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ARTICLE

Neuron Differentiation-Related Genes Are Up-regulated in the Hypothalamus of Odorant-Inhaling Rats Subjected to Acute Restraint Stress

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To elucidate some physiopsychological effects of a pleasant odor, we analyzed gene expression profiles in the hypothalamus of rats which, under a restraint-stressed condition, inhaled (*R*)-(−)-linalool. Consequently, 697 probe sets showed significant expression changes in the odorant-inhaling rats subjected to 2 h of restraint stress (false discovery rate < 0.05). We observed up-regulation of 594 among them, including genes related to neuron differentiation and transcriptional regulatory factors. Another important result was that inhalation of (*R*)-(−)-linalool returned the expression of 49 restraint-regulated genes to a normal condition. In contrast, the inhalation also further up-regulated the expression of 16 restraint-up-regulated genes that included those encoding heat shock proteins as factors to induce some biological responses against stresses. In the present study we thus found the substantial example that, in the hypothalamus involved in feeding behaviors, an inhaled pleasant odor acts to regulate the gene expression related to the functions of neuronal developments to cope with stresses.

KEYWORDS: Odorant; linalool; differentiation; DNA microarray; hypothalamus; restraint

INTRODUCTION

It has long been empirically known that several odorants bring about psychophysiological effects. These include sedative, stimulative, antistress, and anticonvulsant effects. In recent years, particular interest has been paid to the effects of inhaled odorants on health maintenance and promotion. However, research assessing these effects in vivo is still quite limited. Previously, we showed through DNA microarray analysis that (*R*)-(−)-linalool inhalation in rats during a 2 h restraint period repressed stress-induced effects on the gene expression profiles of their whole blood cells (*1*).

Linalool (3,7-dimethyl-1,6-octadien-3-ol) has been identified in numerous foods and flowers, including tea (*2*,*3*), oranges (*4*,*5*), grapes (*6*), mangoes (*7*), lemons (*8*), tomatoes (*9*), basil (*10*), and lavender (*11*). The characteristic odor of this compound is important for the formulation of a variety of fruit-like flavors and fragrances. Also, a large number of studies have been directed at certain kinds of psychophysiological effects that are elicited by this odorant (*12*−*17*). It has been suggested that the effects are elicited by the actions of the compound on the central nervous systems (CNS) (*12*−*14*). There are two optical isomers of linalool ((*R*)- and (*S*)-configurations) that act on different psychophysiological parameters (*15*). (*R*)-Linalool has a sedative effect, inducing a significant decrease in heart rate under stressed conditions (*16*). Bilateral lesions of the hypothalamic suprachiasmatic nucleus (SCN) eliminate linalool-induced changes in autonomic neurotransmission and blood pressure (*17*). These studies suggest that linalool inhalation affects some psychophysiological parameters via the hypothalamus, which is the center of stress response as well as a region of the brain that integrates different stress pathways.

The hypothalamus is activated under restraint-stressed situations to initiate a stress response, and it coordinates a variety of bodily changes needed for putting up with or avoiding stress (*18*). A restraint stressor is known to induce both physical and psychological stresses, resulting in a wide range of behavioral and physiological alterations, including reduced locomotor activity, anxiety, secretion of stress hormones, and neuronal cell death in the brain (*19*−*21*). Moreover, transcriptional profiling using DNA microarray analysis on the hypothalamus of restraint-stressed mice has shown that apoptosis-, tumor-suppression-, DNA-binding-, and protein-folding-related genes are induced by repeated restraint (*22*), whereas immune-related genes are activated by acute restraint (*23*).

Our previous research showed that gene expression profiling of whole blood was able to detect odorant-induced alterations and that (*R*)-(−)-linalool inhalation repressed the effects of stress on blood cell gene expression profiles over the course of a 2 h restraint period (*1*). However, it remained unclear how the complicated relationships of the gene network mechanisms are induced

