical environment.

In conclusion, this study provides information on the quantitative relation between plasma concentration of donepezil and brain AChE inhibition measured *in vivo* using PET and [^{11}C]MP4A. The major difference of *in vivo* experiment from *in vitro* and *ex vivo* experiments is the ability to evaluate the effects of intrinsic acetylcholine as an inhibitor and the strong tissue-binding effects of donepezil. Therefore, PET evaluation would provide unique information on the *in vivo* pharmacology of AChE inhibitors and novel drugs.

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No association of the brain-derived neurotrophic factor (BDNF) gene polymorphisms with panic disorder

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Abstract

Several lines of evidence suggest that genetic factors might contribute to susceptibility to panic disorder. Our previous studies show that the brain-derived neurotrophic factor (BDNF) may play a role in the pathophysiology of major depressive disorders and eating disorders. Assuming that BDNF may be implicated in the putative common pathophisiology of depression and anxiety, we analyzed the association of two BDNF gene single nucleotide polymorphisms (SNPs), 132C>T (formerly named C270T) in the noncoding region of exon V and 196G>A (val66met) in the coding region of exon XIIIA, with panic disorder. In this study, 109 patients with panic disorder diagnosed according to the DSM-IV criteria, and 178 control subjects were recruited. There were no significant differences in the frequency of the genotype or allele in these two SNPs between patients and controls [132C>T in exon V: genotype, $p=1.0$, allele, $p=0.59$; 196G>A (val66met) in exon XIIIA: genotype, $p=0.77$, allele, $p=0.78$]. Furthermore, no significant associations of agoraphobia with the two SNPs were detected. This study suggests that the BDNF gene polymorphisms are not associated with panic disorder in our Japanese population.

Keywords: Agoraphobia; Anxiety; Brain-derived neurotrophic factor; Panic disorder

1. Introductions

Panic disorder is an anxiety disorder characterized by unexpected and repeated episodes of intense fear accompanied by physical symptoms including palpitations and difficulty breathing. It is often associated with agoraphobic avoidance stemming from fear of further attacks. It has a lifetime prevalence between 1.5 and 3.0%. Family and twin studies have indicated that genes influence susceptibility to panic disorder (Hettema et al., 2001), but the genes involved remain unknown. Recent evidences suggest genetic and neurobiologic similarities between depressive and anxiety disorders, which may belong to the same spectrum (Kendler et al., 1992).

Brain-derived neurotrophic factor (BDNF), the most abundant of the neurotrophins in the brain, plays important roles in the enhancement of the growth and maintenance of several neuronal systems, in the modulation of neurotransmission and in the plasticity mechanisms such as long-term potentiation and learning (Lindsay et al., 1994). Several lines of evidence suggest BDNF as a candidate molecule involved in the pathophysiology of mood disorders. As researchers have reported that antidepressant treatment increases the expression of BDNF in the rat hippocampus (Shirayama et al., 2002), the relationship between low BDNF levels and major depressive disorder has been suggested (Duman et al., 1997). Recently, we have shown that serum BDNF levels are significantly lower in the antidepressant-naive depressed patients than in the treated patients or in the healthy control subjects (Shimizu et al., 2003a; Hashimoto et al., 2004). We have also reported that serum BDNF levels in patients with anorexia nervosa and

Abbreviations: BDNF, brain-derived neurotrophic factor; SSRI, selective serotonin reuptake inhibitor.

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bulimia nervosa are significantly decreased as compared to normal controls (Nakazato et al., 2003). On the contrary, there was no significant difference between patients with schizophrenia and normal controls (Shimizu et al., 2003b). On the other hand, BDNF-deficient mice developed aggressiveness and hyperphagia (Lyons et al., 1999) and BDNF conditional mutant mice were hyperactive after exposure to stressors and had higher levels of anxiety when evaluated in the light/dark exploration test (Rios et al., 2001). These behavioral abnormalities in the mutant mice are known to correlate with serotonergic dysfunction. Thus, it seems that BDNF deficiency influences the regulation of anxiety-related behavior based on the serotonergic system. Proven effects of the selective serotonin reuptake inhibitors (SSRIs) for a broad range of mood spectrum disorders, including major depressive disorder, anxiety disorders, bulimia nervosa, posttraumatic stress disorder, and dysthymia, suggest that they share one or more common etiologies. Taken together, it is likely that the BDNF gene may be implicated in the putative common pathophisiology of depression and anxiety. Therefore, we focus on a hypothesis that neurotrophin signaling plays a pivotal role, not only in neuronal survival and differentiation, but also in the regulation of mood and anxiety.

