

Figure 1. Group averaged time-frequency maps of ASSR-power for each hemisphere. The color scales signify ASSR-power. HC, healthy controls; BD, patients with bipolar disorder.
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Dipole Moments and Locations

Table 4 shows the group mean dipole moments for each group. A repeated measures ANOVA demonstrated significant main effects of group ($F [1,37] = 18.9, p = 0.03$), frequency ($F [3,35] = 53.8, p < 0.0001$), and hemisphere ($F [1,37] = 8.8, p = 0.005$), and significant frequency-by-group ($F [3,35] = 4.9,$

$p = 0.003$) interactions, with no other significant interactions ($0.32 \leq p \leq 0.64$). To delineate the significant frequency-by-group interaction, group differences were compared with t -tests using the average of both hemispheres for each frequency. Participants with BD showed significantly reduced dipole moments at 30-Hz ($t [37] = 2.0, p = 0.05$), 40-Hz ($t [37] = 3.1, p = 0.003$), and 80-Hz ($t [37] = 2.0, p = 0.05$), while no significant group differences were observed for 20-Hz ($t [37] = -0.66, p = 0.51$).

With respect to dipole locations, a multivariate ANOVA (MANOVA) demonstrated no group effect and no interactions related to group, indicating that there were no significant group differences for dipole locations of the ASSR (see Table 5).

Discussion

The current study investigated the MEG-ASSR elicited by click trains of 20, 30, 40 and 80 Hz, and symptom-ASSR associations in patients with BD. The major findings in this study were: [1] BD patients exhibited bilaterally reduced mean ASSR power and PLF to 30-, 40- and 80- Hz stimulation, with no significant reduction to 20- Hz stimulation; [2] there was a significant negative correlation between right hemisphere 80 Hz-ASSR-power values and SIGH-D scores in patients with BD; [3] No significant group differences were observed in the dipole locations of ASSR.

To our knowledge, this is the first study to demonstrate both high and low gamma band ASSR deficits in patients with BD. Previous EEG studies reported reduced 20-, 30-, 40-, and 50-Hz

Table 2. Mean ASSR-power.

		HC (n = 25)	BD (n = 14)	df	t	p
		(fT/cm)	(fT/cm)			
20 Hz	Left	253.9±162.1	220.0±235.8	37	0.53	0.6
	Right	285.4±193.2	272.6±252.0	37	0.18	0.86
30 Hz	Left	264.4±176.0	152.5±80.1	37	2.2	0.03
	Right	318.3±187.0	166.8±112.2	37	3.2	0.003
40 Hz	Left	505.8±299.7	292.1±240.9	37	2.3	0.028
	Right	625.1±302.2	370.5±275.3	37	2.6	0.013
80 Hz	Left	76.6±73.5	46.1±35.7	37	1.7	0.09
	Right	96.5±84.8	48.9±39.7	37	2.4	0.023

Data are given as mean ± SD.
ASSR: auditory steady state response, HC: healthy controls, BD: patients with bipolar disorder.
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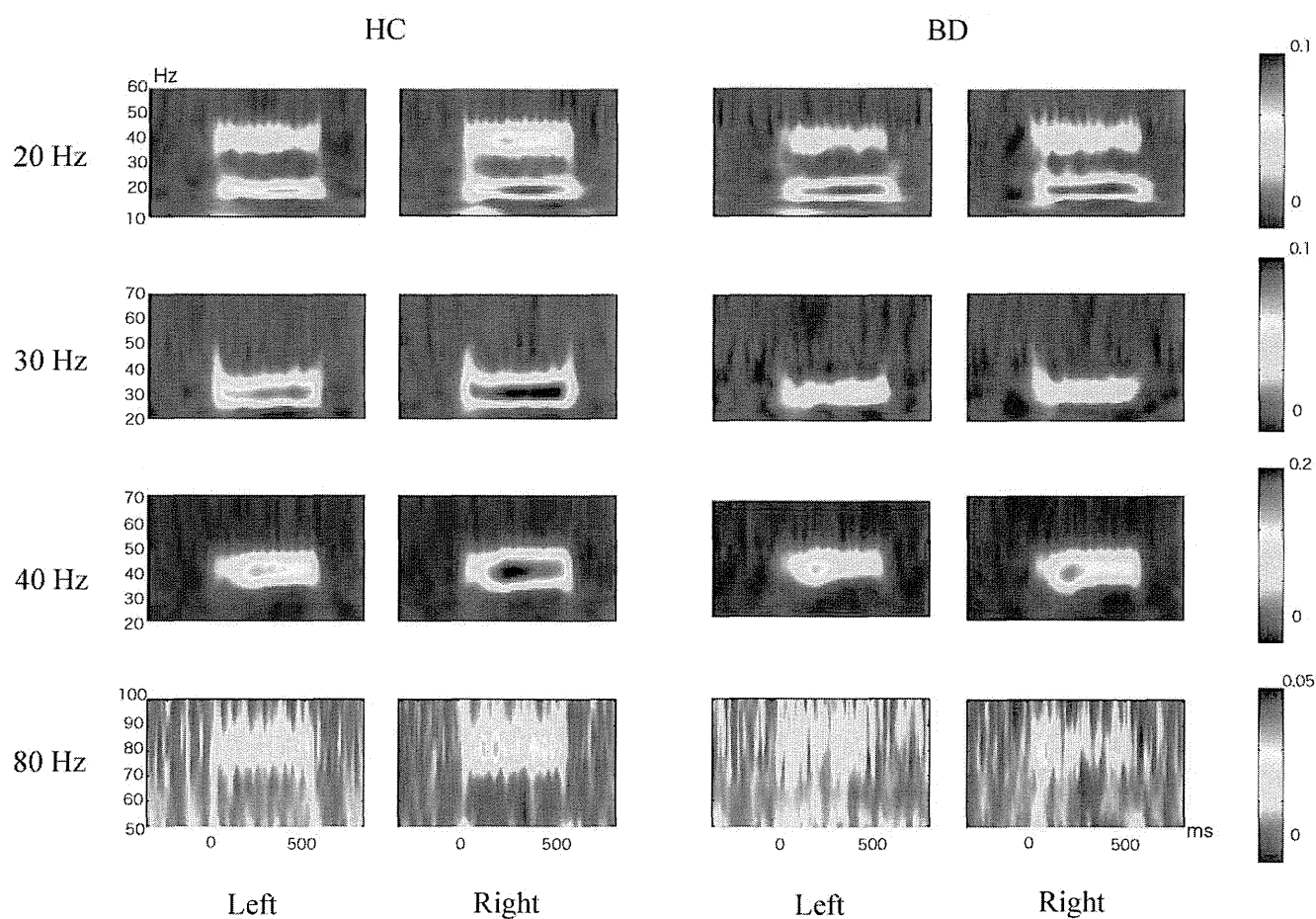


Figure 2. Group averaged time-frequency maps of ASSR-PLF for each hemisphere. The color scales signify ASSR-PLF value. HC, healthy controls; BD, patients with bipolar disorder.
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ASSR in people with BD [12] and reduced ASSR at 30 and 40 Hz in people with psychotic BD [23]. Rass et al. reported reduced ASSR power at 40 Hz and reduced ASSR synchronization to 40 Hz- and 50 Hz- stimulation in BD patients [13]. One MEG study reported that patients exhibited reduced right ASSR to 40 Hz- stimulation [11]. The present results partially support these previous findings. For the high gamma band, oscillations can be

useful markers of cortical activity during a variety of cognitive tasks [21] and may reflect a fundamental aspect of temporal coding in cortical networks [22]. Additionally, different functions between beta and gamma oscillations have been suggested. Beta oscillations are related to sensory gating, attention and perception, and gamma oscillations are associated with memory and consciousness

Table 3. Mean ASSR PLF.

		HC (n = 25)	BD (n = 14)	df	t	p
20 Hz	Left	0.038±0.022	0.027±0.026	37	1.3	0.2
	Right	0.043±0.026	0.031±0.028	37	1.4	0.17
30 Hz	Left	0.044±0.031	0.022±0.011	37	3.1	0.004
	Right	0.053±0.03	0.023±0.012	37	4.3	<0.001
40 Hz	Left	0.091±0.051	0.052±0.038	37	2.5	0.018
	Right	0.11±0.046	0.063±0.042	37	3.3	0.002
80 Hz	Left	0.013±0.011	0.008±0.007	37	1.3	0.188
	Right	0.016±0.011	0.007±0.006	37	2.6	0.013

Data are given as mean ± SD.
ASSR: auditory steady state response, PLF: phase locking factor,
HC: healthy controls, BD: patients with bipolar disorder.
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Table 4. Dipole moments of the ASSR.

		HC (n = 25)	BD (n = 14)	df	t	p
		(nA/m)	(nA/m)			
20 Hz	Left	3.5±1.2	3.8±1.6	37	−0.55	0.59
	Right	3.6±1.4	3.9±1.3	37	−0.47	0.64
30 Hz	Left	2.9±1.1	2.5±1.1	37	1.1	0.29
	Right	3.9±1.4	2.9±0.9	37	2.4	0.02
40 Hz	Left	3.7±2.2	2.2±1.0	37	2.3	0.03
	Right	3.9±1.4	2.8±1.0	37	2.6	0.01
80 Hz	Left	1.3±0.8	1.1±0.3	37	1.0	0.31
	Right	1.6±0.9	1.2±0.4	37	1.5	0.14

Data are given as mean ± SD.
HC: healthy controls, BD: patients with bipolar disorder.
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Table 5. Dipole locations of the ASSR.

