JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

In Vitro Neuropeptide Y mRNA Expressing Model for Screening Essences That May Affect Appetite Using Rolf B1.T Cells

Shiau-Wei Chen, Po-Ju Wu, and Been-Huang Chiang*

Institute of Food Science and Technology, National Taiwan University, Number 1, Section 4, Roosevelt Road, Taipei 106, Taiwan

ABSTRACT: Neuropeptide Y (NPY) is the most important appetite regulator. This study aimed to establish an *in vitro* NPY mRNA expression model for screening essences to determine if they are an appetite stimulator or inhibitor. We cultured the olfactory nerve cells Rolf B1.T for 2 days and then treated the cells with the known appetite inhibitor limonene and stimulator linalool. It was found that linalool could significantly stimulate NPY mRNA expression in 10 min, and limonene had the opposite effect. Similar results were also found in primary olfactory ensheathing cells isolated from rats. Further clinical trials using human subjects found that, when 10 min of treatment was applied, linalool indeed increased the serum NPY level in human peripheral blood. Limonene, on the other hand, decreased the serum NPY level. Thus, NPY mRNA expression in Rolf B1.T cells could be used as an *in vitro* model for screening essences that may affect appetite.

KEYWORDS: *NPY, olfactory nerve cells, appetite, essences, linalool, limonene*

INTRODUCTION

Appetite is regulated in part by a neuropeptide produced in the brain or gut.[1] In the brain, expression and regulation of a hypothalamic neuropeptide are controlled by the hormonal- and nutrient-related signals to maintain energy homeostasis in mammals. Neuropeptide Y (NPY) and agouti-related peptide (AGRP) are anabolic neuropeptides that promote weight gain by increasing appetite and reducing energy expenditure. They are co-expressed in arcuate nucleus (ARC) neurons in the hypothalamus but inhibited by insulin and leptin.[2]

NPY, a 36 amino acid peptide, is a powerful orexigenic agent, even in satiated animals.[3] The intracerebroventricular administration of NPY could increase short-term (2 h) food intake. NPY is widely distributed in the central and peripheral nervous systems, with high levels in the paraventricular nuclei of the hypothalamus, olfactory bulb, cortex, and spinal cord.[4,5] NPY also participates in various physiological functions, including regulation of neuroendocrine secretion,[6] blood pressure, circadian rhythms,[7] and sexual behavior,[8] and plays a significant role in the response to stress and the control of anxiety and depression.[9−12] In the olfactory system, NPY is expressed in the olfactory ensheathing cells. The olfactory ensheathing cells are a special type of glial cells surrounding the axons of olfactory sensory neurons, which are on their way from the olfactory epithelium to the glomeruli in the olfactory bulb.[13,14] NPY can readily cross the blood−brain barrier (BBB) to enter the brain from blood by diffusion, suggesting that NPY in periphery can also stimulate food intake.[15]

The volatile compounds may exert various effects on humans and other mammalian species when inhaled or ingested, and many of these compounds have been investigated.[16,17] Effects, such as the control of emotion and mood,[16] antispasmodic, control of the autonomic nervous system activity and endocrine system, strengthening of the immune system by stimulating the production of white blood cells, pain mitigation, antitumor, increase of lipolysis, etc., have been documented.[18,19] Odor stimuli by volatile compounds also play a major role in eating behavior. Food-related odors have been shown to increase appetite, to induce salivation, and to release gastric acid and insulin.[20] Because food intake and sympathetic activity are closely related, Shen et al. examined the effects of the scents of grapefruit oil, lavender oil, limonene, and linalool on food intake and body weight.[21−23] They suggested that the smell of grapefruit oil, particularly its primary compounds, limonene, affected autonomic nerves and enhanced lipolysis through a histaminergic response, resulting in the reduced appetite and body weight.[22] Scents of lavender oil and its active component, linalool, have the opposite effects.[23]

There was no report concerning the stimulation of the olfactory nervous system to express NPY and, in turn, influence appetite by volatile compounds. In the present study, we assumed that the appetite regulation effect of essences might be, in part, dependent upon the secretion of NPY by the olfactory system. Therefore, we used adult rat olfactory nerve cells Rolf B1.T as an *in vitro* model and treated the cells with the essences that are known to be able to increase or decrease appetite to see if they could also stimulate or inhibit NPY mRNA expression of the cells. Furthermore, a clinical trial was implemented to study the effects of these essences on the NPY concentration in peripheral blood in human subjects. Thus, the usefulness of the developed *in vitro* model can be confirmed.

