Neuroscience Letters 653 (2017) 314–319

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Research article

Inhalation of a racemic mixture (*R*,*S*)-linalool by rats experiencing restraint stress alters neuropeptide and MHC class I gene expression in the hypothalamus

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HIGHLIGHTS

- Inhalation of (*R*,*S*)-linalool affected overall gene expression in the hypothalamus.
- (*R*,*S*)-linalool inhalation altered the expression of genes related to synaptic transmission.
- Oxytocin and neuropeptide Y were upregulated by (*R*,*S*)-linalool inhalation.
- Several MHC class I molecules were also upregulated by inhaled (*R*,*S*)-linalool.

ARTICLE INFO

Article history:
Received 29 December 2016
Received in revised form 16 May 2017
Accepted 22 May 2017
Available online 6 June 2017

Keywords:
(*R*,*S*)-linalool
Oxytocin
Neuropeptide Y
MHC class I
Microarray
Hypothalamus

ABSTRACT

Some odorants have physiological and psychological effects on organisms. However, little is known about the effects of inhaling them, particularly on the central nervous system. Using DNA microarray analysis, we obtained gene expression profiles of the hypothalamus from restraint stressed rats exposed to racemic (*R*,*S*)-linalool. Hierarchical clustering across all probe sets showed that this inhalation of (*R*,*S*)-linalool influenced the expression levels of a wide range of genes in the hypothalamus. A comparison of transcription levels revealed that the inhalation of (*R*,*S*)-linalool restored the expression of 560 stress-induced probe sets to a normal status. Gene Ontology (GO) analysis showed that these genes were associated with synaptic transmission via neurotransmitters including anxiolytic neuropeptides such as oxytocin and neuropeptide Y. These genes also included several major histocompatibility complex (MHC) class I molecules necessary for neural development and plasticity. Moreover, Upstream Regulator Analysis predicted that the hormone prolactin would be activated by the inhalation of (*R*,*S*)-linalool under stress. Our results reveal some of the molecular mechanisms associated with odor inhalation in the hypothalamus in organisms under stress.

1. Introduction

Research has shown that some odorants can influence both psychological and physiological states of organisms including humans. For example, odors affect autonomic responses such as heartbeat and blood pressure [1,2]. However, the molecular mechanisms in the central nervous system (CNS) that underlie the effects of inhaled odorants are still poorly understood.

Linalool (3,7-dimethyl-1,6-octadien-3-ol), an odorant identified in numerous foods and flowers, elicits certain psychological and physiological effects including anticonvulsant, analgesic, and anxiolytic effects [3–8]. Linalool has two enantiomers, (*R*)- and (*S*)-linalool, which react differently within certain physiological parameters [4]. These two enantiomers exhibit anticonvulsant effects to similar extents, while racemic (*R*,*S*)-linalool displays more potent activity than each individual enantiomer [5]. These effects

Abbreviations: MHC, major histocompatibility complex; CNS, central nervous system; RP, rank products; FDR, false discovery rate; GO, Gene Ontology; Oxt, oxytocin; Npy, neuropeptide Y; PVN, paraventricular nucleus; ARC, arcuate nucleus; AgRP, agouti-related protein; PRL, prolactin.

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http://dx.doi.org/10.1016/j.neulet.2017.05.046
0304-3940/

are considered to be responsible, in whole or in part, for the actions of the compound on the CNS including the hypothalamus, which responds to stress [6–9].

The hypothalamus is activated by restraint stress to initiate various physiological changes [10]. Physical and psychological stresses induced by restraint result in a wide range of behavioral and physiological responses including reduction of locomotor activity, increased anxiety, stress hormone secretion, and cell death in the brain [11–13]. It has been reported that acute restraint stress alters gene expression profiles in the murine hypothalamus [14]. In this study, we performed a DNA microarray analysis of rat hypothalamus after exposure to restraint stress with inhalation of racemic (*R*,*S*)-linalool. Our goal was to determine whether this odorant alters stress-related gene expression in the stress response center of the CNS.

2. Materials and methods

2.1. Animals and odor inhalation under stress

Seven-week-old male Wistar rats were housed in a room where a 12 h light/dark cycle was maintained; lights on at 8:00 a.m. and off at 8:00 p.m. The rats were allowed free access to food and water. After acclimatization to the environment and the investigators for one week, 18 rats were divided into three groups (n = 6 each): group C, control; group S, exposure to stress only; and group SO, exposure to both stress and odor.