		Left (mm)			Right (mm)		
		x	y	z	x	y	z
20 Hz	HC (n = 25)	−45.6±4.9	6.6±12.7	61.6±9.0	49.1±7.3	9.5±9.5	61.4±12.8
	BD (n = 14)	−47.3±6.8	0.16±17.4	58.0±7.4	49.5±5.5	5.1±13.8	57.2±8.9
30 Hz	HC	−45.6±4.3	3.9±11.4	63.0±10.7	48.2±5.9	8.7±9.5	60.1±9.6
	BD	−45.8±5.1	3.0±16.4	60.9±8.0	47.5±7.8	5.7±14.7	59.4±8.0
40 Hz	HC	−47.0±5.2	4.3±13.0	60.9±10.9	50.6±11.8	9.3±10.5	58.3±14.3
	BD	−49.3±7.7	2.2±16.0	52.4±16.2	51.2±6.9	2.7±15.4	56.8±7.3
80 Hz	HC	−48.3±6.5	3.0±14.2	51.9±15.8	51.9±7.1	5.6±16.2	54.4±12.3
	BD	−46.7±5.4	−0.33±17.2	49.7±17.6	50.7±6.1	5.8±11.9	44.1±20.3

Data are given as mean ± SD. HC: healthy controls, BD: patients with bipolar disorder. The zero point was the mid-point of the line connecting the bilateral preauricular points. The x-axis was the line from the left to the right with positive values toward the right, the y-axis was the postero-anterior line with positive values presented anteriorly, and the z-axis was the ventro-dorsal line with positive values located dorsally. doi:10.1371/journal.pone.0039955.t005

as well as attention and perception [4]. Future studies should investigate the relationship between ASSRs and neural oscillatory activities during cognitive tasks in patients with BD, to clarify ASSR-cognitive related oscillations.

It has been suggested that GABAergic dysfunction plays a role in BD patients [9], [10]. The administration of mood stabilizers, such as valproate, carbamazepine, lithium, and lamotrigine, has been reported to increase GABA turnover in the mouse and rat brain [24–27]. In addition, valproate has been shown to increase plasma GABA levels in humans, suggesting that it enhances GABA activity in the human brain [28–30]. Recent *in vitro* studies have suggested that beta2 (20–30 Hz) oscillations are different from gamma oscillations in terms of generation. For instance, Cunningham et al. reported that the fast rhythmic bursting neurons in layer II/III play a crucial role in the generation of gamma oscillations [31]. GABAergic neurons have been reported to play a crucial role in the primary generation of gamma oscillations and their local synchronization [32]. In addition, direct electronic coupling through gap junctions between inhibitory neurons also contributes to the synchronization of gamma oscillations [33]. Both low and high gamma band oscillations can be generated by recurrent inhibition, but differ in their relationship to the spiking activity of parvalbumin-containing interneurons; in terms of their pharmacological modulation profiles as well as their layer specificity [5]. Conversely, an *in vitro* study by Roopun et al. reported that beta2 oscillations occurred in layer V pyramidal cells [34]. Moreover, this study indicated that beta2 oscillations are involved in gap junctional coupling and are independent of chemical synaptic transmission. The present study reported gamma band ASSR reduction and no significant reduction of beta band ASSR in BD patients, suggesting that BD might be characterized by hypofunction of GABA interneurons related to the fast rhythmic bursting neurons in layer II/III.

The present results revealed a significant negative correlation between right hemisphere 80 Hz-ASSR-power values and SIGH-D scores, indicating that BD patients with more severe depressive symptoms exhibited more reduced 80 Hz-ASSR power in the right hemisphere. However, this correlation should be confirmed in a larger sample. Rass et al. recorded 20-, 30-, 40-, and 50-Hz ASSRs in BD, and investigated associations between ASSRs and clinical status, cognitive function, and pharmacological treatment [13]. They reported that BD patients taking psychotropic

medication exhibited decreased PLF relative to BD patients who had withdrawn from medication. In this study, mood state, psychotic features, cognitive performance, smoking, or history of substance use disorder were unrelated to ASSRs. Future studies that incorporate an assessment of patients before and after medication would be helpful in clarifying the associations between clinical symptoms and ASSR deficits in people with BD.

Reite et al. investigated ASSR source locations in people with BD. In normal control subjects the right hemisphere source was superior to the left, but no such hemisphere asymmetry was observed in BD patients [1]. However, the present results revealed no significant group differences in the dipole locations of ASSR. The heterogeneity of BD patients may account for this discrepancy. For example, the BD patients in the present study had never experienced psychotic symptoms and the sample was predominantly female, while Reite et al. examined 10 individuals with BD who had a history of psychosis and seven with no history of psychosis [1]. The ASSR of BD patients with a history of psychosis requires further investigation.

Several potential limitations of the current study should be considered. We were unable to exclude any treatment effects of mood stabilizers, neuroleptics or antidepressants on ASSR abnormalities in BD patients, and we found significant negative correlations between right hemisphere 40 Hz-ASSR and valproate dosage. Cross-sectional studies with more homogenous patient groups (drug-free vs medicated), as well as studies that assess participants before and after treatment with specific medications (thus controlling for health status) are required in future. Moreover, the effects of gender, and the ASSR of BD patients with a history of psychosis require further investigation.

Overall, the current study showed that BD patients exhibit reduced low and high gamma ASSR power and PLF bilaterally, with no significant beta band ASSR reduction. BD is characterized by gamma band ASSR deficits, which may be associated with dysfunctions of GABA inhibitory interneuronal activity.

Materials and Methods

Subjects

MEG data obtained from 14 (4 males, 10 females) individuals with BD and 25 (10 males, 15 females) healthy controls (HC) were analyzed in the present study. The data from 22 of the 25 HC participants were analyzed in our previous study [18]. The data

from 14 BD and 3 HC participants were newly recorded and analyzed for the present study. MEG recording was conducted between September 2007 and December 2009 for the HC group, and from July 2007 to May 2010 for the BD patients. We used the same recording equipment for both groups. All participants had normal hearing, were aged 20–60 years and were right-handed [assessed via Edinburgh Inventory [35]]. After being given a complete description of the study, all participants gave written informed consent in accord with the regulations of the Ethics Committee of the Graduate School of Medical Sciences, Kyushu University. Two senior clinical psychiatrists confirmed that all subjects had the ability to consent to participate in the examination. The exclusion criteria were: 1) neurological illness or major head trauma that would result in abnormal electroencephalography; 2) electroconvulsive therapy; 3) alcohol or drug dependence; 4) alcohol or drug abuse within the past five years; or 5) a verbal intelligence quotient below 75. HCs were screened using the Structured Clinical Interview (SCID), non-patient edition. No HCs exhibited any Axis-I psychiatric disorders, nor did their first-degree relatives.

All patients were recruited from Kyushu University Hospital and were diagnosed based on the SCID-DSM IV and medical records. No BD patients exhibited psychotic episodes. The patients were assessed using the Young Mania Rating Scale (YMRS) [36] and SIGH-D [37]. Demographic data for all subjects are presented in Table 1. Based on the criteria for depression [38] and euthymia [39], seven patients showed mild depression and seven were euthymic. Eight patients were receiving neuroleptic medication [typical neuroleptics (1/8 patients), atypical (7/8)], with a mean daily dose equivalent to 314 ± 201 mg of chlorpromazine [40]. Regarding mood stabilizers, lithium was administered with a mean daily dose of 750 ± 141 mg in eight BD patients, and valproate was administered with a mean daily dose of

844 ± 445 mg in nine BD patients. The footnote in Table 1 lists the patients' medication.

Stimuli

The stimuli consisted of 1-msec clicking sounds, presented binaurally as trains of clicks for each stimulus frequency (20, 30, 40 and 80 Hz). The duration of each click train was 500 msec, and the intensity of the click trains was 80 dB sound pressure level. The inter-train interval was 500 msec. The mean number of presented click trains in one block was 313.9 ± 105.7 for HC and 306.4 ± 60.4 for BD, and there was no significant group difference ($t[37] = 0.24$, $p = 0.81$). The order of blocks was randomized across subjects.

Data Acquisition and Processing

The MEG signals were acquired using a whole-head, 306-channel sensor array (Vectorview; ELEKTA Neuromag, Helsinki, Finland). In this study, we analyzed MEG data recorded from 22-channel, planar-type gradiometers located at the sensor exhibiting the strongest response. This procedure was conducted for each hemisphere (Figure 3) based on our previous methods [18]. Prior to recording, four head position indicator (HPI) coils were attached to the scalp, and a three-dimensional (3D) digitizer was used to measure the anatomical landmarks of the head with respect to the HPI coils. The precise location of the head with respect to the sensor array was determined using the HPI coils. A band pass filter for recording was set to 0.01–330 Hz, and the sampling rate was 1 kHz. The subjects were instructed to keep their eyes open, remain attentive and listen to the trains of clicks presented through earphones. A spatio-temporal signal space separation (tSSS) method was applied off-line to the recorded raw data [41]. tSSS-reconstructed raw data with signal variations exceeding 4000 fT were excluded, and 200 responses were averaged for each type of stimulus as a result. The data were

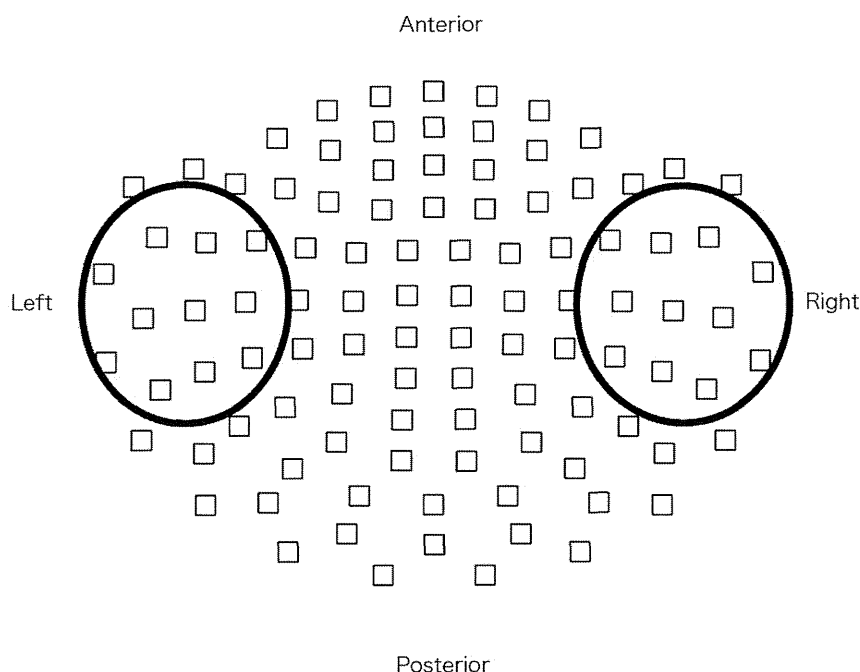


Figure 3. Layout of the measured channels. The MEG signals were acquired using a whole-head, 306-channel sensor array comprised of 102 identical triple-sensor elements. Each sensor consisted of two orthogonal planar-type gradiometers and one magnetometer. We used 11 sensors (a 22-channel orthogonal gradiometer) around the location that elicited the strongest response in each hemisphere. Circled squares indicate the sensors used for analysis.

