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Effects of Coffee Bean Aroma on the Rat Brain Stressed by Sleep Deprivation: A Selected Transcript- and 2D Gel-Based Proteome Analysis

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The aim of this study was 2-fold: (i) to demonstrate influences of roasted coffee bean aroma on rat brain functions by using the transcriptomics and proteomics approaches and (ii) to evaluate the impact of roasted coffee bean aroma on stress induced by sleep deprivation. The aroma of the roasted coffee beans was administered to four groups of adult male Wistar rats: 1, control group; 2, 24 h sleep deprivation-induced stress group (the stress group); 3, coffee aroma-exposed group without stress (the coffee group); and 4, the stress with coffee aroma group (the stress with coffee group). Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of some known genes responsive to aroma or stress was performed using total RNA from these four groups. A total of 17 selected genes of the coffee were differently expressed over the control. Additionally, the expression levels of 13 genes were different between the stress group and the stress with coffee group: Up-regulation was found for 11 genes, and down-regulation was seen for two genes in the stress with coffee group. We also looked to changes in protein profiles in these four samples using two-dimensional (2D) gel electrophoresis; 25 differently expressed gel spots were detected on 2D gels stained by silver nitrate. Out of these, a total of nine proteins were identified by mass spectrometry. Identified proteins belonged to five functional categories: antioxidant; protein fate; cell rescue, defense, and virulence; cellular communication/signal transduction mechanism; and energy metabolism. Among the differentially expressed genes and proteins between the stress and the stress with coffee group, *NGFR*, *trkC*, *GIR*, thiol-specific antioxidant protein, and heat shock 70 kDa protein 5 are known to have antioxidant or antistress functions. In conclusion, the roasted coffee bean aroma changes the mRNA and protein expression levels of the rat brain, providing for the first time clues to the potential antioxidant or stress relaxation activities of the coffee bean aroma.

KEYWORDS: Brain; coffee aroma; proteomic analysis; sleep deprivation; transcriptomics analysis

INTRODUCTION

Coffee is the most widely consumed beverage by a large proportion (approximately 70−80%) of the human population worldwide (*1*). Because coffee has been a part of the human diet for over a thousand years (*1*), the effects of coffee on the human species have been investigated by numerous researchers. However, the beneficial or adverse effects of coffee extracts on human health are still controversial as discussed in the literature (*2, 3*). Some beneficial effects of coffee consumption via liquid type on human psychological aspects including the potential reduction of suicide risk and depression (*4*) and the alleviation of stress (*5, 6*) of human as well as animal models have been reported. These "beneficial" effects may be due to caffeine, a key ingredient studied in most of the cases.

Despite numerous studies, most of the experiments have focused on nonvolatile compounds of brewed coffee, especially caffeine. Although about 900 volatile compounds with a wide variety of functional groups have been identified in roasted coffee (*7*), there are few studies that deal with the beneficial effects of coffee volatiles or aroma. Our group is interested in the effects of roasted coffee bean aroma because of the following three merits. First, coffee is consumed for its pleasing and

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attractive aroma, which is the result of roasting; a large number of different volatiles are produced by the Maillard reaction, Strecker degradation, and other chemical reactions that occur during roasting (*7*). Second, the sound backgrounds on the coffee volatile compounds can save the time and costs to identify the potential compounds to certain effects or mechanisms, although the volatile compounds and intensities of coffee varied depending on several conditions such as species, roasting, and extraction (*7, 8*). Czerny et al. (*9*) reported that the following compounds, 2-furfurylthiol, 4-vinylguaiacol, several alkyl pyrazines, furanones, acetaldehyde, propanal, methyl-propanal, and 2- and 3-methylbutanal out of the 28 potent odorants of medium-roasted Colombian coffee (the similar species with the coffee beans of this study), have greater contributions on sensory perception than the others. Finally, in spite of being the most popular beverage, approximately 20−30% of population does not drink coffee due to several reasons. Interestingly, a single study using rats revealed its preference for coffee aroma; the study employed an artificial aroma experiment (*10*).

To study the effects of coffee bean aroma, we designed an experiment to investigate the potential effects therein, by using an animal model and molecular "omics" approaches, namely, genomics, transcriptional profiling of gene expression (*11*), and proteomics, protein complement of the genome (*12, 13*). The animal model selected for the present study was the rat, which has been used by the group of Yoshinori Masuo (HSS, AIST) for investigating the mechanisms of psychiatric and neurological disorders and the effects of stress on the central nervous system (*14–16*). Moreover, rats are inherently advantageous: They are genetically homogeneous, experimental controls are provided easily, and there is a large body of literature for stress, brain function, and gene expression (*14–20*). As odor deals with sense and emotion in the brain (*21*), it was reasoned that by examining molecular (mRNA expression and protein levels) responses in the brain of rat exposed to coffee bean aroma, we might get insight on the aroma-induced changes associated with brain function. Especially, as coffee is also considered a stress reliever (*5, 6*), we incorporated a stress condition via sleep deprivation of 24 h in our experimental design with or without the presence of coffee bean aroma to see whether coffee can counteract the effects of stress at the level of gene/protein expression.

Results from the present study demonstrate the influence of coffee bean aroma on the rat's brain functions at both genome and proteome levels, and the paper discusses these differentially expressed genes and proteins. This study is the first effort to elucidate the effects of coffee bean aroma on the sleep deprivation-induced stress of the rat brain.

MATERIALS AND METHODS

Coffee and Preparation of Coffee Bean Aroma. Green coffee beans (*Coffea arabica* var. Colombia) were roasted using a drum roaster at medium-dark roasting degree (Full city level) at the local roastery shop in Seoul, Korea. One hundred grams of ground roasted coffee beans was heated using an electric heater for 15 h at 38 °C in the draft chamber. The draft was sealed to prevent the release of coffee bean aroma from the chamber. Because of the staling process of coffee aroma with time, ground roasted coffee beans were exchanged with new beans 10 min prior to administering the coffee aroma to rats. After initial heating for 5 min at 50 °C, coffee beans were not heated to prevent a temperature increase in the inner atmosphere of the draft chamber.

