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## Effect of serotonin transporter genotype on self-reported efficacy and activity changes of brain prefrontal area in response to placebo

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Patients benefit from drug therapy not only through pharmacological mechanisms, but also through non-pharmacological action (placebo effect), which may be mediated in part by the prefrontal area of the brain. We consider that the difference between responders and non-responders to placebo might be related to polymorphisms in the serotonin transporter-linked polymorphic region (5-HTTLPR). To study this idea, we performed a randomized double-blind clinical trial using caffeine and lactose (placebo). Activity in the prefrontal area of the brain was measured in terms of blood flow by means of near-infrared spectroscopy (NIRS) as an objective indicator. Self-reported feelings of drowsiness on established scales were used as subjective indicators. Twenty-one subjects in block A took caffeine on the first day and placebo on the third day, and 21 in block B took placebo on the first day and placebo on the third day. After placebo administration, improvement of sleepiness was significantly enhanced, a similar extent to that after caffeine medication. Among the 42 subjects, 22 showed S/S type polymorphism in the serotonin transporter (52.4 %), 17 showed S/L type (40.5 %) and 3 showed L/L type (7.10 %). Statistical analysis of the results indicate that subjects with L/L genotype showed a significantly greater placebo response in terms of both self-reported feeling of drowsiness and blood flow in the prefrontal area of the brain associated with working memory (46 area). Our results indicate that the L/L genotype of 5-HTTLPR, which is rare in Japanese (3.2 %) but common in Americans (32.2 %), may be associated with a greater placebo effect.

### 1. Introduction

The placebo effect is well-recognized (Tilburt et al. 2008; Benedetti and Amanzio 2013), and may involve expectation based on previous experience (conditionality) (Finniss et al. 2010). In terms of the placebo effect, individuals can be grouped into two categories: responders and non-responders (Benedetti et al. 2004). It has been suggested that gene polymorphism, e.g., in the serotonin transporter (5-HTT) and catechol-O-methyltransferase (COMT) genes, play a role in the difference between the two groups (Hall et al. 2015). In particular, the serotonin transporter gene (SLC6A4) contains a region known as the serotonin transporter-linked polymorphic region (5-HTTLPR), and polymorphisms in this location seem to influence brain cognition function (Borg et al 2009). 5-HTTLPR has a short (S) allele and a long (L) allele version, and there are two homozygous and one heterozygous genotypes: S/S, S/L and L/L (Lesch et al. 1996).

A clinical trial of caffeine reported a placebo effect (Hammani et al. 2010), which may be related to expectation and conditionality. Therefore, we decided to conduct a clinical trial using caffeine in order to examine whether responder or non-responder to placebo is associated with particular polymorphisms of 5-HTTLPR. In addition to using subjective self-reported responses, it would be desirable to have an objective indicator, and a possible parameter for this purpose is blood flow changes in the prefrontal area of the brain, which can be estimated noninvasively using near-infrared spectroscopy (NIRS) (Tsujii and Watanabe 2009). Therefore, it is possible that working memory has a role in the placebo effect (Baddeley 1992). In our previous studies, we used these approaches to show that counseling enhances the effect of aromatherapy. In that work, we found that aromatherapy influenced blood flow volume in the laterodorsal side of the prefrontal area corresponding to the 46 field of the prefrontal cortex area (Brodmann's brain map) (Isawa et al. 2016) and the frontal pole

of cerebrum corresponding to the 10 field of the prefrontal cortex area (Brodmann's brain map) (Tsujii et al. 2011; Tujimoto et al. 2010). The laterodorsal side of the prefrontal area is thought to be involved in cognition, while the frontal pole of the cerebrum may be associated with conditionality (Kern et al. 2004). If the placebo effect could be verified objectively using NIRS, this might enable a broader utilization of the effect in clinical practice.

### 2. Investigations and results

#### 2.1. Study design

The study was designed to compare the effects of a 200 mg dose of placebo (lactose) and caffeine (Fig. 1). Participants were block-randomized (block size of two) to randomized single studies. The block A participants took active drug (caffeine group) on the first day, and placebo on the third day (caffeine-conditioned placebo group), while the block B participants took placebo (placebo group) on the first and third days (placebo-conditioned placebo group). The second day was a washout period. This was considered sufficient, as the half-life of caffeine is 3–7 hours for adults (Kraus and Pham 2016).

#### 2.2. Preparation of test drugs

We prepared one capsule containing 100 mg lactose and caffeine. We used Japanese Pharmacopoeial Standard anhydrous caffeine (Lot RK-8, Kozakai Pharmaceutical CO.LTD, Tokyo) and lactose (Lot.436AKR, Nichi-Iko Pharmaceutical CO.LTD, Tokyo).

