2nd Workshop Argentina-Japan
- November 8th and 9th 2010 -

Stress Regulation by (*R*)-(-)-Linalool as Seen from Blood Gene Expression Analysis

-Development of functional foods and evaluation of their effects by sophisticated methodologies -

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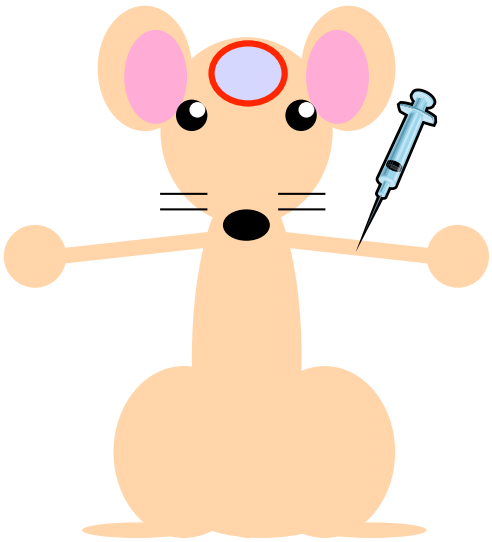
Outline

- Introduction:
  - Olfactory System
  - Effect of Aroma on Stress Responses
- Profiles of blood cells and gene expressions in whole blood of aroma-inhaling rats under restraint stress
- Regulation of gene expressions in hypothalamus of aroma-inhaling rats under restraint stress
- Conclusion



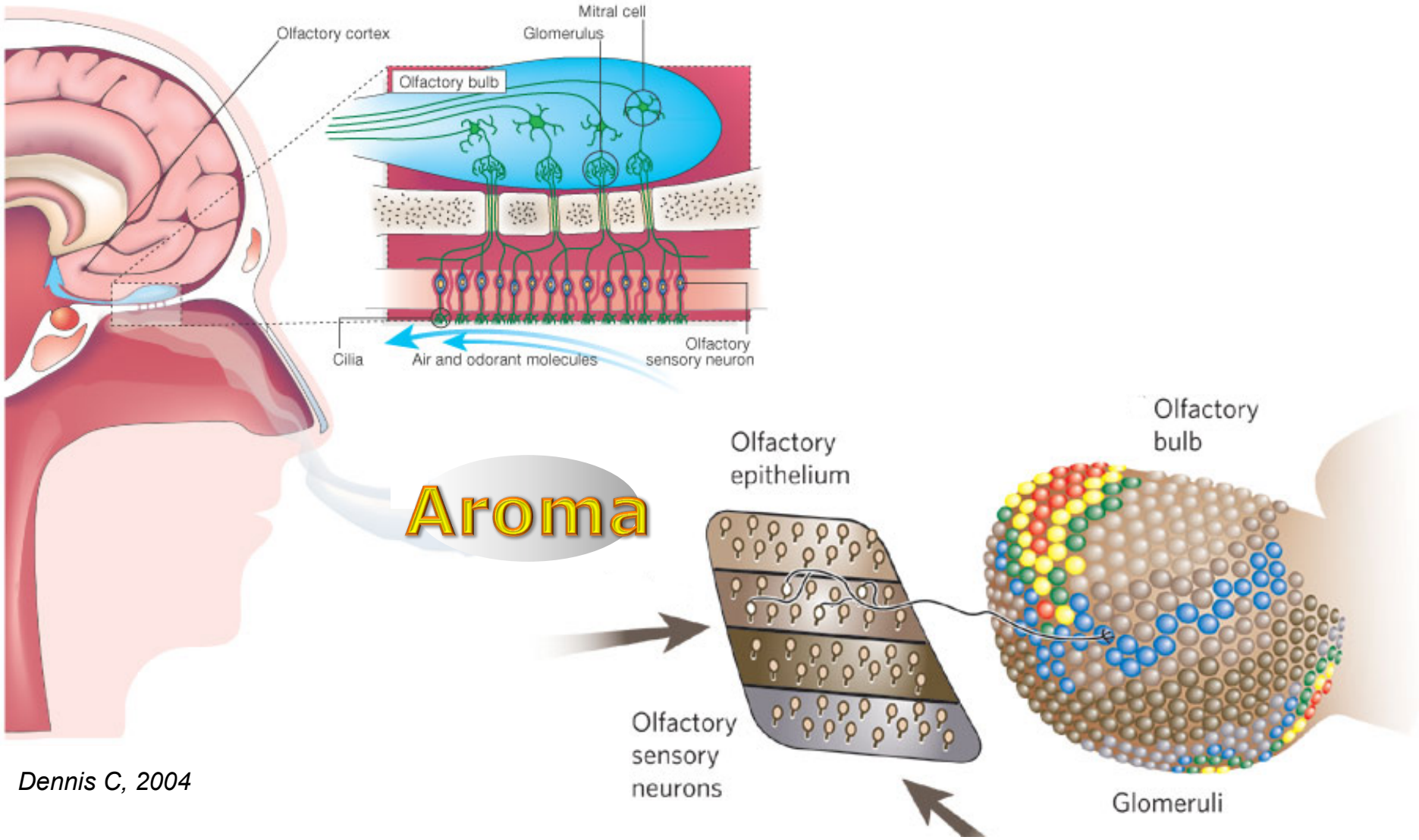
FLAVOR

T.Hasegawa Co., Ltd. from its founding in 1903 has been dedicated to the enhancement of daily living and dietary patterns in the development, manufacture and marketing of aroma products.

Aroma products may be broadly divided into two kinds, natural and synthetic, being further divided into "fragrances" for perfumes, cosmetics, soaps and detergents, and "flavors" for processed foods.

FRAGRANCE

Olfactory System



Dennis C, 2004

Mori K. et al., 1999

Psycho-physiological Effects

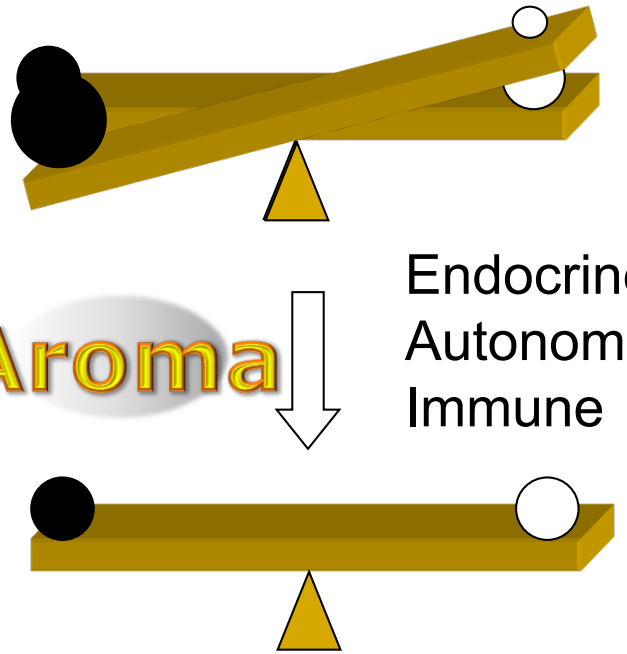
- Sedative
- Stimulative
- Antistress
- Anticonvulsant
- Aromatheraphy

...

Olfactory System

+psychological (preference,・・)

+pharmacological



Endocrine
Autonomic nerve
Immune
} System

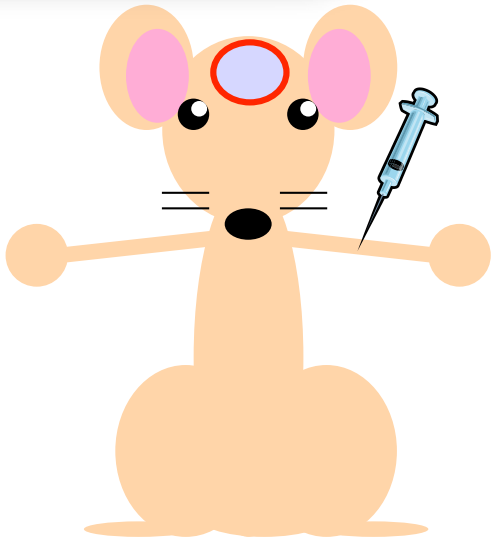
Homeostasis

Backgrounds

- In recent years, there has been interested in the psycho-physiological effects elicited by aroma, because of their expected contribution to health maintenance and promotion.
- Research for assessing these effects of inhaled aroma *in vivo* is still quite limited.
- The effects are considered to be caused by complex psychological and physiological interactions.
- Existing reports, contrary to this study, cover the phenomena elicited by aroma using but limited biomarkers.

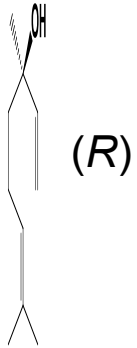
Aims

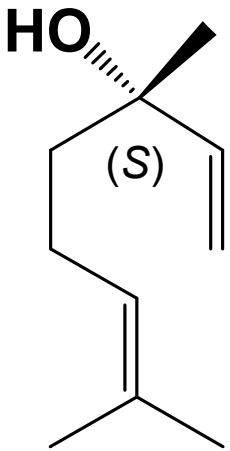
- Quantify *in vivo* the effects of inhaled aroma.
- Elucidate whether aroma inhalation alters stress-induced gene expression.



Linalool

- Found in numerous foods and flowers
- Its characteristic aroma is important
  - in formulating varieties of fragrances and flavors.
  - in eliciting certain kinds of psycho-physiological effects.



