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A Study of Differentiation Analysis of Metabolites in the Bilateral Insular Area of Smoker/Non-smokers and Identification of Differences in Brain Connectivity Between Male University Students in Their 20s

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The aim of this study was to investigate difference in brain metabolites between smokers and non-smokers in the brain insular region related to addiction for men in their twenties. Differences in brain connectivity between the two groups were also determined. A total of 20 males volunteers (10 smokers and 10 non-smokers) were enrolled for this study. Magnetic resonance spectroscopy (MRS) was performed using a 3.0 Tesla MRI scanner. MRS data of left/right insular brain areas were acquired via point-resolved spectroscopy. A total of 3,096 functional magnetic resonance imaging (fMRI) images were obtained under axial conditions in 2D mode. Non-smokers showed significant differences in glycerophosphorylcholine, total choline, free creatine, and total creatine between left and right insular cortical regions. Differences in concentrations of metabolites in the islet cortex between smokers and non-smokers were higher in the left islet region than in the right insular region of non-smokers. Concentrations of tCr metabolites in the left insular area of non-smokers were higher than those of smokers. In addition, smokers showed higher connectivity in the right gyrus and occipital fusiform regions than non-smokers, whereas non-smokers had stronger connectivity between the left insular cortex area and the frontal role right than smokers. This study is meaningful in that it provides a new strategy using fMRI as well as MRS to identify differences in metabolism between smokers and non-smokers in a specific age group.

Keywords : magnetic resonance spectroscopy, magnetic resonance imaging, insular cortex, smoking cessation

1. Introduction

Smoking is known to affect the brain and the nervous system because various chemicals including nicotine, tar, and carbon monoxide are inhaled during smoking [1-3]. Smokers cannot easily quit smoking, even though they are well aware of harmful effects of smoking due to addiction to nicotine contained in cigarettes [4-6]. As a result, various studies have investigated nicotine addiction and a wide range of physiological effects across the nervous system [7-9]. In studies related to addiction, experiments related to the insular area have been conducted. In particular, meaningful experiments related to nicotine addiction concerning smoking have been conducted [10]. Insula is an area involved in addiction. For patients with damaged insula, recurrence and impulsive smoking are not persistent [11].

Morphological diagnosis of the brain can be precisely performed through magnetic resonance imaging. It is difficult to explain brain diseases solely as anatomical damage to specific brain regions. This is because understanding the synaptic pattern of neural circuits is very important to physiologically explain brain functions [12, 13]. Functional magnetic resonance imaging (fMRI) can be used for functional diagnosis. That is, it can be used to visualize the activation of a specific area of the brain [14-16]. fMRI is based on blood oxygen level dependent (BOLD) technique, which increases local blood oxygen partial pressure in a specific active brain region but decreases concentration of deoxy Hb when the activity of the cranial nerve is increased [17]. Thus, it is an imaging modality used to visualize and reveal the activity of cranial nerves as changes in magnetic resonance signals used in the functional analysis of the brain. Structural

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(anatomical) connections of neural elements such as white matter pathways and the degree of coupling between specific cortical regions are actual physical connections. Unlike functional connections, they represent pathways for mutual communication (chemical synapse and electrical junction) [18]. Evaluation of fMRI-based connectivity based on chemical synapse and electrical junctions is effective for *in vivo* monitoring. However, fMRI connectivity cannot be used to observe changes in brain metabolites. Therefore, fMRI and proton magnetic resonance spectroscopy (^{1}H-MRS) have been utilized [19]. In addition, ^{1}H-MRS technique can be used to non-invasively screen metabolites in the brain. MRS is used as a very important test method clinically by selecting a specific brain region for quantifying metabolites [20, 21]. Although MRS is commonly used for the diagnosis of diffuse brain injury, encephalopathy, and metabolic disorders, it can also be used for tumor imaging in specific areas of the brain for positive and differential diagnosis, extension, and treatment follow-up [22, 23]. Accordingly, it represents a gold standard of *in vivo* metabolite quantification.