It has been reported that the gene encoding BDNF might be an important candidate for susceptibility of psychiatric disorders including bipolar disorders (Neves-Pereira et al., 2002; Sklar et al., 2003) and eating disorders (Koizumi et al., 2004). In the studies reporting possible association of BDNF and these disorders, two single nucleotide polymorphisms (SNPs) of the BDNF gene has been reported. One is 196G>A (val66met) SNP in exon XIIIA (GENBANK: AF411339; at position 95422) located within the propeptide region of BDNF (SWISS.PROT: P23560.VAR 004626). The A of the ATG-translation initiation codon is denoted nucleotide +1 in exon XIIIA (GENBANK: AF411339; at position 95227). Recently, Egan et al. (2003) have indicated that this SNP may be functional and the variants affect activity-dependent secretion of BDNF. In addition, Chen et al. (2004) found that variant BDNF (met66) may alter the intracellular trafficking and activity-dependent secretion of wild-type BDNF (val66).

The other SNP frequently analyzed is 132C>T in the noncoding region of exon V (GENBANK: AF411339; at position 53620). This SNP is located and numbered at position 132 from the start of exon V (GENBANK: AF411339; at position 53488; Itoh et al., 2004). It was detected and named C270T by Kunugi et al. (2001) after their searching for a novel nucleotide substitution in the noncoding region of the BDNF gene reported by Shintani et al. (1992). It has been reported that 132C>T SNP may be associated with late-onset Alzheimer's disease (Kunugi et al., 2001), schizophrenia (Szekeres et al., 2003), or Parkinson's disease (Parsian et al., 2004).

Assuming that BDNF is a candidate gene in panic disorder, we analyzed the association of these SNPs, 132C>T (C270T named formerly) in the noncoding region of exon V and 196G>A (val66met) in the coding region of exon XIIIA, with panic disorder in Japan.

2. Methods

2.1. Subjects

This study was approved by the ethics committee of the Chiba University Graduate School of Medicine. One hundred and one unrelated patients with panic disorder [39 males and 70 female; age, 37.4±13.3 (mean±S.D.)] were recruited from Chiba University Hospital. All patients were diagnosed according to the DSM-IV (American Psychiatric Association, 1994) criteria. The ratio of panic disorders with agoraphobia ($n=75$) to without agoraphobia ($n=34$) was 6.9:3.5. The 23 patients suffered from a comorbid second disorder; depressive disorders ($n=16$), somatoform disorders ($n=5$), eating disorders ($n=2$), social anxiety disorder ($n=1$), or obsessive–compulsive disorder ($n=1$). One hundred and seventy-eight volunteers [75 males and 103 females; age 28.7±10.2 (mean±S.D.)] who did not have any medical or psychiatric diagnosis after our clinical interview were selected as healthy controls. All subjects including patients and controls were ethnic Japanese.

2.2. Procedure

Blood samples were obtained after the completion of informed consent forms. The genomic DNA was extracted from peripheral leukocytes by standard procedures.

Table 1
BDNF 132C>T in exon V genotype distributions and allele frequency in 178 healthy control subjects, 109 patients with panic disorder (total), and 75 patients with agoraphobia

	n	Genotype			p	Allele		p
		CC	CT	TT		C	T	
Controls	178	170 (95.5%)	7 (3.9%)	1 (0.6%)		347 (97.5%)	9 (2.5%)	
Panic (total)	109	105 (96.3%)	4 (3.7%)	0 (0.0%)	1.0	214 (98.2%)	4 (1.8%)	0.59
		$x^2=0.63$, $df=2$				$x^2=0.29$, $df=1$		
Agoraphobia	75	72 (96.0%)	3 (4.0%)	0 (0.0%)	1.0	147 (98.0%)	3 (2.0%)	0.72
		$x^2=0.42$, $df=2$				$x^2=0.13$, $df=1$		

Numbers in parentheses indicate percentages. Statistical analysis was performed by Fisher's Exact Test.

The BDNF 196G>A (val66met) SNP was assayed by PCR using constructed primers (forward: 5′-GGTGAGAA-GAGTGATGACCA-3′ and reverse: 5′-GCCAGCCAATTC-TCTTTTTG-3′) and enzymatic digestion with restriction enzyme *Pma*CI (Takara Shuzo Ltd., Kyoto, Japan) followed by 2% agarose gel-electrophoresis, as described previously (Itoh et al., 2003; Koizumi et al., 2004).