MATERIALS AND METHODS

Chemicals. Limonene (≥94% purity), phosphate-buffered saline (PBS) (8 g of NaCl, 0.2 g of KCl, 1.44 g of Na_2HPO_4, and 0.24 g of KH_2PO_4 dissolved in 1000 mL of distilled water at pH 7.4), and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Linalool (≥97% purity) was purchased from Fluca (St. Louis, MO). Diethylpyrocarbonate (DEPC), chloral hydrate, Type IX trypsin, and 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2*H*-tetrazolium

Received: June 3, 2012
Revised: July 2, 2012
Accepted: July 3, 2012
Published: July 3, 2012

ACS Publications © 2012 American Chemical Society

bromide (MTT) were purchased from Sigma (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), Ham's F-12, fetal bovine serum (FBS), glutamine, and gentamicin were obtained from Gibco (Grand Island, NY). The antibiotic penicillin−streptomycin was from Biofluids (Rockville, MD). Reagents for quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) were ordered from Bio-Rad (Hercules, CA).

Rolf B1.T Cells. The adult rat olfactory nerve cells Rolf B1.T were purchased from the European Collection of Cell Culture (ECACC, Proton Down, Salisbury, U.K.). For routine culture maintenance, Rolf B1.T cells were grown in DMEM (Gibco, Grand Island, NY) with 2 mM of glutamine and 10% FBS at 37 °C and 5% CO_2. Cells were subcultured using trypsin/ethylenediaminetetraacetic acid (EDTA), at a split ratio of 1:5.

***In Vitro* Cytotoxicity of Essences.** The cytotoxicity of limonene and linalool was evaluated. Cells were seeded on 96-well plates (5×10^4 cells in each well), and two essences were applied after 24 h. The viability of cultured cells was determined by the reduction of MTT to formazan. After incubation of cells with various concentrations of two essences for 15 min at 37 °C and 5% CO_2 atmosphere, 100 μL of MTT (2 mg/mL) was added to each well. Cells were incubated at 37 °C for another 3 h, and DMSO was then added to dissolve the formazan crystals. At the end of incubation, tetrazolium dye was added as an indicator to convert tetrazolium salts to formazan. The absorbance was then measured at 570 nm using a microplate reader.[24] The entire experiment was performed in triplicate.

Expression of NPY mRNA in Rolf B1.T with Different Essences. For sample treatment, Rolf B1.T cells (5×10^5 cells/mL) were cultured for 2 days and treated with different essences for another 10 min at 37 °C and 5% CO_2 atmosphere. For NPY mRNA level determination, cells (after 10 min of exposure) were washed with DEPC-treated PBS (DEPC−PBS) at 4 °C and RNA was extracted using Geneaid RNA Mini Kit (Taipei, Taiwan). Later, the quality of RNA samples was measured by Nanodrop-100 (Wilmington, DE), and cDNA was synthesized by Bio-Rad iScript cDNA Synthesis Kit (Hercules, CA). For all cDNA samples, a qRT-PCR (iQ5, Bio-Rad, Hercules, CA) for NPY and β-actin was performed. The primer design for the NPY gene was performed using GenBank sequence NM_012614.1. The sequence of NPY primers was 5′-CCCAGAG-CAGAGCACC-3′ (forward) and 5′-CCCCTCAGCCAGAATG-3′ (reverse), and the amplicon length was 132 base pairs (bp). The primer design for the β-actin gene was performed using GenBank sequence NM_031144. The sequence of β-actin primers was 5′-ATGGGTCAGAAGGACTCCTACG-3′ (forward) and 5′-AGTGG-TACGACCAGAGGCATAC-3′ (reverse), and the amplicon length was 309 bp. β-Actin was used as the housekeeping gene because its expression was not influenced by any of the samples. Therefore, the β-actin gene was used as an internal standard for NPY gene amplification.

Expression of NPY mRNA in Primary Olfactory Ensheathing Cells. Male, 8-week-old Sprague−Dawley rats (200−220 g) were obtained from the National Laboratory of Animal Breeding and Research Center, National Science Council of the Republic of China (ROC) and were used for primary olfactory ensheathing cell isolation in the experiment. The animal use protocol was conducted in accordance with the Institutional Animal Care and Use Committee (IACUC) at the National Taiwan University (NTU95134).