As described above, for a study related to nicotine addiction, it is possible to evaluate connectivity in the resting state using fMRI of the insular region and analyze brain metabolites using the MRS technique. For the evaluation of connectivity, the path for communication (chemical synapse, electrical junction), which is a physical connection of other brain regions, can be revealed by setting the insular area as a seed. In addition, brain metabolites can be discovered as biomarkers related to addiction due to differences in specific metabolites. While a previous study has evaluated smoking dependence in patients with brain injury, studies investigating smoking associated with insular damage in the general population without under-lying disease have not been reported yet.

Therefore, the objective of this study was to determine the relationship between brain connectivity and metabolites in the bilateral insular area of early smokers legally permitted in our country. Differences in brain metabolites and brain connectivity between smokers and non-smokers in men in their twenties in the brain insular region related to addiction were investigated.

2. Experiment and Method

Following a detailed explanation of the study procedures (approved by the Committee on the Ethics of Experiments of the Korea Basic Science Institute, KBSI-2016-0555-015), all participants gave written informed consent.

2.1. Smoking and non-smoking group characteristics and nicotine addiction assessment

A total of 20 volunteers were selected to observe differences in brain connectivity and metabolite concentration between smokers and non-smokers. Their average age was 23.2 ± 3 years old (Table 1). Twenty healthy volunteers without underlying disease were selected. All applicants were males with similar education level (university students). For smokers, the Fagerström test for nicotine dependence (FTND) was performed before the magnetic resonance test to analyze the correlation between nicotine addiction dependence and the concentration of metabolites in the future. The questionnaire for evaluating nicotine dependence had a total of 11 points, with higher score indicating more severe addiction [24].

2.2. Insular area magnetic resonance spectroscopy (MRS) data acquisition methods and quantification

The experiment for acquiring MRS data in the insular area of this study was designed with reference to the study about identifying the change in the concentration of metabolites before and after rapid withdrawal of nicotine in heavy smokers [25]. All MRS experiments were conducted using a 3.0 Tesla MRI scanner (gradient strength: 200 mT/m). The 32-channel array coils for the brain (AchivaTx 3.0 T; Philips Medical Systems, Netherlands) were used for data acquisition. Using T2-weighted fast spin echoes (repetition time/echo time: 3,000 ms/104 ms; matrix: 256 × 256 pixels; slice thickness: 1 mm; number of excitations: 1), cross-sectional, sagittal, and coronal images were obtained as shown in Fig. 1. Using these acquired T2-enhanced images, the pulse sequence used to acquire ^{1}H-MRS of the left and right insular areas of volunteers was point-resolved spectroscopy (PRESS). Parameters used for data acquisition were set as follows:

Table 1. Volunteer demographic information.

Group	Age	Sex	Education levels	Regions
Smoker	23.8 ± 2.57	male 10, female 0	undergraduate (Same University)	South Korea 10 people
Non-Smoker	22.6 ± 3.24	male 10, female 0	undergraduate (Same University)	South Korea 10 people