#### 2.3. NIRS measurements

NIRS noninvasively measures relative changes in the concentrations of oxygenated and deoxygenated hemoglobin (Hb) in

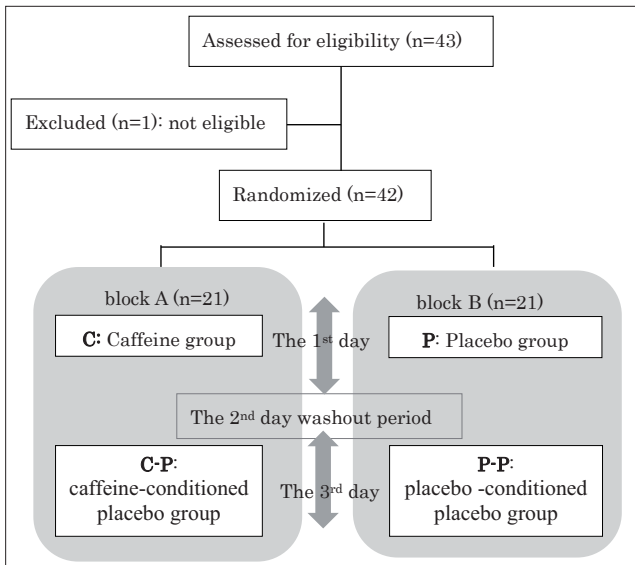


Fig. 1: Flow diagram of study procedures

localized brain tissues (Fig. 2, Maki et al. 1995). We focused on oxygenated Hb (Oxy-Hb), because Oxy-Hb responds to regional cortical activation (Obrig et al. 2002) and blood flow is increased in the corresponding area (Aoki et al. 2011). The NIRS headset fits on the front of the head for multichannel monitoring 22 channels, and we observed channel 5 to channel 18, which exhibited stable signals. The channels from 5 to 9 correspond to 46 field right, those from 14 to 18 correspond to 46 field left, and those from 10 to 13 correspond to 10 field according to Brodmann’s brain map. The Oxy-Hb responses were sampled 20 times per second and smoothed by taking a 5-sec moving average (Sato et al. 2009). We eliminated data blocks with particularly low or high signal-to-noise ratio, which were considered likely to have been influenced by obstruction by hair and those with movement artifacts. We calculated the area under the observation value of the median 20 s for the task and median 20 s for each of two base states before and after the task. To obtain stable data, we omitted

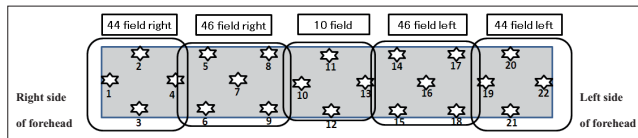


Fig. 2: Schematic illustration of the NIRS set-up  
NIRS was used to measure blood volume at 22 points, marked with stars. The 22 points are located in the following fields of Brodmann’s brain map.  
1~4 stars : 44 field (right)  
5~9 stars : 46 field (right)  
10~13 stars : 10 field  
14~18 stars : 44 field (left)  
19~22 stars : 46 field (left)

the 20 s at the beginning and end of the task state, and 10 seconds at the beginning and end of each base state. Baseline values were subtracted from the observed values to determine the hemodynamic responses in the measurement channels. In our previous study, we used least-squares linear fitting (Homae et al. 2012; Toyada et al. 2008) in order to calculate NIRS activity. Here, we confirmed that the results of the area under observation analysis closely matched those obtained by least-squares linear fitting. The verbal fluency task (VFT) during NIRS measurement is shown in Table 1. The VFT is a test used to examine frontal cortex function (Pu et al. 2012; Kono et al. 2007; Watanabe et al. 2003). In the task state, participants have to provide words using a given initial letter within a total time period of 60 s. The initial letter is changed 3 times (20 s per letter). Participants are not permitted to use words given as preliminary examples. In the base state before and after the task state, participants repeat “a, i, u, e, o” for 30 s in order to avoid any effect of illocutionary force. There was a rest time of 30 s between the base state and task state. Participants were given a 1-minute resting period before and after the VFT.

2.4. Stanford sleepiness scale (SSS)

The results for SSS mean and median values are shown in Table 2. In the caffeine group, placebo group and placebo-conditioned placebo group, there were significant changes of sleepiness between pre and post medication. The caffeine group showed a tendency for reduced drowsiness compared to the placebo group

Table 1: Time course of verbal fluency task (VFT)

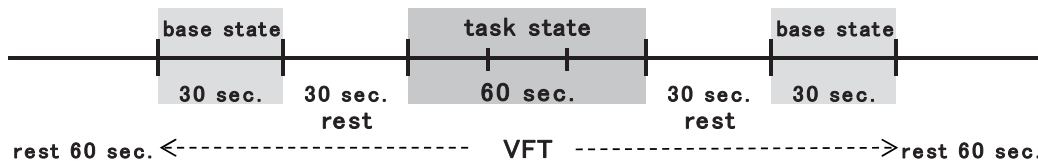


Table 2: Mean and median data from Stanford Sleepiness Scale (SSS) and Feeling of drowsiness-Visual Analogue Scale (VAS)