Rat Growth and Exposure to Coffee Bean Aroma. This study was carried out in accordance with the Guidelines for the Care and Use of Laboratory Animals in the National Institute of Advanced Industrial Science and Technology (AIST) (Japan). Adult male Wistar rats, weighing 270−320 g, were obtained from Clea Japan (Tokyo, Japan). The rats were housed in acrylic cages at controlled temperature, 22 °C, and were given access to tap water and laboratory chow ad libitum. The breeding rooms were illuminated from 07:00 to 19:00 h in 12 h cycles. Thirty rats at 10 weeks of age were randomly divided into four groups at 09:00−10:00: (i) control group without stress, control group ($n = 7$); (ii) control group with stress, stress group ($n = 8$); (iii) coffee aroma exposure group without stress, coffee group ($n = 7$); and (iv) coffee aroma exposure group with stress, stress with coffee group ($n = 8$). Rats of each group were separated into two acrylic cages, three or four rats per cage. The cages were placed in the two split drafts, with and without coffee aroma. The rats of control group, that is, i and ii, were laid in the draft without coffee aroma, whereas the rats of the coffee aroma exposure group, that is, iii and iv, were placed in the draft with coffee aroma. For stress treatment, the rats were deprived of sleep via forced locomotion in an acrylic cage that was filled with water to a level of 2 cm from the bottom. Tap water and laboratory chow were available ad libitium.

Dissection of Brain. After 24 h under conditions described above, the rats were decapitated, and whole brains were rapidly removed, and when required, brain regions were dissected on ice (*14, 15*). At each group, a total of three whole brains were used for this study and the other whole brains (three or four) were dissected into the 11 regions for further study according to the method of Glowinski and Iversen (*22*) with some modifications. Whole brains and dissected regions were immediately weighted, flash frozen in liquid nitrogen, and stored at −80 °C.

Total RNA Extraction and Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR). Prior to the RT-PCR, total RNA samples were isolated from the finely powdered brain samples that were ground in liquid nitrogen using a prechilled motar and pestle as described previously (*20*). Total RNA samples were DNase-treated with an RNase-free DNase (Stratagene, La Jolla, CA). After determination of the RNA quality, that is, purity using a spectrophotometer (NanoDrop, Wilmington, DE) and formaldehyde-agarose gel electrophoresis, first-strand cDNA was synthesized in a reaction mixture with a StartaScript RT-PCR Kit (Stratagene) in accordance with the protocol provided by the manufacturer. The 50 μL reaction mixture (10× buffer) of this kit consisted of 1.0 μL of the first-strand cDNA, 100 mM dNTPs, 10 pmol of each primer, and 0.1 U of taq polymerase (TaKaRa Ex Taq Hot Start Version, TaKaRa Shuzo Co. Ltd., Shiga, Japan). Specific primers were designed to form the 3′-UTR regions of each of the genes as described in **Table 1**. FBJ murine osteosarcoma viral oncogene homologue (*c-fos*), early growth response 1 (*egr-1*), early growth response 2 (*egr-2*), immediate early gene transcription factor NGFI-B (*NGFI-B*), PP1 inhibitor 1 (*Ppp1r1a*), adenosine A_{2A} receptor (*Adora2a*), fast nerve growth factor receptor (*NGFR*), neurotrophic tyrosine kinase, receptor, type 2 (*trkB*), neurotrophic tyrosine kinase, receptor, type 3 (*trkC*), monoamine oxidase A (*Maoa*), glucocorticoid-induced receptor (*GIR*), activity regulated cytoskeletal-associated protein (*Arc*), 5HT3 receptor 5HT3 receptor, cannabinoid receptor 1 (brain) (*Cnr1*), cholecystokinin 1 receptor (CCK_1R), cholecystokinin 2 receptor (CCK_2R), and Jun oncogene (*Jun*) were obtained using the NCBInr database search. Thermal-cycling parameters of PCR (TaKaRa PCR Thermal Cycle Dice, model TP600, Tokyo, Japan) were as follows: After an initial denaturation at 97 °C for 5 min, samples were conducted to a PCR cycling regime of 25 cycles at 95 °C for 45 s, 55 °C for 45 s, and 72 °C for 1 min. At the end of the final cycle, an additional step was performed at the condition of 72 °C for 10 min. After completion of the PCR, the total reaction mixture was mixed with 2.0 μL of 10× loading buffer, followed by vortexing, and 8.0 μL was loaded into wells of a 1.8% agarose gel (Agarose ME, Iwai Chemicals, Tokyo, Japan). Electrophoreses was performed for ca. 30 min at 100 V in 1× TAE buffer, using a Mupid-ex electrophoresis system (Advance, Tokyo, Japan) and stained with ethidium bromide for ca. 10 min. The stained bands were visualized using an UV transilluminator (ATTO, Tokyo, Japan), and the intensity of each band was calculated using the ATTO lane and spot analyzer version 6.0 (ATTO).

Extraction of Total Protein. Extraction of total protein was carried out following the method developed by Hirano et al. (*20*). Briefly, the frozen whole brains were placed in liquid nitrogen and then ground thoroughly to a very fine powder with a prechilled motar and pestle. The tissue powder (100−150 mg) was transferred to sterile tubes

Table 1. Gene Expression Levels of the Rat Brain over the Control in the Stress, Coffee, and Stress with Coffee Groups[a]