For the establishment of primary cultures, rats were anesthetized with 10% chloral hydrate (10 mL/kg) and decapitated. A skin incision was made in the scalp, extending from the tip of the nose to the occipital bone of the skull, and the nasal as well as frontal bones were removed. The olfactory nerve rootlets and olfactory bulbs were detached and placed in 10 mL of chilled calcium- and magnesium-free Hank's balanced salt solution (HBSS, Biofluids, Rockville, MD). The tissue was prepared for seeding based on the technique described by Ramón-Cueto and Nieto-Sampedro.[25] After harvest, the tissue was divested of all meninges and blood vessels and placed in 10 mL of chilled HBSS. The outer two layers of the olfactory bulb, the olfactory nerve and glomerular layers, were dissected. These two layers along with the olfactory rootlets were pooled in 10 mL of chilled HBSS. The tissues were minced with a razor blade, triturated using a 20-gauge needle, and trypsinized at 37 °C for 15 min using 0.1% (w/v) trypsin in a 5% CO_2 incubator. The trypsinization was quenched using a complete media consisting of DMEM and Ham's F-12 (1:1), with 10% FBS, 1% glutamine, 2% penicillin−streptomycin, and 1% gentamicin. The cells were centrifuged twice (1000 rpm for 10 min). The number of viable cells in the pellet was estimated using a hemocytometer after suspending 1 mL of the pellet in complete media and stained with one drop of trypan blue. The isolated primary olfactory ensheathing cells (5×10^5 cells/mL) were cultured for 7 days, planted in flasks, and fed with fresh complete DMEM supplemented with 10% FBS. Then, cells were treated with different essences, and the expression of NPY mRNA was determined following the same protocol as that for Rolf B1.T cells.

Clinical Trial. The human subjects including 37 healthy adults in ages ranging from 22 to 31 were divided into three groups (Table 1).

Table 1. Baseline Characteristics of Experimental Subjects

	n	sex[a]	BMI[b]	body fat (%)	age (year)	education
water	11	4M and 7F	23.2	27.8	27.8	graduated
linalool	13	4M and 9F	21.1	23.6	24.2	graduated
limonene	13	7M and 6F	21.1	21.8	23.8	graduated

[a]M, male; F, female. [b]BMI = body mass index.

An agreement was signed by each subject in which all procedures and possible risks of the study were indicated beforehand. The subjects were required to abstain from alcohol, coffee, or tea and excessive eating 1 day before the experiment. On the day of the experiment, the subjects were required to fast for 12 h and abstain from exercise 2 h prior to the experiment. All of the blood samples and measurements were taken between 8:00 and 10:00 a.m. The subject use protocol was conducted in accordance with the Institution Hospital Research Ethics Committee (HREC) at the National Taiwan University (200904033R).

The participants were randomly assigned to the control group (water; $n = 11$), limonene group ($n = 13$), and linalool group ($n = 13$). To examine the effects of limonene and linalool on the NPY level in the peripheral blood, water containing 1000 ppm of essences was soaked onto a piece of filter paper ($\varphi = 40$ mm). These filter papers were hidden in the gauze masks, which could fully cover the subject's nose, for him/her to inhale the essences. Before the experiment began, all subjects rested for at least 10 min in a sitting position with a quiet and relaxed manner. Blood samples were collected before and after the experiment into serum tubes for serum NPY concentration assay.

Human serum NPY concentrations were measured using commercially available NPY (human, rat, and mouse) EIA Kit (Millipore, Billerica, MA). All samples were analyzed in duplicates. The results are presented as percent of serum NPY change = {(serum NPY concentration after essence stimulation) − (serum NPY concentration before essence stimulation)}/(serum NPY concentration before essence stimulation).

Statistical Analysis. The statistical significance of the data were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's test using SPSS software. Significance was defined by $p < 0.01$ or $p < 0.05$.

RESULTS AND DISCUSSION

NPY mRNA Expression Model Using Rolf B1.T Cells. Because the regulation of appetite is largely associated with NPY, we proposed that some essences may also regulate appetite at least in part through NPY secretion by the olfactory cells. Because of the immortal variant subpopulation, Rolf B1.T can express most of the phenotypic characteristics of

ensheathing cells (a NPY synthesizer) both *in situ* and in primary culture,[13] suggesting that this cell may synthesize NPY as well. Therefore, we decided to establish an *in vitro* model using rats' olfactory cells Rolf B1.T.

Panels A–C of Figure 1 show the epithelial-like Rolf B1.T cells cultivated for 1, 2, and 3 days after inoculation (5×10^5 cells/mL). After 24 h of cultivation, the cells were in the adaptation period and began to attach to the plate and the cell number was still pretty low (Figure 1A). When the cells were cultivated for 48 h, the spindle-shaped cells were observed and the cells proliferated to an extent that many of them aggregated together. After further cultivation of the cells to 72 h, the plate was fully occupied by the cells and an individual cell was difficult to observe because they overlaid each other. In addition, the color of the pH indicator, phenol red, turned yellow, suggesting that the pH of the cultivation medium DMEM was decreased because of the overgrowth of the cells and excessive waste accumulation. Therefore, it appeared that the proper cultivation period for preparing the cells for treatment was 48 h.