	Caffeine group (C)			Placebo group (P)			Caffeine-conditioned placebo group (C-P)			Placebo-conditioned placebo group (P-P)		
	Pre*	Post*	p-value	Pre*	Post*	p-value	Pre*	Post*	p-value	Pre*	Post*	p-value
SSS mean	3.05	2.43	0.029	2.647	2.41	0.031	2.57	2.24	0.096	2.64	2.27	0.042
$\Delta$ SSS( $\pm$ S.E.)		0.62 $\pm$ 0.29			0.23 $\pm$ 0.14			0.33 $\pm$ 0.16			0.36 $\pm$ 0.18	
median	3	2		3	2		2	2		3	2	
VAS mean	45.4	32.8	0.027	34.5	30.0	0.030	36.2	30.5	0.173	33.1	26.6	0.029
$\Delta$ VAS( $\pm$ S.E.)		12.6 $\pm$ 5.73			4.48 $\pm$ 3.44			5.71 $\pm$ 2.65			14.4 $\pm$ 12.7	
median	42.3	31.8		31.3	25.5		33.5	27.5		34.8	20.0	

\*pre/post: data of pre administration/post administration  
Scores for SSS and VAS in the caffeine group compared with placebo group (first day), caffeine-conditioned placebo group and placebo-conditioned placebo group.  $\Delta$ SSS = pre SSS value – post SSS value.  $\Delta$ VAS = pre VAS value – post VAS value. P-values for differences between pre and post value are shown..

in the first day. But no significant difference was found in any intergroup comparison.

### 2.5. Visual analogue scale (VAS)

The results for VAS of means and median values are shown in Table 2. The results of VAS were indicated similarly to those of SSS. The caffeine group showed a tendency for reduced drowsiness compared to the placebo group in the first day, but no significant difference was found in any intergroup comparison.

### 2.6. NIRS measurement

It should be noted that NIRS measurement may be influenced by body motion, breathing, headset position, etc. We rejected charts with well-defined artifacts. The average  $\Delta\text{Oxy-Hb}$  (mM $\cdot$ mm) values for caffeine (C) and placebo (P) administration on the first day are shown in Fig. 3. Average  $\Delta\text{Oxy-Hb}$  values for the caffeine-conditioned placebo (C-P) and placebo-conditioned placebo (P-P) at the third day are shown in Fig. 4. No significant difference was found in any comparison

### 2.7. Genotyping

Genotyping frequencies were 52.4 % for S/S (n=22), 40.5% for S/L (n=17), and 7.10 % for L/L (n=3) for all participants. Since the L/L genotype was segregated to one side of block B, statistical analysis was carried out only for the P and P-P groups. Genotyping frequencies in block B were 61.9 % for S/S (n=13), 23.8 % for S/L (n=5), and 14.3 % for L/L (n=3).

The average SSS values for P at the first day are shown in Fig. 5 (A-left side) and the average SSS values for P-P at the third day are shown in Fig. 5 (A-right side). The value for P was significantly increased in L/L versus S/S (average  $\pm$  standard error of L/L:  $1.00 \pm 0.0$ ,  $P=0.022$  vs. S/S:  $0.15 \pm 0.15$ ), and also versus S/L (average  $\pm$  standard error of L/L:  $1.00 \pm 0.0$ ,  $P=0.024$  vs. S/L:  $0.20 \pm 0.20$ ). The value for P-P was significantly increased in L/L versus S/S (average  $\pm$  standard error of L/L:  $1.33 \pm 0.33$ ,  $P=0.0092$ , vs. S/S:  $0.0 \pm 0.20$ ). The average VAS values for P at the first day are shown in Fig. 5 (B-left side), and those for P-P at the third day are shown in Fig. 5 (B-right side). No significant difference was found in any comparison. For NIRS we analyzed the following regions: 46 right area (channels 5-9), 10 area (channels 10-13), and 46 left area (channels 14-18) as shown in Fig. 6. The average blood flow volumes for P and P-P are shown in Fig. 6 (A) and Fig. 6 (B), respectively. The value for P was significantly increased in S/L versus S/S in the 46 right area (average  $\pm$  standard error of S/L:  $0.53 \pm 0.78$ ,  $P=0.018$  vs. S/S:  $-0.15 \pm 1.2$ ), while it was significantly decreased in L/L versus S/L (average  $\pm$  standard error of L/L:  $-0.25 \pm 1.25$ ,  $P=0.037$  vs. S/L:  $0.53 \pm 0.78$ ). The value for P was significantly increased in S/L versus S/S in the 10 area (average  $\pm$  standard error of S/L:  $0.50 \pm 1.4$ ,  $P=0.045$  vs. S/S:  $-0.27 \pm 1.03$ ). In the 46 right area, the value for P-P was significantly increased in L/L versus S/S (average  $\pm$  standard error of L/L:  $1.03 \pm 1.40$ ,  $P=0.58 \times 10^{-5}$  vs. S/S:  $-0.38 \pm 0.79$ ), and also versus S/L (average  $\pm$  standard error of L/L:  $1.03 \pm 1.40$ ,  $P=0.0059$  vs. S/L:  $-0.11 \pm 0.78$ ).