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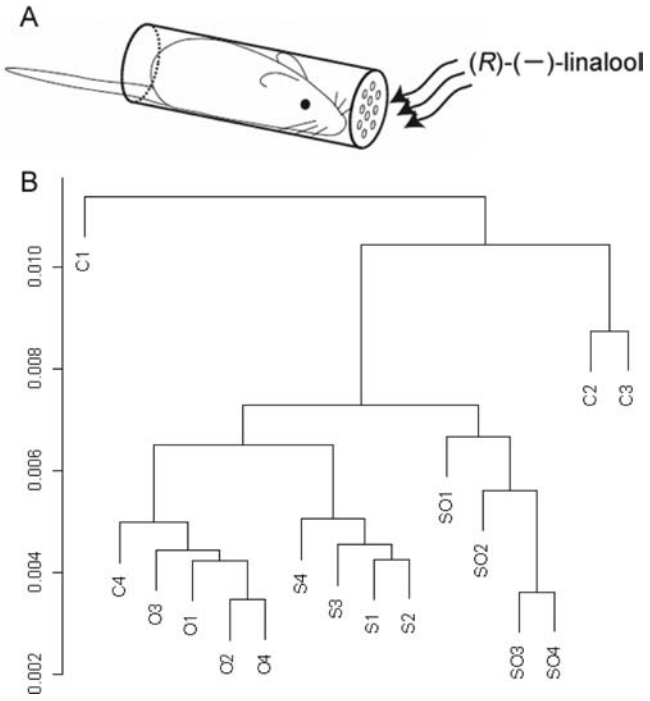

Figure 1. Illustration of the experimental setup (**A**) and gene expression profiles altered by (*R*)-(−)-linalool inhalation during 2 h of restraint (**B**). Cluster dendrogram showing similarities among the gene expression patterns of the 16 experimental rats. The group average method was used for the construction. The vertical scale corresponds to the distance between clusters. Group C is the control (C1−C4) in which rats were exposed to neither stress nor odor; group S (S1−S4) is rats exposed to stress only; group SO (SO1−SO4) is rats exposed to both stress and odor; and group O (O1−O4) is rats exposed to odor only.

in the CNS just by odor inhalation for 2 h. In the present study, we used DNA microarray analysis of rats subjected to acute restraint in the presence or absence of (*R*)-(−)-linalool to determine whether this odorant is able to alter stress-induced gene expression in the center of the stress response in the CNS. We found that neuron differentiation-related genes were significantly up-regulated in the hypothalamus of odor-inhaling rats under restraint stress.

MATERIALS AND METHODS

Animals. Male Wistar rats aged 7−8 weeks were housed individually in stainless steel cages and kept in an isolated room at controlled temperature (23 ± 2 °C) and humidity (55 ± 5%). Lighting was maintained on a light/dark cycle with lights turning on at 9:00 a.m. and off at 9:00 p.m. They were allowed free access to food and water. After acclimatization to the environment and to investigators for 1 week, 16 rats were divided into four groups (n = 4 each): group C (control) was exposed to neither stress nor odor; group S was exposed to stress only; group SO was exposed to both stress and odor; and group O was exposed to odor only. The 12 animals in groups C, S, and SO were the same as those used in our previous study (*1*).

Stress and Odor Inhalation. Rats in groups S and SO were placed in a restraining plastic tube for 2 h (10:00 a.m.−12:00 p.m.); they were able to breathe freely but unable to turn around head to tail. Twenty microliters of (*R*)-(−)-linalool (92% ee) was evaporated and allowed to spread throughout a 40 L box in which the rats with the tubes (group SO) and those without the restraint (group O) were placed. The experimental setup of group SO is illustrated in **Figure 1A**. All of the experiments were done in a room at controlled temperature (23 ± 2 °C) and humidity (55 ± 5%). After the exposure, all 16 rats were immediately sacrificed by decapitation, and then the brains were dissected to isolate the hypothalamic blocks. To look at the brain from the ventral side, two coronal cuts were placed at the apex of the optic chiasm and at the rostral margin of the mammillary bodies using a razor blade. This slab was then placed flat, and two cuts were placed on either side of the optic chiasm. The last cut was placed just above the third ventricle. The block was then put in ice-cold RNAlater (Ambion, Austin, TX). The protocol for the animal experiments was approved by the Animal Use Committee of the Faculty of Agriculture at The University of Tokyo.

Microarray Hybridization. Total RNA was extracted from each hypothalamic block according to a method that combined TRIzol RNA extraction (Invitrogen Life Technologies, Carlsbad, CA) with an RNA-easy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. The quality and quantity of the total RNA were checked by agarose gel electrophoresis and spectrophotometry. DNA microarray analysis was performed as described previously (*1*). In brief, biotinylated cRNA was obtained from 2 μg of purified total RNA using a One-Cycle Target Labeling Assay Kit (Affymetrix, Santa Clara, CA). It was then transcribed, fragmented, and hybridized to an Affymetrix Rat Genome 230 2.0 GeneChip, which contained 31099 probe sets representing transcripts and variants from over 28000 rat genes. After hybridization at 45 °C for 16 h, the arrays were washed and labeled with phycoerythrin. The fluorescent signals were scanned using the Affymetrix GeneChip System. Affymetrix software (Microarray Suite 5.0) was used to reduce the array images to the intensity of each probe (CEL files). All microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/, GEO series ID GSE19589).