The genotyping of 132C>T SNP was performed with an ABI PRIZM 7000 Sequence Detection System (Applied Biosystems, Foster City, CA), using ABI Universal PCR Master Mix, the primers and the probes designed by Custom TaqMan SNP Genotyping Assays, Human (Applied Biosystems, Foster city, CA), according to the manufacture's protocol. Then, the data were extracted by ABI PRISM 7000 SDS Software (Applied Biosystems, Foster City, CA).

2.3. Data analyses

The data are presented as the mean ± standard deviation (S.D.). Statistical calculations were performed using the statistical software package SPSS 12.0 Base System and Exact tests for Windows (SPSS Inc., Chicago, IL, USA), and the extended Fisher's Exact Test was used for categorical comparisons. In order to calculate the exact probability value for the relationship between patients and controls among the three genotype groups, we used the extended Fisher's Exact Test for a two by three crosstable with small expected frequencies, rather than the chi-square test. Significance for the results was set at $p<0.05$.

3. Results

There were no significant differences in the frequency of the genotype or allele in these two SNPs between patients and controls [132C>T in exon V: genotype, $p=1.0$, allele, $p=0.59$; 196G>A (val66met) in exon XIIIA: genotype, $p=0.77$, allele, p=0.78; Table 1; Table 2]. The genotype distribution for the patients and the controls did not deviate significantly from the Hardy–Weinberg equilibrium. From the viewpoint that anxiety sensitivity is heritable in women (Jang et al., 1999), we divided our subjects into the male and the female group. However, there were also no significant differences in the frequency of the genotype or allele between the female patients and the female controls (data not shown). Moreover, we analyzed the effects of agoraphobia on the BDNF gene polymorphisms within patients. The genotypic and allelic distributions of the two SNPs were not significantly different between the patients with agoraphobia and the healthy controls [132C>T in exon V: genotype, $p=1.0$, allele, $p=0.72$; 196G>A (val66met) in exon XIIIA: genotype, $p=0.95$, allele, $p=0.92$; Table 1; Table 2].

Table 3
Ethnic difference of allele and genotype frequencies of the BDNF 132C>T in exon V in Japan and USA

	Japan ($n=178$)[a]	USA ($n=199$)[b]
132C>T allele		
Allele T	9 (2.5%)	44 (11.1%)
Allele C	347 (97.5%)	354 (88.9%)
$x^2=20.9$ ($df=1$), $p<0.0001$		
132C>T genotype		
T/T	1 (0.6%)	0 (0.0%)
C/T	7 (3.9%)	44 (22.0%)
C/C	170 (95.5%)	155 (78.0%)
$x^2=27.5$ ($df=2$), $p<0.0001$		

Numbers in parentheses indicate percentages. Statistical analysis was performed by Fisher's Exact Test.
[a] Our control group in this study (Japanese).
[b] Parsian et al., 2004 (Whites of non-Hispanic origin).

In comparison with the data by American researchers (Parsian et al., 2004; Egan et al., 2003), the significant ethnic differences between Japan and USA in allele and genotype frequencies of the 132C>T in exon V (T allele 2.5% in Japan versus 11.1% in U.S.A.) were found (Table 3), and those of the 196G>A (val66met) in exon XIIIA (A allele 41.3% in Japan versus 18.0% in U.S.A.) were confirmed again (Shimizu et al., 2004) in healthy controls.

4. Discussion

The present study suggests that these two BDNF gene polymorphisms may not be associated with panic disorder in Japanese samples. The ethnic differences in the allele and genetic distributions of the two SNPs between Japanese and American populations were detected. However, no ethnic difference in epidemiological studies of panic disorder has

Table 2
BDNF 196G>A (val66met) in exon XIIIA genotype distributions and allele frequency in 178 healthy control subjects, 109 patients with panic disorder (total), and 75 patients with agoraphobia

	n	Genotype			p	Allele		p
		GG	GA	AA		G	A	
Controls	178	59 (33.1%)	91 (51.1%)	28 (15.7%)		209 (58.7%)	147 (41.3%)	
Panic (total)	109	33 (30.3%) $x^2=0.45$, $df=2$	56 (51.4%)	20 (18.3%)	0.81	122 (56.0%) $x^2=0.42$, $df=1$	96 (44.0%)	0.54
Agoraphobia	75	25 (33.3%) $x^2=0.12$, $df=2$	37 (49.3%)	13 (17.3%)	0.95	87 (58.0%) $x^2=0.02$, $df=1$	63 (42.0%)	0.92

Numbers in parentheses indicate percentages. Statistical analysis was performed by Fisher's Exact Test.

been found until now. Therefore, it is unlikely that the ethnic differences of the BDNF gene polymorphisms may be a risk factor of the development of panic disorder.