## 3. Discussion

In our previous studies, we confirmed the existence of good responders and non-responders to placebo, in terms of both subjective and objective indicators (Itoh et al. 2010), although subjective indicators were more responsive to cognitive actions such as counseling and placebo effects, compared with the objective indicator (Isawa et al. 2013). In the present study, the caffeine group showed a tendency for reduced drowsiness compared to placebo on the first day in terms of SSS and VAS, confirming the expected action of caffeine. On the other hand, placebo medication significantly improved drowsiness compared to pre-administration, confirming the existence of a placebo effect. Two hundred mg caffeine is equivalent to two cups of brewed coffee. Participants who reported that they felt the effect of caffeine were 12 persons, while those

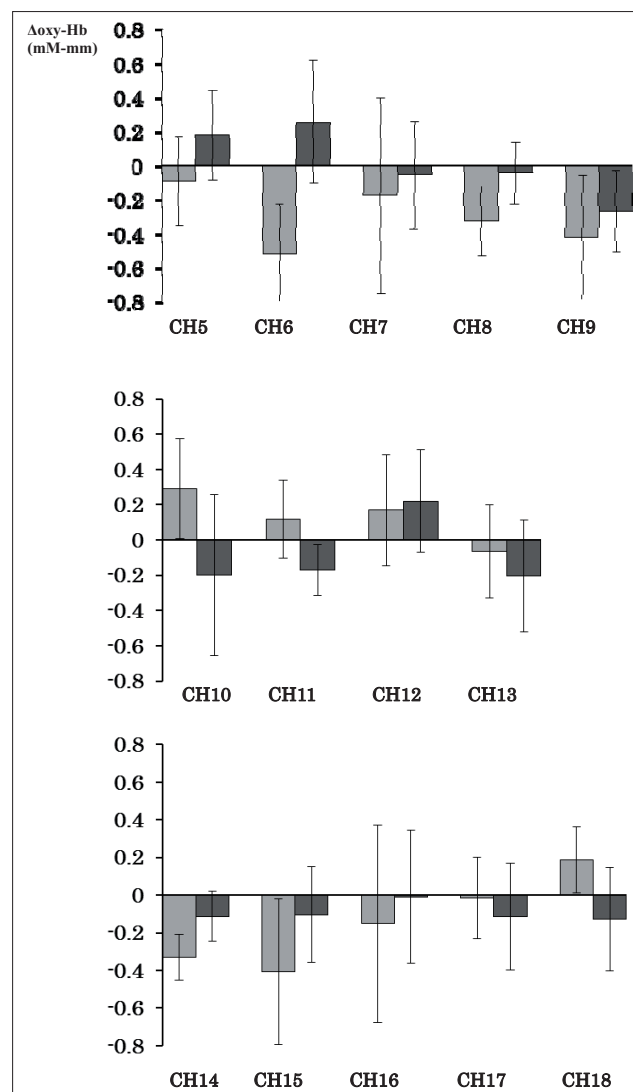


Fig. 3:  $\Delta\text{Oxy-Hb}$  in the inferior frontal cortex region for caffeine and placebo in the first day

- Legend:  
 Light gray bar: Caffeine group  
 Dark gray bar: Placebo group
- (A)  $\Delta\text{Oxy-Hb}$  in the inferior frontal cortex region (channels 5, 6, 7, 8 and 9). The error bars represent the standard errors of oxy-Hb values.  
 Channel (Ch) X = Y (C group), Z (P group)  
 Ch5: n=14,11, Ch6: n=16,17, Ch7: n=15,15, Ch8: n=16,16, Ch9: n=15,16.
- (B)  $\Delta\text{Oxy-Hb}$  in the inferior frontal cortex region (channels 10, 11, 12 and 13). The error bars represent the standard errors of oxy-Hb values.  
 Channel X = Y (C group), Z (P group)  
 Ch10: n=11,8, Ch11: n=16,14, Ch12: n=15,16, Ch13: n=11,9
- (C)  $\Delta\text{Oxy-Hb}$  around the inferior frontal cortex region (channels 14, 15, 16, 17 and 18). The error bars represent the standard errors of oxy-Hb values.  
 Channel X = Y (C group), Z (P group)  
 Ch14: n=17,16, Ch15: n=17,17, Ch16: n=8,8, Ch17: n=9,7, Ch18: n=16,11.

who did not were 5 persons. In the case of SSS, the former group showed a significant improvement compared with the latter group ( $p < 0.01$ , data not shown). Conditionality (memory) may be correlated with different effects of medicine.

On the other hand, the NIRS results confirmed the expected action of caffeine on the first day. The reduction of brain blood flow was considered to be due to caffeine-induced coarctation of brain arterioles, i.e., the pharmacological action of caffeine. However it failed to show significant placebo effects overall.

