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L-theanine and caffeine improve target-specific attention to visual stimuli by decreasing mind wandering: a human functional magnetic resonance imaging study

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Abbreviations

fMRI; functional magnetic resonance imaging

BOLD; blood oxygenated hemoglobin level-dependent

FSL; FMRIB Software Library

VCSDRT; visual color stimulus discrimination reaction time

WF; white-colored flashes

RF; red-colored flashes

RT; reaction time

ACCEPTED MANUSCRIPT

Abstract

Oral intake of L-theanine and caffeine supplements are known to be associated with faster stimulus discrimination, possibly via improving attention to stimuli. We hypothesized that L-theanine and caffeine may be bringing about this beneficial effect by increasing attention-related neural resource allocation to target stimuli and decreasing deviation of neural resources to distractors. We used functional magnetic resonance imaging (fMRI) to test this hypothesis. Solutions of 200 mg of L-theanine, 160 mg of caffeine, their combination or the vehicle (distilled water; placebo) were administered in a randomized four-way crossover design to nine healthy adult males. Sixty minutes after administration, a 20-minute fMRI scan was performed while the subjects performed a visual color stimulus discrimination task. L-theanine and L-theanine-caffeine combination resulted in faster responses to targets compared to placebo ($\Delta = 27.8$ ms, $P = .018$; and $\Delta = 26.7$ ms, $P = .037$ respectively). L-theanine was associated with decreased fMRI responses to distractor stimuli in brain regions that regulate visual attention, suggesting that L-theanine may be decreasing neural resource allocation to process distractors, thus allowing to attend to targets more efficiently. L-theanine-caffeine combination was associated with decreased fMRI responses to target stimuli as compared to distractors in several brain regions that typically show increased activation during mind wandering. Factorial analysis suggested that L-theanine and caffeine seem to have a synergistic action in decreasing mind wandering. Therefore, our hypothesis that L-theanine and caffeine may be decreasing deviation of attention to distractors (including mind wandering), thus enhancing attention to target stimuli was confirmed.

Key words

Caffeine; Echo-planar Magnetic Resonance Imaging; Clinical Trial; Tea; Attention

1. Introduction

L-theanine and caffeine are two natural compounds that are purported to improve multiple cognitive functions including alertness and attention [1]. L-theanine is a non-protein forming L-amino acid that has structural similarities to glutamic acid. Caffeine is a natural plant alkaloid. Caffeine is primarily consumed as an ingredient in coffee and both L-theanine and caffeine exist naturally in tea. Both of these compounds are freely available for purchase over the counter in tablet and powder forms as nutritional supplements in the United States. Moreover, both L-theanine and caffeine are used as additives in a variety of food and beverages, including some beverages that claim to improve alertness [2, 3, 4, 5]. Thus, both L-theanine and caffeine are present in the food chain in varied forms.

Pharmacokinetics of both L-theanine and caffeine have been extensively studied. In animal studies, L-theanine has been shown to be readily absorbed from the small intestine and peaks in the plasma within 30 minutes of oral administration [6]. Forty minutes after oral administration of 200 mg of L-theanine, a significant increase in alpha electroencephalographic activation was observed, suggesting that L-theanine level in the brain reaches a therapeutic concentration within 40 minutes of administration [7]. L-theanine is completely cleared from the plasma and the brain within 24 hours of administration [8]. Similarly, caffeine is almost entirely absorbed from the intestines within 45 minutes of oral administration [9] and reaches a peak level in the plasma within 30 – 75 minutes [10]. Overall elimination half-life of 200 mg of caffeine is 3.46 hours [11], thus caffeine can also thought to be completely cleared from plasma within 24 hours of oral administration. However, metabolism of caffeine could be influenced by smoking and cyclical changes in estrogen and progesterone levels in females [12, 13]. Taken together, both L-theanine and caffeine enter the brain and affect brain functions within 45

minutes of oral administration and both substances are almost completely cleared from the body within 24 hours of oral administration.

Despite the fact that L-theanine and caffeine are often believed and at times advertised to improve alertness and attention, little work has been conducted to examine the effects of these compounds and their combination (as they occur naturally in tea; *Camellia sinensis*) on attention in human subjects. Our previous findings indicate that L-theanine, caffeine and their combination improve visual color stimulus discrimination reaction time and increase the P300 event-related potential amplitude in an auditory oddball paradigm (which is an electrophysiological marker of allocation of attention-related neural resources for target stimulus identification and stimulus discrimination [14]) compared to a placebo [15]. Our subsequent analyses also revealed that L-theanine and caffeine have additive effects in increasing neural resource allocation for stimulus discrimination (as indicated by auditory P300 amplitude) and also in increasing visual color stimulus discrimination [15, 16]. These findings are compatible with behavioral and electrophysiological findings of many others, suggesting that L-theanine, caffeine and their combination improve cognitive processing, visual stimulus discrimination and possibly visual attention [1, 15, 16, 17, 18, 19, 20].

While informative, these data provide an incomplete picture because there is no direct evidence demonstrating neural activation in specific brain regions that would support conclusions regarding mechanisms of action of L-theanine and caffeine in mediating the observed cognitive effects. First, during a visual choice reaction time task, the brain regions that are known to process color stimuli (e.g. the primary visual cortex, the lateral occipital cortex, V4) are likely to be involved in differentiating the color stimuli at the visual cortical level [21, 22]. Thus, it is logical to speculate that the primary visual cortex as well as the lateral occipital

cortex could be potential targets of L-theanine and caffeine, if the compounds act on the visual cortical level. Second, a theoretical network of brain regions known as the default-mode network is known to show decreased activity when attention is focused on processing task-related stimuli, while showing increased activity when an individual's attention drifts from processing task-related stimuli to task-independent thoughts (known as mind wandering) [23, 24, 25]. Faster reaction times associated with L-theanine, caffeine and their combination could theoretically be due to decreased mind wandering as reflected by decreased task-related activity in brain regions forming the default mode network (e.g. ventromedial prefrontal cortex, posterior cingulate cortex and precuneus). Third, alteration in neuronal activity in a brain regions known as the anterior cingulate cortex is thought to generate the auditory P300 event-related potential, during the shifting of attention from a standard stimulus to a target stimulus [14]. Furthermore, there is converging evidence to suggest that the anterior cingulate cortex is involved in detecting and allocating attentional resources to resolve conflicts in response selection in reaction time tasks, while being minimally influenced by the characteristics of stimuli *per se* [26, 27]. Therefore, if the mechanisms of action of L-theanine and caffeine involve response selection and allocation of attentional resources for response selection, the anterior cingulate cortex is likely to be potential target of L-theanine and caffeine. Taken together, improved (i.e. faster) reaction times observed in a visual stimulus discrimination task following administration of L-theanine, caffeine and their combination could be due to either 1) increased activity of brain regions that process visual stimuli (e.g. primary visual cortex and visual association areas) in response to target stimuli as compared to distractor stimuli; 2) decreased activity in the brain regions involved in mind wandering (i.e. the default mode network); or 3) increased activity of the anterior cingulate cortex.