description	accession	group: stress	group: coffee	group: stress + coffee	forward primer (5′−3′)	reverse primer (5′−3′)	product size (bp)
FBJ murine osteosarcoma viral oncogene homologue (c-fos)	NM_022197	++	+	++	ACGTCTTCCTTTGTCTTCACCT	TGTTTCACGCACAGATAAGGTC	253
early growth response 1 (egr-1)	NM_012551	+++	+++	+++	CTCCTCTACCTACCCGTCTCCT	TCTTCCCTCCTGTCCTTTATTG	260
early growth response 2 (egr-2)[b]	X06746	++	++	+++	CAGAAGGAACGGAAGAGCAGT	AACAGGGAAGGGTGGTAGTGTT	240
immediate early gene transcription factor NGFI-B (NGFI-B)	U17254	+	++	+++	TTGCTAGCTGTCTGAAGGAACA	ACAAGAAGAACATGCACGTGAG	252
PP1 inhibitor (Ppp1r1a)	AY648296	+++	++	+++	AGAATCCAAACCCAAGACTCAG	GAAAGAAGAAAAGCACCAAGGA	228
adenosine A_{2A} receptor (Adora2a)	NM_053294	+	+	++	GGGATGTGGAGCTTCCTACC	CTTTCTTACTGGGCTTCATGCT	259
fast nerve growth factor receptor (NGFR)	X05137	—	–	–	CTGGGTTACCAGCCTGAACATA	GCTGGCTAGAACATCAGTCGTC	249
neurotrophic tyrosine kinase, receptor, type 2 (trkB)	M55291	+	+	++	GAACCACACACAAGGAAGAACA	ATACTGTCTGTGGATGGGGAAG	254
neurotrophic tyrosine kinase, receptor, type 3 (trkC)	L14445	+	–	–	CGAGTCTGCCCAAAAGAAGTAT	TCAAAGATGGACGAAAGGAGTT	241
monoamine oxidase A (Maoa)[b]	NM_173740	+++	+++	+++	CAGTATCACAGGCCACATGTTC	GGGTAAGTTTTCCCCTTGACAT	300
glucocorticoid-induced receptor (GIR)	AY029071	—	–	+	AGATCCAGTCTGGGAAGACAGA	TACAACTGACAGGGGTTGACTG	298
activity regulated cytoskeletal-associated protein (Arc)	U19866	—	–	–	CCAAGTTCAAGCGCTTTCTG	CCCAGCTCAATCAAGTCCTAGT	279
5HT3 receptor	U59672	+	+	+	CTGCTGTTTCGCATCTACCTG	GAGGAGTCTTGGTTCCTCTTGA	267
cannabinoid receptor 1, brain (Cnr1)	NM_012784	+	+	+	ATCATGGTGTATGACGTCTTCG	CGATCTTAACGGTGCTCTTGAT	286
cholecystokinin 1 receptor (CCK_1R)	NM_012688	—	—	+	CTCCTGTGTTAACCCCATCATC	GTGAGAAATGGGTCTTCCCTCT	299
cholecystokinin 2 receptor (CCK_2R)	X79209	++	+	++	TTTTGCTTTTCTTCCTGTGTTG	GGTATAGCTTAGCCTGGACAGC	302
Jun oncogene (Jun)	NM_021835	++	+	+++	CGGCTAGAGGAAAAAGTGAAAA	AACCCAGTCCATCTTGTGTACC	295
glyceraldehyde 3-phosphate-dehydrogenase (gapdh)	X02231	+	+	+	TCCCTCAAGATTGTCAGCAA	AGATCCACAACGGATACATT	308

[a] Specific 3′-UTR primers were designed based on the available nucleotide sequence databases at NCBI. +, ++, and +++ represent expression levels in the range of 101−149, 150−199, and more than equal to 200%, over the control (100%), respectively. − and −− represent expression levels less than 30 and 70% of the control, respectively. [b] These nucleotide sequences are from mouse.

containing cold (−20 °C) TCAAEB [acetone containing 10% (w/v) trichloroacetic acid (TCA), and 0.07% mercaptoethanol], and the proteins were precipitated for 1 h at −20 °C, followed by centrifugation at 15000 rpm for 15 min at 4 °C. After the supernatant was decanted, the pellet was washed twice with chilled wash buffer [acetone containing 0.07% mercaptoethanol, 2 mM EDTA, and two EDTA-free proteinase inhibitor cocktail tablets (Roche Diagnostics GmbH, Mannheim, Germany) in a final volume of 100 mL of buffer], and then, all of the acetone was removed. The pellet was solubilized in lysis buffer (LB-TT) [7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 18 mM Tris-HCl (pH 8.0), 14 mM trizma base, two EDTA-free proteinase inhibitor cocktail tablets in a final volume of 100 mL of buffer, 0.2% (v/v) Triton X-100 (R), containing 50 mM dithiothreitol (DTT)], subsequently incubated for 20 min at 4 °C with occasional vortexing, and centrifuged at 15000 rpm for 15 min at 10 °C. The supernatant was used for protein determination using a Coomassie Plus (Pierce, Rockford, IL) protein assay kit and stored in aliquots at −80 °C.

Two-Dimensional Gel Electrophoresis (2D-GE). 2D-GE was performed following the method described by Hirano et al. (*20*) with a slight modification. Briefly, the 120 μg of total soluble protein was mixed with LB-TT containing 0.5% (v/v) pH 4−7 IPG buffer (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) to make a final volume of 460 μL. After bromophenol blue (BPB) was added into the mixture, the whole one was kept at room temperature (RT) for 5 min. The mixture was vortexed and then centrifuged at 15000 rpm for 15 min at 10 °C followed by pipetting into a 24 cm strip holder tray (GE Healthcare). Precast IPG gel strips (24 cm, pH 4−7; GE Healthcare) were carefully placed onto the protein samples in the strip holder, covered with a lid, and placed into the IPGphor unit (GE Healthcare). The IPG gel strips were placed gel-face down onto the protein samples and rehydrated with the protein samples for 90 min, followed by overlaying the IPG gel strips with 1400 μL cover fluid. These procedures were conducted by an automated five-step active rehydration and focusing protocol (24 cm strip) of the IPGphor unit. The whole procedure was carried out at 20 °C, and a total of 76908 Vh was used for the 24 cm strip. In the case of 18 cm IPG gel strips (pH 4.5−5.5), a final volume of 360 μL containing 110 μg of the total soluble protein was used, followed by IEF (69608 Vh). On completion of the IEF, the IPG strips were removed from the strip holder and processed for the second dimension.

The gel strips were incubated in equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, and 2% (w/v) SDS)] containing 2% (w/v) DTT for 10 min (×2 times) with gentle agitation, followed by incubation in the same equilibration buffer supplemented with 2.5% (w/v) iodoacetamide for the same time periods at RT. Before the second dimension separation of the IPG gel strips (pH 4−7; 18 cm) using polyacrylamide gels on a Nihon Eido (Tokyo, Japan) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) vertical electrophoresis unit, the IPG gel strips were immersed to rinse in cathode running buffer [0.025 M Tris, 0.192 M glycine, and 0.2% (w/v) SDS], laid on top of polyacrylamide gels, and covered with agarose solution [60 mM Tris-HCl, pH 6.8, 60 mM SDS, 0.5% (w/v) agarose, and 0.01% (w/v) BPB]. The lower anode buffer contained 0.025 M Tris, 0.192 M glycine, and 0.1% (w/v) SDS. SDS-PAGE (4% T, 2.6% C stacking gels, pH 6.8, and 12.5% T, 2.6% C separating gels, pH 8.8) as the second dimension was carried out at a constant current of 40 mA/gel for ca. 4.5 h. The % T is the total monomer concentration expressed in g per 100 mL, and the % C is the percentage cross-linker. The stacking and separating gel buffer concentrations were 0.125 M Tris-HCl, pH 6.8, and 0.375 M Tris-HCl, pH 8.8, respectively. In the case of the 18 and 24 cm IPG gel strips, the horizontal electrophoresis system, Multiphor II (GE Healthcare), was used. The IPG gel strips were placed onto the gradient gels after equilibration, followed by placement of the cathode and anode buffer strips and electrodes, respectively. SDS-PAGE was carried out following the recommended protocol of the manufacturer. Molecular masses were determined by running standard protein markers (2.5 μL/gel; DualColor Precision-Plus Protein Standard; Bio-Rad). Gels were at a current of 40 mA/gel at 20 °C until the marker reached the bottom of the gel. For each sample, a minimum of two IPG strips and polyacrylamide gels was run under the same conditions.