When we investigated the influence of polymorphism of the serotonin transporter (5-HTT), we found that subjects with the L/L type were significantly more susceptible to the placebo effect than the others in terms of subjective indicators. It is noteworthy that the multidimensional approach of SSS indicated greater effectiveness than the single-dimensional measurement of VAS. NIRS

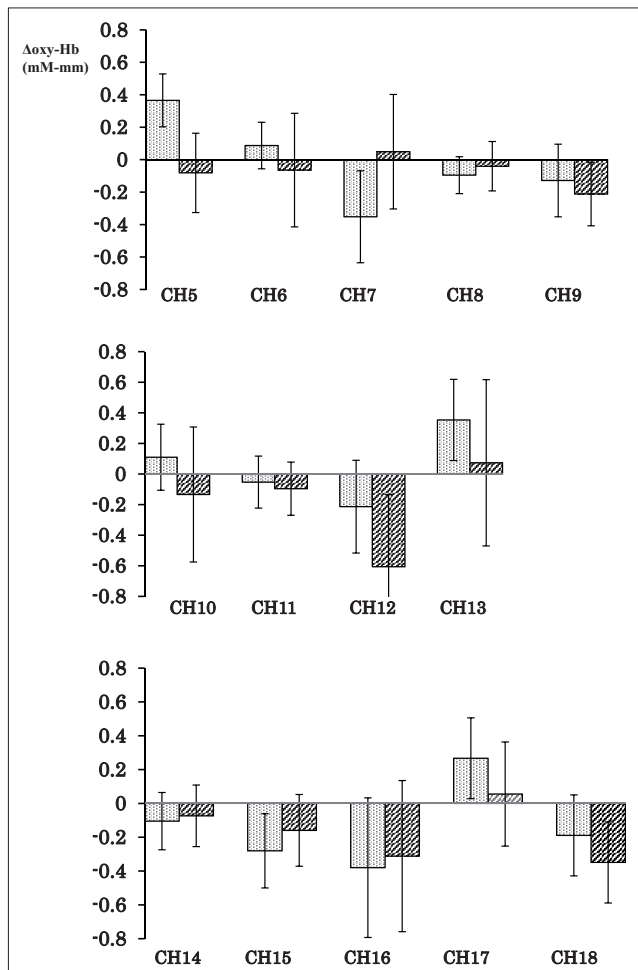


Fig. 4:  $\Delta$ oxy-Hb in the inferior frontal cortex region for caffeine-conditioned placebo and placebo-conditioned placebo group. In the third day, all subjects received placebo

▨ : Caffeine conditioned placebo group  
▨ : Placebo conditioned placebo group

- (A)  $\Delta$ oxy-Hb in the inferior frontal cortex region (channels 5,6, 7, 8 and 9). The error bars represent the standard errors of oxy-Hb values.  
Channel (Ch) X = Y (C group), Z (P group)  
Ch5: n=8,3, Ch6: n=12,5, Ch7: n=11,4, Ch8: n=11,5, Ch9: n=12,4
- (B)  $\Delta$ oxy-Hb in the inferior frontal cortex region (channels 10, 11, 12 and 13). The error bars represent the standard errors of oxy-Hb values.  
Channel X = Y (C group), Z (P group)  
Ch10: n=6,2, Ch11: n=10,4, Ch12: n=11,5, Ch13: n=6,3
- (C)  $\Delta$ oxy-Hb around the inferior frontal cortex region (channels 14,15,16,17 and 18). The error bars represent the standard errors of oxy-Hb values.  
Channel X = Y (C group), Z (P group)  
Ch14: n=11,5, Ch15: n=12,5, Ch16: n=6,2, Ch17: n=5,2, Ch18: n=7,4

measurement of L/L type in the placebo conditionality group on the third day also showed increased blood flow in the 46 field area of the brain prefrontal region as compared with S/S and S/L type. The conditionality data indicate that homozygous long allele carriers performed better in cognitive tasks compared with homozygous carriers for the short allele. This finding is consistent with previous work (Furmark et al. 2008). It is interesting that although the placebo effect could not be objectively confirmed overall (Fig. 4), it was detected in the L/L genotype both subjectively and objectively. As for the NIRS results,  $\Delta$ Oxy-Hb in the 46 field area was associated with working memory capacity and concentration (Tsujii et al. 2007). However, care is needed in interpreting this result, as we had only three L/L subjects, and they were all in the placebo group. Also, in considering the pharmacological action of caffeine, we should take account of the activity of CYP or affinity of adenosine receptor for caffeine. Thus, although our results suggest that the L/L genotype may be associated with placebo responder status, further studies with larger numbers of

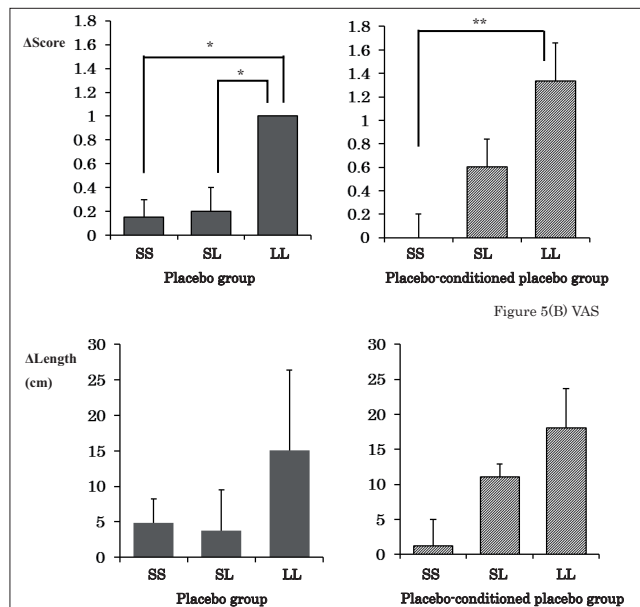


Fig. 5: Estimated SSS and VAS scores as a function of serotonin transporter (5HTT) genotype. Comparison of performance between homozygous short carriers (S/S), heterozygous carriers (S/L) and homozygous long carriers (L/L). Scores for SSS and VAS in the placebo group (first day) compared with placebo-conditioned placebo group (placebo administration in third day). The error bars represent the standard errors of scores. Genotyping numbers were n=13 for S/S, n=5 for S/L, and n=3 for L/L.