Advances in functional magnetic resonance imaging (fMRI) have provided a means to test hypotheses regarding neurophysiological processes underlying improvements in the performance of neuropsychological tasks (e.g. a visual color stimulus discrimination task) by visualizing the alterations in blood flow to specific regions in the brain while the subjects engage in such tasks [28]. This tool is ideal for taking the next logical step in examining the mechanism through which L-theanine, caffeine and their combination improve attention, especially in relation to stimulus discrimination, by allowing for examination of the brain regions that show activations / deactivations during stimulus discrimination in subjects who have ingested these compounds.

Improving human attention in relation to stimulus discrimination has implications for day-to-day functioning (e.g. driving), skilled professions (e.g. pilots, military) and education as well as certain clinical conditions (e.g. attention deficit hyperactivity disorder). Given that there is evidence to suggest that L-theanine, caffeine and their combination are likely to improve the speed of stimulus discrimination, outlining the potential neurophysiological mechanisms leading to such improvements could result in use of these substances and possibly food or beverages containing these substances as performance enhancers as well as therapeutic agents.

Therefore, in a pilot four-way, cross-over fMRI study, we aimed to examine the neurophysiological responses of the human brain while the subjects performed a visual color stimulus discrimination reaction time task, 60 minutes after ingestion of a 150 ml solution of distilled water containing either 1) 200 mg of L-theanine, 2) 160 mg of caffeine or 3) their combination compared to 4) a distilled water only placebo. We specifically hypothesized that L-theanine and caffeine may be improving the speed of stimulus discrimination by the brain, via increased allocation of attention-related neural resources to process target stimuli and decreased

deviation of neural resources in the brain to distractor stimuli (i.e. external distractors) as well as mind wandering (i.e. internal distractors) as measured by blood-oxygen level dependent (BOLD) fMRI responses while the subjects engage in a visual color stimulus discrimination reaction time task. We further hypothesized that L-theanine and caffeine may have additive effects in allocating attention-related neural resources to process target stimuli and in decreasing deviation of neural resources to internal and external distractors.

2. Methods and Materials

2.1. Ethics

All materials and methods were approved by the Human Research Protection Program of the Texas Tech University. All procedures were conducted in accord with the Helsinki Declaration revised in 2000 [29]. Informed written consent was obtained from all subjects who met the eligibility criteria. The protocol was Clinical Trials Registry Number NCT02770105 (<https://clinicaltrials.gov/ct2/show/NCT02770105>).

2.2. Subjects

Nine adult men (age 18-60 years) were recruited from Texas Tech University and or surrounding Lubbock Texas community. Potential subjects were screened for eligibility in an initial telephone pre-screening interview conducted using a standard script. Individuals who had absolute contraindications to undergo magnetic resonance imaging; gross visual, auditory or motor impairments; severe physical, psychiatric or severe neurological impairments; a current or past history of exposure to medications, toxins or substances known to affect neurological function; history of alcohol or substance abuse; and diseases that could be aggravated by L-

theanine or caffeine consumption (e.g. tea or coffee) were excluded from the study. Individuals who were on medications that included caffeine, adenosine receptor blockers or phosphodiesterase inhibitors and individuals who were on medications that are known to interact with caffeine were also excluded. Finally, individuals who were not willing to participate in the interventional study within the given time period were excluded.

2.3. Design

A repeated measures placebo-controlled four-way cross over design was implemented that included 5 weekly in-person visits [Figure 1]. During visit 1, the subjects were re-screened for eligibility and informed consent was obtained. The subjects were subsequently trained to perform a recognition visual reaction time task on a laptop computer and were allowed to practice the task for 10 minutes.

On arrival for each of visits 2 through 5, the subjects consumed either 200 mg of L-theanine; 160 mg of caffeine; a combination of 200 mg of L-theanine and 160 mg of caffeine; or distilled water (placebo) during each consecutive visit. The doses of the substances were determined based on the results of our prior study and studies of several others that suggested administration of 200 mg of L-theanine (equivalent to the dose found in many commercially available supplements or 8 cups of tea), 160 mg of caffeine (equivalent to the dose found in 8 cups of tea) and their combination seem to improve visual stimulus discrimination reaction times and auditory P300 event-related potential amplitudes. Furthermore, evidence derived from a pilot clinical experiment [16] a subsequent dose-response study (unpublished data) also suggested that attention enhancing effects of L-theanine appear to peak at 200 mg. Tea was not administered in a separate arm as our prior studies indicated that a cup of Ceylon black tea is unlikely to elicit

significant neurophysiological or behavioral responses in a stimulus discrimination task compared to a placebo [15]. The order of substance administration by visit was randomized using a random number generator in R statistical software (version 3.2.4). At each visit the subjects arrived and were given the randomly assigned solution to consume. They were then allowed to practice the recognition visual reaction time task for 10 minutes and then remained seated and relaxed until 60 minutes elapsed from the time of administration of the solution. Subsequently, they underwent an approximately 20-minute scanning session.

2.4. Treatment

L-theanine and caffeine were purchased in the purified powder form (Powder City Inc., York, PA, USA) and were stored in opaque air-tight containers in room temperature. The doses of L-theanine and caffeine were measured using a microscale with a precision of 1 mg. L-theanine, caffeine and their combination were administered after dissolving in 200 ml of distilled water to obtain clear solutions. Two-hundred milliliters of distilled water was administered as the placebo. Each solution was prepared within 4 hours of the time of administration. The subjects consumed each 200 ml solution in a disposable plastic cup within 1 minute.