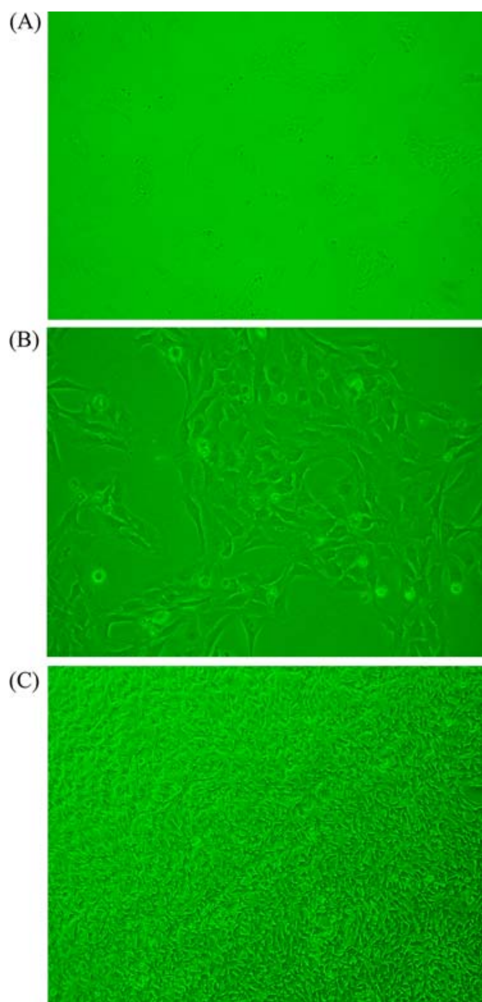

Figure 1. Morphology of Rolf B1.T cells in serum-containing medium after (A) 1 day, (B) 2 days, and (C) 3 days of cultivation.

Figure 2 shows the NPY mRNA expression of the Rolf B1.T cells during cultivation. The highest NPY mRNA expression was also found in the cells cultivated for 48 h, suggesting that the cells were at the most healthy and active status. Therefore, for the *in vitro* NPY mRNA expression model, we decided to adopt the Rolf B1.T cells after inoculation (5×10^5 cells/mL) and cultivation for 48 h.

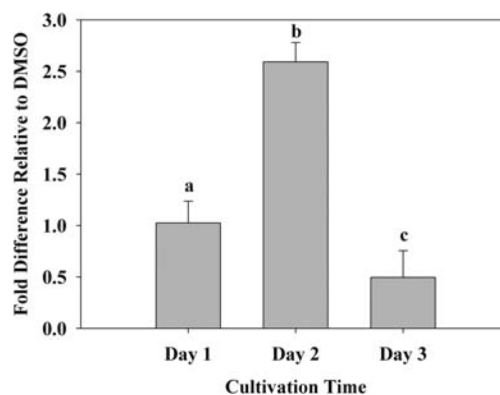

Figure 2. Gene expression of NPY mRNA in Rolf B1.T cells cultivated for different periods at 37 °C and 5% CO_2 atmosphere [$n = 3$; mean ± standard deviation (SD); $p < 0.01$].

Effect of the Appetite Stimulator and Inhibitor on NPY mRNA Expression of Rolf B1.T Cells. It was reported that the scents of grapefruit oil (mainly limonene) and lavender oil (mainly linalool) can influence rats' appetite.[21−23] Therefore, we used these two essences to test the established *in vitro* model. First of all, we determined the cytotoxic effect of linalool and limonene on Rolf B1.T cells. During the *in vitro* cytotoxicity experiments, the essences were dissolved in DMSO, which was used as the control to avoid confounding the expression of NPY mRNA. Figure 3 shows that both 0.08% (v/v) limonene and linalool resulted in the cell viability of 100%. The population of Rolf B1.T cells, however, decreased significantly when 0.17% (v/v) limonene or linalool was applied. According to the cell viability testing result, the dosage of 0.08% (v/v) limonene or linalool was used for the subsequent studies.

In the preliminary study, we could not detect any NPY mRNA expression when the Rolf B1.T cells were treated with linalool for more than 30 min (data not shown). On the other hand, the 15 min linalool treatment resulted in a significant stimulation of NPY expression in Rolf B1.T cells. The observed phenomena may be due to olfactory fatigue. According to the results of the preliminary study, we decided to adopt 5, 10, and 15 min of treatment time to test the effect of linalool on the NPY mRNA expression of Rolf B1.T cells. Meanwhile, we also treated the rat primary olfactory ensheathing cells with linalool under the same condition for comparison. The results are given in Figure 4.