Microarray Analysis. Data were analyzed using the statistical language R (*24*) and Bioconductor (*25*). The CEL files were quantified with the robust multiarray average (RMA) method (*26*). A correlation coefficient was used to define the distance among probe sets, and groups were hierarchically clustered by the package pvclust on the basis of the group average method for working out correlations (*27*). To detect the differentially expressed genes between groups S and SO, as well as between group C and one of the inhalation groups (S, O), the rank products (RP) method was used (*28*) as a nonparametric statistic derived from biological reasoning to detect items that are consistently highly ranked in a number of lists, by use of the RankProd package (*29*). The number of permutations was set to 500. The RP method returned RP statistics with a false discovery rate (FDR) for each of the up- and down-regulated probe sets. The FDR considered the problem of multiple testing and did not require any additional correction method. Probe sets with FDR values of < 0.05 were regarded as differentially expressed. Some probe sets were removed from further analysis; these displayed both up- and down-regulation in comparison to the two groups. The use of the RMA preprocessing algorithm followed by the RP selection method has been proposed to be one of the best combinations for accurate detection of differentially expressed transcription (*30*, *31*).

Gene annotation enrichment analysis of up- or down-regulated probe sets was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) (*32*, *33*) and QuickGO (*34*). The annotation file for the Rat Genome 230 2.0 array was obtained from DAVID (October 21, 2009) and Affymetrix (July 29, 2009). To statistically extract overrepresented gene ontology (GO) terms between groups and to correct the results for multiple testing, the Expression Analysis Systematic Explorer (EASE) score (*35*) and Benjamini and Hochberg FDR correction (*36*) were used, respectively. The over-represented GO terms were selected with the Functional Annotation Chart with a count threshold of 2 (minimum number of genes for the corresponding term) and EASE score threshold of 0.05 (maximum EASE score/p value). GO terms with FDR-corrected p values of < 0.05 were regarded as significant.

RESULTS

Cluster Analysis of Gene Expression Profiles. Gene expression profiles were compared among the control rats, the stressed rats, and the rats that inhaled odor. A hierarchical clustering analysis using the normalized values obtained by the RMA algorithm from all 31099 probe sets revealed that the expression profiles in the SO group (exposed to odor inhalation during the restraint, **Figure 1A**) and the S group (exposed to the restraint) clustered into two respective dendrograms (**Figure 1B**). However, the

Table 1. Significantly Enriched GO Terms Found in 594 Up-regulated Genes following (*R*)-(−)-linalool Inhalation under Stressed Conditions ($p < 0.05$)[a]

GO-ID	GO term	Counts	FDR-corrected *p*-value
0065007	biological regulation	137	8.42E-03
0050789	regulation of biological process	126	8.85E-03
0019222	regulation of metabolic process	75	1.08E-02
0050794	regulation of cellular process	113	8.16E-03
0031323	regulation of cellular metabolic process	68	2.93E-02
0019219	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	63	2.53E-02
0045449	regulation of transcription	62	1.77E-02
0006350	transcription	65	1.96E-02
0010468	regulation of gene expression	67	1.03E-02
0010467	gene expression	85	2.44E-02
0006139	nucleobase, nucleoside, nucleotide and nucleic acid metabolic process	96	9.64E-03
0016070	RNA metabolic process	70	2.44E-02
0009987	cellular process	250	3.27E-02
0030030	cell projection organization and biogenesis	20	2.43E-02
0048858	cell projection morphogenesis	20	2.43E-02
0032990	cell part morphogenesis	20	2.43E-02
0048869	cellular developmental process	65	4.98E-02
0030154	cell differentiation	65	4.98E-02
0031175	neurite development	17	3.14E-02
0048666	neuron development	19	2.49E-02
0030182	neuron differentiation	22	2.44E-02
0048699	generation of neurons	24	2.57E-02
0022008	neurogenesis	25	3.40E-02
0007399	nervous system development	39	3.32E-02

[a] Counts represent the number of probe sets annotated to each gene ontology (GO) term. FDR-corrected *p* values and counts of the categories appearing in the deepest hierarchy are shaded. The up-regulation of the 594 genes was statistically identified by rank products between group S exposed to stress only and group SO exposed to both stress and odor.

profiles of group C (the control) were not clustered. In considering the profiles other than group C, the profiles in the 12 rats were primarily classified into two different subtypes. One included group SO, whereas the other included groups S and O (exposed to odor inhalation). In addition, the expression profiles in group S formed a different cluster from those in group O. These results indicate that inhaled (*R*)-(−)-linalool influenced the gene expression profiles in hypothalamic tissues.