2.5. Imaging Paradigm

All scanning sessions were conducted at the Texas Tech Neuroimaging Institute using a 3.0 T Siemens Skyra scanner equipped with a 20-channel head coil. First, 4 functional magnetic resonance imaging runs were performed followed by a T1-weighted structural magnetic resonance imaging scan. During each functional run, the recognition visual reaction time task was presented on a LCD screen and was reflected using a mirror attached to the head coil of the

scanner. The subjects responded by pressing a button in a hand-held fiber optic device with the index finger of the right hand.

The recognition visual reaction time task adopted from our previous work [15] was programmed and presented using PsychoPy 2.0 (University of Nottingham, UK). A blue-colored background was presented. Within each functional run, 20 white-colored 100-ms flashes (i.e. target stimuli) and 20 red-colored 100-ms flashes (i.e. distractor stimuli) were presented on the background in a random order with a jittered inter-stimulus interval ($5000 \text{ ms} \pm 3000 \text{ ms}$). The subjects were instructed to press the fiber optic response button as quickly as possible, only in response to the white-colored flashes, while ignoring the red-colored flashes. The importance of accuracy and speed was emphasized. The reaction time (i.e. the time between the presentation of the stimulus and the response) of each target stimulus trial and the averaged reaction time to target stimuli within each functional run were displayed on the screen. Responses to distractor stimuli were counted as errors. Target stimuli for which the subjects did not respond within 1000 ms were counted as omissions. Omissions and errors committed within each run were also displayed on the screen. For functional magnetic resonance imaging scans, the stimuli were displayed on an LCD screen and were reflected using a mirror attached to the head coil of the scanner, while the subjects responded by pressing a button in a hand-held fiber optic device with the index finger of the right hand irrespective of the handedness.

fMRI data were acquired using an echo planar imaging sequence with the following parameter settings: repetition time = 2140 ms; echo time = 25; flip angle = 70; field of view = 192 mm X 192 mm; acquisition matrix = 64 X 64; slice thickness = 2.5 mm; and 42 ascending axial slices. Slices were tilted approximately 30 degrees from the anterior commissure – posterior commissure line to minimize orbitofrontal cortical signal dropout [30]. A T1-weighted

MPRAGE scan was also collected using the following parameters: repetition time = 1900 ms; echo time = 2.49; flip angle = 9; field of view = 240 mm X 240 mm; acquisition matrix = 256 X 256; slice thickness = 0.9 mm; and 192 slices in the sagittal plane. Each scanning session was completed within approximately 20-25 minutes.

2.6. Statistical Analyses

Due to the lack of previous comparable fMRI studies to derive effect-sizes, sample size was determined based on the effect-size of the relative increase in auditory P300 event-related potential amplitude (which is an electrophysiological marker of allocation of attention-related neural resources for target stimulus identification and stimulus discrimination) with L-theanine compared to placebo in a previous randomized placebo-controlled crossover trial conducted among healthy adult males [15] using R statistical software (version 3.2.4). Based on the outcomes of the power calculation a minimum of nine subjects was required to confirm the hypothesis of the current study.

All reaction time and error data were imported to R statistical software (version 3.2.4). A mixed-effects model was constructed using the nlme package in R to predict the reaction time using the administered substance, accounting for the variability within the levels of subjects, scans and functional runs. Placebo was used as the reference category, thus the regression coefficients of other categories were interpreted as differences in reaction times between the respective substance and the placebo. A similar mixed effects model was constructed to predict errors. A separate factorial mixed-effects analysis was conducted to examine for the additive and / or synergistic effects of L-theanine and caffeine, including L-theanine, caffeine and their interaction as regressors. $P < 0.05$ was considered significant.

All structural and functional raw data images were converted to NIFTI format using the dcm2nii converter [31]. The Freesurfer (autorecon1) [32, 33] was employed for structural image preprocessing and FMRIB Software Library (FSL; Version 6.00, Oxford, UK) was used for functional image preprocessing and analysis. Preprocessing of functional images included: motion correction by aligning each functional volume to the center volume within each functional run with 6-DOF sinc interpolation using the MCFLIRT tool in FSL [34]; skull-stripping using the BET tool in FSL [35]; registration to high resolution structural space by the BBR algorithm and subsequently to the standard space by 12-DOF using the FLIRT tool in FSL [36]; spatial smoothing using a Gaussian kernel of FWHM 8.0 mm; grand-mean intensity normalization of the entire 4D dataset by a single multiplicative factor; and high-pass temporal filtering (Gaussian-weighted least-squares straight line fitting, with $\sigma=50.0$ s) and FILM pre-whitening [37].

Standard 3-level analysis in the FEAT tool in FSL was used to analyze the fMRI data. In level-1 models, single run functional time series were modeled with task-based regressors for stimuli (i.e. target stimuli, distractor stimuli, correctly identified target stimuli, omissions, errors, and correctly ignored distractor stimuli). Task-based regressors were convolved using a canonical double gamma hemodynamic response function. Confound variables to control for motion effects included the 6 motion parameters, their temporal derivatives, and regressors to scrub (i.e. censor) volumes that exceeded a frame-wise displacement of 0.9 mm [38]. An autocorrelation correction was included to account for serial dependencies between samples not accounted for by the task and confound variables. The contrast between correctly identified target stimuli and correctly ignored distractor stimuli was modeled. In addition, the contrast between correctly identified target stimuli and the baseline and correctly ignored distractor

stimuli and the baseline were examined. In the level-2 analyses, the level-1 experimental variables of all functional runs of each subject were averaged using a fixed effects model [39, 40, 41]. In the third level analysis, the level-2 averages were regressed by 9 variables that represented each subject, and 3 variables that represented L-theanine, caffeine and their combination using a mixed-effects model with subject as a random effect for population inference (FLAME 1). Specifically, the L-theanine vs. placebo, caffeine vs. placebo and L-theanine-caffeine vs. placebo contrasts were examined. A separate level 3 factorial analysis was performed to examine for the additive and / or synergistic effects of L-theanine and caffeine by regressing the level-2 outputs of the scans by variables representing each of the 9 subjects, L-theanine, caffeine and their interaction. The final statistical maps were corrected for multiple comparisons at $P < 0.05$ at a Z-threshold of 1.96 via the Gaussian Random Field Theory-based approach in FSL. The Harvard-Oxford cortical and subcortical structural atlases and the atlasquery tool in FSL were used to identify the brain regions showing statistical significance.