It was found that 5, 10, and 15 min of linalool treatments resulted in a significant stimulation of NPY expression in Rolf B1.T. cells as well as in the primary rat olfactory cell culture. Both Rolf B1.T and the rat olfactory primary culture showed the highest NPY mRNA expression level when they were treated with 0.08% linalool for 10 min. On the other hand, 15 min of treatment resulted in the lowest level of NPY mRNA expression. It appeared that 5 min of stimulation by linalool is not long enough to allow for the cells to fully express the NPY mRNA, but 15 min of stimulation is too long, so that olfactory fatigue may set in. When the Rolf B1.T cells were compared to the primary olfactory cell culture, we found that Rolf B1.T is more sensitive to linalool treatments (1.8- and 2.7-fold at 10 min, respectively). This result demonstrates that the *in vitro*

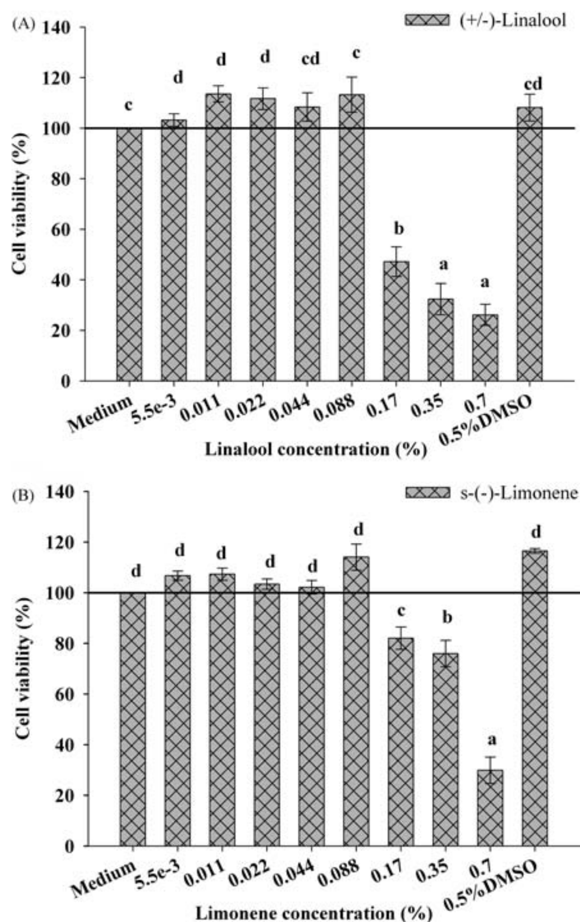

Figure 3. Dose-dependent cytotoxicity of (A) linalool and (B) limonene to Rolf B1.T cells (15 min of exposure) determined by the MTT assay. Error bars indicate the standard deviation (n = 24).

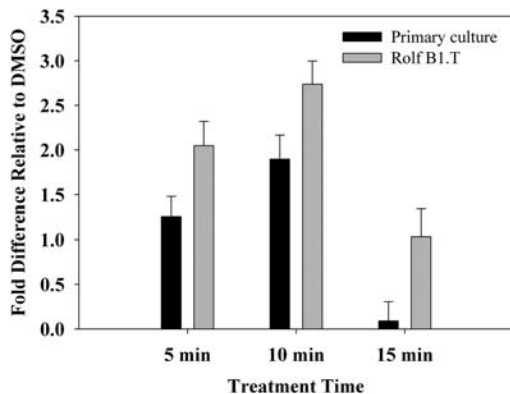

Figure 4. Gene expression of NPY mRNA in Rolf B1.T cells and rat olfactory primary cell culture when treated with 0.08% linalool for different time periods (n = 3; mean ± SD; p < 0.01).