GO Analysis of Differentially Expressed Genes Regulated by (*R*)-(−)-Linalool Inhalation. To identify odorant-induced effects during the 2 h restraint, we used the RP method and extracted probe sets in which the mRNA levels were statistically different between groups S and SO. The probe sets having expression altered by odor inhalation included 594 up-regulated (452 annotated and 142 unknown) and 103 down-regulated probe sets (75 annotated and 28 unknown).

The differentially expressed genes by (*R*)-(−)-linalool inhalation under the restraint stress were classified into functional categories according to GO. The use of DAVID gave no significantly enriched term in the down-regulated gene sets, whereas we identified 24 GO terms in the biological process category significantly enriched in up-regulated gene sets (**Table 1**). The output graph drawn by QuickGO is shown in the Supporting Information. Because the hierarchical structure of GO means that more specific categories appear deeper in the hierarchy, the most important categories exist at the lower end of the graph. As shown in **Table 1**, the most specifically overrepresented categories in the up-regulated genes with the deepest hierarchy involved neurite development, regulation of transcription, cell projection morphogenesis, and RNA metabolic processes. These GO terms fell into two clusters: neuron differentiation and regulation of gene expression. Due to the hierarchical GO character, the same gene can be associated with multiple GO terms. To show the association of genes with multiple GO terms and the resulting complex interdependencies of processes involving the same genes, Venn and Euler diagrams were used to summarize the genes involved (**Figure 2**). The greatest number of changes were observed in transcription factors, such as nuclear receptor subfamily 4 group A (*Nr4A1*, *Nr4A2*, *Nr4A3*), *Atf3* (activating transcription factor 3), and *Egr1* (early growth response 1). These genes are also known as immediate early genes (IEGs), representative markers of neural activation (*37*) such as *Fos* and *Jun*.

Comparing transcription levels in groups C and O using the RP method led to the extraction of 63 probe sets for which expression was up-regulated by odor inhalation under nonstressed conditions. However, analysis of the 63 probe sets did not reveal any significantly enriched GO terms (data not shown). Moreover, these 63 probe sets did not include the 17 genes related to neurite development (**Figure 2**), which was the most strongly represented category in the genes up-regulated following exposure to an odorant under stressed conditions. These results indicate that the inhalation of (*R*)-(−)-linalool under stressed conditions induced the up-regulation of genes related to neurite development. Therefore, we focused on the difference of transcription levels in groups S and SO to assess the effect of the odor inhalation.

Effects of (*R*)-(−)-Linalool Inhalation on Gene Expression under Stressed Conditions. Comparing transcription levels in groups C and S using the RP method led to the extraction of 372 probe sets for which expression was altered by the applied restraint. To further assess the odorant-induced effects on these stress-related gene expression, the effect of inhaled (*R*)-(−)-linalool on the stress responses induced by the 2 h restraint was investigated. For this purpose, we statistically examined the genes that were differentially expressed between groups C and S, as well as those that were differentially expressed between groups S and SO. The results showed that 104 of the 372 stress-regulated probe sets were also included in the 697 odor-regulated probe sets (594 odor-up-regulated and 103 odor-down-regulated probe sets), indicating significantly alteration by restraint plus (*R*)-(−)-linalool inhalation (79 annotated and 25 unknown probe sets). A list of 33 of the 104 probe sets for which *p* values of the differences in expression levels between groups were < 0.01 is shown in **Table 2**. To clarify the influence of inhaled (*R*)-(−)-linalool on the stress