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averaged with the following conditions: the analyzed period included the duration 400 ms before and 900 ms after stimulus onset.

Frequency Analysis

We used an estimation of the time-frequency energy based on the wavelet transform of the signal. The signal was convoluted by complex Morlet wavelets $\omega(t, f_0)$ having a Gaussian shape with the wavelet being centered at the center frequency f_0 and time t : $\omega(t, f_0) = A \cdot \exp(-t^2/\sigma^2) \exp(2i\pi f_0 t)$, where $2\pi f_0 \sigma = 7$ in 1-Hz steps. Wavelets were normalized so that their total energy was 1, with the normalization factor A equal to $(\sqrt{2\pi}\sigma)^{-1}$. We defined the squared modulus of the result of the convolution of a complex wavelet $\omega(t, f_0)$ with the averaged responses $s(t)$: power $(t, f_0) = |\omega(t, f_0) \otimes s(t)|^2$ as the ASSR-power, where the symbol \otimes indicates convolution. The square-root transform was applied to the ASSR-power for normalization. We also calculated the ASSR-phase-locking factor (PLF) using the following formula: PLF

$(t, f_0) = \frac{|\sum_i \frac{\omega(t, f_0) \otimes s(t)}{|\omega(t, f_0) \otimes s(t)|}|}{N}$. The PLF ranges from 0 (purely non-phase-locked activity) to 1 (strictly phase-locked activity). In calculating the power and PLF, we applied a baseline correction (from -200 to -100 msec). The mean power and PLF from 0–500 msec for each stimulus were averaged across 10-Hz bands.

Dipole Moments and Source Localization

The averaged responses were digitally filtered using a Butterworth filter (band pass; 15–25 Hz for the 20 Hz stimulation, 25–35 Hz for 30 Hz, 35–45 Hz for 40 Hz, and 75–85 Hz for 80 Hz). A single moving equivalent current dipole source model was applied, and dipole fits in each hemisphere were calculated by a

least-squares fit. Single source dipole localization was performed for each time-point for 0–500 msec after stimulus onset. Only dipoles with goodness-of-fit criteria (>0.9) were chosen. The dipole locations were expressed by x, y, and z-coordinates.

Statistical Analysis

The mean ASSR powers and PLF were analyzed using a repeated measures ANOVA with group (BD or HC) as a between-subjects factor, and frequency (20, 30, 40 or 80 Hz) and hemisphere (left or right) as within-subjects factors. When significant interactions involving the group factor were identified, *post-hoc* analyses were conducted using t-tests. Additionally, 40 Hz harmonic ASSR powers and PLF to 20 Hz stimulation were analyzed using a repeated measures ANOVA with group as a between-subjects factor, and hemisphere as a within-subjects factor. For dipole locations, MANOVA was performed with group as a between-subjects factor, and frequency, hemisphere and axis (x, y or z) as within-subjects factors. Degrees of freedom were adjusted with the Huynh-Feldt epsilon for factors with more than two levels. Spearman's rho was used for correlation analyses. All results were considered significant at $p \leq 0.05$.

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Author Contributions

Conceived and designed the experiments: TO RT SK. Performed the experiments: YO NO IN. Analyzed the data: YO RT SH YH. Contributed reagents/materials/analysis tools: TU TM. Wrote the paper: YO TO SK.

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Minocycline Modulates Human Social Decision-Making: Possible Impact of Microglia on Personality-Oriented Social Behaviors

Takahiro A. Kato^{1,2*}, Motoki Watabe^{3*}, Sho Tsuboi⁴, Katsuhiko Ishikawa⁵, Kazuhide Hashiya⁵, Akira Monji¹, Hideo Utsumi², Shigenobu Kanba¹

1 Department of Neuropsychiatry, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan, **2** Innovation Center for Medical Redox Navigation, Kyushu University, Fukuoka, Japan, **3** Graduate School of Economics, Waseda University, Waseda, Japan, **4** Department of Psychology, Graduate School of Letters, Kyoto University, Kyoto, Japan, **5** Graduate School of Human-Environment Studies, Kyushu University, Fukuoka, Japan

Abstract

Background: Microglia, one of the glial cells, play important roles in various brain pathologies including psychiatric disorders. In addition, microglia have recently been proved to monitor synaptic reactions via direct-touching even in normal brain. Human microglia may modulate various social/mental functions, while microglial social/mental roles remain unresolved especially in healthy humans. There is no known drug with the specific effect of modulating microglia. Therefore, using minocycline, a tetracycline antibiotic and the most famous microglial inhibitor, is one of the best alternative approaches to clarify microglial functions on human social/mental activities.

Methodology/Principal Findings: We conducted a double-blind randomized trial of trust game, a monetary decision-making experiment, with ninety-nine human adult males who decided how much to trust an anonymous partner after a four-day administration of minocycline. Our previous pilot trial indicated a positive effect of minocycline, while the underlying mechanisms were not clarified. Therefore, in this trial with larger samples, we additionally measured the effects of anxiety and personality. The monetary score in trust game was significantly lower in the minocycline group. Interestingly, participants' ways of decision-making were significantly shifted; cooperativeness, one component of personality, proved to be the main modulating factor of decision-making in the placebo group, on the other hand, the minocycline group was mainly modulated by state anxiety and trustworthiness.

Conclusions/Significance: Our results suggest that minocycline led to more situation-oriented decision-making, possibly by suppressing the effects of personality traits, and furthermore that personality and social behaviors might be modulated by microglia. Early-life events may activate human microglia, establish a certain neuro-synaptic connection, and this formation may determine each human's personality and personality-oriented social behaviors in later life. To explore these mechanisms, further translational research is needed.

Trial Registration: UMIN clinical trial center UMIN000004803

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* E-mail: takahiro@npsych.med.kyushu-u.ac.jp (TAK); motokiw@gmail.com (MW)

These authors contributed equally to this work.

Introduction

Microglia are one of the glial cells with immunological/inflammatory functions, and contribute to various brain pathologies; not only in neurodegenerative diseases [1,2,3] but also in psychiatric disorders such as schizophrenia and autism [4,5,6]. Minocycline, a tetracycline antibiotic, is known as the most famous microglial inhibitor [7], which has recently been applied to brain diseases such as stroke and neurodegenerative diseases [8,9]. In addition, minocycline has been suggested to be an effective drug for psychiatric disorders [10,11]. These reports suggest that

inhibiting microglial activation may modulate human social and mental activities, and rodent studies have indicated this possibility [12,13].

Rodent microglia have recently been shown to monitor synaptic reactions via direct-touching not only in pathological brain but also in normal brain [14,15,16,17], and have proved to play important roles in normal brain development such as synaptic pruning [18]. Neurons and neuronal networks including synapses have been dominantly believed to play crucial roles in human social/mental activities. The above-mentioned evidence indicates

that human microglia may modulate various social/mental functions, while microglial social/mental roles continue to remain unresolved especially in healthy humans.

There is no known drug with the specific effect of modulating microglia. Therefore, using minocycline, a tetracycline antibiotic and the most famous microglial inhibitor, is one of the best alternative approaches to clarify microglial functions on human social/mental activities. One human study suggests that minocycline attenuates the subjective reward effects of dextroamphetamine [19], while, to our knowledge, the effects of minocycline on human social/mental activities are not well understood.

Crockett et al have revealed that serotonin modulates behavioral reactions to unfairness, via a monetary decision-making game with healthy volunteers who were administered tryptophan-depleted amino acid which induces lower serotonin levels [20]. In order to measure human social/mental activities, these monetary decision-making experiments have been actively applied because such experiments enable the analysis of the interaction between social/mental activities and actual social behaviors [21,22]. Pharmacology-based neuro-economic research is showing that human social behaviors are modulated by neurotransmitters such as serotonin and oxytocin [20,23,24,25]. In addition, a significant link has recently been reported between the dopamine D4 receptor gene and fairness preference in ultimatum game [26]. However, the pharmacological interaction of social decision-making beyond neurotransmitters remains to be clarified [27].

As a first step in this direction, we recently conducted a pilot experiment with trust game, one of the decision-making experiments, with minocycline [28]. The forty-nine participants, healthy adult humans, made a monetary decision about whether or not to trust an anonymous partner after a four-day oral administration of minocycline or placebo. The minocycline group showed a strong and positive correlation between their scores in trust game and their pre-evaluation scores in others' trustworthiness, but the placebo group did not. These pilot data have suggested that inhibitory effects of microglial activation may sharpen a sense of trust in social behavior, and this effect would enhance situation-oriented behaviors according to immediate social situations. In trust game, a player's optimal decision depends on his/her prediction about the other player's decision. Thus, social environment, including the other's behavior, determines what behavior the player should take. In our actual life, however, our decisions are determined not only by social environment but also by our fundamental mental factors such as temperament and character (i.e. personality), which are independent from situational factors and may strongly impact on decision-making. These factors may act as a "noise" in trust game and during similar human decision-making situations [29]. Our pilot data demonstrated that only the minocycline group showed situation-oriented decision-making, which suggests that microglia may be inducing the "noise" consistently and inhibiting microglial activation could reduce this "noise" effect. However, the underlying mechanisms of "noise" were not clarified in our previous trial [28], thus the next appropriate step is the measurement of the effects of not only trustworthiness but also anxiety and personality.