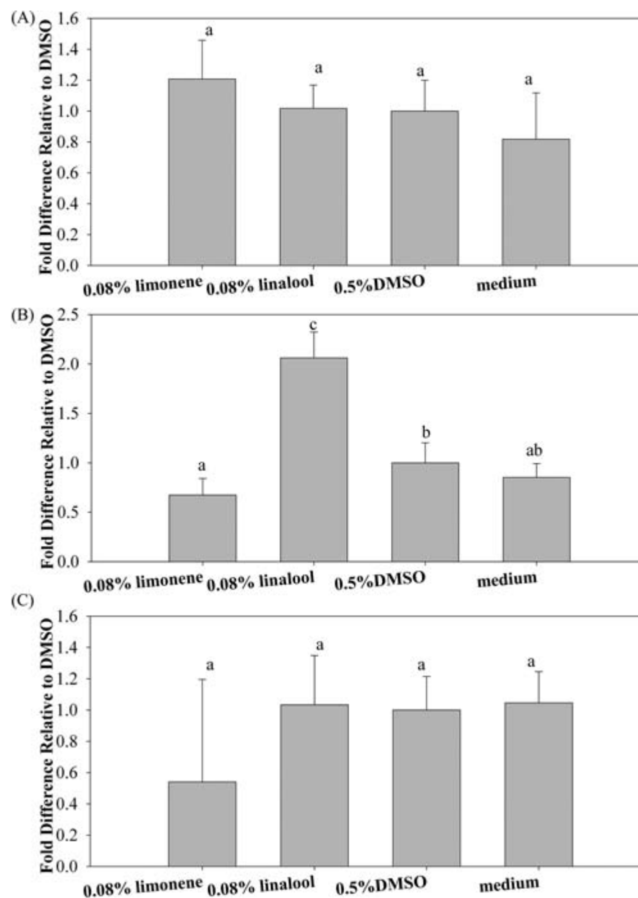

Figure 5. Gene expression of NPY mRNA in Rolf B1.T cells after treating with different samples for (A) 5 min, (B) 10 min, and (C) 15 min (n = 3; mean ± SD; p < 0.01).

NPY mRNA expression model using Rolf B1.T cells instead of primary culture for essence screening is feasible and more convenient.

Figure 5 shows the NPY mRNA expression after treating the Rolf B1.T cells for 5, 10, and 15 min with cultivation medium, DMSO (the solvent for dissolving the essence), linalool, and limonene. Again, only 10 min of stimulation resulted in a significant difference among the treatments. The appetite stimulator linalool enhanced the NPY mRNA expression significantly. Limonene, on the other hand, significantly decreased NPY mRNA expression. Shen et al. observed that, in rats, olfactory stimulation with the scent of grapefruit oil (SGFO) for 10 min elevated the activity of the sympathetic nerve.[22] The SGFO also suppresses gastric vagal (parasympathetic) nerve activity, increases the plasma glycerol concentration, blood pressure, body temperature, and reduces appetite. In contrast, olfactory stimulation with the scent of lavender oil for 10 min increases the appetite of rats.[21] Tanida et al. found that olfactory stimulation with the SGFO or scent of lavender oil for 10 min elevated or lowered, respectively, brown adipose tissue temperature and body temperature through changes in the autonomic nerve activities.[23] In this study, we found that treating the 2 day culture of olfactory nerve cells (Rolf B1.T) with limonene or linalool for 10 min could increase or decrease the NPY mRNA expressions, respectively, which may at least in part explain the mechanism of the aforementioned *in vivo* studies.

Effect of the Appetite Stimulator and Inhibitor on the Human Serum NPY Concentration. Lavender oil is a known appetite stimulator in rats. Therefore, its major flavor compound, linalool, might also increase the appetite of human subjects. Figure 6 shows the serum NPY levels after inhaling linalool for various time periods. It was found that the serum NPY concentration increased significantly after stimulating with linalool for 5 or 10 min compared to limonene or water. Unlike the *in vitro* model, there was no significant difference in the serum NPY concentration between 5 and 10

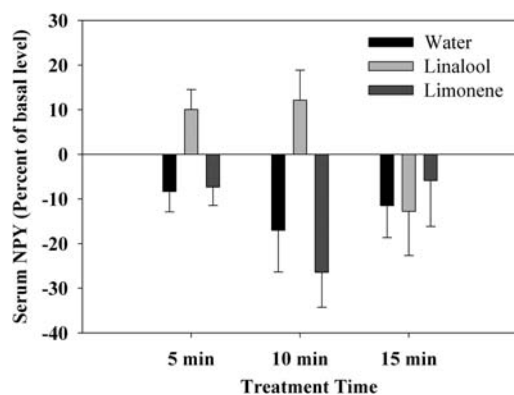

Figure 6. Changes of human serum NPY concentration after inhaling various essences for 5, 10, or 15 min (water, $n = 11$; limonene, $n = 13$; and linalool, $n = 13$; mean ± SD; $p < 0.05$).

min of stimulation. First of all, human subjects are easily influenced by the environmental factors as well as individual emotional changes, which may result in larger experimental error compared to the *in vitro* model. Second, olfactory fatigue may occur around 10 min of stimulation. After 15 min of stimulation with linalool, the serum NPY concentration was even lower than the baseline. Besides olfactory fatigue, we suspect that some of the produced NPY has degraded because its half-life is around 12 min in plasma.[26] Therefore, both olfactory fatigue and NPY degradation may result in a lower NPY concentration in blood when the human subject was exposed to the appetite simulator for longer than 10 min.