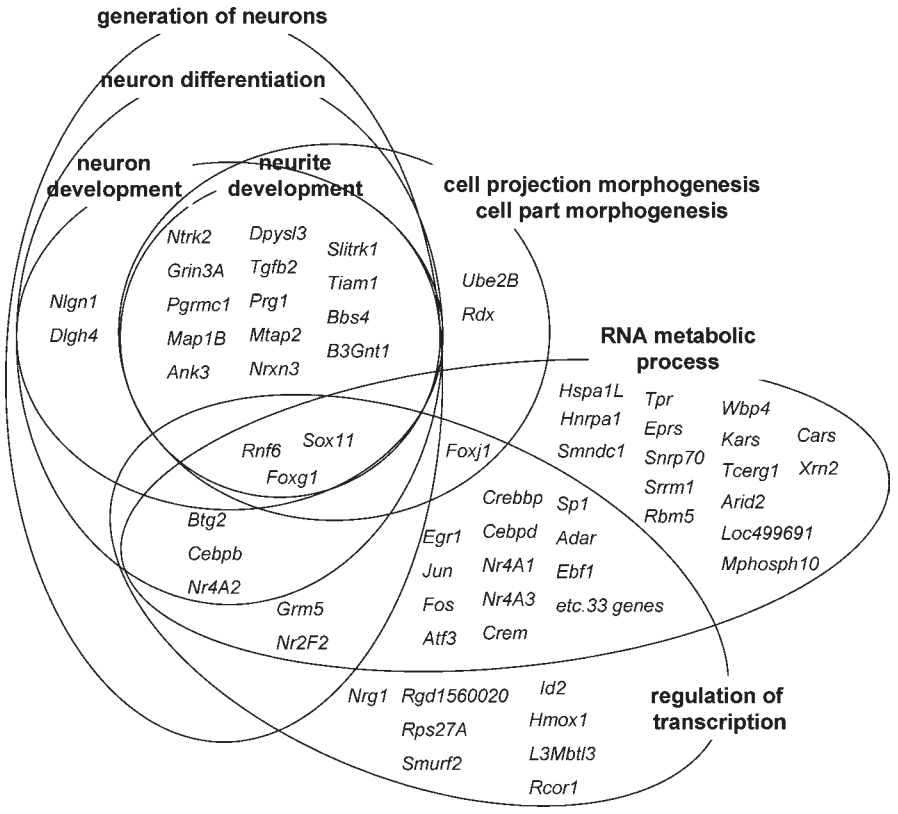

Figure 2. Venn and Euler diagrams representing the association of up-regulated genes with multiple GO terms and the resulting complex interdependencies of categories sharing differentially expressed genes following (*R*)-(−)-linalool inhalation under restraint-stressed conditions. The genes are represented as gene symbols. 33 of the 54 gene symbols which related to both regulations of transcription and RNA metabolic process gene symbols are not included. The upregulated genes were statistically identified by rank products between group S exposed to stress only and group SO exposed to both stress and odor.

responses, we examined the expression patterns of the 104 probe sets that were significantly altered by restraint plus inhalation. The 79 annotated probe sets among the 104 represented 69 genes because of redundancy of the probe sets. The heat map shows the difference in expression levels of these 69 genes among all 16 rats (**Figure 3**). The expression levels of 10 of the genes (**Figure 3**) were the calculated mean values of those from double-probe sets. The 2 h restraint down-regulated 34 of the 69 genes but up-regulated the remaining 35 genes. The inhalation of (*R*)-(−)-linalool during the 2 h restraint restored the expression of 49 genes to their normal state. However, it promoted the stress responses of the remaining 20 genes, especially the expression levels of 16 restraint-up-regulated genes that were more up-regulated by (*R*)-(−)-linalool inhalation. These alterations are consistent with those of leukocyte distribution and stress hormone release as reported in the findings that (*R*)-(−)-linalool restored the levels of both neutrophils and lymphocytes to their normal levels (*1*). By contrast, plasma ACTH and corticosterone levels were increased significantly by inhalation during restraint (*1*). Intriguingly, the 16 up-regulated genes included a number of heat shock proteins (HSPs) (*Hspa1L*, *Hspb1*, *Hsph1*, *Dnajb1*), CCAAT/enhancer binding proteins (*Cebpb*, *Cebpd*), a Homer homologue (*Homer1*), and IEG (*Fos*).

These results suggest that the inhaled (*R*)-(−)-linalool affects stress-related gene expression profiles in the hypothalamus of rats restrained for 2 h.

DISCUSSION

To elucidate whether (*R*)-(−)-linalool inhalation is able to alter stress-induced gene expression in the CNS, we examined gene expression in the hypothalamus in rats using DNA microarray analysis. Inhaled (*R*)-(−)-linalool significantly up-regulated genes related to neuron differentiation under stressful conditions (**Figure 2**). Moreover, it repressed the 2 h restraint-induced expression of 49 genes, whereas it promoted the expression of 20 genes, including HSP-related genes (**Figure 3**).