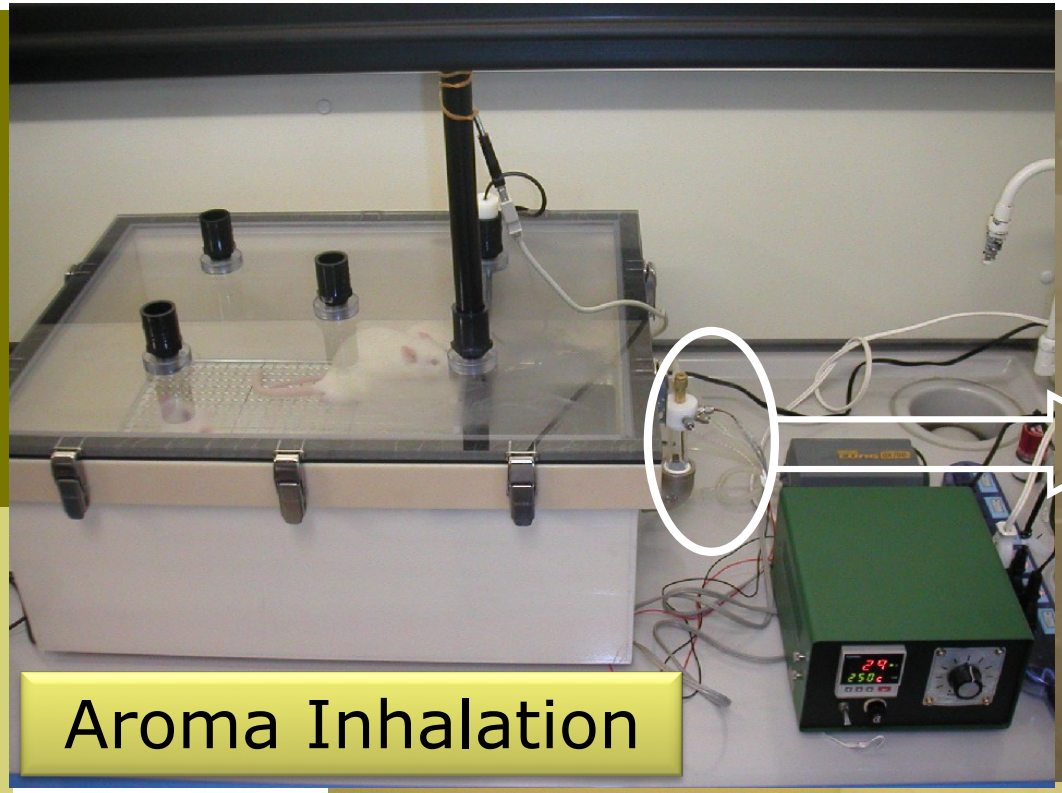


Physiological Effect of Linalool

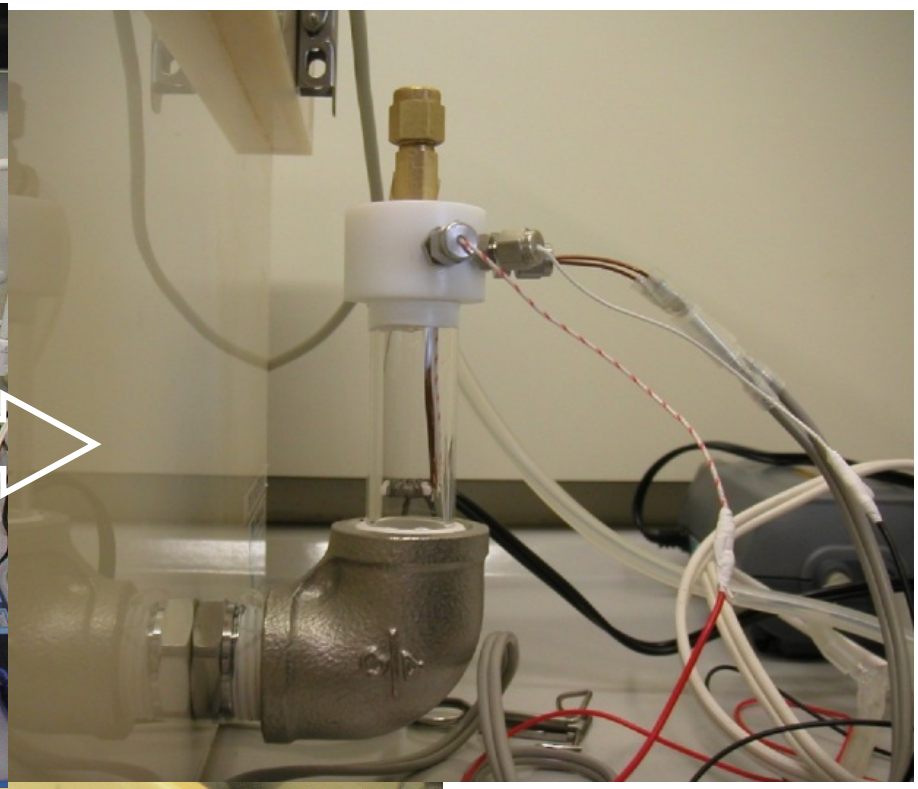
- The effects are elicited by its actions on the central nervous systems.
- Two optical isomers of linalool with (*R*)- and (*S*)-configurations act differently on psycho-physiological parameters (Höferl et al., 2006).
- (*R*)-Linalool elicit a significant decrease in heart rate under stressed conditions because of its sedative effect (Kuroda et al., 2006).

Profiles of Blood Cells and Gene Expressions in Whole Blood of Aroma-inhaling Rats under Restraint Stress

Stress Repression in Restrained Rats by (*R*)-(-)-Linalool Inhalation and Gene Expression Profiling of Their Whole Blood Cells



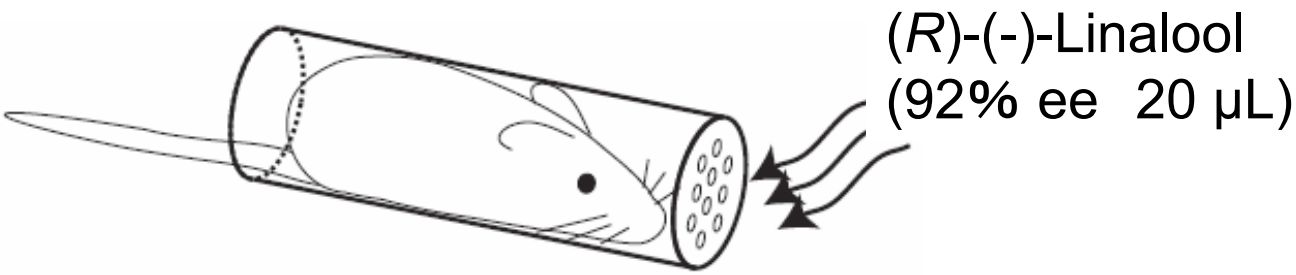
Aroma Inhalation



Acute Restraint Stress

Stress and Aroma Inhalation

(n = 4, Wistar rats (8 weeks, Male))



(*R*)-(-)-Linalool recovers the levels of both neutrophils and lymphocytes to their normal levels

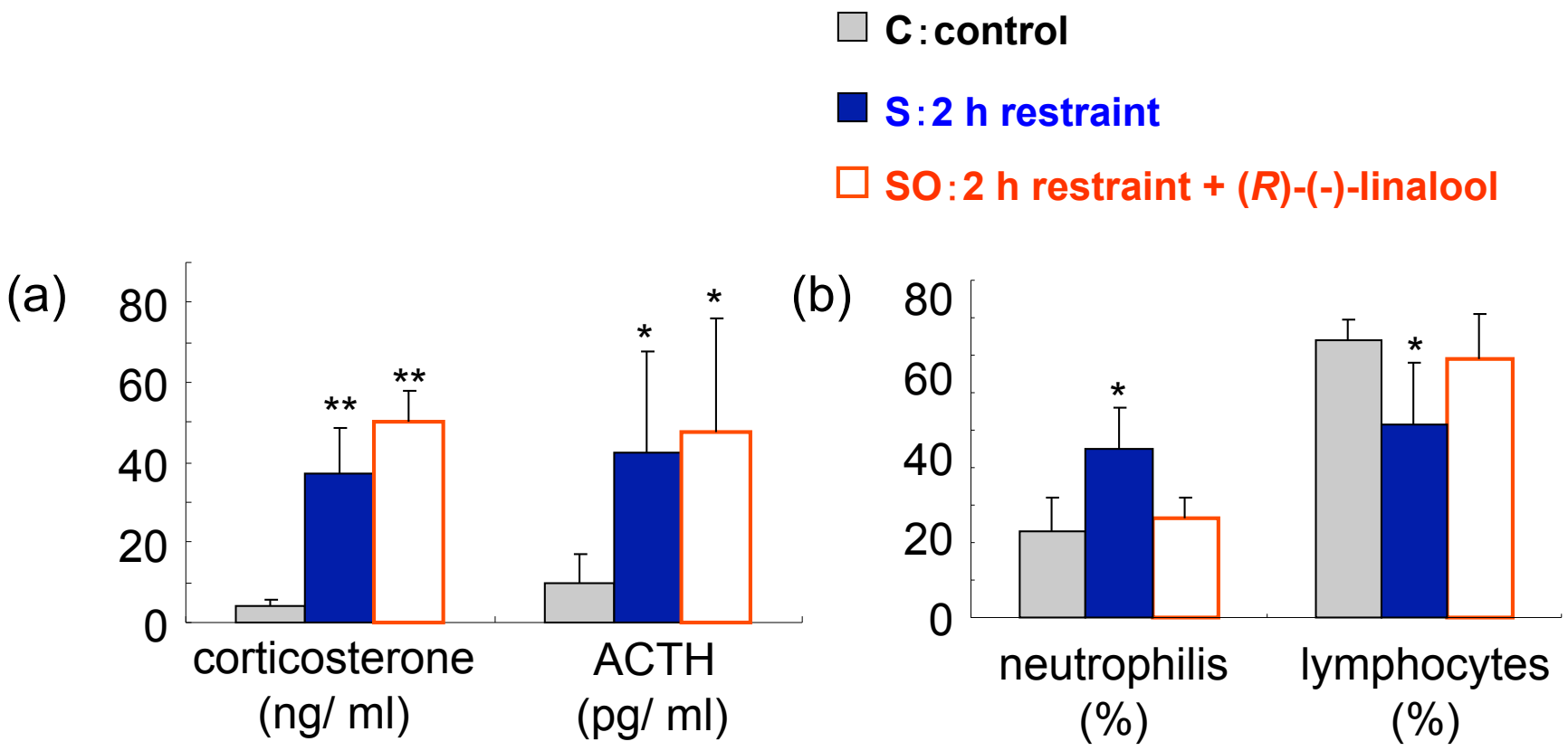
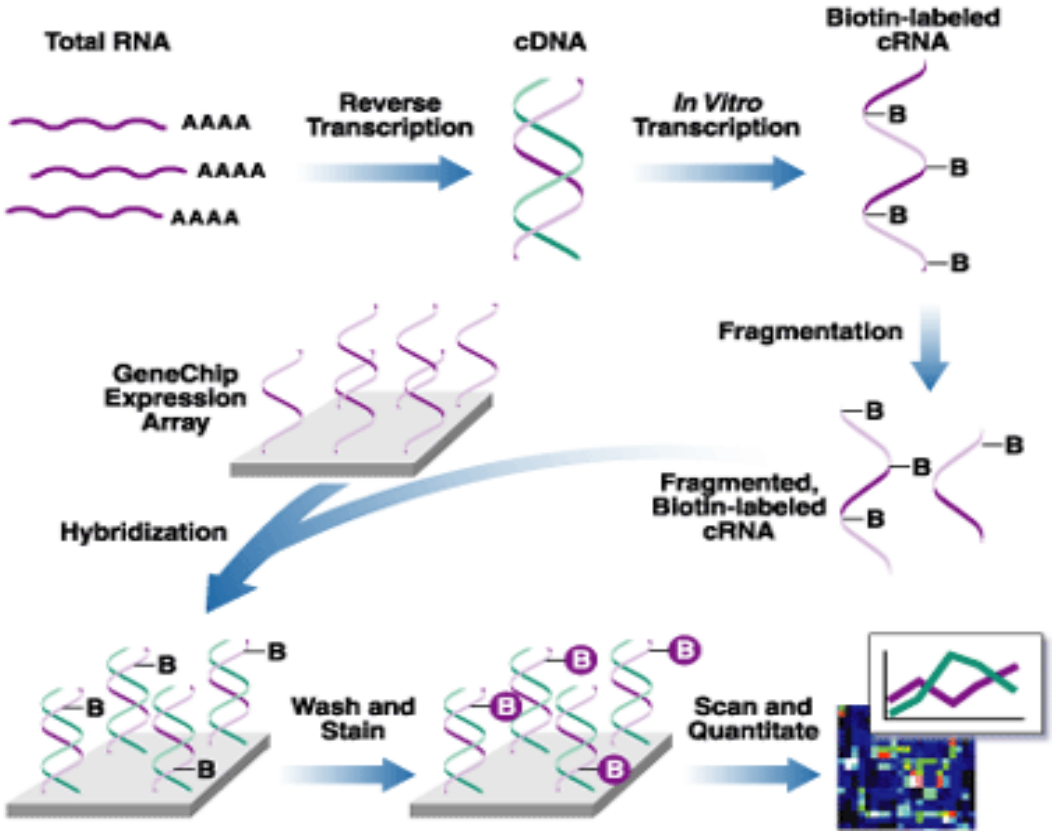