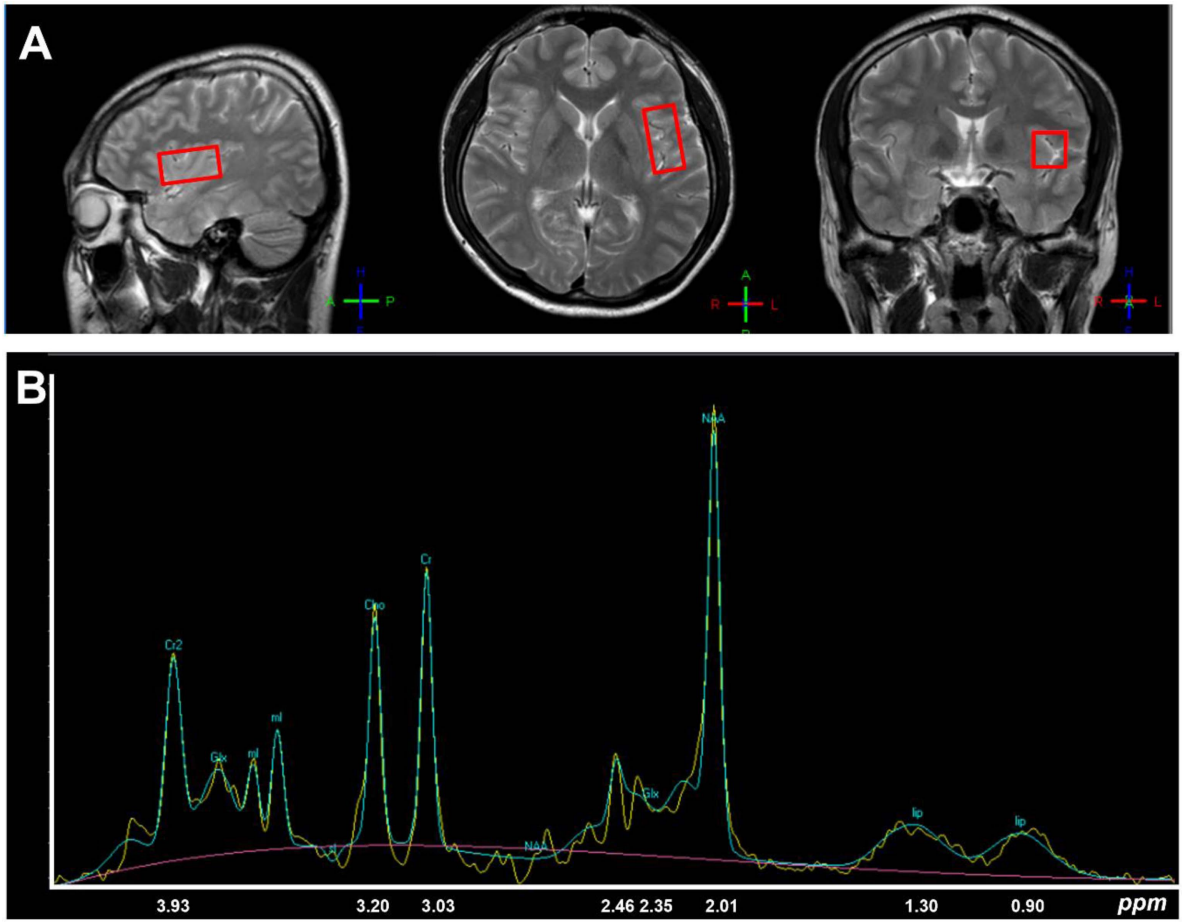

Fig. 1. (Color online) Magnetic resonance spectroscopy acquisition and brain metabolite profile insular area: (a) The volume of interest for 1H-MRS is an area measuring 20 mm × 20 mm × 38 mm^3 located in the left insular area at 3.0T. (b) An example of a spectrum from a smoker subject is shown, including re-sults from the LCModel analysis (red curve). Signals are assigned to Lip, lipids; NAA; NAAG; Glu; Gln; Cr; Ch, choline; mI, myoinositol; and Glx (= Glu + Gln).

TR of 1,500 ms, TE of 35 ms, number of acquisitions of 32, and voxel size 20 mm × 20 mm × 38 mm.

^{1}H-MRS analysis was performed using an automated quantification program LCModel (version 6.31H, Stephen W. Provencher). The applied TE of 35 ms basis was provided directly by the LCMdoel developer for metabolite quantification. Metabolites analyzed in this study were as follows: Asp, Aspartate; Cr, creatine; tCr, creatine compounds; Glu, glutamate; Gln, glutamine; GPC, glucerophosphorylcholine; PCh, phosphorylcholine; tCho, choline-containing compounds; NAA, N-acetyl aspartate; NAAG, N-acetyl aspartyl glutamate; and Tau, taurine (Fig. 1). Less than 20 % standard deviation (%SD) was allowed for metabolite quantification. The %SD called the Cramér–Rao lower bound as a useful reliability indicator was used for error estimation.

2.3. Functional magnetic resonance imaging (fMRI) data acquisition and fMRI analysis

Functional magnetic resonance imaging (fMRI) was performed immediately after acquiring MRS images with the same equipment without any movement of subjects. Gradient echo plannar pulse sequence was used to acquire resting-state fMRI. A total of 3,096 transverse axial 2D images were obtained. Specific fMR parameters were set as follows: TR of 2,200 ms, TE of 30 ms, a flip angle of 90°, matrix of 64 × 64 pixels, slice thickness of 5 mm, and transverse axial of 36 slices. For brain connectivity analysis, the MATLAB software-based "conn" toolbox (http://www.fil.ion.ucl.ac.uk/spm/ext/) was used. The connectivity of the whole brain was analyzed based on the left/right insular area set by the seed. Finally, statistical difference in connectivity between the smoker and non-smoker groups was determined. Region of interest (ROI)-to-ROI connectivity (RCC) matrices were implemented

for connectivity measurement. Related formulas are shown as follows:

$$\gamma(i,j) = \frac{\int R_i(t)R_j(t)dt}{(\int R_i^2(t)dt \int R_j^2(t)dt)^{1/2}}$$

$$Z(i,j) = \tan^{-1}(r(i,j))$$

where R is the BOLD time series within each ROI (for simplicity, time series here is considered centered to zero mean), r is the matrix of correlation coefficients, and Z is the RRC matrix of Fisher-transformed correlation coefficients.