Panic disorders frequently comorbid depressive disorders. However, these results which did not support BDNF may be implicated in the putative common pathophisiology of depression and anxiety. Further research concerning common etiologies of depression and anxiety must be designed. On the other hand, dysfunction of fear-relevant memory has been proposed as a risk factor for the development of panic disorder (Berksun, 1999). In this study, panic disorder is not correlated with BDNF 196G>A (val66met) polymorphism, which particular variants may affect hippocampal function and human memory (Egan et al., 2003). Further association studies of panic disorder with other genes, which particular variants may affect emotional memory and fear-conditioning, should be done to clarify the involvement of the genes in anxiety disorders.

A number of limitations merit consideration. The current sample numbers are relatively large but not enough. These data needed to be confirmed in a much larger sample. Possible stratification resulting from different age groups in the case-control study is a point to be considered. However, we believe that one generation within 10 years may have little effect to the candidate genes of panic disorder. Though we found no significant association in this case-control study, transmission disequilibrium test (TDT) of trio samples was not performed in our study design. To replicate our findings in investigations of triads or families using TDT would be important in future work.

5. Conclusion

No significant association of the two BDNF gene polymorphisms with panic disorder has been found in our Japanese population.

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Serum brain-derived neurotrophic factor (BDNF) levels in patients with panic disorder: As a biological predictor of response to group cognitive behavioral therapy

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Abstract

Little is known about biological predictors of treatment response in panic disorder. Our previous studies show that the brain-derived neurotrophic factor (BDNF) may play a role in the pathophysiology of major depressive disorders and eating disorders. Assuming that BDNF may be implicated in the putative common etiologies of depression and anxiety, the authors examined serum BDNF levels of the patients with panic disorder, and its correlation with therapeutic response to group cognitive behavioral therapy (CBT). Group CBT (10 consecutive 1 h weekly sessions) was administered to the patients with panic disorder after consulting the panic outpatient special service. Before treatment, serum concentrations of BDNF and total cholesterol were measured. After treatment, we defined response to therapy as a 40% reduction from baseline on Panic Disorder Severity Scale (PDSS) score as described by Barlow et al. (2000) [Barlow, D.H., Gorman, J.M., Shear, M.K., Woods, S.W., 2000. Cognitive-behavioral therapy, imipramine, or their combination for panic disorder: A randomized controlled trial. JAMA. 283, 2529–2536]. There were 26 good responders and 16 poor responders. 31 age- and sex-matched healthy normal control subjects were also recruited in this study. The serum BDNF levels of the patients with poor response (25.9 ng/ml [S.D. 8.7]) were significantly lower than those of the patients with good response (33.7 ng/ml [S.D. 7.5]). However, there were no significant differences in both groups of the patients, compared to the normal controls (29.1 ng/ml [S.D. 7.1]). No significant differences of other variables including total cholesterol levels before treatment were detected between good responders and poor responders. These results suggested that BDNF might contribute to therapeutic response of panic disorder. A potential link between an increased risk of secondary depression and BDNF remains to be investigated in the future.

Keywords: Anxiety; Brain-derived neurotrophic factor; Cholesterol; Cognitive behavioral therapy; Panic disorder

1. Introduction

Panic disorder, which encompasses both biological and psychological dimensions, is a common and disabling anxiety disorder. It is often associated with agoraphobic avoidance stemming from fear of further attacks. It has a lifetime prevalence between 1.5 and 3.0% (Katschnig and Amering, 1998).

Several lines of evidence suggest that neurobiological factors might contribute to susceptibility to panic disorder (Van Megen et al., 1994; Coplan and Lydiard, 1998; Hettema

Abbreviations: BDNF, brain-derived neurotrophic factor; CBT, cognitive behavioral therapy; GAF, Global Assessment of Functioning; HAM-A, Hamilton Rating Scale for Anxiety; HAM-D, Hamilton Rating Scale for Depression; NEO-FFI, Neuroticism–Extraversion–Openness Five Factor Inventory; PCT, Panic Control Treatment; PDSS, Panic Disorder Severity Scale.

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et al., 2001). Biological challenge tests by using several agents, named as panicogens (Javanmard et al., 1999; Zedkova et al., 2003), have been shown to provoke panic attacks in susceptible patients (Gorman et al., 1997; Bocola et al., 1998). Plasma MHPG levels and heart rate were reported as correlates of non-response to drug therapy in panic disorder patients (Slaap et al., 1996). However, little is known about biological predictors of treatment response in panic disorder.