Therefore, to clarify the microglial "noise" effect during human social decision-making, we newly explored whether anxiety and personality as a "noise" influences outcomes of trust game on humans with minocycline or placebo. To improve the small sample size and the weaker statistical power of our previous trial, we newly conducted the trial with larger samples (about one hundred participants).

Methods and Materials

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1, Protocol S1 and Protocol S2. This double-blind randomized study was approved by the Kyushu University Ethical Committee under the administration of the UMIN clinical trial center (UMIN000004803). All the participants of the present experiment, which was conducted in December 2010, were unique to this study and differ from the previous participants who enrolled in an earlier experiment in March 2010 under the administration of the UMIN clinical trial center (UMIN000003281; published in Watabe et al. *Psychopharmacology* 2012). Flow diagram of this study is listed on **Figure 1**. All participants gave written informed consent to participate after a complete description of the study. Participants were administered minocycline or placebo for four days, after which they played a trust game with an anonymous partner.

Subjects

Participants were recruited by advertisements on campus. Inclusion criterion was as follows; healthy adult males from 20 to 30 years old who can obtain informed consent. Exclusion criteria were the following five items; 1) those who have had side effects to antibiotics including minocycline, 2) those who have severe heart, liver or kidney disease, 3) those who have a tendency to develop allergies, and 4) those who have been diagnosed with psychiatric disorders. Their mental and physical health was confirmed by interview with a psychiatrist (TAK). All the participants were qualified for this study (**Table S1**).

Drug Administration

Participants received a sheet describing their detailed dosing schedule. They were then asked to write the exact time of every dosing, and to submit every capsule package as evidence of dosing. Participants started to take a capsule in the evening of the first day and twice daily (morning and evening) for four days afterward. On the day of the game experiment (the fifth day), they were instructed to take the last capsule three-hours prior to the appointment time for the experiment so that all participants played the trust game under the similar drug effect. Each capsule contained 100 mg minocycline (in the treatment group) or 100 mg lactose (in the placebo group). This minocycline dose (200 mg/day) is within the range of the usual daily dose used for treatment of infections [30], and this dose has also been applied in recent clinical trials [10,19]. Participants were randomly assigned to the treatment group or to the placebo group in advance, with a double-blind procedure.

Procedure

Prior to drug administration, participants completed a set of questionnaires (details in **Scales**). After four days of drug administration, participants were interviewed by physicians regarding side effects, other medications, and adherence to the drug administration protocol. They then played a trust game [21] and responded to the same set of questionnaires they had completed before administration.

Trust Game

Figure 2 shows the structure of trust game. In this two-player game, each player was initially given 1300 JPY (nine hundred JPY had been used in our previous trial [28], but to let participants recognized clearer incentive and make their decisions more seriously, we used 1300 JPY (about 15 USD) in this new trial so

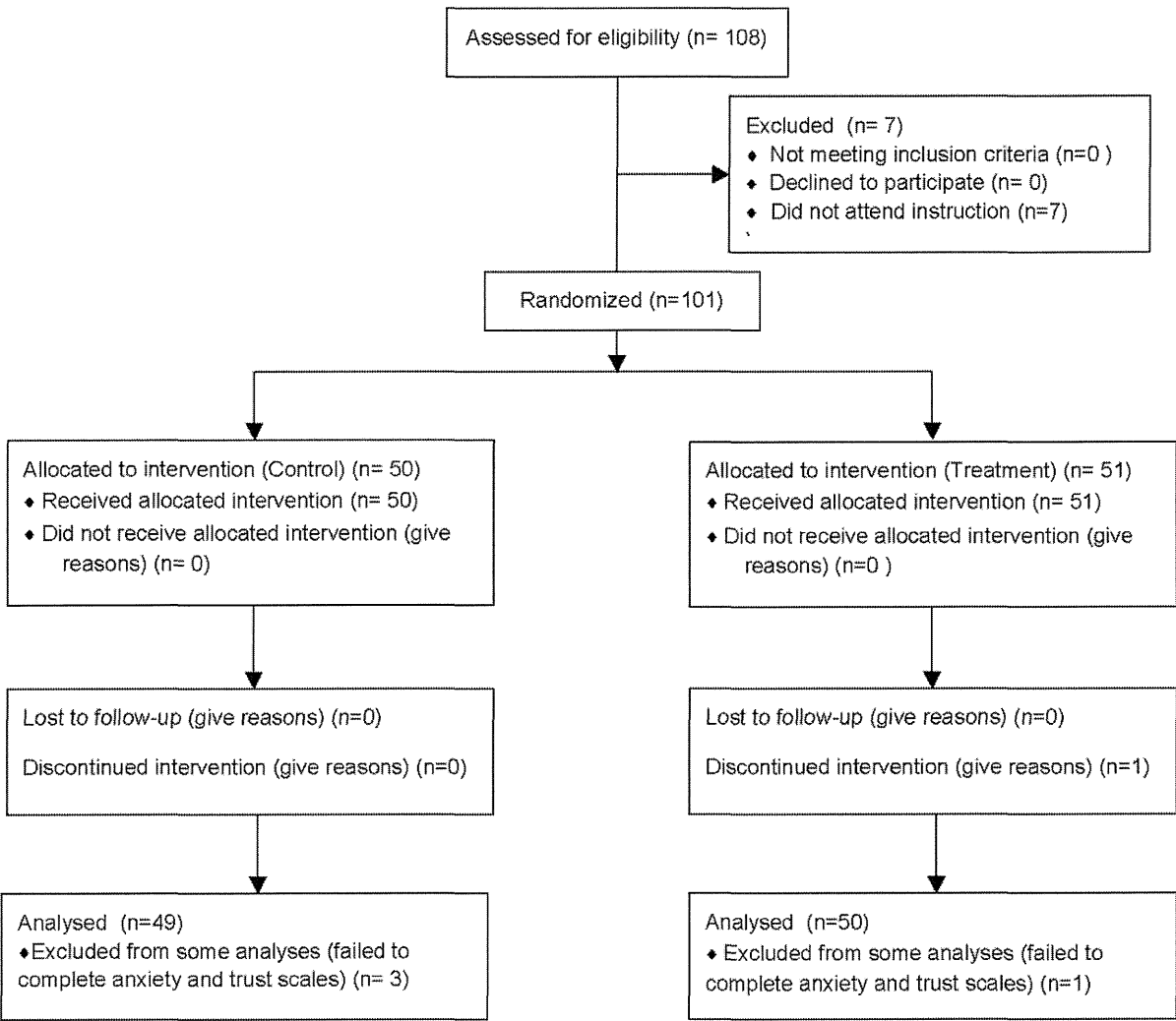


Figure 1. Flow Diagram of This Study.
doi:10.1371/journal.pone.0040461.g001

that we can obtain more reliable behavioral data). The first player then decided how much of the 1300 JPY to give to the second player. The second player then went to another room, where the amount of money given to him by the first player was tripled. The second player then decided whether to split his money equally with the first player or to take all of his money. In this experiment, all the participants were actually assigned to be the first player. The first player's decision as to how much money to give to the second player is thought to be the first player's level of trust in his partner. The amount of money given was expected to be a behavioral measure of the first player's trustfulness.

In this experiment, participants had no information about the partner except that he was male. The participants thus were likely to have made their decisions based primarily on how much they trusted others in general. All the participants' partner was actually a research confederate and always the same person, a 22-year-old Japanese male. In order to control the participant's impression of the partner, the partner acted and talked exactly in the same way throughout all the experimental sessions.

Scales

Our previous trial showed the positive correlation between participants' scores in trust game and their pre-evaluation scores in

others' trustworthiness, while we did not examined other psychological factors and thus the underlying mechanisms were not clarified [28]. Therefore, we examined the effects of anxiety and personality, in addition to the trust scores, in this trial. The following self-rated questionnaires were completed by the participants at pre- and post-treatment.

Temperament and Character Inventory (TCI)

TCI is based on the seven-factor model of temperament and character in personality [31]. According to TCI model, personality is classified into temperament, which consists of Novelty Seeking (NS), Harm Avoidance (HA), Reward Dependence (RD), Persistence (PS), and character, which consists of Self-Directedness (SD), Cooperativeness (C), Self-Transcendence (ST) with a four-point Likert type scale. We used a Japanese version with 125 questions (TCI-125) [32], which was kindly provided for use in this study from the HUMAN CAPITAL CONSULTING Corporation, Tokyo, Japan.

State-Trait Anxiety Inventory (STAI)

This anxiety scale with 20 questions consists of two factors; state anxiety, which refers to relatively unstable emotional threat to

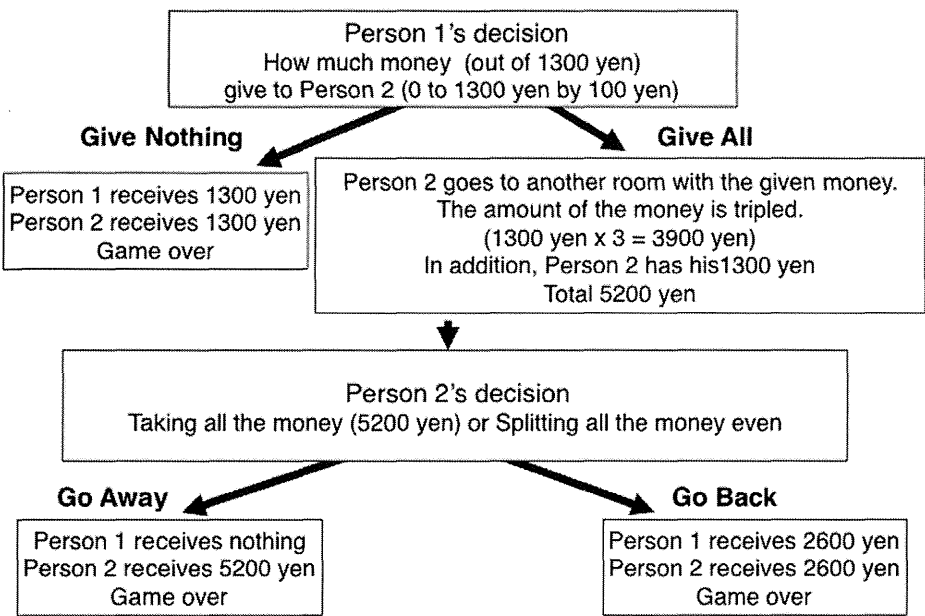


Figure 2. Trust Game Structure.
doi:10.1371/journal.pone.0040461.g002

current situations, and trait anxiety, which refers to relatively stable emotional threat consistently felt in daily life [33].