Protein Visualization and Quantification. Silver staining for visualizing protein spots on polyacrylamide gels was performed as described in the instructions provided along with the Plus One Silver Staining Kit (GE Healthcare). Using a digital scanner (CanoScan 8000F, resolution of 300 dpi), the protein patterns in the gels were recorded

as digitalized images and saved as TIFF file types, and resulting images were analyzed using the ImageMaster 2D Platinum software version 5.0 (GE Healthcare) for the gel spot detection, normalization, and quantification as per the protocol of the imaging analysis software. The dissimilarly expressed gel spots, as compared to that of control, were detected by using the software. These spots were excised from the 2D gels with a gel picker (One Touch Spot Picker, P2D1.5, The Gel Company, San Francisco, CA) and transferred to sterilized eppendorf tubes (1.5 mL), followed by storage at −30 °C.

Mass Spectrometry. Proteins were identified by peptide-mass fingerprinting (PMF) methods (*23*) and tandem mass spectrometry (Q-TOF-MS/MS).

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF-MS). In the case of PMF, the excised gel spots were destained with 100 μL of destain solution [30 mM potassium ferricyanide (Sigma), in 100 mM sodium thiosulfate (Merck)], with shaking for 5 min. After the solution was removed, the gel spots were incubated with 200 mM ammonium bicarbonate (Sigma; and hereafter called AMBIC) for 20 min. The gel pieces were dried in a speed vacuum concentrator for 5 min and then rehydrated with 20 μL of 50 mM AMBIC containing 0.2 μg of modified trypsin (Promega, Madison, WI) for 45 min on ice. After removal of the solution, 30 μL of 50 mM AMBIC was added, and the digestion was performed overnight at 37 °C. The peptides were desalted and concentrated using C18 nanoscale (porus C18) column (IN2GEN, Seoul, Korea). For the analysis of MALDI-TOF-MS by the PMF method, the peptides were eluted by 0.8 μL of matrix solution [70% acetonitrile (Merck), 0.1% TFA (Merck), and 10 mg/mL α-cyano-4-hydroxycinamic acid (Sigma)]. The eluted peptides were spotted onto a stainless steel target plate. Masses of peptides were determined using MALDI-TOF-MS (model M@LDI-R; Micromass, Manchester, United Kingdom). Calibration was performed using internal mass of trypsin autodigestion product (*m/z* 2211.105).

Quadrupole Time-of-Flight Tandem Mass Spectrometry (Q-TOF-MS/MS). For analyses by MS/MS, 15 μL of the peptide solutions from the digestion supernatant was diluted with 30 μL in 5% formic acid, loaded onto the column, and washed with 30 μL of 5% formic acid. Peptides were eluted with 2.0 μL of methanol/H_2O/formic acid (50/49/1, v/v/v) directly into a precoated borosilicate nanoelectrospray needles (EconoTip, New Objective). MS/MS of peptides generated by in-gel digestion was performed by nano-ESI on a Q-TOF2 MS (Micromass). The source temperature was 80 °C. A potential of 1 kV was applied to the precoated borosilicate nanoelectrospray needles in the ion source combined with a nitrogen back-pressure of 0−5 psi to produce a stable flow rate (10−30 nL/min). The mass spectrometer was operated in an automatic data-dependent MS/MS to collect ion signals from the eluted peptides. In this mode, the most abundant peptide−ion peak with doubly or triply charged ion in a full scan mass spectrum (*m/z* 400−1500) was selected as the precursor ion. Finally, an MS/MS spectrum was recorded to confirm the sequence of the precursor ion using collision-induced dissociation (CID) with a relative collision energy dependent on molecular weight. The cone voltage was 40 V. The quadrupole analyzer was used to select precursor ions for fragmentation in the hexapole collision cell. The collision gas was Ar at a pressure of $6-7 \times 10^{-5}$ mbar, and the collision energy was 20−30 V. Product ions were analyzed using an orthogonal TOF analyzer, fitted with a reflector, a microchannel plate detector, and a time-to-digital converter. The data were processed using a MassLynx (version 3.5) Windows NT PC system.

Protein Identification. Protein identification was performed by searching the National Center for Biotechnology Information (NCBI) nonredundant database using the MASCOT search engine (Matrix Science, Inc., London, United Kingdom; www.matrixscience.com), which uses a probability-based scoring system. The following parameters were used for database searches with MALDI-TOF PMF data: monoisotopic mass, 50 ppm mass accuracy, trypsin as digesting enzyme with one missed cleavage allowed, carbamidomethylation of cysteine as a fixed modification, oxidation of methionine, and N-terminal pyroglutamic acid from glutamic acid or glutamine as allowable variable modifications. For database searches with MS/MS spectra, used were the following parameters: average mass, 1.0 Da peptide and MS/MS mass tolerance; peptide charge of +1, +2, or +3; trypsin as digesting enzyme with one missed cleavage allowed; carbamidomethylation of cysteine as a fixed modification; and oxidation of methionine as allowable variable modifications. Taxonomy was limited to mammalian for both MALDI and MS/MS ion searches. For MALDI-TOF-MS data to qualify as a positive identification, a protein's score had to equal or exceed the minimum significant score. Positive identification of proteins by MS/MS analysis required a minimum of two unique peptides, with at least one peptide having a significant ion score. The mass spectra data are presented as Supplementary Figure 1.