Odor inhalation under the restraint-stressed condition significantly up-regulated neurite development-related genes, which encoded *Map1B*, *Ank3*, *Nrxn3*, and *Tiam1* as well as transcriptional regulatory factors, such as *Jun*, *Fos*, *Egr1*, and *Crebbp*, necessary for neuronal maturation (**Figure 2**). No significant changes in gene expression were observed for neuropeptides and secretion system-related factors. These results show that the odor inhalation under a stress-added condition has a positive effect on the gene expressions related to the maturation of neurons by regulating the neuronal connectivity and synaptic formation in the hypothalamus.

It is known that HSPs are important modulators of the apoptotic pathway having cytoprotective functions that are largely explained by their anti-apoptotic function (*38*). In our study, *Hspa1L* (HSP70-1) and *Hspb1* (HSP27-1) were significantly up-regulated by the restraint stress and promoted by the simultaneously inhaled odorant (**Table 2**; **Figure 3**). HSP70 and HSP27 are molecular chaperones the expression of which is increased by many different types of stress. HSP70 is thought to be a key protein in orchestrating the decision to pursue differentiation rather than apoptosis (*38*). HSP27 blocks caspase-dependent apoptotic pathways (*39*). Apoptosis and differentiation are physiological processes that share many common features. This may be the main reason why many recent studies have highlighted the significance of HSPs in the differentiation process. Genes related to a neuroprotective factor involved in the cellular defense response to restraint were also up-regulated by both restraint and odor inhalation. These include Bcl2-associated

Table 2. 35 Probe Sets Show the Effect of (*R*)-(−)-Linalool on Stress-Regulated Gene Expression[a]

		S/C		SO/S		
Genbank accession no.	gene symbol	fold change		fold change		gene name
NM_031971	Hspa1L	19.60	↑	2.17	↑	heat shock 70kd protein 1a
BI278231	Hspa1L	13.12	↑	2.64	↑	heat shock 70kd protein 1a
NM_031970	Hspb1	3.57	↑	2.36	↑	heat shock 27kda protein 1
AF030088	Homer1	2.42	↑	1.79	↑	homer homologue 1 (*drosophila*)
AI031032	Rgd1310127	1.82	↑	1.58	↑	similar to cdna sequence bc017158
BF416264		1.79	↑	1.66	↑	
AB003726	Homer1	1.61	↑	1.71	↑	homer homologue 1 (*drosophila*)

		C/S		SO/S		
Genbank accession no.	gene symbol	fold change		fold change		gene name
F409092		5.37	↓	3.66	↑	
AI548117	Col10A1	3.93	↓	3.12	↑	procollagen, type x, α 1
AF312319	Gabbr1	2.54	↓	1.98	↑	γ-aminobutyric acid (gaba) b receptor 1
BE111706	Marcks	2.53	↓	2.15	↑	myristoylated alanine-rich protein kinase c substrate
AT005664		3.43	↓	1.70	↑	
BF411568		2.16	↓	1.75	↑	
BE107602		2.15	↓	2.18	↑	
AI030231		1.93	↓	1.56	↑	
AI454332	Rgd1563617	1.92	↓	1.89	↑	similar to sry sex-determining region y-box 9 protein
BI288811	Loc499779	1.90	↓	1.63	↑	similar to riken cdna 2900010j23
BE104180	Sox11	1.88	↓	1.59	↑	sry-box containing gene 11
AI231826	Rgs5	1.83	↓	1.84	↑	regulator of g-protein signaling 5
BE107282	Vof16	1.78	↓	2.04	↑	ischemia related factor vof-16
M59859	Marcks	1.75	↓	1.64	↑	myristoylated alanine-rich protein kinase c substrate
BI295188		1.74	↓	1.69	↑	