General Trust Scale (GTS)

GTS consists of six questions with a seven-point Likert type scale developed by Yamagishi and Yamagishi [34]. This scale measures respondents' estimation of others' trustworthiness. The reliability and validity of GTS have been confirmed across many countries [35]. According to past research on GTS, the major confounders of general trust are culture, sex and education level [34]. To eliminate the effects of these confounders, we recruited a homogenous sample as possible. As a result, all the participants were Japanese males and who had collage/university level educations so that we could test the effect of general trust without these confounders.

Data Analysis

Ninety-nine Japanese males, out of 101 entries, completed our experiments (mean age 21.52 years, SD 1.65 years), and analysis was conducted on this data. Among the participants, four (three in the minocycline condition, one in the control condition) failed to complete the questionnaires of STAI and GTS, so the analyses including these two scales were performed with the data of the 95 participants. All of the data analyses were performed with SPSS ver.19.

Results

In our previous trial, the statistical power was 0.766, and the statistical power in the present trial is 0.847. Therefore, the present trial exceeds the suggested efficient power of 0.8. The following analyses are shown with this more appropriate power.

Behavior in Trust Game

We compared the mean amount of participants' monetary offers in trust game by a t-test (Table 1). The monetary score in trust game was significantly lower in the minocycline group

compared to the placebo group ($t(97) = 2.08, p < .05$). This result is consistent with our pilot study [28].

Effects of Minocycline on Personality, Anxiety and Trust

The effects of minocycline on personality, anxiety and trust were evaluated with the seven subscales of TCI, the two subscales of STAI, and GTS. We performed ANOVA with a repeated measure; the scores of the subscales as the dependent variable, and drug condition (Minocycline vs. Control), repeated measure of the subscales' scores (Before vs. After treatment) and their interaction as independent variables (Table 1).

There was no significant interaction term on each of the subscale of TCI. The main effect of time (Before vs. After treatment) was significant for Persistence. The score of Persistence is higher After (mean score = 13.09, SD = .237) than Before (mean score = 13.46, SD = .236). No effect was found on the rest of the items. These results indicate that participants' personality itself was not significantly affected by minocycline.

On STAI, interaction effect and main effect were significant on Before-After for state anxiety. Compared to the control group, the state anxiety score increased steeply in the minocycline group. According to simple main effect test, the score after the treatment was significantly higher in the minocycline group than in the control group ($p < .001$). Thus, this result may explain the cause of the lower trusting behavior for minocycline group in trust game. We found no significant effect on trait anxiety.

On GTS, there were no main or interaction effects.

Effects of Minocycline on Decision-Making Style

Next, to examine the effects of minocycline on decision-making style, we performed a multiple linear regression analysis of the amount of money offered (monetary score) in trust game as the dependent variable, and subscales of TCI, STAI and GTS as independent variables by conditions (Table 2). We revealed that state anxiety ($\beta = -.795, t = -4.42, p = .001$) and trust ($\beta = .321, t = 2.35, p = .023$) have significant effects in the minocycline group ($R^2 = .288, F(3,46) = 9.30, p = .001$) while only cooperation scale of

Table 1. Behavior in Trust Game, and Effects of Minocycline on Personality, Anxiety and Trust.

Category	Subcategory	Before Treatment		After Treatment		Before-After	Control-Minocycline	Interaction
		Control	Minocycline	Control	Minocycline			
Monetary Score in Trust Game (%)	–	N/A	N/A	61.38 (32.43)	48.77 (27.70)	N/A	$t(97) = 2.08, p < .05$	N/A
TCI (from 1 to 20)	Self-Transcendence	10.69 (2.05)	10.82 (2.19)	10.52 (2.47)	10.57 (2.63)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Cooperative-ness	14.98 (1.79)	14.98 (1.89)	14.80 (1.91)	14.79 (1.98)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Self-Directedness	12.87 (0.20)	12.71 (0.22)	12.62 (1.98)	12.22 (2.09)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Persistence	12.86 (2.14)	13.54 (2.53)	13.31 (2.34)	13.76 (2.25)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Reward Dependence	14.07 (1.88)	14.28 (2.38)	14.10 (1.75)	14.10 (2.21)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Harm Avoidance	13.51 (2.47)	13.30 (2.37)	13.55 (2.50)	13.26 (2.56)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
	Novelty Seeking	12.97 (1.69)	12.99 (1.76)	12.94 (1.77)	13.02 (1.62)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
STAI (from 1 to 4)	State Anxiety	2.04 (0.45)	2.00 (0.53)	2.11 (0.51)	2.28 (0.57)	$F(1, 93) = 18.60, p < .01$	<i>ns.</i>	$F(1, 93) = 6.57, p < .05$
	Trait Anxiety	2.33 (0.52)	2.21 (0.56)	2.27 (0.51)	2.27 (0.58)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>
General Trust Score (from 1 to 7) -		4.31 (1.06)	4.51 (1.12)	4.41 (1.07)	4.53 (1.04)	<i>ns.</i>	<i>ns.</i>	<i>ns.</i>

We performed t-test on the behavior (monetary score) in trust game, and the average scores are shown in the Table. The effects of minocycline on personality, anxiety and trust were evaluated with the seven subscales of TCI, the two subscales of STAI, and GTS. We performed ANOVA with a repeated measure; the scores of the subscales as the dependent variable, and drug condition (Minocycline vs. Control), repeated measure of the subscales' scores (Before vs. After treatment) and their interaction as independent variables. As four participants (three for control, one for minocycline group) failed to complete the questions of STAI and GTS, 95 sets of data were analyzed. Significant and/or marginal effects are shown in the Table. Results were expressed as means (S.D.).
doi:10.1371/journal.pone.0040461.t001

TCI ($\beta = .486, t = 2.58, p = .013$) was significant in the control group ($R^2 = .092, F(3,42) = 2.51, p = .078$). Our novel finding in the present study is that the effect of state anxiety was stronger than that of trustworthiness. In sum, for the minocycline group, the less state anxiety and the more trustful, the more trusting behavior; while for the control group, the more cooperativeness, the more trusting behavior.

Discussion

As a first step to explore how microglia modulates human social/mental activities, we showed the novel effect of minocycline, the most famous inhibitor of microglial activation, on human monetary decision-making in trust game. Our previous trial, with smaller sample size and weaker statistical power, indicated the positive effect of minocycline on trust game, while the significant results were limited. In the present trial, we newly revealed that the monetary score in trust game was significantly lower in the minocycline group. Another novel finding was that minocycline treatment itself did not change personality, while, surprisingly participants’ ways of decision-making were significantly shifted; cooperativeness, one component of personality, was the main modulating factor of decision-making in the placebo group, on the other hand, the minocycline group was mainly modulated by state anxiety and trustworthiness, both of which are known to be mainly dependent on real-time environments such as present social situation. In addition, the effect of state anxiety was stronger than that of trustworthiness. These results suggest that minocycline led to more situation-oriented decision-making, supporting our “noise reduction” hypothesis [28]; participants’ personality may act as a “noise” during human social decision-making and minocycline may mimic personality- oriented behaviors.

Impact of Microglia on Personality-Oriented Social Behaviors

The novel effects of minocycline may explain the unknown role of microglia in social/mental activities. Until now, no study has reported microglial activities in healthy human subjects, while microglia have proved to play important roles in normal brain by communicating with neurons via releasing mediators and synaptic direct contact in rodent studies [14,15,16]. Therefore, human microglia may perform actively even in healthy brains, and inhibiting microglial activation with minocycline may create a shift from personality-oriented behaviors to situation-oriented behaviors by modulating neuro-synaptic-microglial networks. Rodent

microglia play essential roles in synaptic pruning [18], which has pointed to the cryptic roles of microglia in human brain development. A recent study suggests that rodent microglial activation by infections during early developmental periods last, and these pre-activated microglia will be re-activated rapidly compared to normal state microglia [36]. Another study has suggested that microglia have a crucial role in the process of early-life memory in rats [37]. Early-life events can significantly modulate normal learning-dependent cytokine activity within the hippocampus, via a specific, enduring impact on brain microglial function, and preventing microglial activation by minocycline during learning prevents memory impairment in neonatally infected rats. Microglia are known to be activated not only by infection but also by physical and psychological stress in rodent studies [12,38,39,40]. In addition, Wei et al. reported that early life stress inhibits expression of a novel innate immune pathway in the developing hippocampus in pups [41]. Based on these recent findings, we suggest the possible existence of the following mechanism on personality and social behaviors; early-life environmental experiences such as psychological stress and traumatic events may activate human microglia, establish a certain neuro-synaptic-microglial connection, which may be memorized unconsciously as a primer for an extended period, and this formation in the human brain may determine each human’s personality and personality- oriented social behaviors in later life (Figure 3). In addition, we can interpret the present results as follows; the control group’s personality- oriented behaviors could be formulated by microglial priming effects, and the minocycline group’s situation- oriented behaviors may be induced by suppressing microglial contribution to social behaviors. Further studies are needed to clarify contributions of microglia to human development including personality formation, and social/ mental activities in later life.