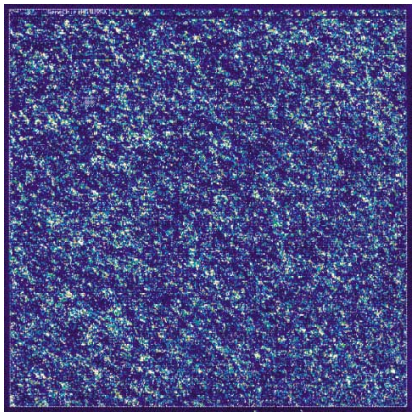
Fig.1 Effects of (*R*)-(-)-linalool on (a) plasma ACTH and corticosterone levels and (b) leukocyte populations in stress model rats. Data are expressed by means ± S.D. of four rats. * $p < 0.05$, ** $p < 0.01$ compared to the control (Student's *t*-test).

Affymetrix Expression Arrays

Affymetrix Rat Genome 230 2.0 GeneChip



http://www.affymetrix.com/technology/ge_analysis/index.affx



Affymetrix Data Flow

Hybridized GeneChip

Scan Chip

DAT file

Process Image (GCOS)

CEL file

MAS5 (GCOS)

CHP file

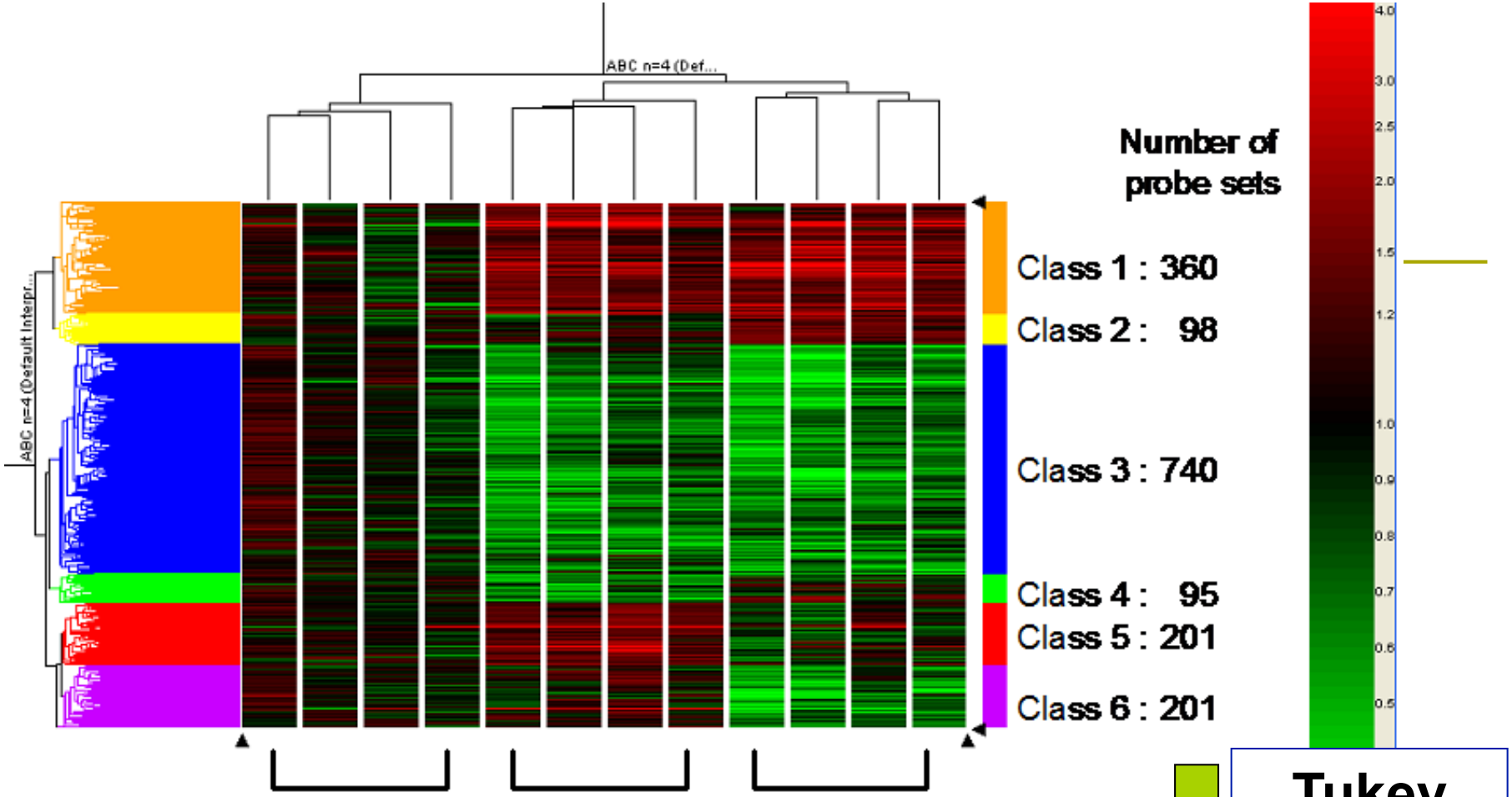
31,099 probe sets

Detection call (>3/4 “present” or “marginal”)

12,263 probe sets

1,695 probe sets

one-way ANOVA (p<0.05, between 3 groups)



similarity measure = pearson correlation
clustering algorithm = average linkage

Tukey
($p < 0.05$)

115 probe sets

Differentially expressed between group C and S, plus S and SO

Fig.2 Hierarchical cluster analysis performed on the 1695 stress and/or aroma responsive genes which passed the filtering criteria. In the heat map, the color indicates high (red) and low (green) relative levels of gene expression, compared with the median for group C.

(*R*)-(-)-Linalool has a Partially Repressive Effect on Changes Induced by the Stress in the Gene Expression Levels