Limonene, the major flavor compound in grapefruit, was used as the appetite inhibitor in this study. A decrease in the serum NPY level was observed in our subjects when limonene was inhaled (Figure 6), which is in agreement with the *in vitro* result, whereby limonene significantly decreased the NPY mRNA level in the Rolf B1.T cells. It was also noticed that the "water group" always had a negative serum NPY level. We suspect that the chemical compounds in the mask had an adverse effect on NPY as well as appetite.

NPY in both the serum and hypothalamus correlates closely with appetite.[27,28] When rats were exposed to cigarette smoke, both their serum NPY and body weight steadily decreased,[29] suggesting that the serum NPY level is strongly associated with food intake. In this study, we established an *in vitro* model using rat Rolf B1.T cells and demonstrated that the appetite stimulator linalool or inhibitor limonene could increase or decrease the NPY mRNA expression by this model, respectively. We also found that olfactory stimulation with linalool or limonene on human subjects had the same effect on their serum NPY concentrations. Therefore, we believe that the *in vitro* model that we have established using rat olfactory Rolf B1.T cells may be a useful tool for screening appetite stimulators or inhibitors of flavor compounds.

■ AUTHOR INFORMATION

Corresponding Author

*Telephone: +886-2-33664120. Fax: +886-2-23620849. E-mail: bhchiang@ntu.edu.tw.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

This research was supported by Grant NSC 98-2313-B-002-042-MY3 funded by the National Science Council of the Republic of China (Taiwan).

■ REFERENCES

(1) Zhang, J. V.; Ren, P. G.; Avsian-Kretchmer, O.; Luo, C. W.; Rauch, R.; Klein, C.; Hsueh, A. J. W. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science* **2005**, *310*, 996−999.

(2) Caminos, J. E.; Bravo, S. B.; González, C. R.; Garcés, M. F.; Cepeda, L. A.; González, A. C.; Cordido, F.; López, M.; Diéguez, C. Food-intake-regulating-neuropeptides are expressed and regulated through pregnancy and following food restriction in rat placenta. *Reprod. Biol. Endocrinol.* **2008**, *2*, 6−14.

(3) Rutkoski, N. J.; Fitch, C. A.; Yeiser, E. C.; Dodge, J.; Trombley, P. Q.; Levenson, C. W. Regulation of neuropeptide Y mRNA and peptide concentrations by copper in rat olfactory bulb. *Brain Res. Mol. Brain Res.* **1999**, *65*, 80−86.

(4) Zhang, L.; Bijker, M. S.; Herzog, H. The neuropeptide Y system: Pathophysiological and therapeutic implications in obesity and cancer. *Pharmacol. Ther.* **2011**, *131*, 91−113.

(5) Brogden, K. A.; Guthmiller, J. M.; Salzet, M.; Zasloff, M. The nervous system and innate immunity: The neuropeptide connection. *Nat. Immunol.* **2005**, *6*, 558−564.

(6) Huda, M. S.; Wilding, J. P.; Pinkney, J. H. Gut peptides and the regulation of appetite. *Obes. Rev.* **2006**, *7*, 163−182.

(7) Sindelar, D. K.; Palmiter, R. D.; Woods, S. C.; Schwartz, M. W. Attenuated feeding responses to circadian and palatability cues in mice lacking neuropeptide Y. *Peptides* **2005**, *26*, 2597−2602.

(8) Ammar, A. A.; Sederholm, F.; Saito, T. R.; Scheurink, A. J. W.; Johnson, A. E.; Soderstent, P. NPY-leptin: Opposing effects on appetitive and consummatory ingestive behavior and sexual behavior. *Am. J. Physiol.* **2000**, *278*, 1627−1633.

(9) Rutkoski, N. J.; Fitch, C. A.; Yeiser, E. C.; Dodge, J.; Trombley, P. Q.; Levenson, C. W. Regulation of neuropeptide Y mRNA and peptide concentrations by copper in rat olfactory bulb. *Brain Res. Mol. Brain Res.* **1999**, *65*, 80−86.

(10) Komiya, M.; Takeuchi, T.; Harada, E. Lemon oil vapor causes an anti-stress effect via modulating the 5-HT and DA activities in mice. *Behav. Brain Res.* **2006**, *172*, 240−249.