Clinical Implication

Minocycline has been suggested to be an effective drug for psychiatric disorders [10,11]. Disturbed decision-making is a common symptom of various psychiatric disorders [42,43], and this disturbance is treated by psychotropic drugs such as antipsychotics and antidepressants, which have proved to inhibit microglial activation from *in vitro* studies [44,45,46,47,48]. In addition, a recent study suggests that effort-based decision-making in rat is modulated by estradiol [49], a sex hormone, which also has inhibitory effects on microglial activation [50,51]. These data support our minocycline results, and indicate that psychiatric

Table 2. Multiple Regression Analysis on Behavior in Trust Game.

Independent Variable	Control Group	Minocycline Group
	Beta	Beta
Cooperativeness (TCI)	.486*	
Reward Dependence(TCI)	-.281	
Self-Directedness (TCI)	-.284	
State Anxiety (STAI)		-.583**
General Trust		.321*
	$N = 46, R^2 = .092, F(3, 42) = 2.51, p < .10$	$N = 49, R^2 = .288, F(2, 46) = 9.30, p < .001$

Note: * $p < .05$, ** $p < .01$. We performed a multiple linear regression analysis of the amount of money offered in trust game as the dependent variable, and subscales of TCI, STAI and GTS as independent variables by conditions. Remarkable effects are shown in the Table. doi:10.1371/journal.pone.0040461.t002

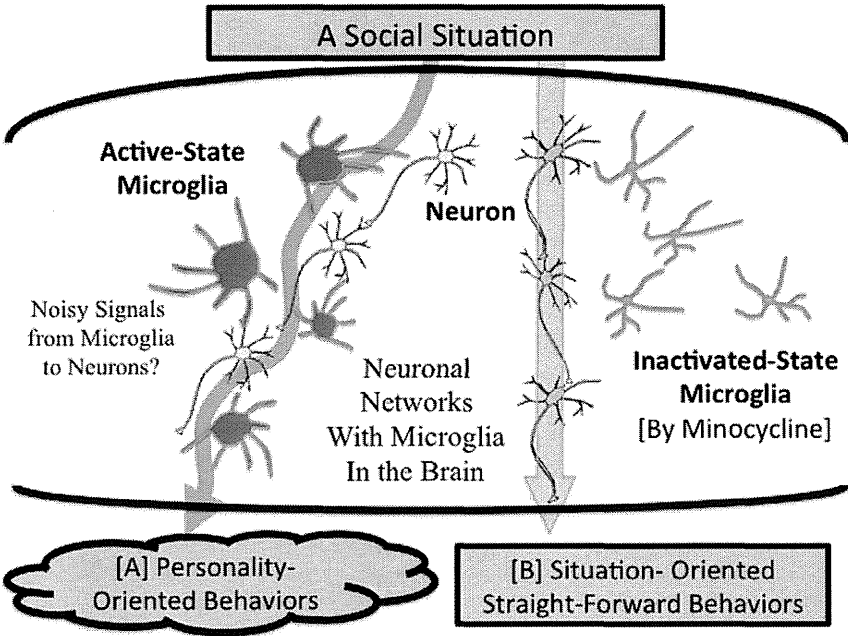


Figure 3. Possible Impact of Microglia on Personality and Social Behaviors. Early-life environmental experiences such as psychological stress and traumatic events may activate human microglia, establish a certain neuro-synaptic-microglial connection, which is memorized unconsciously as a primer for an extended period, and this formation in the human brain determines each human's personality and personality-oriented social behaviors in later life. In sum, neuronal networks with active microglia may induce noisy-decision-making, which is equivalent to personality-oriented behaviors (A). On the other hand, decision-making with neuronal dominant networks may induce straightforward behaviors, which are less affected by personality (B). In the present study, the control group's personality-oriented behaviors could be formulated by microglial priming effects (A), and the minocycline group's situation-oriented behaviors may be induced by suppressing microglial contribution to social behaviors (B).
doi:10.1371/journal.pone.0040461.g003

treatments may modulate microglial contribution to disturbed decision-making in social behaviors. To develop our results and these perspectives, animal based decision-making experiments with minocycline (or other microglial inhibitors) and histological analysis of microglia are called for in the near future. In addition, clinical trials of social decision-making experiments focusing on microglia should be attempted.

Limitation

First, this study did not examine the dose-dependent effects of minocycline. Second, this study was conducted only with adult males, while there may be a difference when players are female. Third, we did not measure microglia activity in the brain via imaging methods, while minocycline may inhibit some brain regional activities which are thought to be linked to trust and social decision-making [52,53]. Therefore, brain imaging studies are needed to clarify these regional activation mechanisms. Finally, other possible minocycline effects should be taken into account. Apart from inhibiting microglial activation, minocycline also has been reported to interact with brain glutamate and dopamine neurotransmission [54,55] and to have direct effects on neuronal cell line, PC12 [56]. Some reports suggest positive links between microglia, glutamate and dopamine interaction [57,58]. Further research should be performed to clarify this interaction mechanism. No specific inhibitor of microglia exists to date, therefore we selected minocycline as the most appropriate and safest drug to be used in humans at present. When a safe, specific inhibitor of microglial activation is developed, microglial human function will be clarified more effectively.

Conclusion

Based on the results of the present human social decision-making experiment, we have proposed a novel microglial contribution to personality and social behaviors. Our present study may shed new light on microglial roles in the social and mental life of healthy humans and also of people with psychiatric disorders. To explore these perspectives, further *in vitro*/*in vivo* studies and translational research are needed.

Ethics Statement

This double-blind randomized study was approved by the Kyushu University Ethical Committee under the administration of the UMIN clinical trial center (UMIN000004803). All participants gave written informed consent to participate after a complete description of the study.

Supporting Information

Table S1 Participants List.
(XLS)

Checklist S1 CONSORT Checklist.
(DOC)

Protocol S1 Trial Protocol.
(DOC)

Protocol S2 Japanese Version of Trial Protocol.
(DOC)

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Author Contributions

Conceived and designed the experiments: TAK MW. Performed the experiments: TAK MW ST KI. Analyzed the data: MW. Contributed reagents/materials/analysis tools: TAK MW KH AM HU SK. Wrote the paper: TAK MW.

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Original Article

Protease-resistant PrP and PrP oligomers in the brain in human prion diseases after intraventricular pentosan polysulfate infusion

Hiroyuki Honda,¹ Kensuke Sasaki,¹ Haruhiko Minaki,¹ Kenta Masui,¹ Satoshi O. Suzuki,¹
Katsumi Doh-ura² and Toru Iwaki¹

¹Department of Neuropathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka and ²Department of Neurochemistry, Tohoku University Graduate School of Medicine, Sendai, Japan.

Intraventricular infusion of pentosan polysulfate (PPS) as a treatment for various human prion diseases has been applied in Japan. To evaluate the influence of PPS treatment we performed pathological examination and biochemical analyses of PrP molecules in autopsied brains treated with PPS (one case of sporadic Creutzfeldt-Jakob disease (sCJD, case 1), two cases of dura mater graft-associated CJD (dCJD, cases 2 and 4), and one case of Gerstmann-Sträussler-Scheinker disease (GSS, case 3). Six cases of sCJD without PPS treatment were examined for comparison. Protease-resistant PrP (PrP^{res}) in the frontal lobe was evaluated by Western blotting after proteinase K digestion. Further, the degree of polymerization of PrP molecules was examined by the size-exclusion gel chromatography assay. PPS infusions were started 3–10 months after disease onset, but the treatment did not achieve any clinical improvements. Postmortem examinations of the treated cases revealed symmetrical brain lesions, including neuronal loss, spongiform change and gliosis. Noteworthy was GFAP in the cortical astrocytes reduced in all treated cases despite astrogliosis. Immunohistochemistry for PrP revealed abnormal synaptic deposits in all treated cases and further plaque-type PrP deposition in case 3 of GSS and case 4 of dCJD. Western blotting showed relatively low ratios of PrP^{res} in case 2 of dCJD and case 3 of GSS, while in the treated sCJD (case 1), the ratio of PrP^{res} was comparable with untreated cases. The indices of oligomeric PrP were reduced in one sCJD (case 1) and one dCJD (case 2).

Although intraventricular PPS infusion might modify the accumulation of PrP oligomers in the brains of patients with prion diseases, the therapeutic effects are still uncertain.

Key words: Creutzfeldt-Jakob disease, oligomer, pentosan polysulfate, prion protein, size-exclusion gel chromatography.