All experiments were done in a room at controlled temperature (24 ± 1 °C) and humidity (55 ± 5%). Individual groups were tested in a 40-L box on different days and derived of food and water during the experiment. Rats in groups S and SO were placed in steel-wire mesh cages inside the 40-L box under restraint for 2 h (10:00–12:00). (*R*,*S*)-Linalool consisting of the (*R*)- and (*S*)-enantiomers at a 1:1 ratio was purchased from BASF Japan Ltd. Spotting twenty microliters of (*R*,*S*)-linalool on a glass-ceramic heated at 200 °C resulted in immediately spreading throughout the 40-L box containing group SO rats. After exposure, all rats were immediately sacrificed by decapitation and their brains were dissected to isolate the hypothalamic blocks as described previously [15]. The isolated blocks were then immersed in ice-cold RNAlater (Applied Biosystems Japan, Japan). This protocol was approved by the Animal Use Committee of the Material Research Center Co., Ltd.

2.2. RNA isolation and microarray hybridization

Total RNA was isolated from each hypothalamic block using the TRIzol reagent (Invitrogen Japan K.K., Japan) and then purified with a Maxwell® 16 Instrument using a Maxwell® 16 Total RNA Purification Kit (Promega Corporation, USA). The concentration and purity of total RNA were measured by spectrophotometry at OD260/280. The quality of total RNA was evaluated with an Agilent 2100 Bioanalyzer using an RNA 6000 Nano Series II Kit (Agilent Technologies Japan, Japan). Total RNA with an RNA integrity number (RIN) of >8.7 was used.

To prepare RNA samples for DNA microarray analysis, the 3′ IVT PLUS Reagent Kit (Affymetrix, USA) was used. The concentration and purity of cRNA were also measured by spectrophotometry at OD260/280. The cRNA size distribution evaluated with the Agilent 2100 Bioanalyzer was distributed from 25 to 6000 nt with a maximum of ∼1350 nt. Fifteen micrograms of biotinylated cRNA was fragmented and hybridized to a GeneChip® Rat Genome 230 2.0 Array (Affymetrix). Following hybridization at 45 °C for 16 h, the array was washed and stained with phycoerythrin using the GeneChip® Fluidics Station 450 (Affymetrix). Fluorescence signals were scanned with a GeneChip® Scanner 3000 7G (Affymetrix). The array images were converted to the intensity values for each probe (CEL files). All microarray data were submitted to the National Center for Biotechnology Information (NCBI) Gene Expression Omnibus section (accession number: GSE90923).

Restraint stress	Inhalation of (*R*,*S*)-linalool	Numbers of probe sets
↓	↑	271
↑	↓	289

Fig. 1. The number of probe sets where expression levels were altered by the restraint stress and altered inversely by (*R*,*S*)-linalool inhalation. White and black arrows represent up- and downregulated gene expressions, respectively.

2.3. Microarray data analysis

The CEL files were quantified by the Factor Analysis for Robust Microarray Summarization (qFARMS) [16] in the statistical language R version 2.12.2. Hierarchical clustering was then performed using the pvclust() function [17] in R. To identify the probe sets that were differentially expressed between the groups, the rank products (RP) method [18] was applied to the qFARMS-quantified data. The number of permutations was set at 500. Probe sets with a false discovery rate (FDR) of <0.05 were considered differentially expressed. The annotation file for the Rat Genome 230 2.0 Array was downloaded from the Affymetrix website (March 30, 2016, Rat230_2.na36.annot.csv).

2.4. GO and upstream regulator analysis

The probe sets inversely regulated by stress and (*R*,*S*)-linalool inhalation, as shown in Fig. 1, were functionally classified according to the Biological Process in GO with the Functional Annotation Tool of the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [19]. Probe set IDs provided by Affymetrix were used as the input data format. In the probe set list manager on the DAVID website (http://david.abcc.ncifcrf.gov/), we selected the species option to limit the annotations exclusively to *Rattus norvegicus*. For the population manager option, the Rat Expression Array 230 2.0 platform was selected as the background. The Functional Annotation Chart was analyzed on the basis of Biological Process in GO, GOTERM_BP_ALL. To extract the statistically overrepresented GO terms within these groups of differentially expressed genes, we used EASE scores, which are modified Fisher's exact test *p*-values [20]. The Benjamini and Hochberg FDR [21] was used to correct for multiple tests. GO terms with an FDR-corrected *p*-value of <0.01 were considered significant. To determine the hierarchical structure of the selected GO terms, the online analysis application QuickGO (https://www.ebi.ac.uk/QuickGO/) was used [22].

The selected probe sets, as shown in Fig. 1, were also imported into Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems, www.ingenuity.com). Upstream Regulator Analysis in the IPA was used to identify potential transcriptional regulators.

3. Results

3.1 (R,S)-Linalool inhalation under restraint influenced the expression of a wide range of genes in the hypothalamus

Gene expression profiles were compared among the three rat groups. A hierarchical clustering analysis using the normalized values obtained by the qFARMS algorithm from more than 31,000