(11) Giesbrecht, C. J.; Mackay, J. P.; Silveira, H. B.; Urban, J. H.; Colmers, W. F. Countervailing modulation of Ih by neuropeptide Y and corticotrophin-releasing factor in basolateral amygdala as a possible mechanism for their effects on stress-related behaviors. *J. Neurosci.* **2010**, *30*, 16970−16982.

(12) Heilig, M. The NPY system in stress, anxiety and depression. *Neuropeptides* **2004**, *38*, 213−224.

(13) Ubink, R.; Hökfelt, T. Expression of neuropeptide Y in olfactory ensheathing cells during prenatal development. *J. Comp. Neurol.* **2000**, *423*, 13−25.

(14) Dumont, Y.; Martel, J. C.; Fournier, A.; St-Pierre, S.; Quirion, R. Neuropeptide Y and neuropeptide Y receptor subtypes in brain and peripheral tissues. *Prog. Neurobiol.* **1992**, *38*, 125−167.

(15) Kastin, A. J.; Akerstrom, V. Nonsaturable entry of neuropeptide Y into brain. *Am. J. Physiol.* **1999**, *276*, 479−482.

(16) Raghavan, S. *Handbook of Spices, Seasonings, and Flavorings*, 2nd ed.; Taylor and Francis Group: New York, 2007.

(17) Haze, S.; Sakai, K.; Gozu, Y. Effects of fragrance inhalation on sympathetic activity in normal adults. *Jpn. J. Pharmacol.* **2002**, *90*, 247−253.

(18) Kuorwel, K. K.; Cran, M. J.; Sonneveld, K.; Miltz, J.; Bigger, S. W. Essential oils and their principal constituents as antimicrobial agents for synthetic packaging films. *J. Food Sci.* **2011**, *76*, 164−177.

(19) Kiecolt-Glaser, J. K.; Graham, J. E.; Malarkey, W. B.; Porter, K.; Lemeshow, S.; Glaser, R. Olfactory influences on mood and autonomic, endocrine, and immune function. *Psychoneuroendocrinology* **2008**, *33*, 328−339.

(20) Yeomans, M. R. Olfactory influences on appetite and satiety in humans. *Physiol. Behav.* **2006**, *89*, 10−14.
(21) Tanida, M.; Shen, J.; Niijima, A.; Yamatodani, A.; Oishi, K.; Ishida, N.; Nagai, K. Effects of olfactory stimulations with scents of grapefruit and lavender oils on renal sympathetic nerve and blood pressure in Clock mutant mice. *Auton. Neurosci.* **2008**, *139*, 1−8.
(22) Shen, J.; Niijima, A.; Tanida, M.; Horii, Y.; Maeda, K.; Nagai, K. Olfactory stimulation with scent of lavender oil affects autonomic nerves, lipolysis and appetite in rats. *Neurosci. Lett.* **2005**, *383*, 188−193.
(23) Tanida, M.; Niijima, A.; Shen, J.; Nakamura, T.; Nagai, K. Olfactory stimulation with scent of lavender oil affects autonomic neurotransmission and blood pressure in rats. *Neurosci. Lett.* **2006**, *398*, 155−160.
(24) Mosmann, T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55−63.
(25) Ramón-Cueto, A.; Nieto-Sampedro, M. Glial cells from adult rat olfactory bulb: Immunocytochemical properties of pure cultures of ensheathing cells. *Neuroscience* **1992**, *47*, 213−220.
(26) Grouzmann, E.; Fathi, M.; Gillet, M.; de Torrenté, A.; Cavadas, C.; Brunner, H.; Buclin, T. Disappearance rate of catecholamines, total metanephrines, and neuropeptide Y from the plasma of patients after resection of pheochromocytoma. *Clin. Chem.* **2001**, *47*, 1075−1082.
(27) Benoit, S. C.; Clegg, D. J.; Wood, S. C.; Seeley, R. J. The role of previous exposure in the appetitive and consummatory effects of orexigenic neuropeptide. *Peptides* **2005**, *26*, 751−757.
(28) Kobeissy, F. H.; Jeung, J. A.; Warren, M. W.; Geier, J. E.; Gold, M. S. Changes in leptin, ghrelin, growth hormone and neuropeptide-Y after an acute model of MDMA and methamphetamine exposure in rats. *Addict. Biol.* **2007**, *13*, 15−25.
(29) Chen, H.; Hansen, M. J.; Jones, J. E.; Vlahos, R.; Bozinovski, S.; Anderson, G. P.; Morris, M. J. Cigarette smoke exposure reprograms the hypothalamic neuropeptide Y axis to promote weight loss. *Am. J. Respir. Crit. Care Med.* **2006**, *173*, 1248−1254.