3. Results

All enrolled subjects completed the study [see **Figure 2** for a CONSORT diagram]. Baseline characteristics of the 9 subjects are shown in **Table 1**. Analysis of reaction time data using a mixed-effects model revealed that L-theanine and theanine-caffeine combination significantly improved visual color stimulus discrimination reaction times compared to the placebo by 27.8 ms (SE = 11.6, $t = -2.40$, $P = .018$) and 26.7 ms (SE = 12.1, $t = -2.21$, $P = .037$) respectively. Improvement observed with caffeine (22.5 ms) compared to the placebo approached significance (SE = 12.1, $t = -1.86$, $P = .075$).

Analysis of errors using a mixed effects model revealed that with placebo, 0.78 errors were committed on average during a scanning session and this was significantly different from zero (SE = 0.02, $t = 3.33$, $P = .001$). Mean of errors caused when L-theanine was administered was also 0.78 (SE = 0.19, $t = 0.00$, $P = 1.000$). Administration of caffeine and L-theanine-caffeine combination was associated with reductions of means of errors by 0.17 and 0.19 vs. placebo respectively, however these improvements in accuracies were not significant (SE = 0.19, $t = -0.89$, $P = .380$ and SE = 0.19, $t = -1.01$, $P = .307$ respectively).

Factorial analyses of reaction times revealed a significant main effect for L-theanine (SE = 11.60, $t = -2.40$, $P = .018$), a trend for caffeine (SE = 12.10, $t = -1.86$, $P = .075$). However, the interaction of L-theanine and caffeine was not significant (SE = 16.9, $t = 1.39$, $P = .176$).

Administration of L-theanine as compared to the placebo was associated with a significant decrease in fMRI BMOLD responses in several brain regions including the bilateral frontal medial cortex, bilateral lateral occipital cortex and the right occipital pole when distractor stimuli were presented [Table 2; Supplemental Table S1, Figure 3; Supplemental Figure S1]. Given that the ventro-medial prefrontal cortex is part of a broader network (known as the default mode network), which shows increased activity during mind wandering (i.e. occurrence of stimulus-independent thoughts), and shows decreased activity when mind wandering decreases and attentional resources are allocated to a specific task-related stimulus [23, 24, 25], our finding indicates that L-theanine may be increasing vigilance to detect distractor stimuli. Decreased responsiveness of the regions that process visual stimuli (e.g. occipital pole and lateral occipital cortex [21, 22]) to distractor stimuli may be another mechanism through which L-theanine, when administered alone or in combination with caffeine, may be associated with faster reaction times to target stimuli in the visual color stimulus discrimination reaction time task. Administration of

L-theanine (vs. placebo) was also associated with increased fMRI BOLD responses when target stimuli were presented as compared to distractor stimuli in the right inferior frontal gyrus, which is also a brain region that is known to be involved in target detection [42]. Therefore, L-theanine appears to improve the speed of visual color stimulus discrimination possibly via decreasing mind wandering, increasing recruitment of neural resources for target detection and decreasing neural resource allocation for processing distractor stimuli at visual cortical level.

Administration of caffeine was not associated with a significant change in fMRI BOLD responses as compared to the placebo when each of target and distractor stimuli were presented. However, as compared to the placebo oral intake of L-theanine-caffeine combination was associated with decreased fMRI BOLD responses of the right precuneus and several regions in the right occipital cortex when target stimuli were presented [Table 2]; and of the left lateral occipital cortex and occipital pole when distractor stimuli were presented. When target vs. distractor stimulus conditions were compared, with administration of the L-theanine caffeine combination, left posterior cingulate cortex showed decreased fMRI BOLD responses. Precuneus and posterior cingulate cortex represents the posterior node of the default mode network that shows increased activity when an individual is engaged in mind wandering [23, 24, 25]. Lateral occipital cortex and occipital pole are involved in early processing of visual stimuli [21, 22]. Therefore, our findings suggest that L-theanine-caffeine combination appears to result in faster visual color stimulus discrimination reaction times, possibly via decreasing mind wandering when target stimuli are presented and decreasing early processing of distractor stimuli.

However, there were several unexpected findings in the L-theanine-caffeine vs. placebo comparison. First, Decreased, rather than increased, fMRI BOLD responses were observed in the anterior cingulate cortex (particularly on the left hemisphere) in response to target stimuli,

despite improved reaction times with L-theanine-caffeine combination compared to the placebo. This finding suggests the possibility of an action of L-theanine-caffeine combination that is independent of the attention regulatory mechanisms in the anterior cingulate cortex, given that the anterior cingulate cortex has been shown to regulate attention and arousal to visual stimuli [43] and attention control over response selection [44, 45]. Second, decreased fMRI BOLD responses in the visual information processing occipital regions (e.g. lateral occipital cortex) were noted with L-theanine-caffeine combination in response to both target and distractor stimuli, also suggest the possibility that when a combination of L-theanine and caffeine is administered, an individual may not have to rely on increasing attentional resources at the stimulus processing level or response selection level, as mind wandering is minimized and attentional resources are focused task-related stimuli (as evidenced by decreased reactivity of the posterior cingulate cortex and precuneus) to a greater extent compared to when either L-theanine or caffeine are administered alone.

In the factorial analysis of BOLD responses to distractor stimuli, the L-theanine was found to be associated with decreased mind wandering when processing target stimuli (as evidenced by decreased fMRI BOLD responses in right precuneus [23, 24, 25]) and also decreased early visual attention to both target and distractor stimuli [**Table 3; Supplemental Table S2**]. Similarly, caffeine was associated with decreased mind wandering when processing target stimuli (i.e. decreased fMRI BOLD responses in right precuneus [23, 24, 25]) and decreased early visual attention to target stimuli only. A significant interaction of L-theanine and caffeine factors were found in the right precuneus cortex and right lateral occipital cortex for target stimuli suggesting that L-theanine and caffeine seem to have synergistic effects in decreasing mind wandering in the presence of target stimuli and decreasing early processing of

target stimuli respectively. The target vs. distractor stimulus contrast also showed a significant interaction in the left posterior cingulate cortex, further substantiating the fact the L-theanine and caffeine seem to have a synergistic effect in decreasing mind wandering especially when discriminating between target vs. distractor stimuli.

4. Discussion

Although previous studies have examined the effects of L-theanine, caffeine and their combination on various electrophysiological and behavioral measures (including reaction time), evidence regarding the specific brain regions that appear to be affected by L-theanine, caffeine and their combination in improving performance in behavioral tasks is scarce. In this first of its kind study, we examined the effects of oral administration of 200 mg L-theanine, 160 mg of caffeine and their combination compared to placebo on reaction times and related BOLD responses of the human brain using fMRI in a four-way crossover trial.