RESULTS

Influence of Coffee Bean Aroma on Responses of Rat Brain at Transcriptional Level. As shown in **Figure 1**, the RT-PCR results demonstrated response changes of corresponding mRNA levels of the selected genes among the four sample groups. In the stress group, 13 genes [*c-fos* (FBJ murine osteosarcoma viral oncogene homologue), *egr-1*, *egr-2*, *NGFI-B*, *Ppp1r1a*, *Adora2a*, *trkB*, *trkC*, *Maoa*, *5HT3 receptor*, *Cnr1*, CCK_2R (formerly called CCK_BR), and *Jun*] were up-regulated over control levels, whereas four genes [*NGFR*, *GIR*, *Arc*, and CCK_1R (formerly called CCK_AR)] were down-regulated. Like the stress group, the coffee group showed similar patterns of expression at mRNA level. That is, 12 genes [*c-fos*, *egr-1*, *egr-2*, *NGFI-B*, *Ppp1r1a*, *Adora2a*, *trkB*, *Maoa*, *5HT3 receptor*, *Cnr1*, CCK_2R, and *Jun*] were up-regulated over control levels, whereas five genes (*NGFR*, *trkC*, *GIR*, *Arc*, and CCK_1R) were down-regulated. Additionally, two genes such as *egr-1* and *Maoa* among the up-regulated genes were overexpressed by over 200%, while the CCK_1R gene was dramatically down-regulated as shown in **Figure 1**.

By comparison of expression levels between the stress and the stress with coffee groups, we elucidated the impacts of coffee aroma on the rat brain under the stress condition. The expression levels of mRNA were increased for 11 genes (*egr-2*, *NGFI-B*, *Ppp1rla*, *Adora2a*, *NGFR*, *trkB*, *GIR*, *Arc*, CCK_1R, CCK_2R, and *Jun*) in the stress with coffee group, as compared to that of the stress group, and the *NGFR* and CCK_1R genes, in particular, were overexpressed.

Influence of Coffee Bean Aroma at the Level of the Proteome. A total of 120 μg of soluble protein was separated by 2D-GE using the 24 cm IPG strip at pH 4−7 and 12–14% gradient polyacrylamide gels (see also Supplementary Figure 2). The number of expressed gel spots was similar between the control and the coffee groups. However, the number of gel spots of the stress group was greater than that of the control group, whereas that of the stress with the coffee group was slightly reduced. To investigate expression changes of the spot in further detail, we performed another 2D-GE analysis using the 18 cm IPG gel strip at a narrow pH range of 4.5−5.5. A total of 953, 984, 1121, and 1107 protein spots were detected on the 2D gels of the control, stress, coffee, and stress with coffee groups, respectively (**Figure 2**). As we expected, the number of expressed gel spots of all groups decreased as compared to that of the 2D-GE at pH 4−7. In particular, the number of gel spots of the coffee exposure groups, that is, the coffee and the stress with coffee groups, increased over that of the control group.

From the comparison of the expression level on the 2D gels using the calculation of the relative ratio of spot volume (using the image analysis software), we selected a total of 25 differentially expressed gel spots. Among these spots, 17 spots (in red) of the coffee group were up-regulated over the control group, whereas eight spots (in yellow) were down-regulated as depicted in **Figure 2** (see also Supplementary Figure 2). Above 25 protein spots were excised from the 2D gels

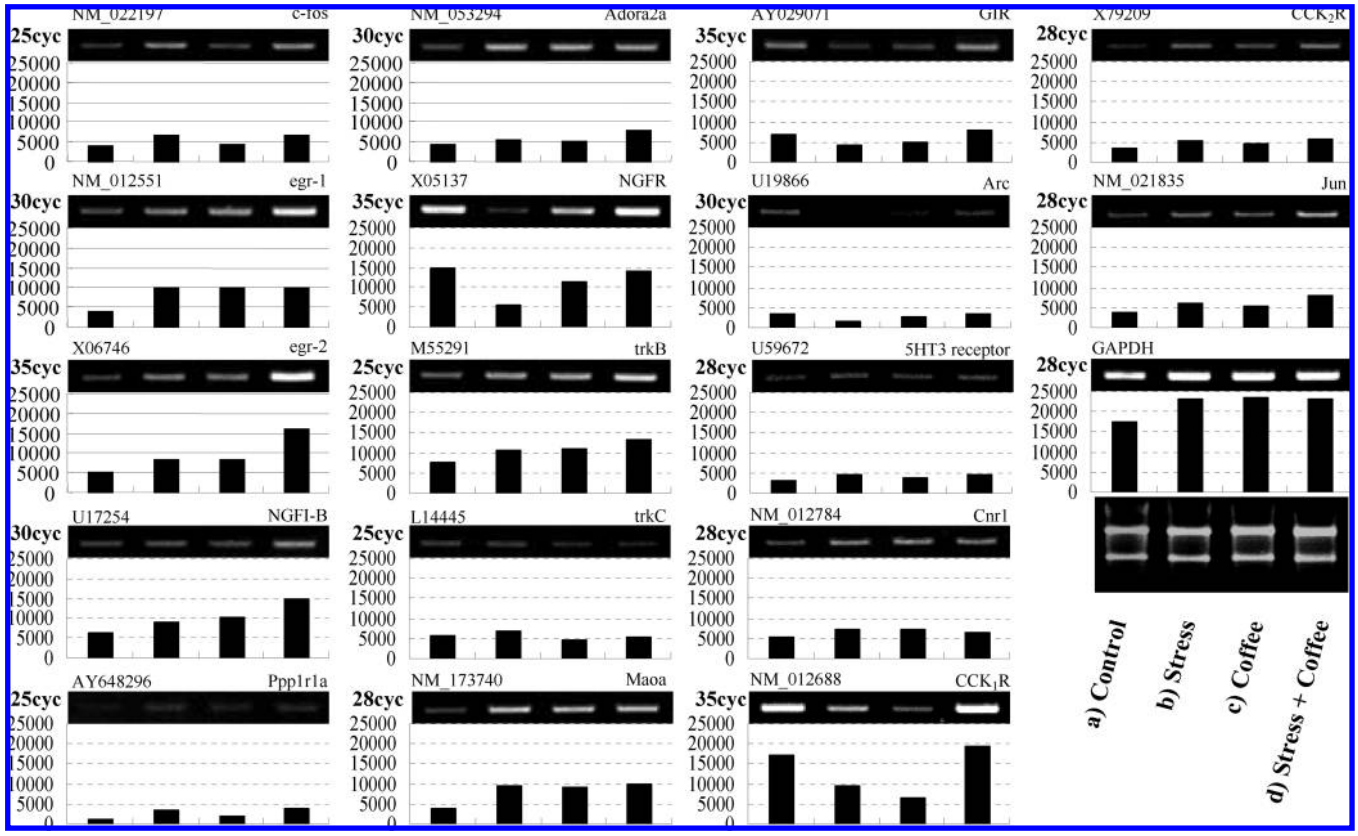

Figure 1. Gene expression levels of the rat brain in the four groups: (**a**) the control group, (**b**) the stress group, (**c**) the coffee group, and (**d**) the stress with coffee group. Gene accession numbers and corresponding gene names are given on the left- and right-hand sides of the gel image, respectively. Gene names were abbreviated as shown in **Table 1**.

and were identified by MALDI-TOF-MS or ESI-MS/MS. In total, from 25 of selected proteins, 12 proteins, including three that were regarded as the same protein, spots 2 and 3, 7 and 8, and 17 and 18, were identified (**Table 2**).