(A): SSS

(B): VAS

\*\* : The SSS score for S/S was significantly different from that for L/L in the placebo conditioned placebo group ( $p=0.0092$ ).

\* : The SSS score for S/S was significantly different from that for S/L in the placebo group ( $p=0.022$ ).

\* : The SSS score for S/L was significantly different from that for L/L in the placebo group ( $p=0.024$ ).

subjects seems necessary. Nevertheless, it is worth noting that polymorphisms of catechol-O-methyltransferase (COMT) also influence drug effects: subjects with Met-type COMT are more susceptible to counseling and placebo effects (Hall et al. 2012). It is necessary to study the influence of polymorphism of COMT along with that of 5-HTT.

Another interesting point is the ethnic difference in the frequency of 5-HTTLPR polymorphisms (Chiao and Blizinsky 2010). The frequency of the L/L genotype in Japanese people is 3.2%, whereas it is 32.2% in Americans. As for S/S type, the frequency is 61.1% in Japanese people, but 18.8% in Americans. There may be an ethnic difference in placebo reaction. Studies on the influence of polymorphisms on placebo effects have been conducted in Europe and the United States (Radua et al. 2014; Pecina et al. 2015; Leuchter et al. 2009), and similar studies may be needed in Japan. Our results support the idea that subjects with L/L type homozygosity of the serotonin transporter-linked polymorphic region are most responsive to the placebo effects. Detailed studies of placebo effects will be useful in deciding optimum pharmacological treatment for patients.

## 4. Experimental

### 4.1. Participants

Candidates, who were 20 to 35 years old, working in the Faculty of Pharmacy in Keio University campus, received a doctor's examination and physical examination (height, weight, temperature, and blood pressure). Clinical evaluations included measurements of aspartate aminotransferase (AST), alanine aminotransferase (ALT), lactate dehydrogenase (LDH),  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GTP) and alkaline phosphatase (ALP). Blood cell and platelet counts were done and a hemogram was obtained. Urine was examined for protein, sugar, and blood.

Persons with hypersensitivity to caffeine or xanthine derivatives, or habitual users of caffeine or xanthine derivatives were excluded. Among 43 screeners, 42 were eligible (male 14, female 18) for this study. Average age of the participants was  $23.5 \pm 1.20$  years (average  $\pm$  standard deviation). All participants were right-handed. The subjects gave prior written informed consent before entry.