		S/C		S/SO		
Genbank accession no.	gene symbol	fold change		fold change		gene name
BG669096	Mpz	4.06	↑	4.90	↓	myelin protein zero
NM_012694	Slc6A3	3.46	↑	2.14	↓	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
M80233	Slc6A3	3.00	↑	2.40	↓	solute carrier family 6 (neurotransmitter transporter, dopamine), member 3
NM_053587	S100A9	2.95	↑	1.61	↓	S100 calcium binding protein a9 (calgranulin b)
AI232414	Pxmp4	2.82	↑	1.79	↓	peroxisomal membrane protein 4
NM_012740	Th	2.22	↑	1.75	↓	tyrosine hydroxylase
BI299174	Aph1B	2.07	↑	1.72	↓	presenilin stabilization factor-like
BI287300	Mgc72973	1.72	↑	1.77	↓	β-glo
BE109618		1.72	↑	2.31	↓	
X05080	Hbb	1.83	↑	1.75	↓	hemoglobin β chain complex
NM_013197	Alas2	1.85	↑	1.95	↓	aminolevulinic acid synthase 2
AI237401	Loc287167	1.90	↑	1.82	↓	globin, α
M10902	Tshb	2.82	↑	5.78	↓	thyroid-stimulating hormone, β subunit

[a] Fold change was calculated from log ratios. Statistical analysis was performed between groups C and S and between groups S and SO with rank products. The 35 gene expression values passed the criteria of $p < 0.01$ between groups C and S as well as between groups S and SO, indicating significant alteration by restraint plus (*R*)-(−)-linalool inhalation. Notably, these genes include the first seven genes, the fold changes of which indicate the promotion of stress by (*R*)-(−)-linalool. (C) Control animals that were not exposed to 2 h of restraint or (*R*)-(−)-linalool; (S) animals that were exposed to 2 h of restraint; (SO) animals that inhaled (*R*)-(−)-linalool during 2 h of restraint.

athanogene 3 (*Bag3*) and CCAAT/enhancer-binding proteins (*Cebpb*, *Cebpd*), which regulate gene expression to control cellular differentiation (*22*). Microarray analysis of the cerebrum and the cerebellum has revealed that *Cepbd* has direct interactions with *Hmox*, *Cebpb*, and *Fos* and also that it is not essential for nonstressed physiological development, although it controls the response to pathological insults as a factor in neuronal homeostasis (*40*). Accordingly, these genes play a protective role in helping the cell to cope with lethal conditions. In our microarray data, these genes were up-regulated by restraint stress and further up-regulated by odor inhalation (**Figure 3**). This result suggests that (*R*)-(−)-linalool at least partially enhanced the defense response.

In addition, (*R*)-(−)-linalool-up-regulated genes include many family members involved in the transforming growth factor (TGF)-β signaling pathway, such as *Bmpr2*, *Rbx1*, *Id2*, *Sp1*, *Crebbp*, *Smurf1*, and *Tgfb2*. Of these genes, *Bmpr2*, *Rbx1*, and *Id2* were significantly down-regulated by restraint, whereas the inhaled odorant restored normal levels (**Figure 3**). *Atf3* is also part of the *Cebpd* network, suggesting potential interactions between these transcription factors (*40*). *Arf3* has been suggested as an "adaptive response" gene as it responds to extra- and intracellular changes induced by stress signals and signals to promote cell proliferation (*41*). *Btg2* (B-cell translocation gene 2, antiproliferative) has also been suggested as a transcriptional coregulator, as well as a differentiation and anti-apoptotic factor in neurogenesis (*42*, *43*). Microtubule-associated proteins (*MAP1B*, *Mtap2*) are major growth-associated and cytoskeletal proteins in neuronal and glial cells. They are essential for stabilizing microtubules during the elongation of dendrites and neurites (*44*). It is likely that the genes up-regulated by odorants (**Table 1**; **Figure 2**) mediate the balance among neuronal apoptosis, cell survival, and differentiation.