Brain-derived neurotrophic factor (BDNF), the most abundant of the neurotrophins in the brain (Lindsay et al., 1994), is suggested as a candidate molecule involved in the pathophysiology of mood disorders (Duman et al., 1997). It is also suggested that the BDNF can cross the blood–brain barrier (Pan et al., 1998) and serum BDNF levels may reflect BDNF levels in the brain (Karege et al., 2002). Recently, we have shown that serum BDNF levels are significantly lower in the antidepressant-naive depressed patients than in the treated patients or in the healthy control subjects (Shimizu et al., 2003a; Hashimoto et al., 2004). We have also reported that serum BDNF levels in the patients with anorexia nervosa and bulimia nervosa are significantly decreased as compared to normal controls (Nakazato et al., 2003). On the contrary, there was no significant difference between patients with schizophrenia and normal controls (Shimizu et al., 2003b). It has been suggested that BDNF deficiency influences the regulation of anxiety-related behavior based on serotonergic system in animals (Lyons et al., 1999; Rios et al., 2001). Taken together, it is likely that BDNF gene may be implicated in the putative common pathophisiology of depression and anxiety. Therefore, we focus on a hypothesis that the neurotrophin signaling plays a pivotal role, not only in neuronal survival and differentiation, but also in the regulation of mood and anxiety.

Cognitive-behavioral approaches to panic disorder are effective, and generally involve a combination of psychoeducation, cognitive restructuring, relaxation training, and exposure (Barlow, 1997; Martinsen et al., 1998). Assuming that BDNF may be implicated in the putative common etiologies of depression and anxiety, we examined serum BDNF levels of the patients with panic disorder, and its correlation with therapeutic response of group cognitive behavioral therapy. We also measured serum cholesterol as a putative biological marker of anxiety (Bajwa et al., 1992) and depression (Maes et al., 1994).

2. Methods

2.1. Treatment conditions

Our group cognitive behavioral therapy (CBT) program consisted of 1-h weekly sessions over a 10-week period. The program was based on Panic Control Treatment (PCT) described by Barlow et al. (2000). We modified it for closed group therapy. During group CBT program, participants were encouraged to keep daily records of dysfunctional thoughts along with self-ratings of anxiety using subjective units of distress. Exercises in each session follow a predetermined program. They include cognitive monitoring; psychoeducation and corrective information about anxiety and panic attacks; relaxation training (breathing retraining, muscle relaxation, and imagery relaxation); coping stressful events; cognitive restructuring of bodily sensations; interoceptive exposure; and situational exposure. Before and after the treatment, clinical symptoms were assessed using the 7-item Panic Disorder Severity Scale (PDSS) (Shear et al., 1997, 2001; Houck et al., 2002), the 14-item Hamilton Rating Scale for Anxiety (HAM-A) (Hamilton, 1959), and the 17-item Hamilton Rating Scale for Depression (HAM-D) (Hamilton, 1960). The DSM-IV axis V Global Assessment of Functioning (GAF) scores (American Psychiatric Association, 1994) were also rated. We defined response to therapy as a 40% reduction (improvement) from baseline on PDSS score, confirmed by questionnaires.

2.2. Subjects

A total of 42 outpatients were recruited from a panic disorder outpatient special service in Chiba University Hospital from May 2001 to May 2003. All of them fulfilled the DSM-IV (American Psychiatric Association, 1994) criteria for panic disorder, without or with agoraphobia. The ratio of panic disorders with agoraphobia to without agoraphobia was 6.9:3.1. The 12 patients suffered from a comorbid second disorder; depressive disorders ($n=11$), social anxiety disorder ($n=1$), or olfactory reference syndrome ($n=1$). A careful diagnostic assessment was performed to rule out other major Axis I disorders, including schizophrenia, bipolar disorders, and substance-related disorders. Subjects with any other diagnosed mental and/or physical illness were excluded from the study.

Healthy individuals who were matched for age were included in the control group ($n=31$; male=5, female=26; age, 32.5 [S.D. 12.6] year-old). We selected healthy control subjects who did not have any medical or psychiatric diagnosis after our clinical interview. No one was a regular drinker or had ever taken any substance except for smoking cigarettes. Written informed consent was obtained from all subjects. All subjects including patients and controls were ethnic Japanese. The ethics committee of Chiba University Graduate School of Medicine approved the present study.