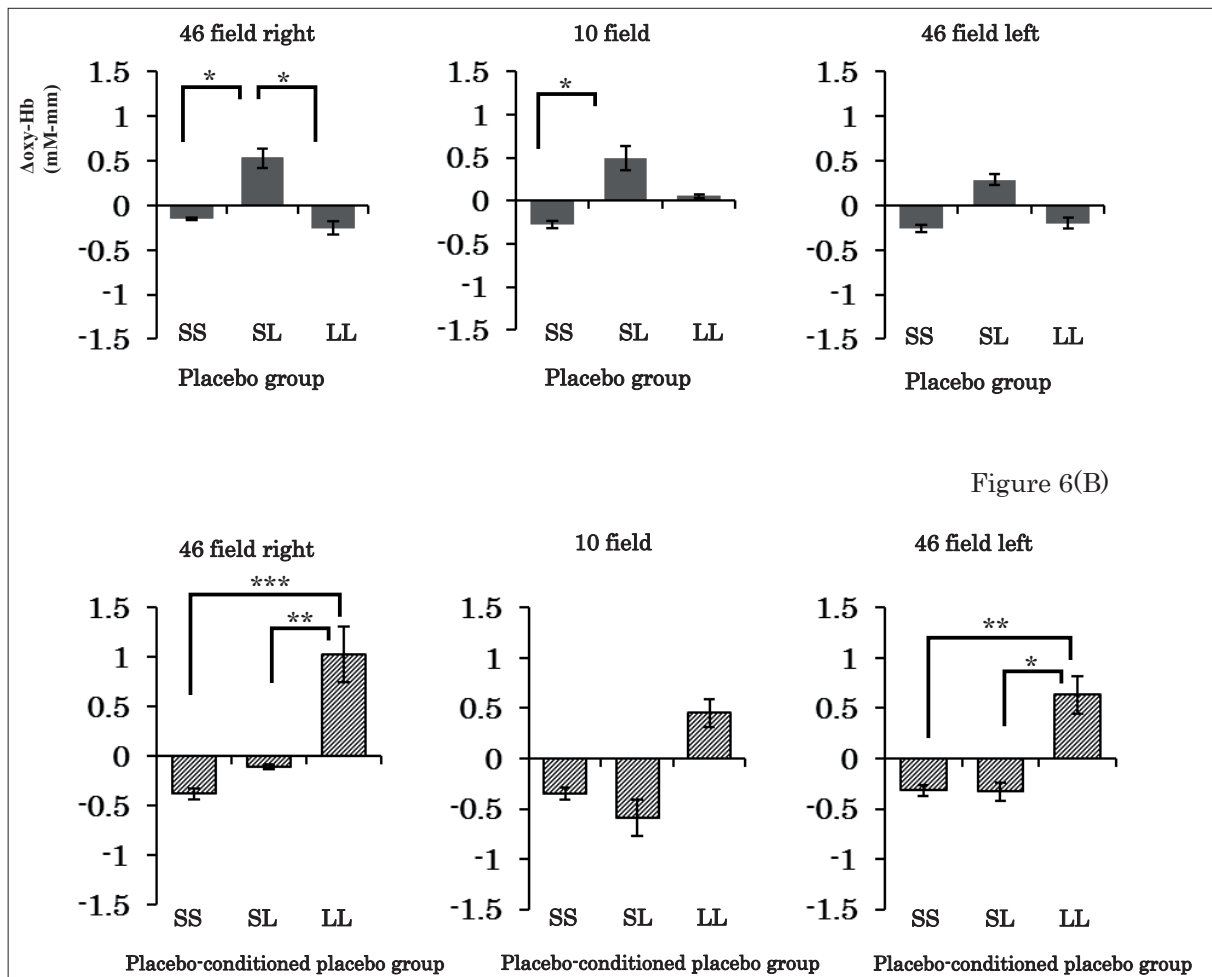


Figure 6(B)

Fig. 6: Estimated NIRS-evaluated blood volume of the brain prefrontal cortex as a function of serotonin transporter (5HTT) genotype. Comparison between homozygous short carriers (S/S), heterozygous carriers (S/L) and homozygous long carriers (L/L) ΔOxy-Hb value for placebo group (first day) compared with placebo-conditioned placebo group (placebo administration in the third day). The error bars represent the standard errors of scores. Genotyping was n=13 for S/S, n=5 for S/L, and n=3 for L/L.

(A): placebo group

(B): placebo-conditioned placebo group

\*\*\*: ΔOxy-Hb value for S/S was significantly different from that for L/L in the placebo-conditioned placebo group in 46 field right ( $p = 0.00058$ ).

\*\*\*: ΔOxy-Hb value for S/L was significantly different from that for L/L in the placebo conditionality group in 46 field right ( $p = 0.0059$ ).

\*\*\*: ΔOxy-Hb value for S/S was significantly different from that for L/L for placebo-conditioned placebo group in 46 field left ( $p = 0.0018$ ).

\*: ΔOxy-Hb value for S/L was significantly different from that for L/L for placebo-conditioned placebo group in 46 field left ( $p = 0.016$ ).

\*: ΔOxy-Hb value for S/S was significantly different from that for S/L in the placebo group in 46 field right ( $p = 0.018$ ).

\*: ΔOxy-Hb value for S/L was significantly different from that for L/L in the placebo group in 46 field right ( $p = 0.037$ ).

\*: ΔOxy-Hb value for S/S was significantly different from that for S/L for placebo group in 10 field ( $p = 0.045$ ).

#### 4.2. Environment

A controlled environment with a temperature of  $24.8 \pm 0.20$  degrees C and a humidity of  $46.8 \pm 2.60$  % (average  $\pm$  standard deviation) was maintained in the test room.

#### 4.3. Evaluation items

The following test items were evaluated in the caffeine group and placebo group (after the first dose), and under caffeine conditionality and placebo conditionality conditions (after the second dose).

- (1) Feeling of drowsiness was self-evaluated by using the Japanese version of the multidimensional Stanford Sleepiness Scale (SSS) (Hoddes et al. 1973) (Table 3).
- (2) Feeling of drowsiness was also self-evaluated by using on the single-dimensional Visual Analogue Scale (VAS).
- (3) Cerebral blood flow in selected brain regions was measured with a NIRS apparatus (compact, wearable near-infrared spectroscopy appliance, Hitachi, Ltd. Tokyo).
- (4) Polymorphisms of 5-HTTLPR were evaluated.

#### 4.4. Experimental schedules

In order to avoid the influence of environmental stress, participants were seated in calm and comfortable individual cabins with air-conditioning for NIRS measurement. The two blocks of the study were separated by an interval of 24 h. All participants were requested to avoid caffeine in all forms for at least 12 h before the start of tests, and to sleep for 6-8 h during the previous night. Participants did not know whether they were receiving active drug or placebo.