We found significantly faster reaction times to the target stimuli with L-theanine and L-theanine-caffeine combination compared to the placebo. We also found a trend of improvement of reaction time for the target stimuli with a moderate effect-size in the caffeine condition. The small sample size in our study may have contributed to our not observing all anticipated effects. Together, these findings do however corroborate our previous findings and findings of others, indicating that L-theanine, caffeine and their combination seem to be associated with faster stimulus discrimination reaction times [1, 15, 16, 17, 18, 19, 20].

Factorial analysis of reaction times revealed a significant main effect for L-theanine and a trend for caffeine. However, the L-theanine X caffeine interaction was not significant. In a

previous study, we have shown that upon oral administration, 200 mg L-theanine and 160 mg caffeine appear to have additive, but not synergistic effects in improving visual color stimulus discrimination reaction times and also the P300 auditory event-related potential amplitudes [15]. Thus, the findings of the factorial analysis of the present study substantiate our previous findings suggesting that in relation to improving (i.e. giving rise to faster) reaction times in a stimulus discrimination task, L-theanine and caffeine seem to have additive effects.

Our fMRI findings suggested that L-theanine appears to improve the speed of visual color stimulus discrimination possibly via decreasing mind wandering, increasing recruitment of neural resources for target detection and decreasing neural resource allocation for processing distractor stimuli at visual cortical level, thus, potentially improving the efficiency of neural resources attending to target stimuli and discriminating target stimuli from distractor stimuli. In two previous studies, Gomez-Ramirez et al. [46, 47] observed decreased background electroencephalographic alpha oscillations in the brain and increased attention related alpha oscillations in subjects who engaged in an inter-sensory and a visuo-spatial attention task following administration of 250 mg of L-theanine as compared to a placebo. Our findings are consistent with the results of these two studies to suggest that L-theanine appear to decrease diverting attention to internal as well as external distractors, thereby allowing an individual to more efficiently attend to target stimuli. Therefore, oral intake of L-theanine supplements and beverages that contain L-theanine as an ingredient at comparable doses (i.e. 200 mg) may improve target discrimination and therefore the performance of day-to-day activities as well as skilled tasks that require vigilance and target discrimination. It should be noted however that L-theanine has been shown to have a vasodilator action in the peripheral circulation, possibly via increasing the production of nitric oxide in the vascular intima [48, 49]. If this effect exists in

cerebral circulation as well, L-theanine may be increasing the BOLD responses irrespective of the status of the task, possibly increasing the baseline BOLD activations and suppressing the BOLD contrast ratio. Future fMRI studies controlling for the vascular effects of L-theanine are needed to fully understand the effects of L-theanine on brain regions that regulate visual attention. Furthermore, more evidence coming from studies that examines the effects of L-theanine on specific day-to-day tasks as well as skilled tasks is required before making recommendations regarding the ability to translate our preliminary finding to practice.

Analysis of fMRI data did not reveal significant differences in BOLD responses in the caffeine condition compared to the placebo. However, the improvement in reaction times to target stimuli in the caffeine condition approached significance as compared to the placebo. This lack of significant findings may have been due to several reasons. First, our study was powered to detect significant improvements in neural resource allocation for attention in L-theanine vs. placebo comparison. Second, caffeine is a well-known cerebral as well as peripheral vasoconstrictor [48]. As a result, caffeine has been shown to suppress the baseline fMRI BOLD responses [48]. Therefore, an adequately powered study needs to be conducted controlling for the vascular effects of caffeine to fully understand and appreciate the neurophysiological effects of caffeine on attention-related neural resources while an individual is engaged in a task that requires stimulus discrimination.

We also observed that L-theanine-caffeine combination appears to result in faster visual color stimulus discrimination reaction times, possibly via decreasing mind wandering when target stimuli are presented and decreasing early processing of distractor stimuli. Therefore, as with L-theanine, L-theanine-caffeine combination also appears to improve the efficiency of neural resource allocation to process target stimuli. Since P300 event-related potentials seem to

decrease with mind wandering [50], it is likely that the action of L-theanine-caffeine combination on the default mode network in the brain on reducing mind wandering may be resulting in increased P300 amplitudes. Therefore, the finding that L-theanine-caffeine combination was associated with decreased BOLD responses in the posterior cingulate cortex and the precuneus is consistent with the increased P300 amplitudes we observed previously in response to administration of the L-theanine-caffeine combination [15]. Similarly, Kelly et al. [51] demonstrated that administration of a combination of L-theanine and caffeine seem to suppress background electroencephalographic alpha activity, supporting our conclusion. Therefore, our findings suggest that oral intake of a combination of 200 mg of L-theanine and 160 mg of caffeine as supplements or as ingredients in food / beverages may be associated with enhance performance in day-to-day tasks as well as skilled tasks that require target discrimination. However, since caffeine has a vasopressor effect on cerebral circulation [52], and as L-theanine may be counteracting this effect [48, 49], it is essential to examine these potential mechanisms in a study that controls for the effects of L-theanine and caffeine on cerebral blood flow.

Factorial analysis of fMRI data provided additional support regarding the specific actions of L-theanine, caffeine and their combination. Both L-theanine and caffeine factors significantly decreased fMRI BOLD responses in the right precuneus (a brain region in the default mode network that is involved in mind wandering [23, 24, 25]) when processing target stimuli and the L-theanine X caffeine interaction was also significant, suggesting that these substances seem to have a synergistic effect in decreasing mind wandering when processing target stimuli. When processing target stimuli as compared to distractor stimuli, L-theanine and caffeine factors showed a significant interaction in the left posterior cingulate cortex, which also shows increased

activity during mind wandering [23, 24, 25], supporting the fact that the actions of L-theanine and caffeine may be synergistic at least at a neurophysiological level. However, only L-theanine was associated with decreasing fMRI BOLD responses in visual information processing regions to distractor stimuli, indicating that this may be a unique action of L-theanine. Therefore, while L-theanine and caffeine appear to act via similar mechanisms showing synergistic effects, L-theanine also appear to act via a mechanism that is independent of the action of caffeine to enhance performance in target stimulus discrimination.