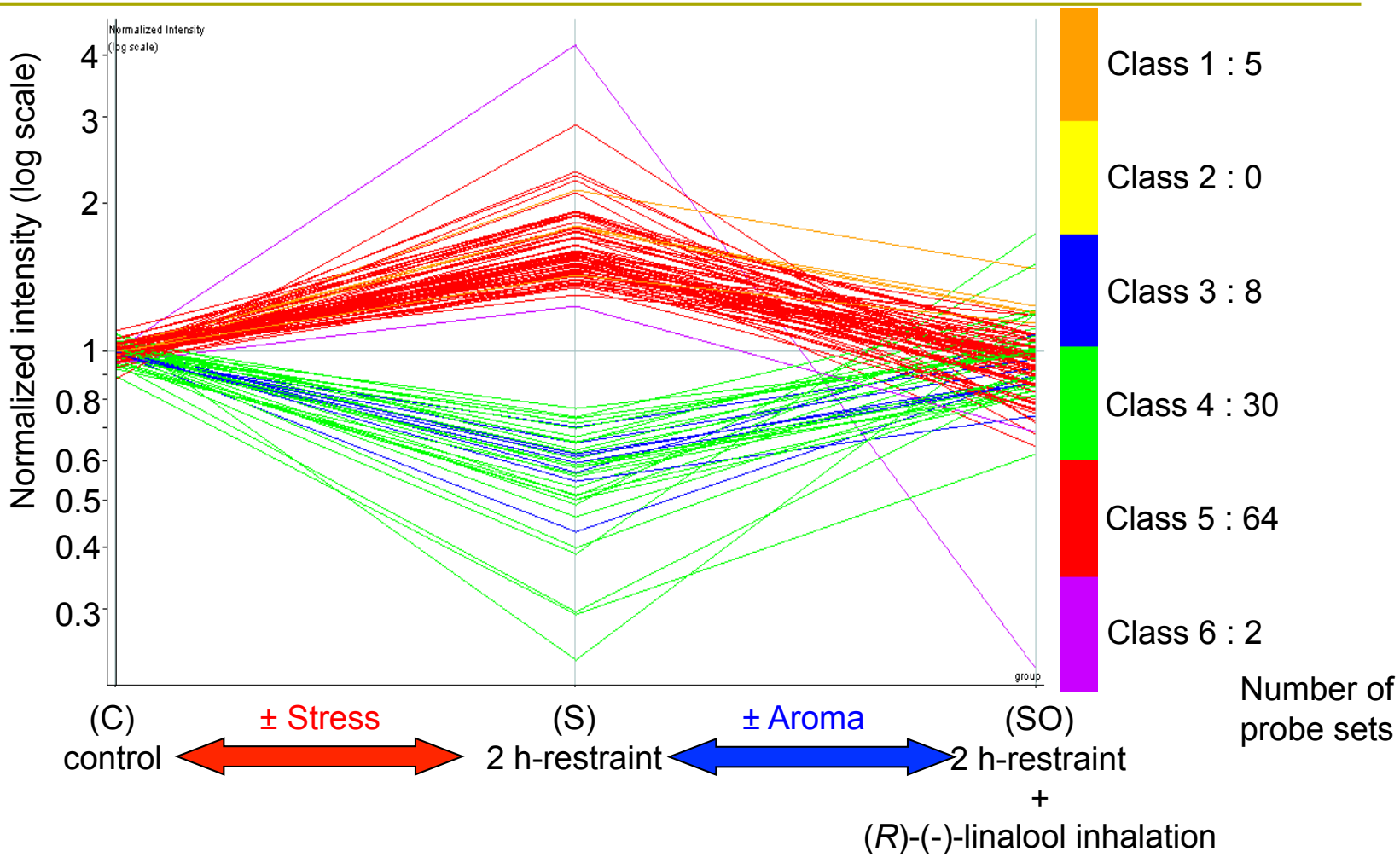


Fig.3 Each line plot shows two-hour-restraint and/or (*R*)-(-)-linalool-induced changes. The 109 gene expression values passed the filtering criteria of both $p < 0.05$ between (C) vs. (S) and (S) vs. (SO) by Tukey's post hoc test.

109/115 probe sets

(*R*)-(-)-Linalool has a Partially Repressive Effect on Changes Induced by the Stress in the Gene Expression Levels

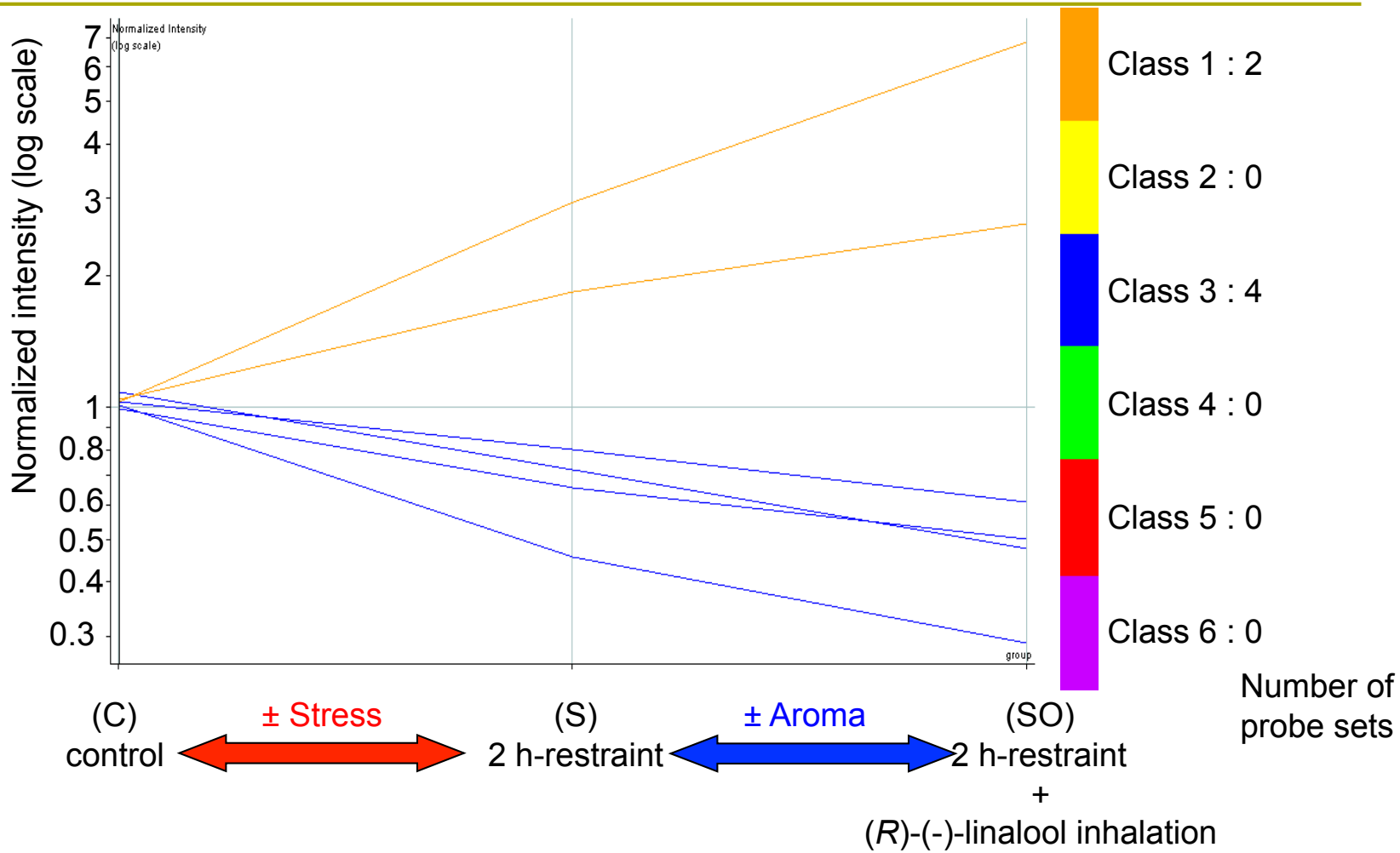


Fig.4 Each line plot shows two-hour-restraint and/or (*R*)-(-)-linalool-induced changes. The 6 gene expression values passed the filtering criteria of both $p < 0.05$ between (C) vs. (S) and (S) vs. (SO) by Tukey's post hoc test.

6/115 probe sets

Summary (1)

- We profiled blood cells and gene expression of restrained rats which inhaled (*R*)-(-)- linalool.

  (1) In neutrophils and lymphocytes, significant changes in quantity caused by the restraint were repressed by exposure to the aroma.

  (2) Significant changes in the stress-induced variations with respect to 115 gene expression levels were observed. Of those, 109 genes were repressed.

- The finding shows that the (*R*)-(-)-linalool inhalation represses the stress-induced effects on the profiles of both blood cells and gene expression.

- The finding suggests the possibility that the aroma-induced effects can be quantitatively evaluated by analyzing the profiles of blood cells and gene expression.

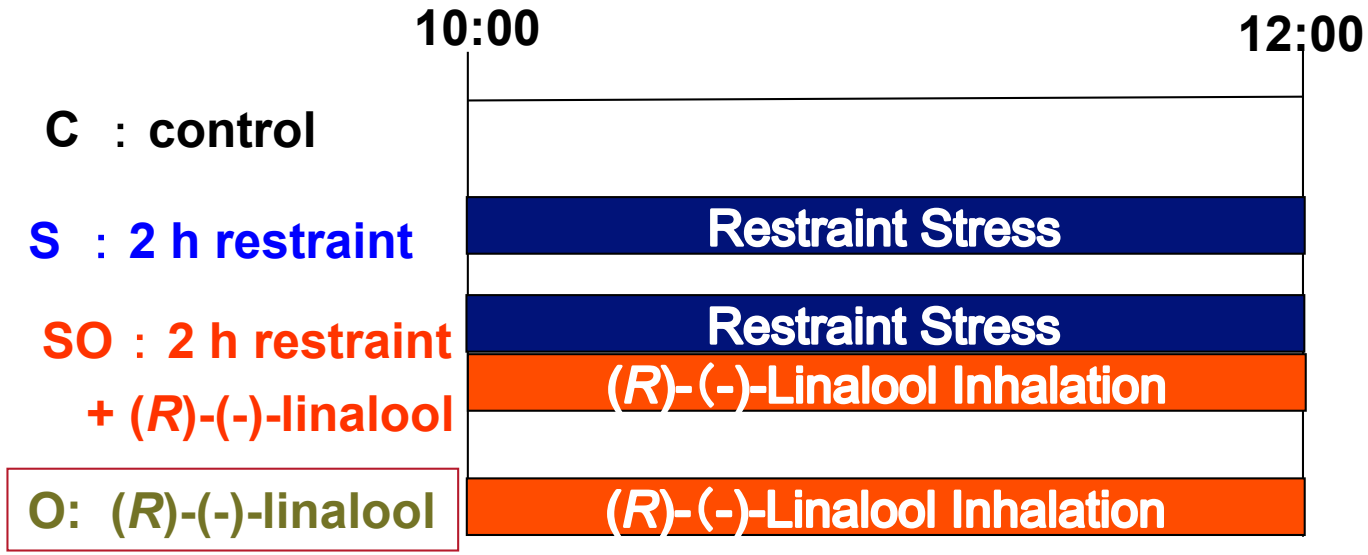
Remaining Questions?