2.4. Statistical Analysis

The LCModel analysis used for quantitative analysis of metabolites in this study provide quantified values by graphically fitting the MRS data of each metabolite. Currently, it is expressed as %SD called the Cramér–Rao of the fitting value of each metabolite. It was denoted by '*' when the Cramér–Rao error was less than 10 % and by '**' when the Cramér–Rao error percentage was less than 5 %. This study's experimental and control groups consisted of 10 volunteers. Therefore, statistical evaluation was performed using a non-parametric method for correlation analysis and means comparison. The accuracy of quantification of each metabolite is indicated in the Table 2 of the results section. FTND score nonparametric correlation analysis expressing nicotine dependency for each metabolite was used. The Wilcoxon Signed rank test was performed to investigate metabolite difference in the bilateral insular region of each group. Mann-Whitney U test was performed to analyze differences in metabolites in the insular area between the smoking group and the non-smoking group.

3. Results

3.1. The nicotine dependence and magnetic resonance spectroscopy (MRS) results

In the present study, the FTND score of the 10 smokers was 4.1 ± 2. Eight brain metabolites were analyzed due to characteristics of 3T in-vivo data obtained. All metabolites were scaled by water signal and used for statistical analysis (Table 2). When nonparametric correlation analysis was conducted based on nicotine dependency and the Glu+Gln/water concentration in the Lt insular cortex, a tendency toward correlation was shown, although it did not reach statistical significance (r = 0.552, p = 0.098). No correlations were observed between concentrations of other metabolites and FTND values.

As a result of analyzing concentrations of eight metabolites, there were no differences in concentrations of metabolites between left and right insular of smokers. In non-smokers, concentrations of tCr, GPC, and tCho were significantly different between left and right insular (Fig. 2). Table 3 shows metabolite differences in left and right insular areas for smokers and non-smokers. There was no difference in metabolites between smokers and non-smokers. However, Tau and tCr concentrations in the left insular cortex region were significantly higher than those in the right insular cortex of non-smokers. In contrast, no statistically significant differences in metabolite

Table 2. Metabolite concentration of the left and right insular areas in the Smoker and Non-Ssmoker Group.

Metabolite	Smoker		Metabolite	non-Smoker	
	Left insular	Right insular		Left insular	Right insular
	Concentration	Concentration		Concentration	Concentration
Asp	1.702 ± 0.261	1.689 ± 0.205	Asp	1.782 ± 0.166	1.727 ± 0.337
Cr*	4.025 ± 0.435	3.904 ± 0.505	Cr*	4.291 ± 0.429	3.970 ± 0.415
Glu*	6.784 ± 0.606	6.653 ± 0.463	Glu*	7.101 ± 0.415	6.895 ± 0.487
GSH	0.845 ± 0.181	0.814 ± 0.108	GSH	0.922 ± 0.138	0.876 ± 0.089
GPC**	0.687 ± 0.072	0.657 ± 0.080	GPC**	0.741 ± 0.039	0.681 ± 0.049
Ins**	2.521 ± 0.286	2.425 ± 0.228	Ins**	2.880 ± 0.151	2.993 ± 1.345
NAA**	6.003 ± 0.602	6.076 ± 0.446	NAA**	6.267 ± 0.332	6.045 ± 0.424
Tau	1.045 ± 0.221	1.053 ± 0.070	Tau	1.218 ± 0.142	1.174 ± 0.168
tCho**	0.687 ± 0.072	0.657 ± 0.080	tCho**	0.741 ± 0.039	0.681 ± 0.049
NAA+NAAG**	6.198 ± 0.642	6.210 ± 0.474	NAA+NAAG**	6.504 ± 0.315	6.279 ± 0.382
tCr**	4.951 ± 0.410	4.441 ± 1.595	tCr**	5.492 ± 0.228	5.067 ± 0.350
Glu+Gln*	8.239 ± 0.883	8.236 ± 0.626	Glu+Gln*	8.805 ± 0.484	8.513 ± 0.617

*Cramér–Rao error percentage is less than 10 %.
**Cramér–Rao error percentage is less than 5 %.