This makes intuitive sense based on the known actions of these compounds on neurotransmitters in the brain. L-theanine is thought to exert cognitive effects by increasing release of dopamine in the brain [53, 54], reducing the release of serotonin, noradrenaline and possibly GABA [55, 56]. L-theanine also functions as a glutamate re-uptake inhibitor and a partial antagonist of glutamate [57, 58]. Caffeine is thought to exert its cognitive effects primarily by competitive inhibition of adenosine receptors A1 and A2A in the brain, resulting in an increased release of dopamine, serotonin, glutamate and noradrenaline [59, 60]. Thus, both L-theanine and caffeine increase dopamine levels in the brain. However, L-theanine decreases serotonin and noradrenaline that are increased by caffeine. Moreover, L-theanine decreases GABA levels in the brain, while caffeine increases glutamate levels. Together, these different mechanisms of action and their interactions may have given rise to the distinct patterns of BOLD responses observed with L-theanine and possibly the L-theanine-caffeine combination.

Being a pilot investigation, the current study has some limitations. First, due to the novelty of using fMRI to examine the differential effects of L-theanine and caffeine on the brain, the sample size was determined using the effect-sizes for increases in auditory P300 event-related potentials in a previous study. Therefore, our study may have been under-powered to

detect changes in BOLD responses in some brain regions. It does however provide valuable data upon which future research can more accurately determine sample size. Second, caffeine is well known to affect cerebral circulation. The effects of L-theanine on human cerebral circulation is not fully understood. Since BOLD fMRI responses depend entirely on changes in regional blood oxygen levels, it is not completely clear whether our observations are due to underlying neural responses or due to vascular responses that are independent of the changes in neural activations [48, 49, 52]. Third, our sample was limited to male subjects to avoid the menstrual cyclical variability in reaction times in female subjects. This limits the generalizability of our findings. Finally, due to being a pilot study with a limited sample size, fMRI data were analyzed and thresholded using a Gaussian Random Field Theory-based approach (an *a priori* decision). Given that thresholding fMRI statistical images using a Gaussian Random Field Theory-based approach have been shown recently to result in higher family-wise error rates than expected, our findings need to be confirmed in a study that is sufficiently powered to use permutation-based thresholding approaches that are robust to the caveats in conventional cluster-wise thresholding options [61]. However, it should be noted that inflation of type I error rates in Gaussian Random Field Theory-based approaches is mainly due to identifying small, near-threshold clusters as significant [61]. All clusters we observed in the current study were larger than 1500 voxels, and most had cluster-wise p-values lower than $p < 0.001$. Thus, despite acknowledged limitations of Gaussian Random Field Theory, our clusters are likely sufficiently improbable to have arisen solely due to chance. Still, future studies building off of this work will want to explicitly evaluate whether our observed clusters also appear in larger, confirmatory samples.

In conclusion, our hypothesis that L-theanine and caffeine may be improving the speed of stimulus discrimination by the human brain, via increased allocation of attention-related neural

resources to process target stimuli and by decreasing deviation of attention-related neural resources to both internal and external distractors was confirmed. Our results also indicated that in addition to the hypothesized additive effects, L-theanine and caffeine may have synergistic effects in decreasing deviation of attention to internal distractors (i.e. mind wandering). Therefore, our study provides preliminary evidence to suggest that oral consumption of 200 mg of L-theanine alone or in combination with 160 mg of caffeine may improve performance in tasks that require stimulus discrimination. Thus, our study provides mechanistic evidence to support the fact the food, beverages and nutritional supplements containing L-theanine and the L-theanine-caffeine combination may enhance stimulus discrimination. However, more evidence is required before these preliminary findings can be translated to clinical practice.

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Table 1: Baseline characteristics of the subjects participating in the four-way crossover trial.

Parameter	Values *
Age (Years)	28.11 ± 9.36
Weight (kg)	86.47 ± 35.68
Height (cm)	176.39 ± 5.84
BMI (kg/m ²)	27.52 ± 10.28

Adult male subjects (N = 9) were recruited to undergo a four-way repeated measures crossover functional magnetic resonance imaging (fMRI) study in which task-related fMRI responses while performing a visual color stimulus discrimination task was examined following administration of each of 200 mg of L-theanine, 160 mg of caffeine, their combination and a placebo (water) in a randomized order.

* Values are means ± SDs

Table 2: Brain regions that showed significant task-related fMRI responses following intake of L-theanine, caffeine and their combination compared to a placebo.

Substance	Stimulus	Response compared to placebo	Brain regions	Functional significance
L-theanine	Target	Not significant		
	Distractor	Decreased (P = 0.001)	Bilateral frontal medial cortex	Decreased mind wandering while processing distractor stimuli
		Decreased (P < 0.001)	Bilateral lateral occipital cortex	Decreased visual attention to distractor stimuli
		Decreased (P < 0.001)	Right occipital pole	Decreased visual attention to distractor stimuli
Target vs. Distractor	Increased (P = 0.049)	Right inferior frontal gyrus	Recruitment of neural resources for target detection	
Caffeine	Target	Not significant		
	Distractor	Not significant		
	Target vs. Distractor	Not significant		
L-theanine-caffeine combination	Target	Decreased (P < 0.001)	Right precuneus cortex	Decreased mind wandering while processing target stimuli
			Right occipital cortex	Decreased recruitment of resources to process target stimuli
	Distractor	Decreased (P < 0.001)	Left lateral occipital cortex	Decreased visual attention to distractor stimuli
			Left occipital pole	Decreased visual attention to distractor stimuli
			Left posterior cingulate cortex	Decreased mind wandering while processing target vs. distractor stimuli
Target vs. Distractor	Decreased (P < 0.001)	Left anterior cingulate cortex	Decreased mind wandering while processing target vs. distractor stimuli	

Adult male subjects (N = 9) consumed 200 mg of L-theanine, 160 mg of caffeine, their combination and a placebo (water) in a randomized order in a four-way repeated measures crossover trial. Functional magnetic resonance imaging (fMRI) was performed while the subjects

engaged in a visual color stimulus discrimination task. Analyses were conducted using FMRIB Software Library (FSL) to examine fMRI responses observed in each stimulus condition (i.e. target vs. distractor) in each treatment condition as compared to the placebo. Third level random effects models were constructed using the FLAME-1 algorithm in FSL. Only the clusters that survived the Z threshold of 1.96 and P threshold of 0.05 after family-wise error correction are reported.

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Table 3: Brain regions that showed significant task-related fMRI responses in a factorial analysis of L-theanine and caffeine factors and their interaction.