INTRODUCTION

Prion diseases, also known as transmissible spongiform encephalopathies (TSEs), are fatal neurodegenerative disorders that include Creutzfeldt-Jakob disease (CJD) and Gerstmann-Sträussler-Scheinker disease (GSS) in humans, and scrapie and bovine spongiform encephalopathy in animals. In these diseases, histopathological changes in the brain are characterized by spongiform change, reactive changes of astrocytes and variable loss of neurons.¹ In addition, deposition of a protease-resistant isoform of prion protein (PrP^{res}) is detected in the brain. This PrP^{res} contains high β -sheet content and is composed of polymerized PrP molecules post-translationally converted from normal, cellular PrP (PrP^c) of 254-amino acid 32–35 kDa glycolipid-anchored, plasma membrane protein that is widely expressed in the CNS.²

There is currently no effective remedy for human prion diseases, but several therapeutic compounds including quinacrine and pentosan polysulfate (PPS) have been tested for patients with prion diseases on experimental trial bases. Quinacrine is reportedly effective in inhibiting PrP^{res} formation in prion-infected cells.^{3,4} However, subsequent studies showed no apparent beneficial effects of quinacrine in either experimental animals or humans.^{5–7} By comparison, PPS has been shown to prevent the propagation of

Correspondence: Hiroyuki Honda, MD, Department of Neuropathology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi-ku, Fukuoka 812-8582, Japan. Email: h-hiroyu@np.med.kyushu-u.ac.jp

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PrP^{res} in prion-infected cells.⁸ Additionally, in experimental animals PPS has been administered directly into the CNS via an intra-hemiventricular canula, resulting in significant prolongation of the incubation periods accompanied with the laterality of neuropathological changes.⁹ PPS inhibits PrP^{res} formation by interfering with the interaction of PrP^c and PrP^{res} with endogenous glycosaminoglycan or proteoglycan.¹⁰ In addition, PPS stimulates endocytosis of PrP^c, reducing the amount of PrP^c present on the cell surface.¹¹ Experimental trials of intraventricular PPS infusion in the patients with prion diseases have been performed on observational bases, and thus it has been difficult to prove its efficacy.^{12,13} In fact, Tsuboi *et al.* reported that PPS treatment showed no apparent improvements of clinical features in Japanese patients with prion disease.¹⁴ In comparison, Bone *et al.* reported that mean survival of seven patients with PPS treatment was longer than reported values for the natural history of prion diseases in the United Kingdom, although possible reasons for this finding remain unclear.¹⁵

The main pathogenic component in prion diseases has been suggested to be PrP^{res} composed of PrP polymers. Recently, PrP oligomers, equivalent to 14–28 PrP molecules were reported as the most infectious units.^{16,17} In addition, Kristiansen *et al.* reported that disease-associated PrP oligomers inhibit the 26S proteasome and cause neurotoxicity.¹⁸ We have previously developed a gel-filtration chromatography method using spin columns to examine PrP oligomers, and revealed that increased PrP oligomers correlated with the degree of histopathological changes such as spongiform change and gliosis.¹⁹ In other neurodegenerative diseases, including Alzheimer’s disease, dementia with Lewy bodies and Parkinson’s disease, soluble oligomers of amyloidogenic proteins were proposed to be the principal neurotoxic agents.^{20,21}

In this report, to clarify the influence of PPS treatment on brains affected with prion diseases, we performed pathological examination and biochemical analyses of PrP,

including its degree of polymerization, in four cases of prion diseases treated with PPS.

MATERIALS AND METHODS

We investigated the degree of polymerization of PrP molecules in four prion diseases cases that received PPS treatment, denoted PPS(+): one case of sporadic CJD (sCJD), two cases of dura mater graft-associated CJD (dCJD), and one case of GSS. We also examined six cases of sCJD without PPS treatment, denoted PPS(–), for comparison. The profiles of the patients are summarized in Table 1. At autopsy the brains were weighed and fixed with 10% buffered formalin. Six micrometer-thick sections of paraffin-embedded tissue from the CNS were stained with HE and the KB staining method. Immunohistochemistry was performed with primary antibodies against anti-prion antibody (mouse monoclonal 3F4, 1:400; Signet, Dedham, MA, USA) and GFAP (rabbit polyclonal, 1:1000; Dako, Glostrup, Denmark). The sections were then treated with appropriate biotinylated secondary antibodies and the reaction products were detected using the avidin-biotinylated peroxidase complex method (ABC; Vector Laboratories, Burlingame, CA, USA) coupled with a diaminobenzidine (Dojindo, Kumamoto, Japan) reaction.

Brain homogenate preparation

Human brains were collected at autopsy from four prion disease cases that had received PPS treatment and six cases of sCJD that had not received PPS treatment. Samples of frontal cortex were frozen fresh and stored at –80°C until used. The brain samples were homogenized to a final concentration of 10% in lysis buffer with sodium dodecyl sulfate (SDS) (100 mM Tris-HCl, 100 mM NaCl, 10 mM EDTA, 1% SDS, pH 7.6) for the size-exclusion gel chromatography assay. Most PrP^c could be solubilized as monomers in lysis buffer with SDS.¹⁹ Samples were homogenized

Table 1 Summary of patient profiles

Case	Diagnosis	Genotype/PrP ^{res} type	Age at death	Sex	Duration of illness (months)	Duration of PPS treatment (months)	Brain weight (g)
1	sCJD	129MM/type 1	74	F	23	20	660
2	dCJD	129MM/type 1	67	M	12	9	950
3	GSS	P102L/8 kDa	70	F	20	14	1055
4	dCJD	129MM/type 1	55	M	14	4	1460
5	sCJD	129MM/type 1	71	M	10	–	562
6	sCJD	129MM/type 1	61	M	30	–	745
7	sCJD	129MM/type 1	69	M	15	–	940
8	sCJD	129MM/type 1	73	F	4	–	1100
9	sCJD	129MM/type 1	68	F	2	–	1260
10	sCJD	NA/type 1	66	M	2.5	–	1435

dCJD, dura CJD; F, female; GSS, Gerstmann-Sträussler-Scheinker disease; M, male; MM, methionine homozygote at prion protein gene codon 129; NA, not available; PPS, pentosan polysulfate; PrP^{res}, proteinase resistant isoform of prion protein; sCJD, sporadic CJD.

at 5000 rpm for 30 s in a bead disrupter homogenizing system (MicroSmash MS-100; Tomy Seiko Co., Ltd, Tokyo, Japan). Homogenates were then clarified by centrifugation at 250 g for 5 min and the supernatant was stored at -80°C .

Detection of PrP^{res}

Conventional procedure for the detection of PrP^{res} was conducted as follows: 1% brain homogenate was prepared in extraction buffer (100 mM Tris-HCl, 100 mmol NaCl, 10 mmol EDTA, 0.5% Nonidet P-40, 0.5% sodium deoxycholate, pH 7.6) and incubated with 50 $\mu\text{g}/\text{mL}$ proteinase K (PK) at 37°C for 1 h. Protease activity was then abolished by the addition of 1 mmol Pefabloc SC (Roche, Indianapolis, IN, USA). Undigested PrP^{res} fragments were separated by SDS-PAGE in 12% NuPAGE Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) and transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Billerica, MA, USA). PrP was detected using anti-PrP antibody (mouse monoclonal 3F4, 1:10 000) as the primary antibody and peroxidase-conjugated anti-mouse IgG as the secondary antibody (AP192P, 1:20 000; Chemicon, Temecula, CA, USA). The immunoreaction was visualized using the ECL plus Western Blotting Detection System (GE Healthcare; Chalfont St. Giles, Buckinghamshire, UK).

Size-exclusion gel chromatography assay

We performed the size-exclusion gel chromatography assay using the spin-column kit CHROMA SPIN-200 (Clontech, San Francisco, CA, USA) that clearly separated oligomeric PrP from monomeric PrP.^{19,22} The samples were first centrifuged at 120 g for 2 min, and the first fraction was collected in the collection tube. Another 40 μL of lysis buffer was added, and the samples were centrifuged at 120 g for 2 min to collect the size-exclusion fractions sequentially. In these operations, we used a centrifuge with a swing-bucket rotor (A-4-62; Eppendorf, Hamburg, Germany). Fractionated PrP was detected without PK treatment by SDS-PAGE and Western blot analysis, as described above.

RESULTS

Case reports and brain pathology

Details of PPS treatment and clinical findings from the patients were described in a previous paper.¹⁴ In all four PPS-treated cases, the PPS infusion catheter was inserted into the right lateral ventricle, and the PPS dose was gradually escalated to the target dosage of 120 $\mu\text{g}/\text{kg}/\text{day}$. PPS treatment showed no apparent improvement of clinical

features in all the cases. Clinicopathological findings from each case that had received PPS treatment are described below.

Case 1

A 72-year-old woman showed truncal ataxia and progressive gait disturbance. She had no family history of prion or neurological disease. Diffusion weighted imaging (DWI) demonstrated diffuse bilateral high-intensity signals in the cerebral cortex and striatum. Periodic synchronous discharge was seen in electroencephalography 2 months after disease onset. She was diagnosed with sCJD. Myoclonus and startle reaction were observed 3 months after the onset. The PPS infusion was started 3 months after the onset; however, no improvement in clinical features was observed. She developed akinetic mutism 6 months after the onset. Tonic seizures in extremities were also seen. The patient died of pneumonia and autopsy was performed 14.5 h after death. The brain weighed 660 g and showed severe atrophy with bilateral subdural hematoma and fluid collection. Microscopy demonstrated severe neuronal loss, rarefaction of neuropil, and gliosis across the cerebral cortices (Fig. 1A). Although astrocytosis was noted in HE staining, GFAP expression was weak in the cerebral cortices, except in subpial astrocytes (Fig. 2A). These pathological changes were also seen in the basal ganglia and thalamus. Synaptic PrP deposition was detected in the cerebral cortices, basal ganglia and thalamus (Fig. 1B). There was no laterality of spongiform change, neuronal loss, gliosis or PrP deposition.