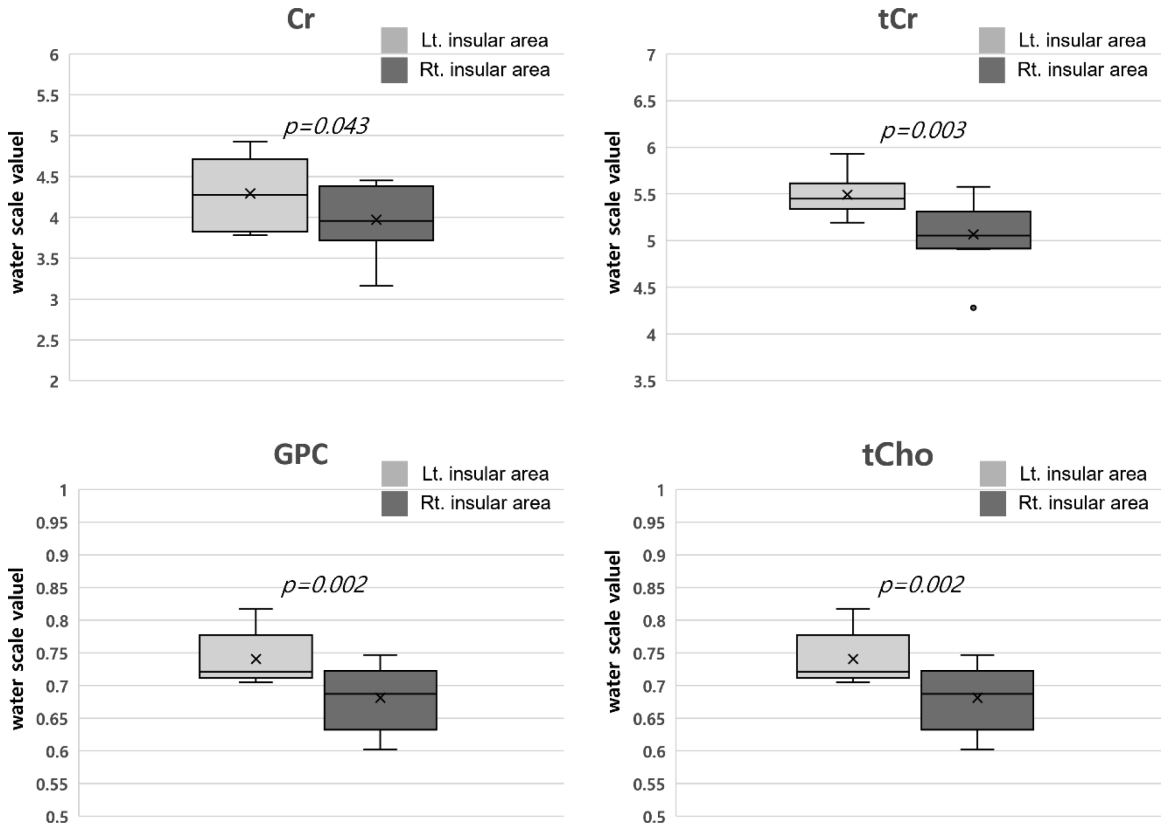

Fig. 2. Metabolites showing significant difference in the left and right insular regions of the non-smoker group: The levels of tCr differed significantly in the non-smoker group, while no significant differences in metabolite concentrations were observed in the smoker group.

Table 3. Mann-Whitney U test results of each of left and right insular area metabolites concentration difference in both groups.