Factor	Stimulus	Response vs. placebo	Brain regions	Functional significance
L-theanine	Target	Decreased (P < 0.001)	Right precuneus cortex	Decreased mind wandering while processing target stimuli
		Decreased (P < 0.001)	Right occipital cortex	Decreased recruitment of resources to process target stimuli
	Distractor	Decreased (P < 0.001)	Left frontal medial cortex	Decreased recruitment of resources to process target stimuli
	Target vs. Distractor	Decreased (P < 0.001)	Bilateral lateral occipital cortex	Decreased visual attention to distractor stimuli
Caffeine	Target	Decreased (P < 0.001)	Right precuneus cortex	Decreased mind wandering while processing target stimuli
		Decreased (P < 0.001)	Bilateral intracalcarine cortex	Decreased recruitment of resources to process target stimuli
		Decreased (P < 0.001)	Right occipital fusiform cortex	Decreased recruitment of resources to process target stimuli
	Distractor Target vs. Distractor	Not significant Decreased (P < 0.001)	Bilateral paracingulate cortex	Decreased mind wandering while processing target vs. distractor stimuli
L-theanine X caffeine interaction	Target	Decreased (P < 0.001)	Right precuneus cortex	Decreased mind wandering while processing target vs. distractor stimuli
		Decreased (P < 0.001)	Right lateral occipital cortex	Decreased recruitment of resources to process target stimuli
	Distractor Target vs. Distractor	Not significant Decreased (P < 0.001)	Left posterior cingulate cortex	Decreased mind wandering while processing target vs. distractor stimuli

Adult male subjects ($N = 9$) consumed 200 mg of L-theanine, 160 mg of caffeine, their combination and a placebo (water) in a randomized order in a four-way repeated measures crossover trial. Functional magnetic resonance imaging (fMRI) was performed while the subjects engaged in a visual color stimulus discrimination task. Factorial analyses were conducted using FMRIB Software Library (FSL) to examine the fMRI responses observed in each stimulus condition (i.e. target vs. distractor) with L-theanine and caffeine factors and their interaction. Third level random effects models were constructed using the FLAME-1 algorithm in FSL. Only the clusters that survived the Z threshold of 1.96 and P threshold of 0.05 after family-wise error correction are reported.

Figure 1: Data collection protocol.

VCSDRT, visual color stimulus discrimination reaction time; fMRI, functional magnetic resonance imaging; WF, white flash (i.e. target stimulus); RF, red flash (i.e. distractor stimulus); RT, reaction time.

Figure 2: Eligibility and randomization of study participants and completion of data collection – CONSORT diagram.

Figure 3: Brain regions showing significant task-related fMRI responses following intake of L-theanine, caffeine and their combination compared to a placebo.

Compared to the placebo, a) brain regions that showed greater fMRI responses to target stimuli as compared to distractor stimuli with L-theanine (coronal section); b) brain regions that showed lesser fMRI responses to distractor stimuli with L-theanine (sagittal section on the right side); c) brain regions that showed lesser fMRI responses to target stimuli as compared to distractor stimuli with L-theanine-caffeine combination (horizontal section); d) brain regions that showed lesser fMRI responses to target stimuli as compared to distractor stimuli with L-theanine-caffeine combination (mid sagittal section); e) brain regions that showed lesser fMRI responses to target stimuli with L-theanine-caffeine combination (horizontal section); and f) brain regions that showed lesser fMRI responses to distractor stimuli with L-theanine-caffeine combination (horizontal section).

Adult male subjects ($N = 9$) consumed 200 mg of L-theanine, 160 mg of caffeine, their combination and a placebo (water) in a randomized order in a four-way repeated measures crossover trial. Functional magnetic resonance imaging (fMRI) was performed while the subjects engaged in a visual color stimulus discrimination task. Analyses were conducted using FMRIB Software Library (FSL) to examine fMRI responses observed in each stimulus condition (i.e. target vs. distractor) in each treatment condition as compared to the placebo. Third level random effects models were constructed using the FLAME-1 algorithm in FSL. Only the clusters that survived the Z threshold of 1.96 and P threshold of 0.05 after family-wise error correction are shown.