Functional Categorization and Subcellular Localization of Differentially Expressed Brain Proteins. Finally, the nine identified proteins excluding the three proteins that were identified as the same proteins were divided into five functional categories: antioxidant; protein fate (folding, modification, and destination); cell rescue, defense, and virulence; cellular communication/signal transduction mechanism; and energy metabolism (**Table 2**). Use of a subcellular localization tool (PSORT II, http://psort.nibb.ac.jp/) revealed that the majority (six out of nine proteins) of identified proteins were cytoplasmic, whereas the one protein, spot 7, was categorized into extracellular including cell wall. The other two proteins (spots 4 and 17) were localized in the nucleus (**Table 2**).

DISCUSSION

Coffee Bean Aroma Affects Responses of Rat Brain at the Transcriptional Level. In this study, we first examined the impacts of coffee aroma at the gene transcriptional level by using RT-PCR. Here, we focused on a total of 17 selective genes, which were related to "olfaction", "odor", and "stress" according to information available in the databases (PubMed). Because mRNA is not the functional end point of gene expression and gene expression may not necessarily correlate with protein expression (*24*), we relatively emphasize the proteomic assessment done later in the study.

In line with the earlier reports (*25*), the *c-fos* gene, a representative marker of neural activation, was up-regulated with presentation of the coffee bean aroma. Contrary to previous reports (*26*), the mRNA expression level of the *Arc*, which encodes a cytoskeleton-associated protein that is affluent in neuronal dendrites (*27*), was down-regulated with coffee bean aroma, which may result from different odor samples or olfactory experiences (*26*). Of interest, the mRNA expression level of the *Arc* was increased in the stress with coffee group, as compared to that of the stress group, and a similar pattern was observed in the *NGFR*, *GIR*, and CCK_1R. This result implies that the association between odor and brain function, especially mRNA expression level, could be influenced by the subject's status (e.g., stressed condition) during the odor presentation.

Coffee Bean Aroma Impacts on Responses of Rat Brain Stressed by Sleep Deprivation at the Transcriptional Level. It has been reported that sleep deprivation causes stress to animal models such as rat (*28, 29*), and this sleep deprivation or waking stress influences the expression of various genes categorized into the immediately early genes/transcription factors, energy metabolism/balance, growth factors/adhesion molecules, chaperones/heat shock proteins (HSPs), vesicle- and synapse-related genes, neurotransmitter receptors, neurotransmitter transporters, enzymes, and others (*28, 30*). Cirelli, however, reported that a small number of the genes, about less than 1% out of approximately 10000 examined transcripts, showed different expressions between sleep and wakefulness or sleep deprivations, and most of genes that were differentially expressed demonstrate higher mRNA levels by 3−8 h of forced wakefulness or by some periods of sleep deprivation (*30*). In line with the previous studies (*19, 28*), the majority of expressed genes (13 of 17) were overexpressed in the stress group as observed in **Figure 1**. Moreover, four genes such as *NGFR*, *GIR*, *Arc*, and CCK_1R were down-regulated.