2.3. Procedures

Serum samples from the patients and normal controls were collected between 11:00 and 12:00 a.m., and were stored at −80 °C until needed for the assay. Serum BDNF levels were measured using the BDNF Emax Immunoassay System kit (Promega; Madison, WI, USA) according to the manufacturer's instructions described previously (Shimizu et al., 2003a; Nakazato et al., 2003). Briefly, 96-well plates were coated

with anti-BDNF monoclonal antibody and incubated at 4 °C for 18 h. The plates were incubated in a blocking buffer for 1 h at room temperature. The samples and BDNF standards were maintained at room temperature under conditions of shaking for 2 h, followed by washing with the appropriate washing buffer. The plates were incubated with antihuman BDNF polyclonal antibody at room temperature for 2 h, washed, and incubated with anti-IgY antibody conjugated to horseradish peroxidase for 1 h at room temperature. The plates were incubated in peroxidase substrate and tetramethylbenzidine solution to produce a color reaction. The reaction was stopped with 1 M hydrochloric acid; the absorbance at 450 nm was measured with an automated microplate reader (Emax, Molecular Devices; USA).

2.4. Data analyses

The data are presented as the mean±standard deviation (S.D.). Statistical calculations were performed using the statistical software package SPSS 12.0 Base System and Exact tests for Windows (SPSS Inc., Chicago, IL, USA). The extended Fisher's Exact Test was used for categorical comparisons. Homogeneity of variance was assessed by F test. The student's *t*-test was employed for the continuous variables with Bonferroni correction for multiple pairwise comparisons (total time of compare was five.). For the multiple group parametric comparisons, analysis of variance (ANOVA) was used. The significance of individual differences was evaluated using Schefee test, provided that the ANOVA was significant. Each parameter in this study had an approximately normal distribution, and correlations were examined with a parametric method that can be applied to situations in which at least one variable has a normal distribution (Altman, 1991). Thus, the relationship between two variables was examined using Pearson's correlation coefficient. The p values < 0.05 were considered statistically significant.

3. Results

After 10-week group CBT, the patients were divided into two groups; a good-outcome group and a poor-outcome group. There were 26 good responders ($n=26$; male=4, female=22; age, 31.5 [S.D. 8.1] years) and 16 poor responders ($n=16$; male=5, female=11; age, 33.6 [S.D. 9.2] years).

Table 1 shows the participant characteristics and serum BDNF levels of all subjects. Age- and sex-matching was found to be successful, since there were no significant differences among the three groups (Table 1). The mean PDSS scores indicate a moderate-to-average severity. There were no significant differences on baseline (at pre-treatment) PDSS, HAM-A, HAM-D, and GAF scores between the two groups (Table 1). On the other hand, the scores of PDSS and HAM-A at post-treatment in the poor response group were significantly higher than those in the good response group (Table 1). In addition, the scores of GAF at post-treatment in the poor response group were significantly lower than those in the good response group (Table 1). These differences of PDSS, HAM-A, and GAF were consistent with the treatment response in the two groups.

Table 1
Characteristics of the patients with panic disorder and the normal controls

	Good response	Poor response	Normal controls	p value
Sex: F/M	22/4	11/5	26/5	n.s.[a]
Age, years	31.5±8.1	33.6±9.2	32.5±12.6	n.s.[b]
Onset, years	25.9±6.7	27.0±9.6		n.s.[c]
Duration, years	5.1±6.0	7.2±10.3		n.s.[c]
PDSS (pre)	1.40±0.70	1.28±0.77		n.s.[c]
PDSS (post)	0.57±0.37	1.27±0.62		0.0005[c]
HAM-A	12.5±5.6	12.5±5.3		n.s.[c]
HAM-D	12.6±6.6	12.9±5.0		n.s.[c]
GAF	58.2±7.2	55.2±7.5		n.s.[c]
Serum cholesterol	191.2±35.2	189.1±32.0		n.s.[c]
Serum BDNF	33.7±7.5	25.9±8.7	29.1±7.1	0.0094[d,e]

PDSS, Panic Disorder Severity Scale; pre, pre-treatment; post, post-treatment; HAM-A, Hamilton Rating Scale for Anxiety; HAM-D, Hamilton Rating Scale for Depression; GAF, Global Assessment of Functioning; BDNF, brain-derived neurotrophic factor.
Normally distributed data are presented as mean±standard deviation (S.D.).
[a] The comparison among three groups was performed using the extended Fisher's Exact Test.
[b] The comparison among three groups was performed using analysis of variance (ANOVA).
[c] The comparison between two groups was performed using *t*-test (two-tailed).
[d] The significant difference between good response and poor response.
[e] The comparison among three groups was performed using analysis of variance (ANOVA). The significance of individual differences was evaluated using Schefee test.