- It remained unclear how the complicated relationships of the gene network mechanisms are induced just by aroma inhalation for two hours.
- The variation in the expression levels of the cell-specific transcripts may have been caused simply by the changes in proportions of blood cell subsets in whole blood.
- Elucidation of the effect of aroma on transcription at a cellular level will be of interest, because it will provide deep insights into the mechanisms for physio-psychological effects of aroma.

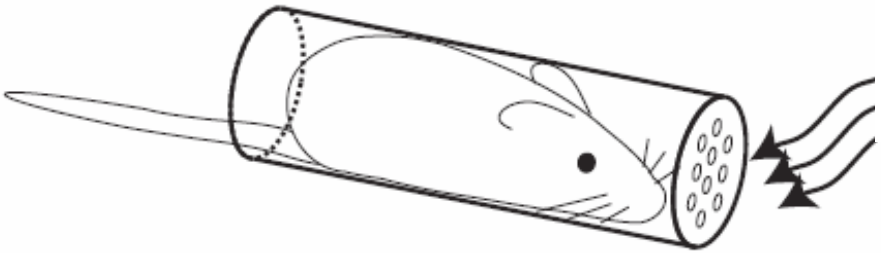
Regulation of Gene Expressions in Hypothalamus of Aroma-inhaling Rats under Restraint Stress

Neuron Differentiation-related Genes are Upregulated in the Hypothalamus of Aroma-inhaling Rats Subjected to Acute Restraint Stress

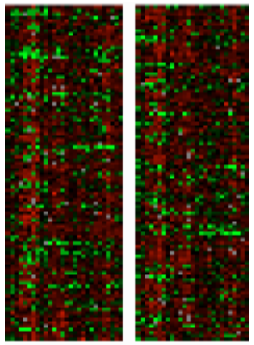
Stress and Aroma Inhalation

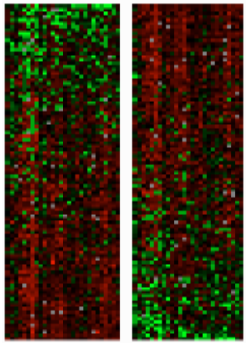


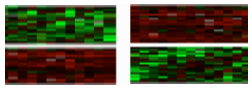
(n = 4, Wistar rats (8 weeks, Male))



DNA Microarray Analysis of Hypothalamus







Scaning Data

GCOS

CEL files

R

RMA

Normalized 31,099 probe sets

R

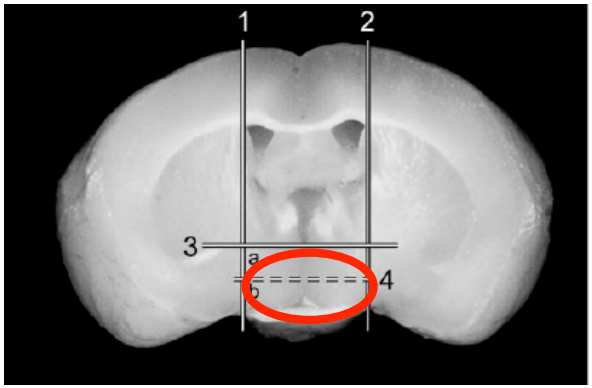
Rank Products

Ranking 31,099 probe sets

Excel

FDR < 0.05

Differentially Expressed Genes



Clustering

Gene Ontology
(GO)

DAVID、QuickGO

Gene Expression Profiles in Hypothalamus Altered by (*R*)-(-)-Linalool Inhalation During 2-h Restraint

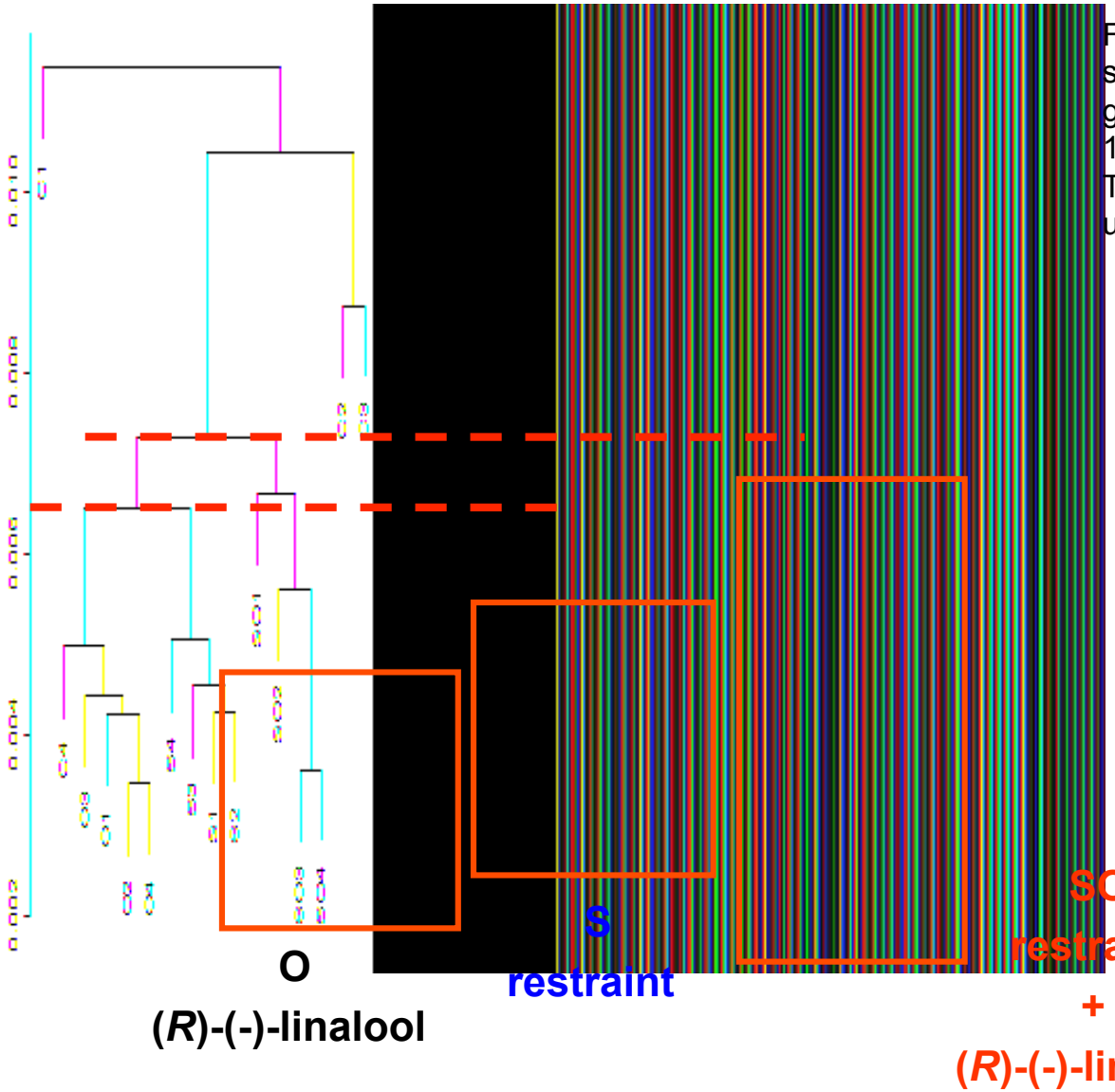
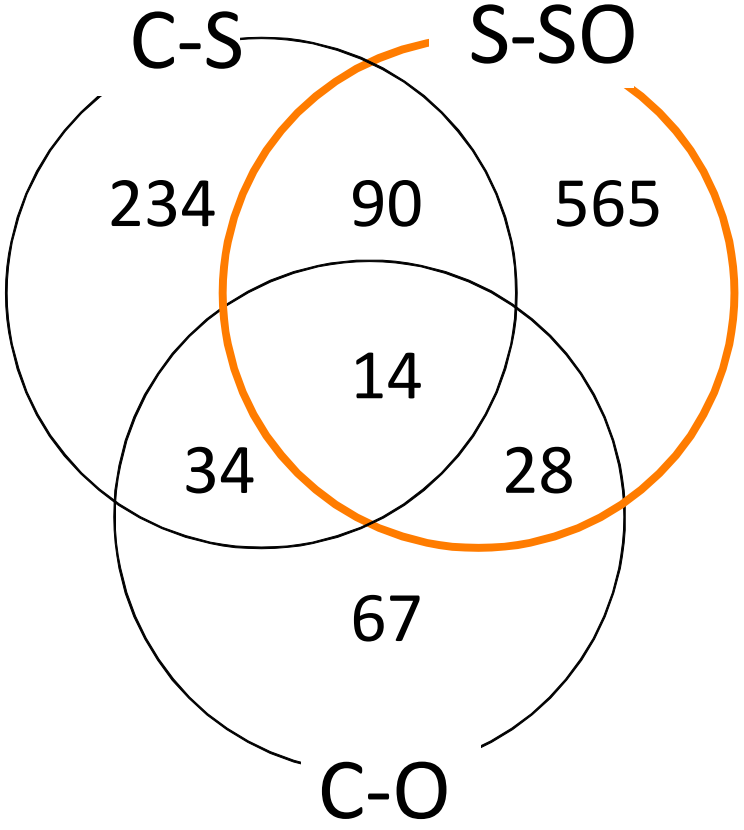


Fig. 5 Cluster dendrogram showing similarities among the gene expression patterns of the 16 experimental rats. The group average method was used for the construction.