Case 2

A 66-year-old man showed dysarthria 25 years after dura mater graft implantation because of cerebral hemorrhage. He manifested right hand clumsiness and progressive gait disturbance. Myoclonus was also seen in the right extremities. He had no family history of prion disease or neurological disease. DWI showed abnormal high-intensity signals in bilateral temporal cortices. In addition, brain biopsy was performed and revealed a type 1 PrP^{res} accumulation. The patient was diagnosed with dCJD. The electroencephalogram showed no periodic synchronous discharges. He developed akinetic mutism 2 months after disease onset. PPS infusion was started 3 months after the onset, but no improvement of clinical features was observed. The patient died of pneumonia and autopsy was performed 9 h after death. The brain weighed 950 g and showed severe atrophy with bilateral subdural fluid collection. Spongiform change, severe neuronal loss and gliosis were seen in the cerebral cortices (Fig. 1C). Astrocytosis was found in all layers of the cerebral cortex; however GFAP expression was weak (Fig. 2B). Synaptic PrP

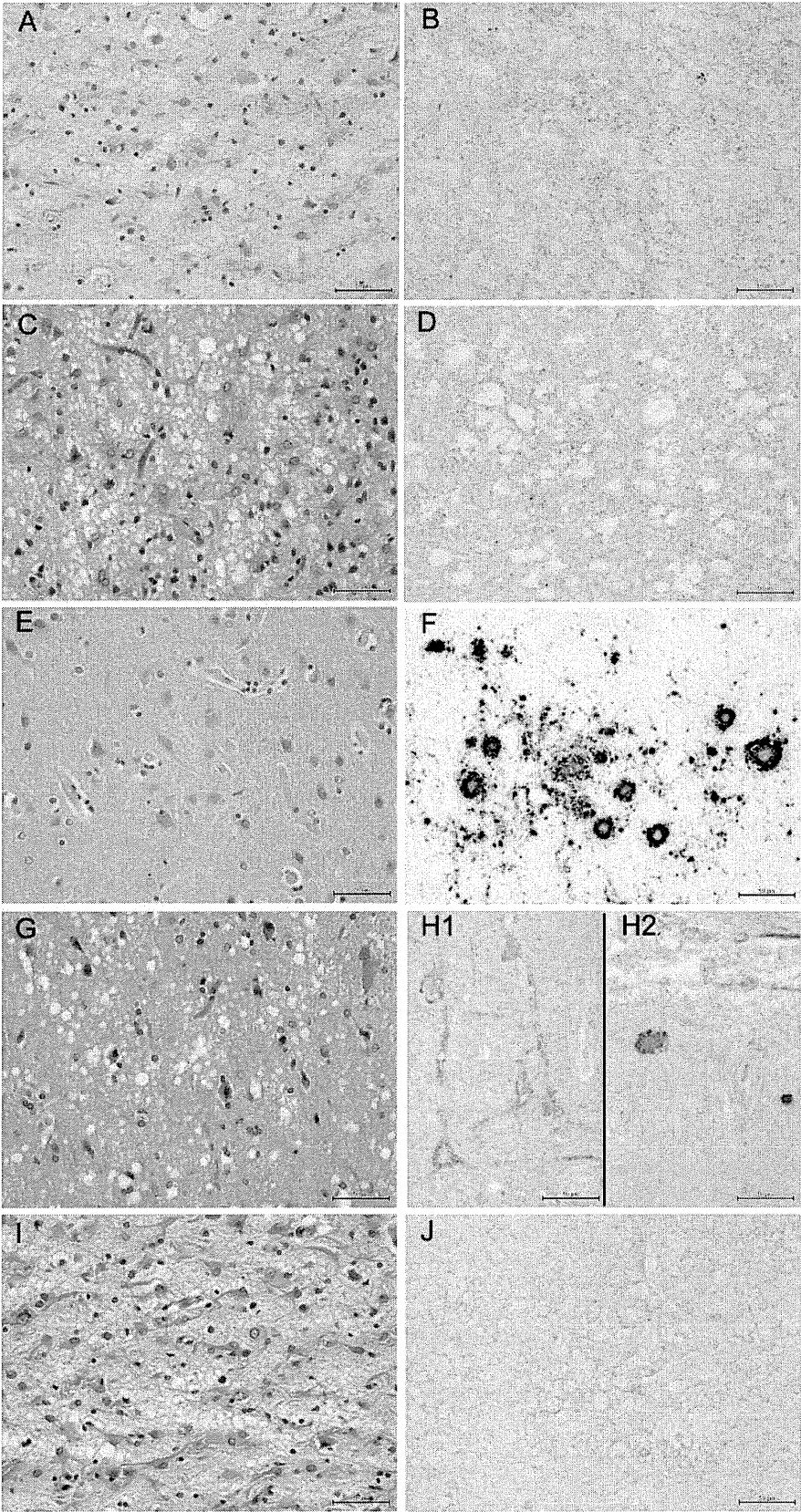


Fig. 1 Light micrographs of frontal cortices in prion diseases. A, C, E, G, I: HE stain. B, D, F, H1, H2, J: immunohistochemistry for PrP. Case 1 shows severe neuronal loss and significant rarefaction of neuropil (A) and synaptic PrP deposition (B). Case 2 shows typical spongiform change (C) and synaptic PrP deposition (D). Case 3 shows several amyloid plaques, neuronal loss and gliosis; however spongiform change is very mild (E). Both plaque-type deposition and synaptic deposition of PrP are detected (F). Case 4 shows neuronal atrophy and spongiform change (G). Synaptic deposition of PrP is mainly seen around pyramidal neurons of the deep cortical layer (H1) and plaque-type depositions are mainly found in the subpial layer (H2). Case 5 without pentosan polysulfate (PPS) treatment shows severe neuronal loss and remarkable rarefaction (I) and synaptic PrP deposition (J).

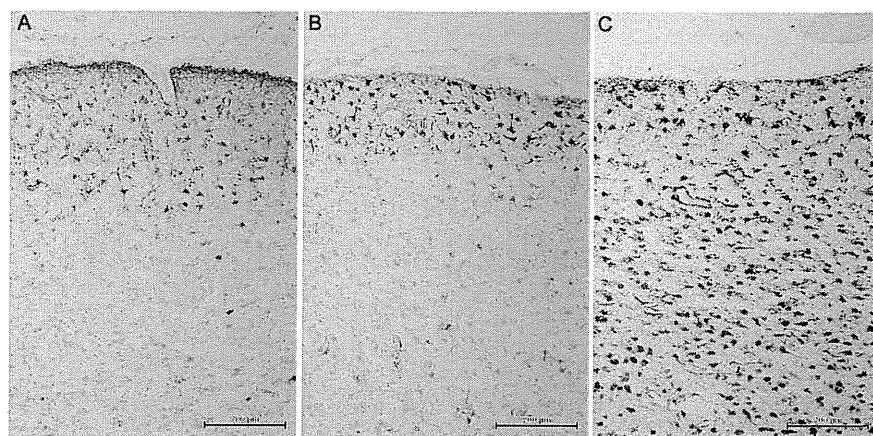


Fig. 2 Immunohistochemistry for GFAP of the frontal cortices. In case 1 (A) and case 2 (B) with pentosan polysulfate (PPS) treatment, subpial astrocytes show strong immunoreactivity for GFAP, but most cortical astrocytes show negative or weak immunoreactivity for GFAP despite their reactive morphology. In comparison, cortical astrocytes in all layers show strong GFAP immunoreactivity in case 5, which did not receive PPS treatment (C).

deposition was also detected in the cerebral cortices (Fig. 1D). No plaque-type deposition of PrP was noted. There was no laterality of spongiform change, neuronal loss, gliosis or PrP deposition.

Case 3

A 68-year-old woman showed progressive gait disturbance and dysarthria. Upper limb ataxia was also observed 5 months after disease onset. DWI showed no apparent intensity changes. The electroencephalogram showed no periodic synchronous discharges. She had a family history of prion disease. Analysis of the PrP gene revealed a P102 L mutation. She was diagnosed with GSS and the PPS infusion was started 6 months after the onset. No clinical improvement was observed. The patient died of pneumonia and autopsy was performed 27 h after death. The brain weighed 1055 g and showed moderate atrophy with bilateral subdural fluid collection. Spongiform change and neuronal loss were mild (Fig. 1E). Although astrogliosis was found in all layers of the cerebral cortices, GFAP immunoreactivity was seen mainly in the superficial cortical layers. Numerous plaque-type PrP depositions were noted in all layers of the cerebral cortices (Fig. 1F), the basal ganglia, thalamus and cerebellar granular layer. Synaptic PrP deposition was also seen in the molecular layer of the cerebral cortices, basal ganglia and thalamus. No laterality of pathological change was seen.

Case 4

A 55-year-old man showed dizziness 19 years after dura mater graft implantation. He developed dysarthria, memory deficits and character changes 8 months after disease onset. Myoclonus and startle reaction were also observed 10 months after the onset. The electroencephalogram showed no periodic synchronous discharges. DWI demonstrated high-intensity signals in the right caudate nucleus and the right thalamus. He had no family history of

prion disease or neurological disease. Brain biopsy was performed and showed a type 1 PrP^{res} accumulation. The patient was diagnosed with dCJD. Although PPS infusion was started 10 months after the onset, no clinical improvement was observed. The patient died of pneumonia and autopsy was performed 13 h after death. The brain weighed 1460 g. Right subdural hematoma was noted, but brain atrophy was not apparent. Spongiform change, severe neuronal loss and gliosis were apparent in the precentral gyrus, entorhinal cortex, anterior cingulate gyrus, thalamus and putamen (Fig. 1G). Even in regions where astrogliosis was found in all layers, GFAP immunoreactivity was seen exclusively in the superficial layer of the cerebral cortices. Both synaptic deposition of PrP (Fig. 1H1) and plaque-type deposition of PrP (Figs 1,2) were noted in the cerebral cortices, basal ganglia and hippocampus. There was no laterality of spongiform change, neuronal loss, gliosis or PrP deposition.

In PPS(–) cases, HE staining revealed that the levels of neuronal loss, spongiosis and gliosis advanced in accordance with the loss of brain weight. Various levels of synaptic PrP deposition were noted in each case. In case 5, which showed severe brain atrophy, neuronal loss and rarefaction of neuropils were evident (Fig. 1I). Both astrogliosis and marked GFAP immunoreactivity were noted in all layers of the cerebral cortices (Fig. 2C). Synaptic PrP deposition was also detected in cerebral cortices (Fig. 1J).

The ratio of PrP^{res}/total PrP

Western blot analysis detected PrP^{res} and total PrP. Cases 1 and 2 showed a type 1 pattern (Fig. 3A,B). Case 3 showed PrP^{res} fragments with molecular masses of around 8 kDa (Fig. 3C). Case 4 showed PrP^{res} fragments with intermediate size between types 1 and 2 (Fig. 3D). We calculated the ratio of PrP^{res}/total PrP based on the signal intensities of the immunoblots. In PPS(–) cases, the ratio of PrP^{res}/total PrP was already increased in case 9 with mild brain