No significant difference of serum total cholesterol between good responders and poor responders was detected (Table 1). Table 1 shows the serum BDNF levels in the good response group, the poor response group, and the control group. One-way ANOVA indicated significant differences in serum BDNF among groups ($F=5.344$, $df=2,70$, $p=0.0069$). Scheffe tests showed that serum BDNF levels in the poor response group (mean, 25.9 ng/ml, [S.D. 8.7]) were significantly lower than those in the good response group (mean, 33.7 ng/ml, [S.D. 7.5], $p=0.0094$). However, there were no significant differences between control group (mean, 29.1 ng/ml, [S.D. 7.1]) and either good responder or poor responder.

There were no significant correlations between serum BDNF levels and other clinical variables including age at onset, duration of illness, PDSS, HAM-A, HAM-D, and GAF score.

4. Discussion

4.1. Serum BDNF

This is the first study to examine serum BDNF levels in patients with panic disorder. The major findings of this

study were that (1) no difference of serum BDNF levels between patients and controls and (2) reduced serum BDNF levels in poor responders, compared to good responders. As the advantage of combined treatment of CBT and pharmacotherapy may vary across the anxiety disorders (Foa et al., 2002), the reduced serum BDNF in poor responders suggested that there might be the differences of biological backgrounds between good and poor responders for group CBT.

4.2. Serum cholesterol

On the other hand, there was no significant difference of serum cholesterol level between good and poor responders. The effects of reduced serum total cholesterol and other lipids have been implicated as a predictor of suicidal behavior in major depression (Maes et al., 1994). In contrast to depression, several researchers reported that the patients with panic disorder had significantly higher serum cholesterol levels than did the normal control subjects (Hayward et al., 1989; Bajwa et al., 1992), whereas other studies have resulted in inconsistent results (Tancer et al., 1990). The result suggested no effect of serum cholesterol level on treatment response for panic disorder patients.

4.3. BDNF, serotonin, and anxiety

The panic-enhancing effect of tryptophan depletion and the panic-protective effect of tryptophan administration in patients with panic disorder suggest that the serotonergic system is causally involved in anxiety-related mechanisms (Maron et al., 2004); Klaassen et al., 1998). BDNF conditional mutant mice had higher levels of anxiety correlated with serotonergic dysfunction (Rios et al., 2001). Thus, it seems that BDNF deficiency influences the regulation of anxiety-related behavior based on serotonergic system. From this viewpoint, low serum BDNF levels may keep patients' anxiety to higher levels and result in poor response of panic treatment. On the other hand, no associations of the BDNF gene polymorphisms with panic disorder have been found (Lam et al., 2004; Shimizu et al., in press). These findings suggest that the BDNF gene variants may not play a major role in the pathogenesis of panic disorder. Further studies exploring the relationship between BDNF gene polymorphisms and treatment response in panic disorder may be appropriate.

4.4. BDNF and comorbid depression

Proven effects of the selective serotonin reuptake inhibitors (SSRIs) for a broad range of mood spectrum disorders, including major depressive disorder, anxiety disorders, bulimia nervosa, posttraumatic stress disorder, and dysthymia, suggest that they share one or more common etiologies. Moreover, genetic and neurobiological similarities between depressive and anxiety disorders suggest that they may belong to the same spectrum (Kendler et al., 1992). Our data do not directly support the view that BDNF is involved in comorbid biology of depression and anxiety. However, it is possible that there is a subgroup of panic patients who have the biological vulnerability to depression, while others do not. Low serum BDNF levels may reflect the biological vulnerability to depression. Lang et al. (2004) have found a negative correlation between the BDNF serum concentration and the depression-related factor neuroticism of the Neuroticism–Extraversion–Openness Five Factor Inventory (NEO-FFI). They stated that low BDNF levels in healthy humans with depressive personality traits might constitute a risk marker, reflecting a personality profile that is linked to vulnerability to mood disorders, and that BDNF may be central to the development of depressive mood states. Panic disorder and major depressive disorder were associated with high neuroticism (Bienvenu et al., 2001). Moreover, panic disorder is often accompanied by depression, and it is not uncommon for patients with panic disorder to suffer from major depression in the future (Gorman and Coplan, 1996; Kaufman and Charney, 2000). Comorbid depression was reported as putative predictors of non-response for panic treatment (Lecrubier, 1998; Slaap and den Boer, 2001). However, several studies have found that major depression is not necessarily associated with a poor response to treatment for panic disorder (Lesser et al., 1998; Maddock and Blacker, 1991; McLean et al., 1998). The result of no difference in HAM-D score at the baseline between poor and good responders in this study suggested that severity of depressive symptoms might not influence outcomes of group CBT responses. It is possible that panic patients with low serum BDNF levels have biological vulnerability to anxiety and depression. Further large-scale follow-up studies about serum BDNF of patients with panic disorder will be necessary to evaluate the response of panic treatment and the onset of comorbid depression.