Differential Expressed Genes

Expressions were altered by
(*R*)-(-)-linalool under the restraint stress



697
／31,099 probe sets

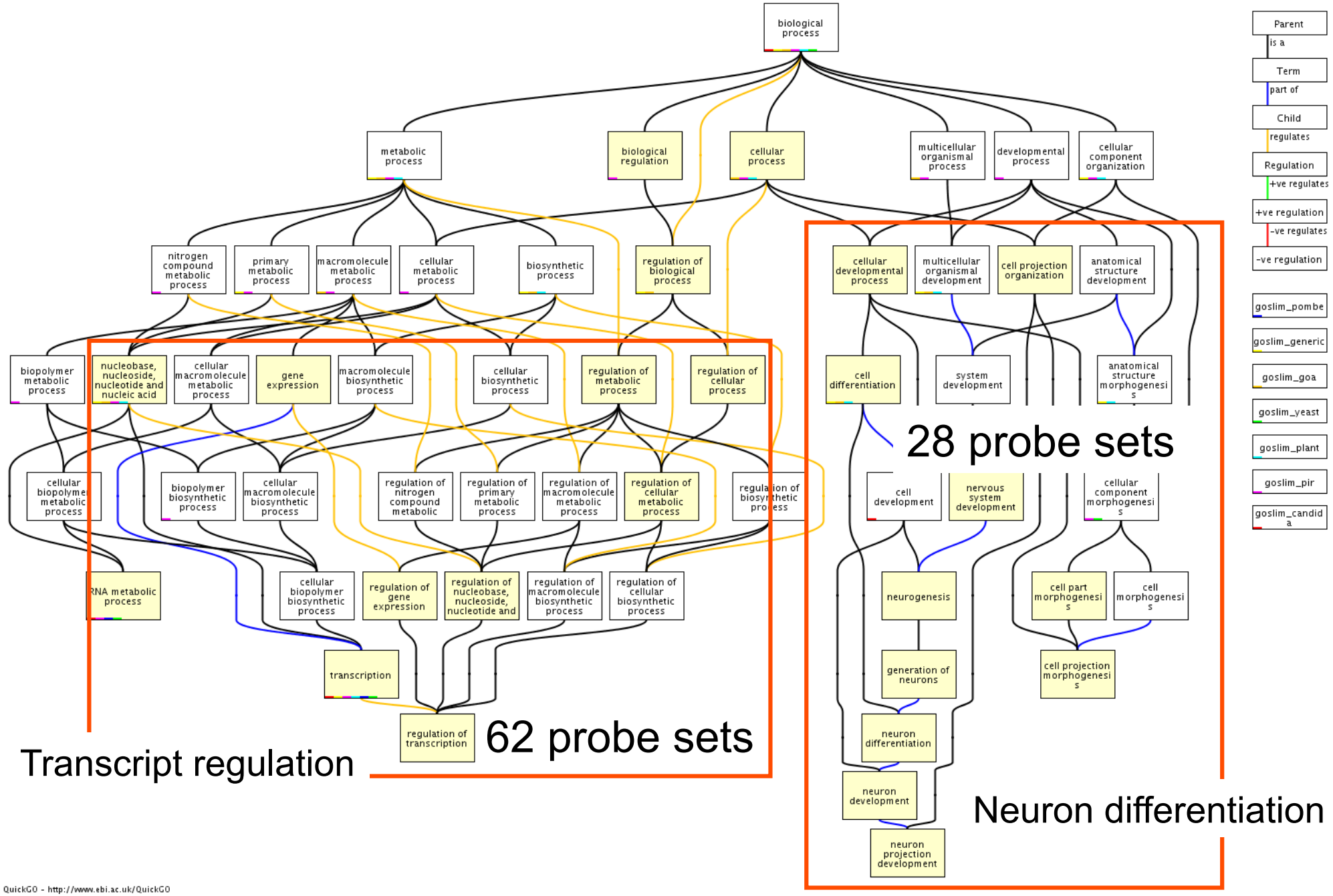
S
SO

594（including 142 unknown）

GeneOntology

103（including 28 unknown）

Upregulated Gene Ontology by (*R*)-(-)-Linalool under Restraint



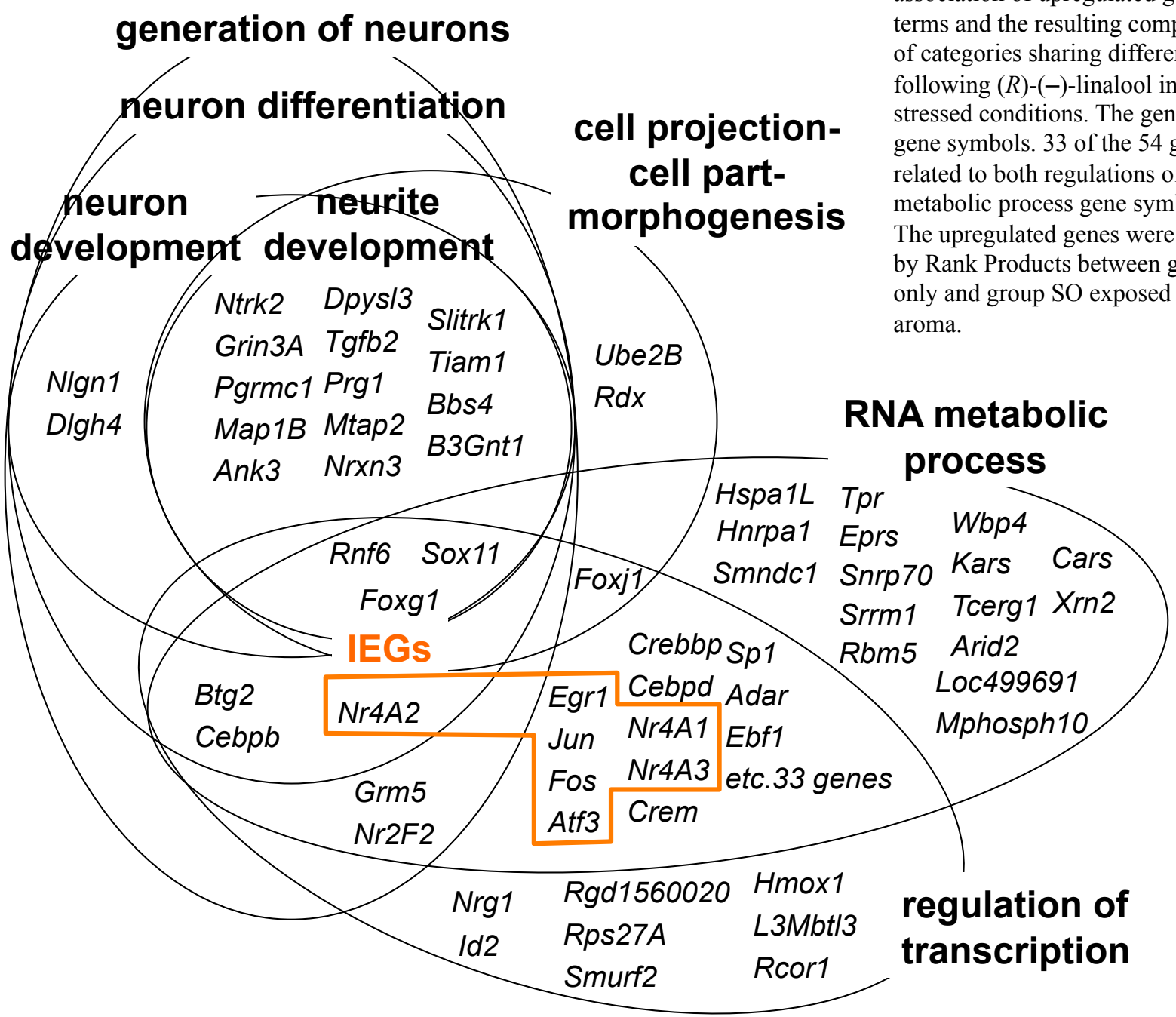
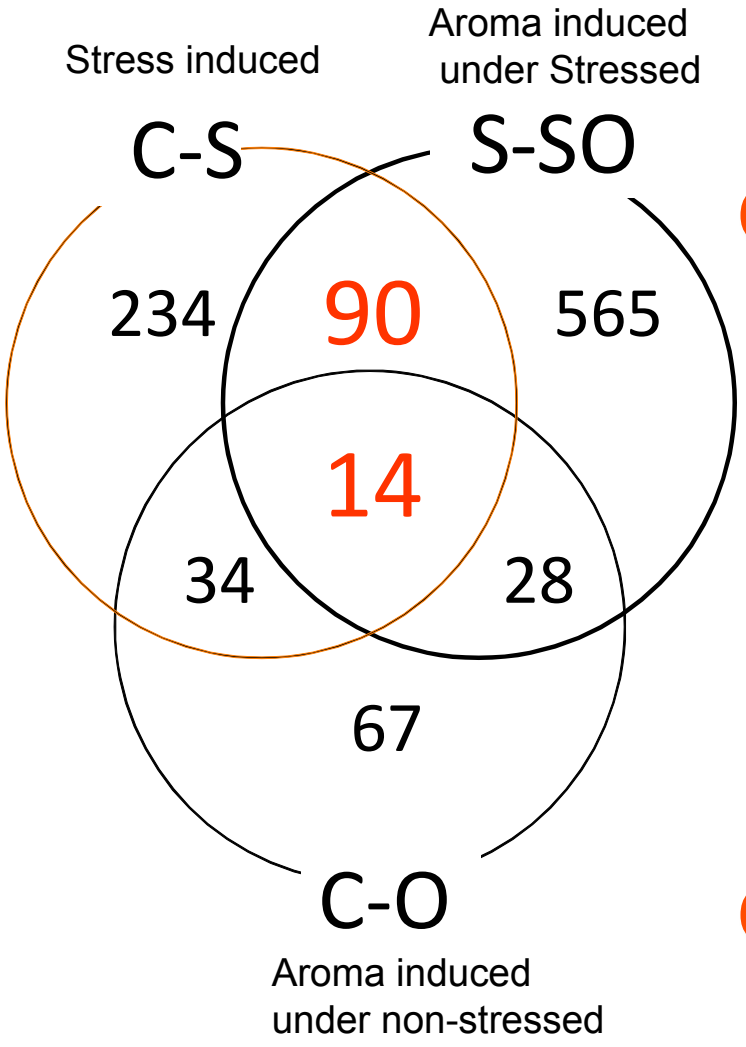


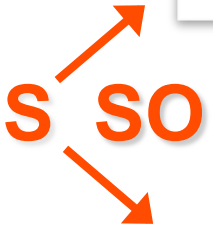
Fig. 7 Venn and Euler diagrams representing the association of upregulated genes with multiple GO terms and the resulting complex interdependencies of categories sharing differentially expressed genes following (*R*)-(−)-linalool inhalation under restraint-stressed conditions. The genes are represented 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 aroma.