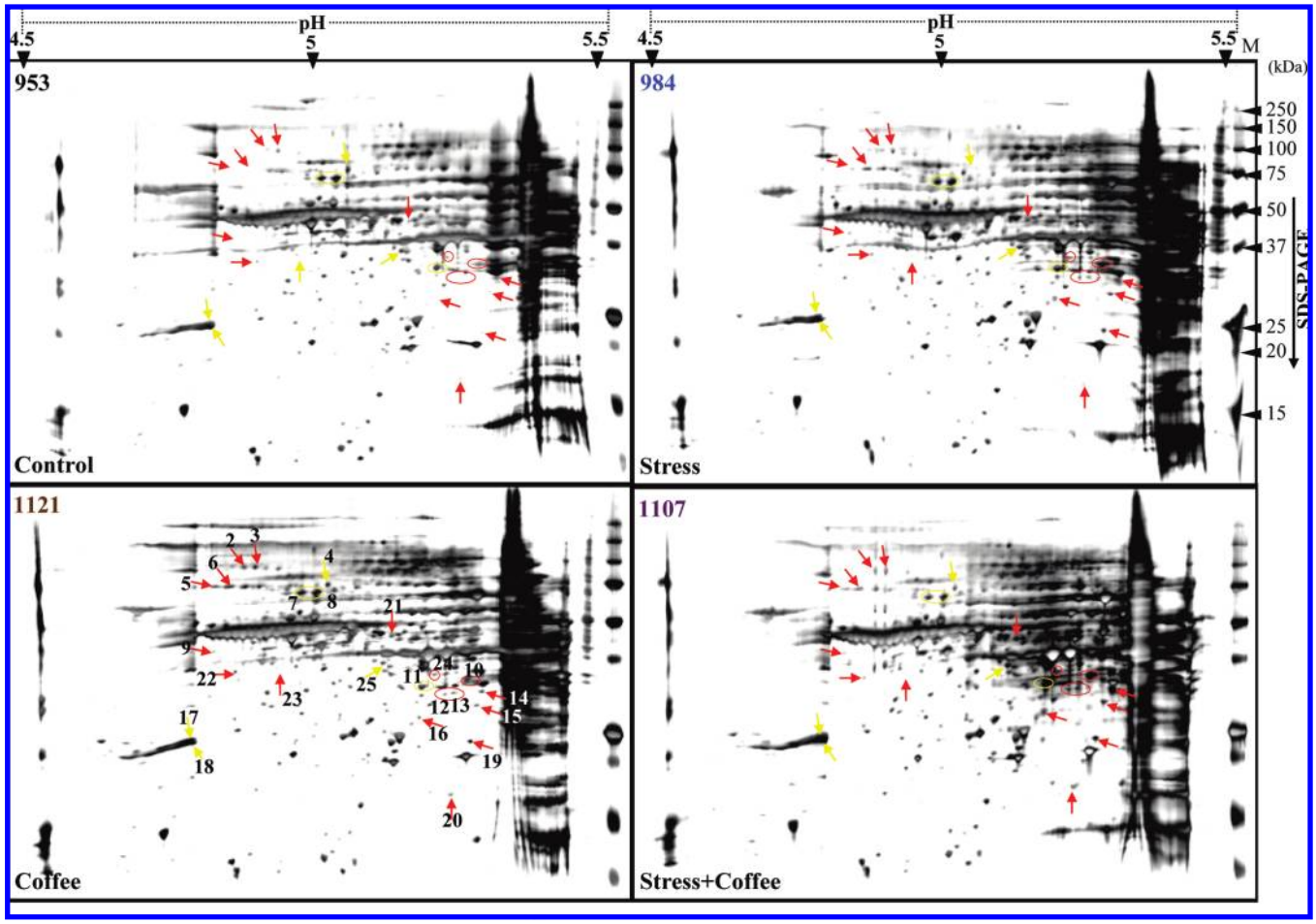

Figure 2. Representative 2D gel protein profile of the four groups: the control group, the stress group, the coffee group, and the stress with coffee group, at the condition of precast IPG strips (18 cm, pH 4.5−5.5). Brains from three individual rats were pooled for each group, and the total protein was extracted. Total soluble proteins of 110 μg were loaded on precast IPG strips. For visualization of separated proteins, silver nitrate staining was performed in the first dimension and followed by IEF and SDS-PAGE as described in the Materials and Methods. Two high-quality technical replicates were analyzed for differential spot expression by the image analysis software. The total number of expressed gel spots is shown at the top left-hand corner of each gel. Twenty-five differentially expressed gel spots were detected between the control and the coffee groups (marked in red color, up-regulation over the control group; and yellow color, down-regulation over the control group). These colors of the stress and the stress with coffee groups mean not up/down-regulation of each group but corresponding spots of the differentially expressed spots of the coffee group. Molecular masses (kDa) of protein markers (Precision Plus Protein Standards) are indicated at the right side.

Nerve growth factor (*NGF*) has been shown to promote resistances to oxidative stress and to enhance cell survival by inducing activities of the free radical scavenging enzyme and glutathione peroxidase (*31*), and *NGFR* mediates its effects of *NGF* (*32*). The up-regulation of the *NGFR* mRNA of the stress with coffee implies the potential antioxidant activity of the coffee bean aroma. In addition, the expression level of the glucocorticoid-induced receptor gene (*GIR*), an orphan G-protein-coupled receptor, of the stress with coffee group was markedly increased than that of the stress group. In that the *GIR* gene may be involved in mediator of anxiety and neuroendocrine control (*33*), the up-regulation of mRNA of GIR implies the potential anxiolytic effect of coffee bean aroma.

As compared to the stress group, only two genes, *trkC* and *Cnr1*, of the stress with coffee group were decreased at the mRNA expression level. The expression activities of coffee bean aroma between neurotrophic tyrosine kinase receptor type 2 (*trkB*) and type 3 (*trkC*) genes were opposite, increasing effect of *trkB* and decreasing effect of *trkC*. Of interest, the coffee bean aroma reduced the expression level of *trkC*, a receptor of the neurotrophin-3, which was reported as an inhibitor of the proliferation of the rat cerebral endothelial cell and as a stimulator of the nitric oxide production (*34*).

Coffee Bean Aroma Influences Responses of Rat Brain Stressed by Sleep Deprivation at the Level of the Proteome. The relationship between odor and rat brain has been emphasized mainly on response changes of specific regions associated with olfactory function than those of the whole regions (*35*). We, therefore, applied the newly developed method (*20*) for gel-based proteomics of rat whole brain to this study for screening overall changes of the rat whole brain with coffee aroma presentation. Also, as previously described, the nine proteins identified from a total of 25 differentially expressed gel spots were divided into five functional categories (**Table 2**).

As shown in Supplementary Figure 2, the expression level of the thiol-specific antioxidant protein, also called peroxiredoxin (PRx) or thioredoxin peroxidase (*36*), of the coffee group was down-regulated, as compared to that of the control group. Contrary to our expectations, the protein of the stress group did not have a distinctive difference of the expression level with that of the control group. This discrepancy may be a result from the expression variations of the six subtypes of the protein corresponding to oxidative stress (*36*) or may be related to differences of the response mechanism (*37*). It is of interest, however, that the protein of the stress with coffee group showed higher expression levels than that of the stress group, and this

Table 2. Differentially Changed Proteins and Their Identification by MS

spot no.	MW (kDa)/pI observed	protein name	MW (da)/pI theoritical	method	accession	score	sequence coverage (%)	functional category	subcellular localization
1	23/6.0	thiol-specific antioxidant protein	24860/5.64	MALDI	CAA76732	93	40	antioxidants	cytoplasmic
2	98/4.9	similar to ubiquitin carboxyl-terminal hydrolase 5	96631/4.89	MALDI	XP_238380	80	14	protein fate (folding, modification, destination)	cytoplasmic
3	98/4.9			MALDI					
4	69/5.0	similar to ubiquilin 2	67275/5.16	MALDI	XP_228806	75	15	protein fate (folding, modification, destination)	nuclear
5	67/4.8	no signal		MALDI					
6	67/4.9	no signal		Q-TOF					
7	65/5.0			MALDI					
		heat shock 70 kDa protein 5	68569/5.15		BAE31281 (AAH62017)	226	35	cell rescue, defense and virulence	extracellular, including cell wall
8	65/5.0			MALDI					
9	42/4.8	no signal		Q-TOF					
		chain B, heterotrimeric complex							
10	36/5.3	of phosducintransducin β-γ	38145/5.47	MALDI	1A0R_B (AAC72249)	137	33	cellular communication/Signal transduction mechanism	cytoplasmic
11	33/5.2	similar to serine/threonine protein phosphatase 2A	32220/5.54	MALDI	XP_858464 (AAH72531)	134	49	cellular communication/Signal transduction mechanism	cytoplasmic
12	31/5.2	no signal		MALDI					
13	32/5.3	no signal		MALDI					
14	32/5.3	no signal		Q-TOF					
15	29/5.3	pyridoxal phosphate phosphatase	33493/5.44	MALDI	Q8VD52	141	34	energy metabolism	cytoplasmic
16	27/5.2	no signal		Q-TOF					
17	24/4.8			MALDI					
		14-3-3 protein	28272/4.75		S13610 (NP_062250)	104	30	cellular communication/Signal transduction mechanism	nuclear
18	23/4.8			MALDI					
19	24/5.3	no signal		MALDI					
20	17/5.3	no signal		Q-TOF					
21	47/5.1	no signal		MALDI					
22	37/4.9	no signal		MALDI					
23	37/4.9	no signal		Q-TOF					
24	36/5.2	no signal		Q-TOF					
25	39/5.1	galactokinose 1	42806/5.24	MALDI	AAM85919	129	24	energy metabolism (galactose metabolism)	cytoplasmic