5. Conclusions

In conclusion, our data suggested that low serum BDNF levels might be associated with poor responses of group CBT, though those in panic disorders were indistinguishable from controls. It would be of interest to study the mechanisms underlying peripheral changes in BDNF, and to search for the relationship between BDNF and the biological vulnerability to anxiety and depression.

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Dysfunction of Glia-Neuron Communication in Pathophysiology of Schizophrenia

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Abstract: Multiple lines of evidence suggest that genetic factors and environmental factors as well as a dysfunction in the glutamatergic neurotransmission contribute to the pathophysiology of schizophrenia. Communication between neurons and glia is essential for axonal conduction, synaptic neurotransmission, and information processing, and thus is required for normal functioning of the nervous system during development and throughout adult life. A number of studies have demonstrated that the substances which communicate between neurons and glia are altered in the blood, cerebrospinal fluid (CSF), and postmortem brain samples of schizophrenic patients. These findings suggest that neuron-glia communication might be impaired in the brains of schizophrenic patients. In this article, we review the imbalance of neuron-glia communication presented in the neurodevelopmental hypothesis as well as the glutamate hypothesis of schizophrenia. First, we discuss the role of growth factors (e.g., basic fibroblast growth factor (bFGF), epidermal growth factor (EGF), midkine), brain-derived neurotrophic factor (BDNF) and cytokines in the pathophysiology of schizophrenia. Second, we focus on the role of endogenous substances (glutamate, glutamine, D-serine, kynurenic acid, and glutathione), which modulate the NMDA receptor function, in the pathophysiology of schizophrenia.

Keywords: NMDA receptor, growth factors, neurotrophic factors, cytokines, D-serine, schizophrenia.

INTRODUCTION

Schizophrenia is a chronic, debilitating psychotic mental disorder that affects about one percent of the world's general population. This illness has varied and ominous symptoms that generally begin in late adolescence or early adulthood and usually continue throughout life. The cause of schizophrenia is unknown, but multiple lines of evidence suggest that genetic factors and environmental factors (eg, prenatal and perinatal events - including maternal influenza, rubella, malnutrition, diabetes mellitus, smoking during pregnancy and obstetric complications) contribute to the pathophysiology of this illness [1-9]. Furthermore, schizophrenia may be a neurodevelopmental and progressive disorder with multiple biochemical abnormalities involving the dopamine, serotonin, acetylcholine, glutamate, and γ-aminobutyric acid (GABA) systems [8-16].

Glia are the support cells of the nervous system. They fill the interstices between neuronal cell bodies, ensheathe axons, and surround the blood vessels of the brain. The two principal types in the central nervous system (CNS), astrocytes and oligodendroglia, are differentiated by their appearance and functions. Astrocytes are an ubiquitous type of glia cells that are defined in part by what they lack: axons, action potentials, and synaptic potentials [17,18]. Astrocytes greatly outnumber neurons, often by 10:1, and occupy 25 to 50% of brain volume. Several lines of evidences have revealed some of the functions of astrocytes and the essential nature of interactions between neurons and astrocytes. Communication between neurons and glia is essential for axonal conduction, synaptic neurotransmission, and information processing, and thus is required for normal functioning of the nervous system during development and throughout adult life [17,18]. In this article we review the role of glia, especially astrocytes, in the pathophysiology of schizophrenia. In particular, based on the neurodevelopmental hypothesis and/or glutamate hypothesis of schizophrenia, we discuss the role of communication between neurons and glia in the pathophysiology of schizophrenia.

NEURODEVELOPMENTAL HYPOTHESIS

Neurodevelopmental models for schizophrenia have proposed both genetic (polygenetic) and environmental risk factors for the development of the disorder, and the process of the illness is postulated to involve early (pre/perinatal) and late (usually adolescent) developmental brain abnormalities [4,19-22]. Several lines of evidence suggest that cytokines, growth factors, and neurotrophic factors play particular signaling roles within the brain to produce neurochemical, neuroendocrinological, neuroimmunological and behavioral changes, and that these substrates also play a key role in the pathophysiology of schizophrenia [23-26]. During development and in adulthood, astrocytes exhibit a regionally selective ability to synthesize and release a host of growth factors, neurotrophic factors, and cytokines. Therefore, it is of great interest to examine the role of these substances in the pathophysiology of schizophrenia.

Basic Fibroblast Growth Factor (bFGF)

Basic fibroblast growth factor (bFGF, also called FGF-2 or heparin-binding growth factor 2) is the most abundant

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