Metabolite	Mann-Whitney U test			
	Left insular area		Right insular area	
	Mann-Whitney U value	p value	Mann-Whitney U value	p value
Asp	35.500	0.280	47.000	0.853
Cr	32.000	0.190	46.000	0.796
Glu	33.000	0.218	35.000	0.280
GSH	37.000	0.353	35.000	0.280
GPC	24.000	0.052	36.500	0.315
NAA	36.000	0.315	41.000	0.529
Tau	21.000	0.029	24.000	0.095
tCho	24.000	0.052	36.500	0.315
NAA+NAAG	34.000	0.247	50.000	1.000
tCr	18.000	0.016	34.000	0.400
Glu+Gln	32.000	0.190	32.000	0.190

concentration were found in the right insular cortex area between smokers and non-smokers. Although other study groups reported changes in Glu concentrations of smokers during smoking cessation [25], no statistically significant change was observed in the present study.

3.2. fMRI connectivity

In smokers, as shown in Fig. 3a, a strong connectivity was detected in 12 brain areas. In non-smokers, seed (insular area) connectivity was found in 9 brain areas, which was slightly fewer than that in smokers. Smokers had stronger connectivity between the right insular cortex

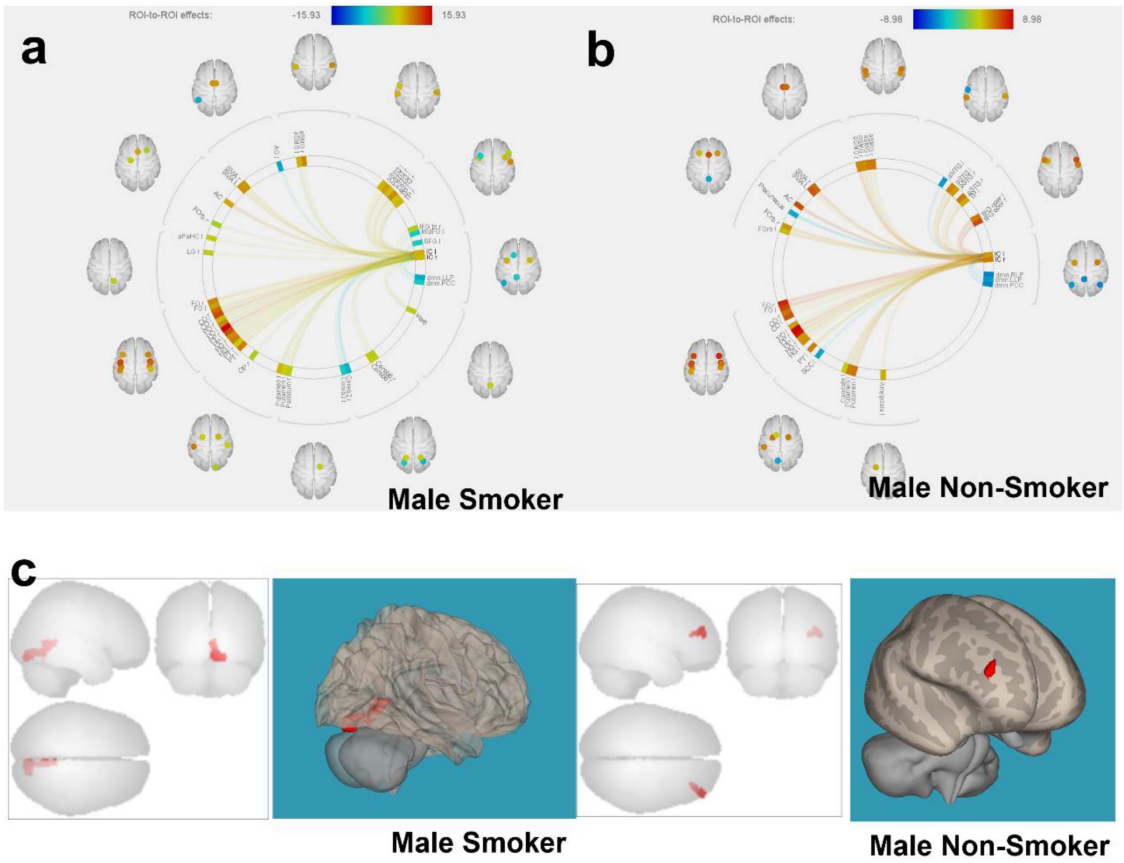

Fig. 3. (Color online) Connectivity between insular cortex and brain regions: (a) Connectivity with each brain re-gion is shown by seeding the left and right insular cortices. (b) The region in which both insular cortices of non-smokers have higher connectivity than that of the smoker (c) is the region where smokers have higher connectivity than non-smokers.