overexpression indicates a potential antioxidant effect of the coffee bean aroma considering that this protein is involved in the reduction of oxidative damage by catalyzing decompositions of hydrogen peroxide, organic hydroperoxides, hydroxyl radical, and peroxynitrite (*37, 38*). To our knowledge, no studies are available about the relation between the activity of this protein, PRx, and odor or sleep deprivation-induced stress in the brain. Concerning the odor, the 28 kDa thiol-specific antioxidant protein was found in the olfactory epithelium of the rat (*39*), and this protein distributed air-contacting areas and reduced the ROS caused by the air-borne oxidant (*38, 39*). Considering its localization, olfactory mucus, this protein may play a critical role in associations with the volatile antioxidants.

Moreover, the ubiquilin 2 protein, spot 4, of the coffee group was down-regulated in comparison to the control group (**Figure 2**). The expression level of this protein of the stress group was also down-regulated, and that of the stress with coffee group was more down-regulated than that of the stress group. In the case of humans, there are three following ubiquilin genes: ubiquilin 1, which is expressed in all of the cells and tissues reported; ubiquilin 2, which is a more restricted expression pattern than ubiquilin 1; and ubiquilin 3, whose expression is shown only in the testis (*40, 41*). The ubiquilin is identified as an interacting protein of presenilin proteins: regulation of presenilin biogenesis and endoproteolysis (*40, 42*). In particular, it is known that mutations (presenilin-1 and presenilin-2) of homologous presenilin genes are associated with early onset Alzheimer's disease (AD) (*41*). Also, it has been reported that overexpression of ubiquilin 2 as well as ubiquilin 1 increases the level of coexpressed presenilin proteins in cells (*41*). Considering two points, the association of genetic variants of ubiquilin 1 with the risk of AD and the strong similarity/identity (80%) between ubiquilin 1 and 2 (*40, 42*), it is worthy to note that the ubiquilin 2 level of the stress with coffee group was more down-regulated than that of the stress group.

Heat shock 70 kDa protein 5, also called 78 kDa glucose-regulated protein, from spot 7 (and 8) is one of the HSPs, which is known as a protector of cells from diverse forms of stress and injuries (*43*). Its expression is induced by glucose-deprived cells and is also up-regulated by a wide range of oxidative stress and endoplasmic reticulum stress such as calcium depletion from the endoplasmic reticulum, prohibition of asparagine (N)-linked glycosylation, reduction of disulfide bonds, and expression of mutant protein or protein subunits (*44, 45*). Accordingly, this heat shock 70 kDa protein 5 has been reported to protect neurons against apotosis as well as excitotoxic and oxidative stressors via reducing the oxy-radical accumulation and stabilizing mitochondrial functions (*45*). Although a big difference in the expression level was not apparent, the expression level of this protein of the coffee group was down-regulated as compared to that of the control group (**Figure 2**). Additionally, the expression level of the stress group was increased as compared to that of the control group, and this tendency was consistent with the earlier reports (*19, 28*). Interestingly, the expression

of this protein was reduced in the stress with coffee group. These results may suggest the potential antioxidant activity of the coffee aroma.

The chain B, heterotrimeric complex of phosducintransducin β-γ protein (spot 10), also called G protein β 1 subunit, is involved in signal transduction, and the phosducin plays a crucial role as a regulator by switching the membrane association/disassociation (*46*). The expression level of this protein of the stress, coffee, and stress with coffee groups were all up-regulated, as compared to that of the control group (**Figure 2**). These results are consistent with the previous mRNA expression results, overexpression of most genes related to immediate early genes in the three groups.

As shown in **Figure 2**, the expression level of the galactokinase 1 protein (spot 25) of the coffee group was down-regulated, while that of the stress group was up-regulated, and these results are consistent in that the sleep deprivation or waking requires more energy (*28*). This galactokinase catalyzes the second step of the Leloir pathway, that is, the conversion of α-D-galactose to galactose 1-phosphage, and the deficiency of this enzyme causes the galactosemia, which is a genetic disease involved in intellectual retardation, liver dysfunction, and cataract (*47*).

This study was the first investigation on the following points: (i) influences of roasted coffee bean aroma on the brain functions at the transcript and protein levels, (ii) impact of sleep deprivation induced stress on brain functions at a molecular level, and (iii) effects of the potential antioxidant or stress alleviation effects of the roasted coffee bean aroma on the brain stress caused by sleep deprivation. Therefore, the results of this study allowed us to improve our knowledge involved in relationships among coffee bean aroma, brain function, and stress via sleep deprivation. However, in the current study, we conducted our experiments using limited techniques as a first step toward our goal of unraveling the molecular effects of aroma on the brain. Thus, further studies using complementary techniques including immunohistochemical assay for protein and DNA microarray for gene expression at the global level are needed for better understanding and verification of these results.

Moreover, of great interest among the differentially expressed genes and proteins, some such as NGFR, trkC, GIR, thiol-specific antioxidant protein, and heat shock 70 kDa protein 5, were involved in the antioxidant or stress alleviation functions, and these results imply the potential beneficial effects of the coffee bean aroma. Fuster et al. demonstrated that heterocyclic compounds, pyrroles, furans, thiophenes, and thiazoles, found in general brewed coffee, showed the antioxidant activity in vitro (*48*). Additionally, Koga revealed that the α wave of the human electro-encephalogram changed with various coffee bean species (*49*). These results indirectly explain why so many people use coffee for staying up all night, although the volatile compounds of coffee beans are not fully consistent with those of the coffee extracts. In other words, the stress caused by sleep loss via caffeine may be alleviated through smelling the coffee aroma. When we stay up all night, is it better for us to smell coffee bean aroma than to drink coffee, or would any other food-related odor produce similar effects? Further studies are needed for not only obtaining the correct answer for this question but also identifying the potential volatile compounds for this beneficial effect.

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Supporting Information Available: MS spectra of the excised spots from 2D gels and peptide sequence information for differentially expressed and identified protein spots on 2D gels. This material is available free of charge via the Internet at http://pubs.acs.org.

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