Table 3: Stanford Sleepiness Scale

Degree of Sleepiness	Scale Rating
Feeling active, vital, alert, or wide awake	1
Functioning at high levels, but not fully alert	2
Awake, but relaxed; responsive but not fully alert	3
Somewhat foggy, let down	4
Foggy; losing interest in remaining awake; slowed down	5
Sleepy, woozy, fighting sleep; prefer to lie down	6
No longer fighting sleep onset soon; having dream-like thoughts	7

Instructions: Using the 7-point scale below, pick what best represents how you are feeling and note the corresponding number on the chart below

First stage of block A: participants take 2 capsules containing caffeine. Before and after taking the medication, participants received SSS, VAS and NIRS. Second stage of conditioning for caffeine in block A: participants take 2 capsules filled lactose. Before and after taking the medication, participants received SSS, VAS and NIRS. First stage of control of block B: participants take 2 capsules containing lactose. Before and after taking the medication, participants received SSS, VAS and NIRS. Second stage of non-conditioning for caffeine of block B: participants take 2 capsules

filled lactose. Before and after taking the medication, participants received SSS, VAS and NIRS.

#### 4.5. Explanation about caffeine

Before administration in the first stage, all participants received the same explanation about caffeine by the same examiner, with the aid of a brochure, for about five minutes. The information covered the expected sleep-averting effect of caffeine, action mechanism, maximal efficacy time (peak pharmacological action), and a comparison of the effect of 200 mg caffeine with coffee and energy drink intakes.

#### 4.6. Assay of polymorphisms of 5-HTTLPR (genotyping)

Genotyping was conducted according to the method of Anderson et al. (2012).

- (1) Samples of buccal mucous membrane were collected on a Whatman Omni swab (Sigma-Aldrich, St. Louis, MO, Tokyo). Genotyping was conducted at the Division of Pharmaceutics, Faculty of Pharmacy, Keio University. DNA was isolated using the QIAamp DNA Investigator kit (QIAGEN, Tokyo).
- (2) DNA Amplification  
Polymorphic regions of 5-HTTLPR were amplified by PCR according to Camilleri et al. (2002). The polymerase chain reaction was modified to include 0.2  $\mu$ M of each primer, 1.75 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.64 M betaine, 0.05 U/ $\mu$ L, and Taq polymerase in (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> reaction buffer. The amplification was performed on a C1000 Thermal cycler (Bio-Rad Laboratories).
- (3) Genotyping  
The amplified fragments were separated on a 2.5% agarose gel (Wako). Agarose electrophoresis was performed using a Mupid-X (ADVANCE).

#### 4.7. Methods of evaluation

##### 4.7.1. Stanford sleepiness scale (SSS) <U3e>

The primary subjective outcome measure was the SSS, which consists of seven questions about drowsiness, each with an ascending scale of 1 to 7. Participants selected and checked the point that they considered the most suitable.

##### 4.7.2. Feeling of drowsiness - Visual analogue scale (VAS)

A ten centimeter scale had the statement "I do not feel drowsy at all" on the left, and "I am falling asleep" on the right. Participants were asked to pick a point on the scale that matched their state. Scales were measured from the left side, so that a larger value corresponds to a greater feeling of drowsiness.

##### 4.7.3. NIRS

Average values of  $\Delta$ Oxy-Hb for the task state were calculated after baseline correction. We focused on channels 5 to 18 of the NIRS instrument, because data from other channels were rather unstable.

##### 4.7.4. Polymorphisms in 5-HTTLPR

The S/S, L/L and S/L homo- and hetero-genotypes were identified.

#### 4.8. Statistics

All data fixed steady, and there was no change to data after analysis and mapping commencement. Data were expressed as mean  $\pm$  standard error and median values of SSS/VAS. The significance of differences between conditions was evaluated by means of Student's *t*-test procedure (PAIRED statement in Excel). The criterion of significance was  $p < 0.01$  or  $p < 0.05$ . We also employed one-way analysis of variance (one-way ANOVA) where appropriate.

##### 4.8.1. SSS

SSS was completed by participants using numerical values on a 7-point scale, before and after caffeine or placebo administration. The score before administration was subtracted from the score after administration.

As a baseline, caffeine (C), caffeine-conditioned placebo (C-P) in block A and placebo (P), placebo-conditioned placebo effect (P-P) in block B were compared between pre and post medication. Also, the effect of C was evaluated by comparing the results with that for P on the first day. To examine the influence of conditionality, the effect of C was evaluated by comparing the result with that for C-P in the same participants. Finally, P-P was evaluated by comparing the result with that for C-P in different participants.

##### 4.8.2. Feeling of drowsiness-VAS

Feeling of drowsiness-VAS was measured from the point of "I do not feel drowsy at all". The results were compared with those of SSS.

##### 4.8.3. Oxy-Hb derived from NIRS measurement

Average values of  $\Delta$ Oxy-Hb for the task state were calculated after baseline correction. We focused on channels 5 to 18 of the NIRS instrument, because the data from other channels were rather unstable. The results were compared with those of SSS.

##### 4.8.4. Genotyping

Results of genotyping were compared using one-way ANOVA, followed by Tukey's test.

#### 4.9. Ethics statement

This study was approved by the Keio University Faculty of Pharmacy Medical Ethics Board (permission number 140425-1 the Keio University Faculty of Pharmacy Ethics Board for human genome and gene analysis (permission number G140425-1), and was registered at University Hospital Medical Information Network (<http://www.umin.ac.jp>, registration number UMIN000022301).

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