Effects of Inhalation on Gene Expression under Stressed Conditions



Stress-induced genes

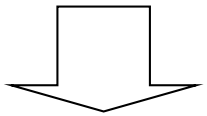
372

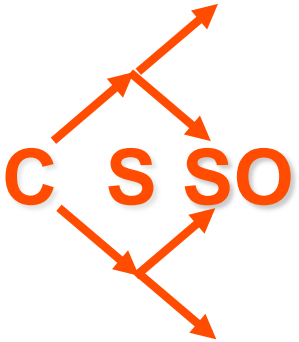


Aroma-induced genes

697

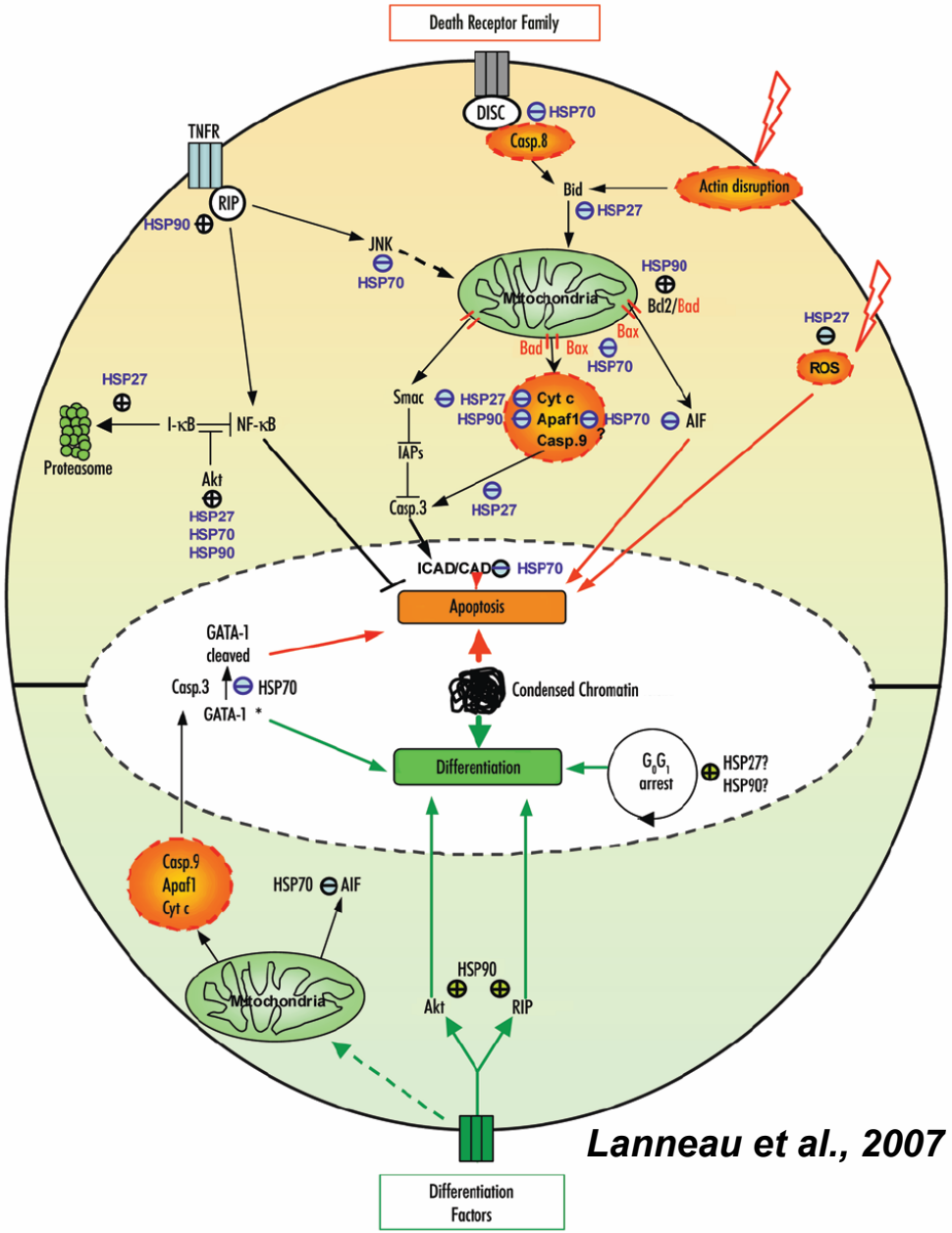
／31,099 probe sets





104 probe sets
(69 gene symbols)

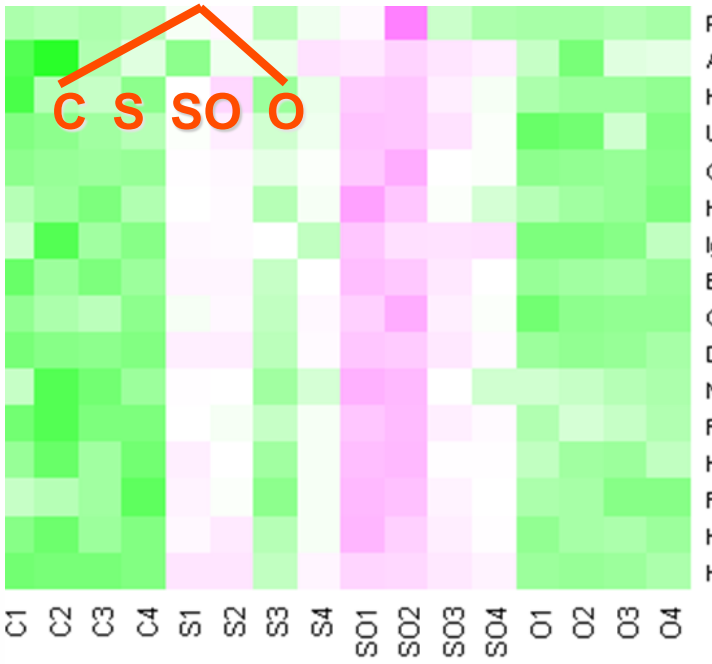
Apoptosis vs. Differentiation



Lanneau et al., 2007

Heat Shock Protein（HSP）

Hspa1L（HSP70-1）
Hspb1　（HSP27-1）



Ptgs2
Aurkb
Hsph1
Ube2B
Cebpd
Hmox1
Igsf11
Bag3
Dnajb1
Cebpb
Nr4A3
Rgd1310127
Homer1
Fos
Hspb1
Hspa1L

Summary (2)

- Another study, aiming to explain one of molecular logics of stress relaxation by (*R*)-(–)-linalool inhalation, was carried out by gene expression profiling with a sample of hypothalamus as a stress response center.

- The Inhalation under a restraint stress-added condition

  (1) Upregulated a number of neuron differentiation-related genes toward activating the processes of neuronal maturation.

  (2) Upregulated restraint stress-inducible, heat shock protein-related genes that can be associated with the suppression of stress-caused apoptosis.

Conclusion

- (*R*)-(–)-linalool inhalation
  - returns stress-elevated levels of neutrophils and lymphocytes to near-normal levels.
  - reduces the activity of more than 100 genes overdriven in stressful situations for the whole blood.

- The possibility that olfactory input can modify the gene expressions in the hypothalamus neural network involved in feeding behaviors of animals, as well as their blood chemistry is clarified.

- Our findings
  - has elucidated a physiological effect of an inhaled pleasant aroma, (*R*)-(–)-linalool in this case, by an in-depth analysis of gene expressions.
  - could largely contribute as a new method for assessing effects of aroma on stress regulation.

Future Prospective

- Aroma sometimes have an effect to relax our stress. Relaxation by inhaling some aroma has been practiced in our life for a long time.
- Our method to inhale aroma under stressed condition is effective for assessing the effects which the inhaled aroma has *in vivo*.
- Given that blood sampling is the most easily accessible in humans as well as animals, our method can be easily applied to tests in humans.
- Our research can be an initial step to determine which kinds of aroma have or do not have psycho-physiological effects.

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End of Presentation

Thank you

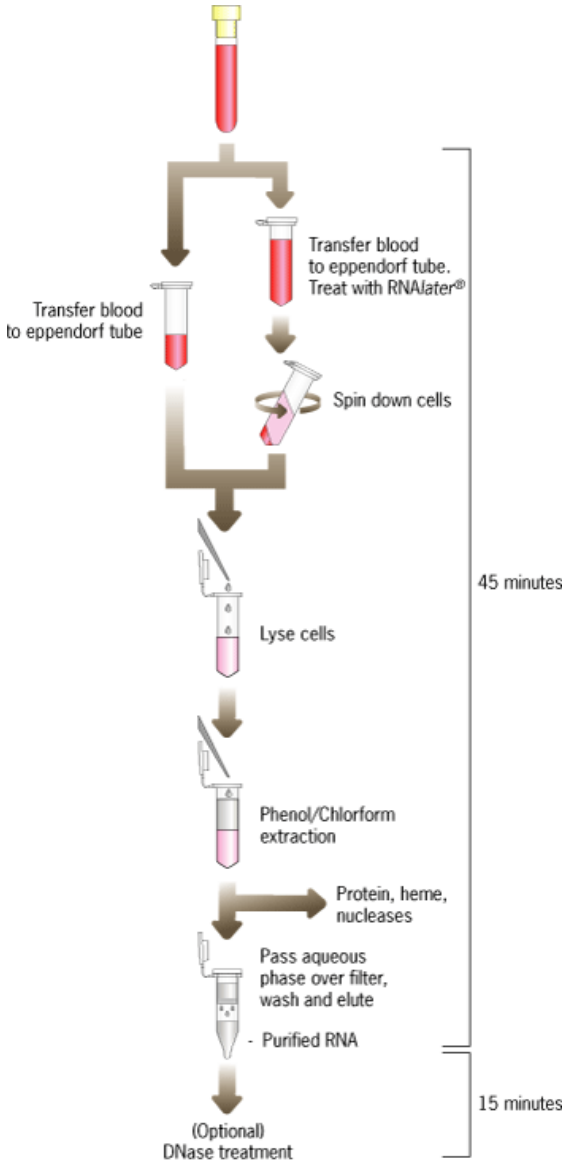
Effect of Fragrance Compounds and Essential Oils on the Motility of Mice after a 1-h Inhalation Period