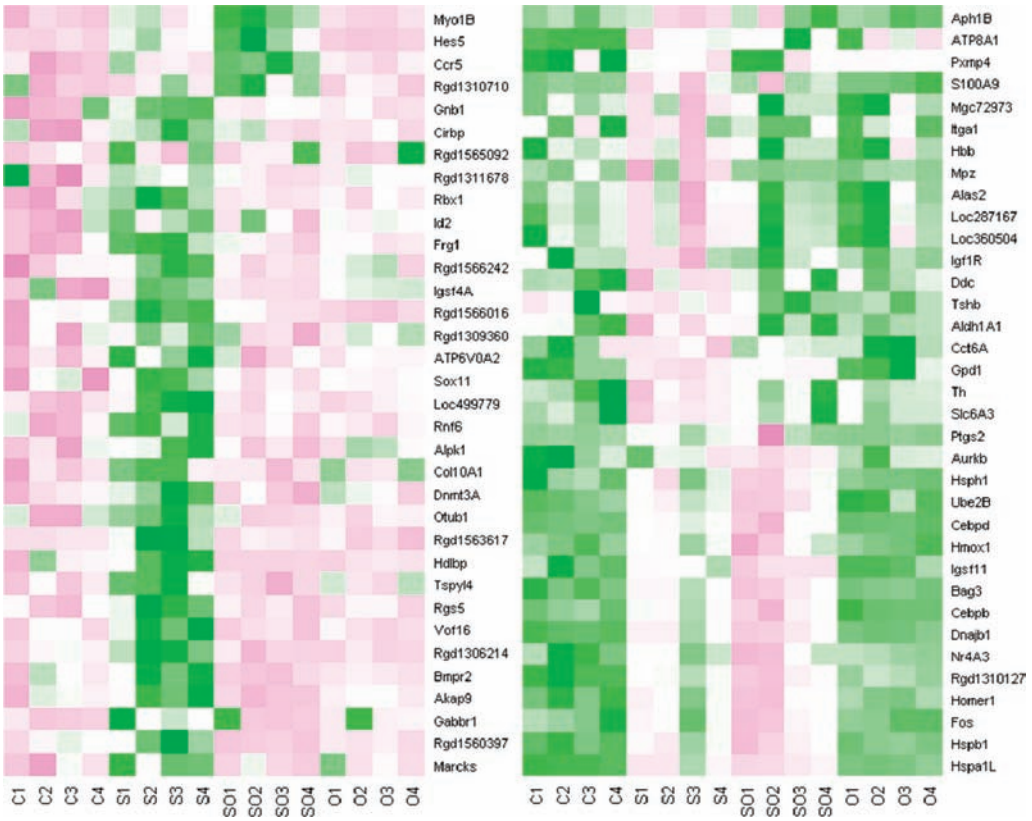

Figure 3. Heat map of expression levels of the 69 genes induced by (*R*)-(−)-linalool inhalation as well as 2 h of restraint. Restraint down-regulated 34 of the 69 genes (left), whereas it up-regulated the remaining 35 genes (right). The colors indicate higher (red) and lower (green) levels of gene expression. The genes are represented as gene symbols. The 69 genes were statistically identified by rank products which differentially expressed between groups C and S, as well as those that were differentially expressed between groups S and SO. Group C is a control (C1−C4) in which rats were exposed to neither stress nor odor; group S (S1−S4) is rats exposed to stress only; group SO (SO1−SO4) is rats exposed to both stress and odor; and group O (O1−O4) is rats exposed to odor only.

The inhalation of coffee bean aroma induces expression of HSP70-5 (*45*), which protects neurons against apoptosis (*46*). Inhalation of this aroma also up-regulates the expression of IEGs, such as *NGFI-B* (*Nr4A1*) and *Jun*, without affecting the expression of other IEGs/transcription factors, such as *c-fos*, as determined by a study performed on whole brains from rats under a one-day sleep deprivation-stressed condition (*45*). This discrepancy may be a result of variation in the stress types and in the duration of the experiment. However, inhalation of some odorants under stressful conditions may affect the expression of CNS genes related to HSPs and IEG/transcription factors to cope with apoptosis caused by various types of stress.

A day−night difference in the effects of the scent of lavender oil on brown adipose tissue temperature and body temperature in conscious mice has been reported, suggesting that the SCN might be involved in these effects (*47*). Our finding that a significant effect of the inhaled odorant does appear under stressed conditions indicates that the effect is condition-dependent.

In conclusion, (*R*)-(−)-linalool inhalation up-regulates the expression of genes related to neuron differentiation and the regulation of transcription in the CNS. Thus, the analysis of gene expression profiles under stressful conditions is necessary to determine odorant-induced effects, as exemplified by the finding that (*R*)-(−)-linalool inhalation under a 2 h restraint-stressed condition affected the commitment to gene expression related to neuronal differentiation as opposed to apoptosis in the hypothalamus involved in feeding behaviors of animals as well as stress responses.

ABBREVIATIONS USED

CNS, central nervous system; SCN, suprachiasmatic nucleus; GO, gene ontology; RMA, robust multiarray average; RP, rank products; FDR, false discovery rate; DAVID, database for annotation, visualization, and integrated discovery; IEG, immediate early gene; HSP, heat shock protein; TGF, transforming growth factor; HPA, hypothalamic−pituitary−adrenal.

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Supporting Information Available: Hierarchical structure of the enriched GO terms of the gene sets up-regulated by (*R*)-(−)-linalool inhalation under restraint stress drawn by QuickGO (Figure S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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