area and the occipital fusiform gyrus right, lingual gyrus right, vermis 4 5, cerebellum 6 right, vermis 6, cerebellum crus 1 right, cingulate gyrus posterior division, precuneus cortex, and cerebellum 4 5 right than non-smokers. The area of the left insular cortex in smokers did not show particularly higher connectivity with other brain areas than nonsmokers (Fig. 3b). In non-smokers, only the right frontal pole area showed higher connectivity with the left insular as a seed than smokers (Fig. 3c). The right insular area of nonsmokers did not show a particularly strong connection with other brain regions.

4. Discussions

Previous studies have reported that the insular cortex is related to nicotine addiction [11, 26, 27] because the proportion of smoking cessation is higher than in those without damage to the insular area [28]. A number of studies have reported that smoking is closely related to nicotine addiction, which is related to the insular cortex [11, 28]. Although many studies on smoking have focused on the duration of smoking, few studies have used MRS and fMRI focusing on specific regions, ages, and educational levels. Therefore, we evaluated nicotine addiction of smokers in their twenties using the FTND questionnaire and evaluated differences in metabolites of the insular cortex and brain connectivity using the insular cortex as a seed area.

In this study, concentrations of GPC, tCho and tCr were significantly higher in the left insular area than in the right insular area of the non-smoker group. In particular, a large difference in concentration of tCr was observed. Creatine is a metabolite required for the synthesis of ATP from brain metabolites [29, 30]. It is used to normalize metabolites and characterize specific diseases because of similar metabolite concentration distribution in all areas of the brain [31, 32]. Yadav *et al*. have shown changes in metabolite of the insular cortex in patients with sleep apnea [33]. In particular, correlations were found among metabolite ratio during sleep, sleep and oxygen saturation characteristics, and neuropsychological scores. A concomitant decrease in NAA metabolite in both insular

areas was attributed to a decrease in neurons due to apnea. Thus, in the absence of adequate oxygenation in the brain, an abnormal accumulation of neuronal substances in the brain can affect brain metabolite concentration. In the present study, non-smokers showed higher creatine concentrations in the left insular region than in the right insular region, suggesting a higher metabolic activity in the left insular area. In the case of smokers, there was no difference in specific metabolites between the two insular regions. According to Faulkner *et al.* [34], in the case of daily and intermittent smoking, concentrations of Cr and Glu (glutamate) were lower in the prefrontal region. These findings were analyzed in our study because the prefrontal volume was smaller in the smoking group. In our study, nonsmokers showed higher connectivity with left insular and frontal right than smokers. Although our study did not directly measure the prefrontal volume, it might suggest that in addition to volume, a low concentration of the specific metabolite might be related to other factors. In the case of smokers, when insular cortex was set as a seed and connectivity was observed, higher correlations with the gyrus right, occipital fusiform, and gyrus right were found compared with non-smokers. Several researchers, including Caron *et al.*, have investigated the relationship between nicotine and neurological parameters [5, 7]. In the case of nicotine, studies have analyzed the effectiveness of nicotine in the prevention of Parkinson's disease, albeit partially. The higher brain connectivity in smokers than in non-smokers found in the current study can be explained by improved neuronal survival.

This study also has some limitations. First, only 10 participants were enrolled for each group. Thus, scarce experimental data were obtained, although a control group was selected and similar educational background as those in their twenties was ensured. Second, during volunteer selection, other addictions such as games and work as well as drug addiction could not be screened. However, in our country, drug addiction is strictly controlled. Given that all participants are college students, the possibility of drug addiction is very slim. Since the demand for computer games is increasing rapidly with social issues about game addiction among college students, additional studies analyzing the connectivity with the insular area and metabolites in other addictions, including game addiction, are needed in the future.

In conclusion, concentrations of tCr metabolites in the left insualr area of non-smokers were higher than those of smokers. In addition, smokers showed higher connectivity in the right gyrus and occipital fusiform regions than non-smokers, whereas non-smokers had stronger connectivity between their left insular cortex area and the frontal role right than smokers. This study is meaningful in that it provides a new strategy using fMRI as well as MRS to determine differences in metabolism between smokers and non-smokers in a specific age group.

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