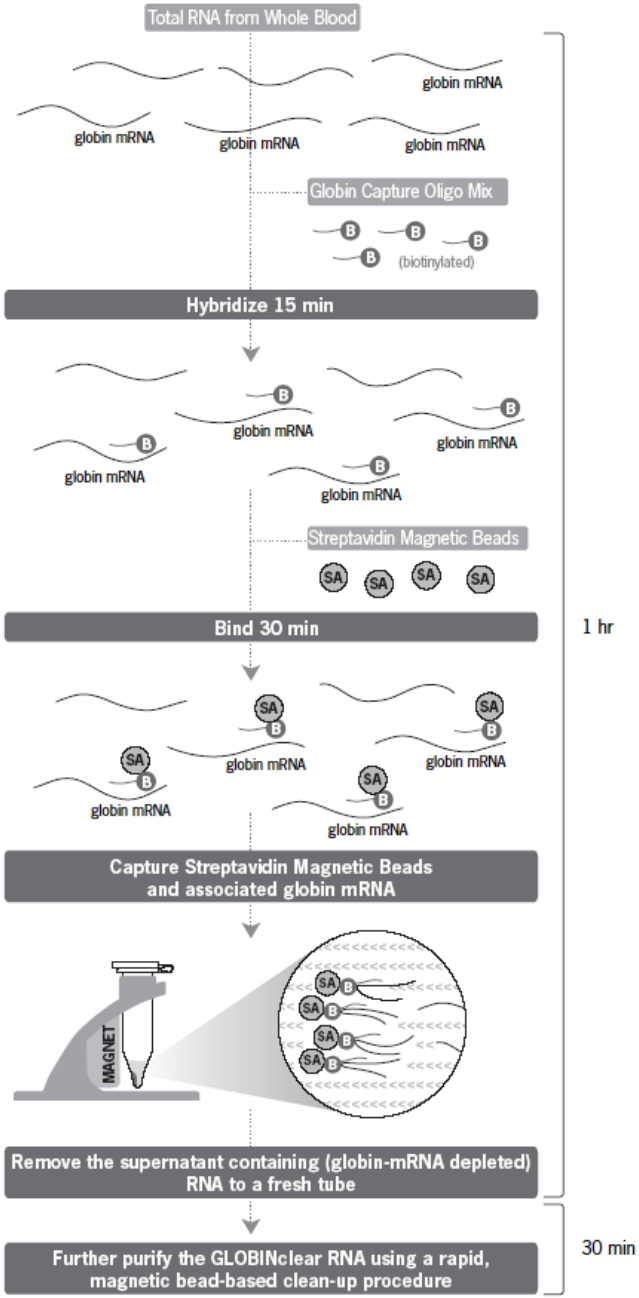
Compound	Effect on Motility (%)	Effect on Motility after Caffeine (%)
Isoborneol	46.90	-11.23
Orange terpenes	35.25	-33.19
Thymol	33.02	19.05
Isoeugenol	30.05	-74.34
Geraniol	20.56	1.20
Anthranilic acid methyl ester	17.70	38.22
Methyl salicylate	16.64	-49.88
β-Ionone	14.20	-27.97
α-Pinene	13.77	4.73
Maltol	13.74	-50.04
Nerol	12.93	29.31
Ethylmaltol	9.73	2.09
Passion flower oil (USA)	8.15	-27.93
Farnesol	5.76	36.34
Dimethyl vinyl cabinol	5.36	-2.11
Farnesyl acetate	4.62	-30.71
Isobornyl acetate	3.16	-22.35
Furfural	3.04	-4.51
2-Phenyl ethanol	2.67	-30.61
Eugenol	2.10	-38.73
Citral	-1.43	17.24
Carvone	-2.46	-47.51
Valerian root oil (Chine)	-2.70	-12.01

Compound	Effect on Motility (%)	Effect on Motility after Caffeine (%)
Borneol	-3.05	-1.88
Citronellol	-3.56	-13.71
range flowe oil (Spain)	-4.64	-14.62
Balm leaves oil (Austria)	-5.21	16.29
Bornyl acetate	-7.79	2.27
Rose oil (Bulgaria)	-9.50	4.31
Anethole	-10.81	-1.26
Benzyl alcohol	-11.21	-23.68
Coumarin	-15.00	-13.75
Bornyl salicylate	-17.29	-2.99
Geranyl acetate	-29.18	-7.46
Lime blossoms oil (France)	-34.34	30.41
Sandalwood oil (East India)	-40.00	-20.70
Benzaldehyde	-43.69	-34.28
α-Terpineol	-45.00	-12.50
2-Phenylethyl acetate	-45.04	12.42
Citronellal	-49.82	-37.40
Neroli oil	-65.27	1.87
Linalyl acetate	-69.10	-46.67
Linalool	-73.00	-56.67
Lavender oil (Mont Blanc)	-78.40	-91.67

G. Buchbauer *et al.* J. Pharm. Sci. 1993, 82, 660-664

mRNA purification

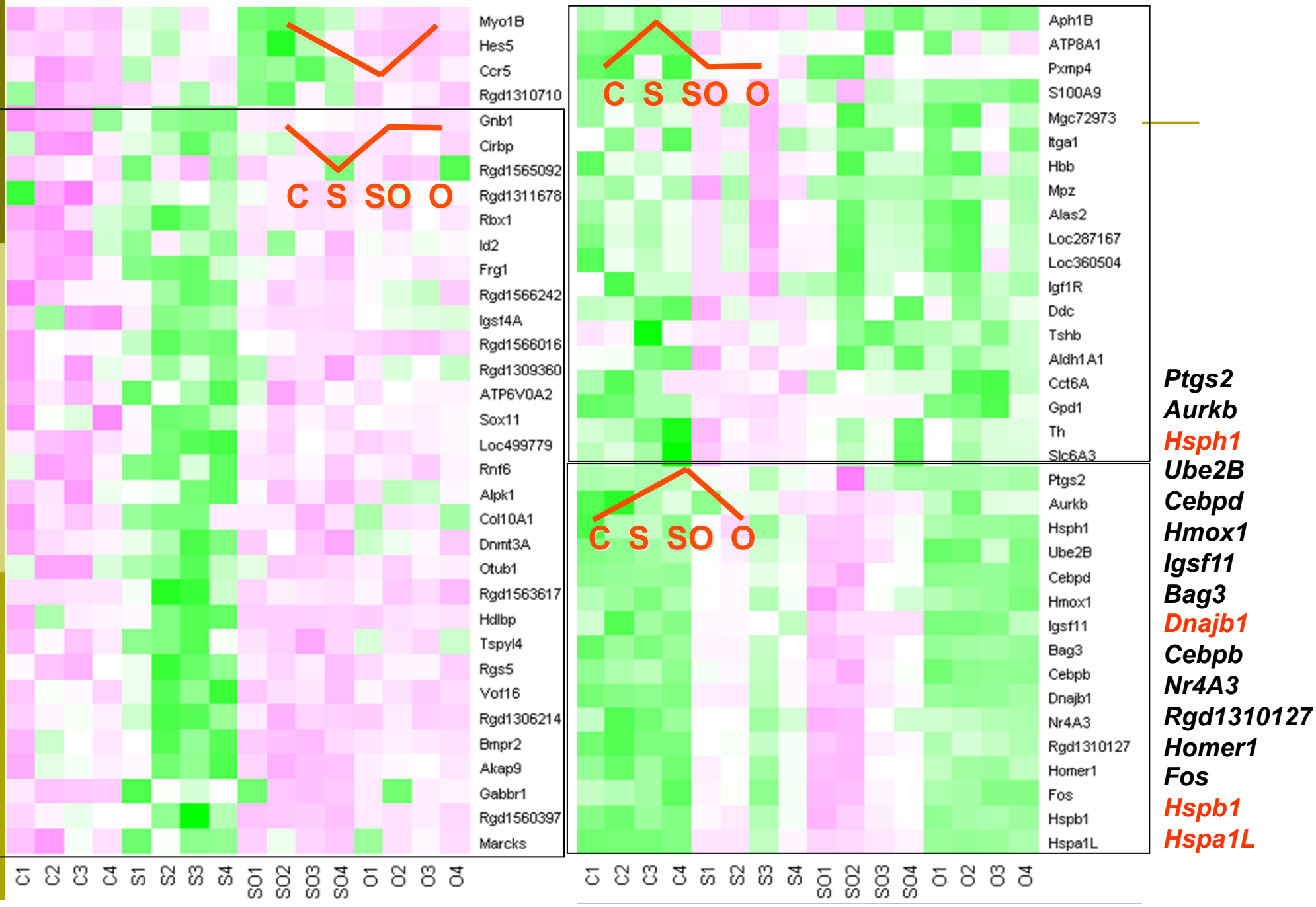




Hypothalamus

- Is activated under restraint-stressed situations to initiate a stress response
- coordinates a variety of bodily changes needed for putting up with or avoiding stress.
- apoptosis-, tumor suppression-, DNA-binding-, protein-folding-related, and immune-related genes are induced by restraint.

Expression levels of the stress and (*R*)-(-)-linalool induced genes



Ptgs2
Aurkb
Hsph1
Ube2B
Cebpd
Hmox1
Igsf11
Bag3
Dnajb1
Cebpb
Nr4A3
Rgd1310127
Homer1
Fos
Hspb1
Hspa1L

Profiles of Blood Cells and Gene Expressions in Whole Blood of Odorant-inhaling Rats under Restraint Stress

- Objectively quantify the effects the inhaled odorant has *in vivo* by multidisciplinary profiling of stress hormones, blood cells, and gene expression.
- Focused on differences in whole blood obtained from normal Wistar rats exposed to a 2 hours restraint, which was defined as a combination of physical and psychological stressors, with or without exposure to (*R*)-(-)-linalool.
- Does the inhaled odorant alter the stress-induced gene expression profiles in 2 hours?

Neuron Development