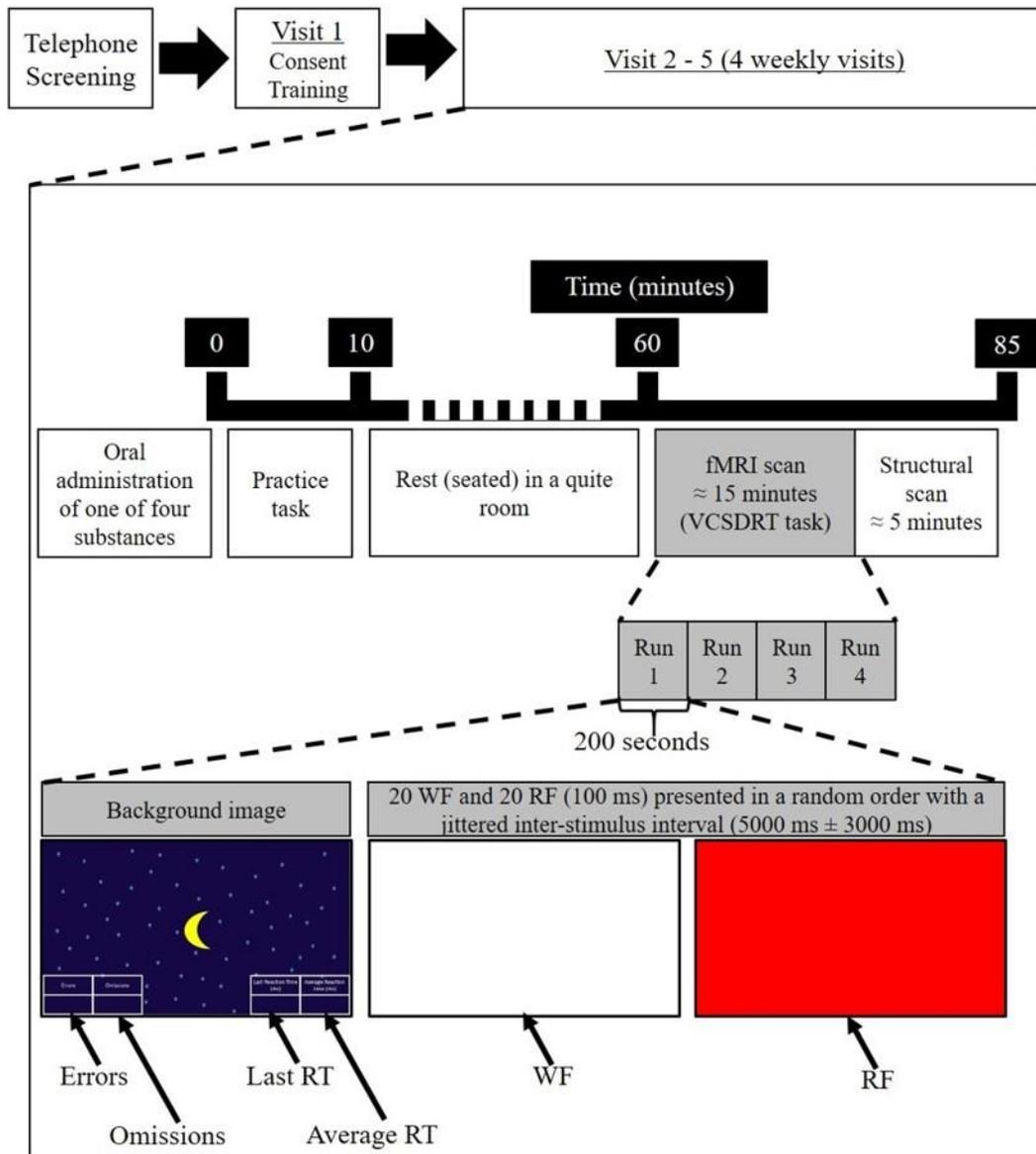


Figure 1

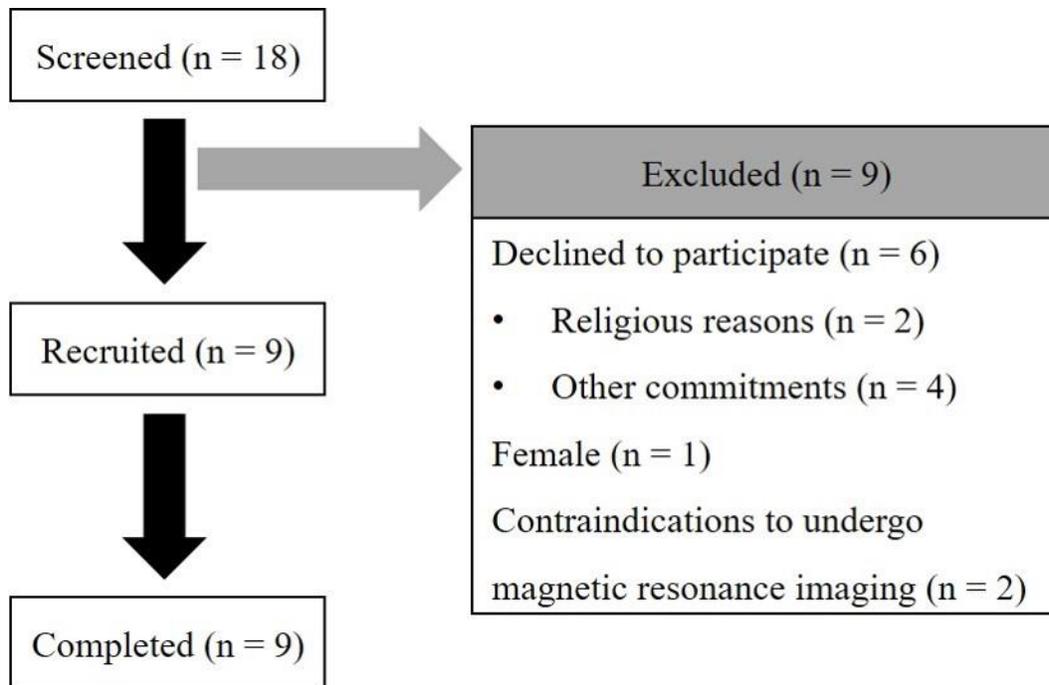


Figure 2

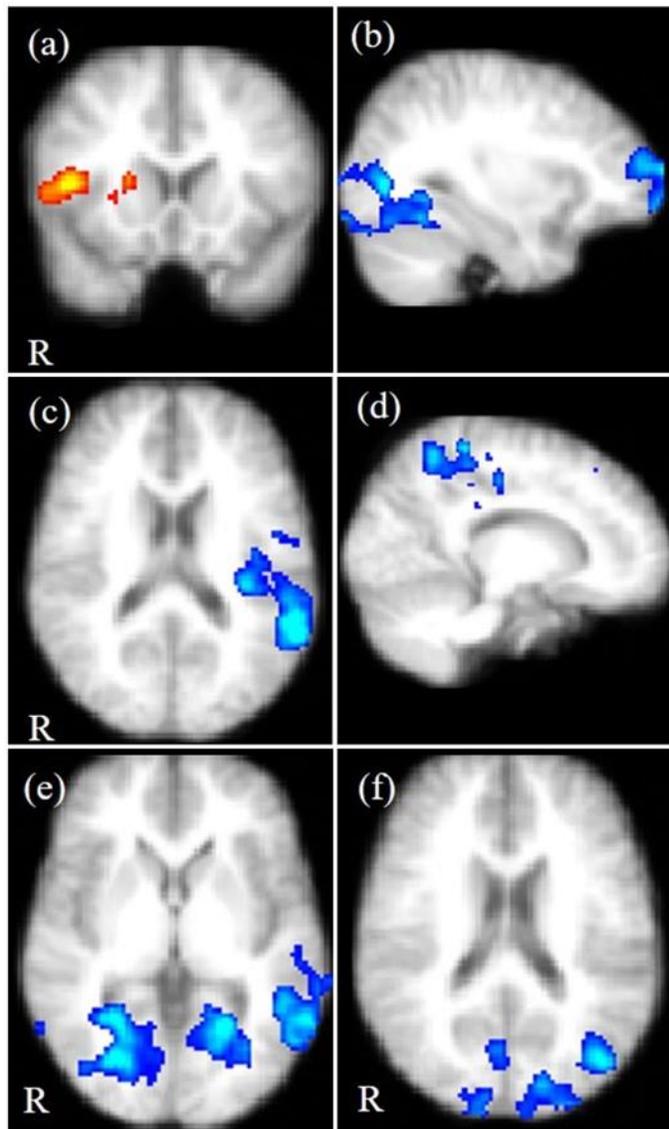


Figure 3