Positive emotion-specific changes in the gene expression profile of tickled rats

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Abstract. The aim of this study was to investigate changes in gene expression after tactile stimulation (tickling) accompanied by positive emotion in the adolescent rat brain. We observed a positive emotional response (50-kHz ultrasonic vocalizations) after tickling using a modified version of the Panksepp method, and then comprehensively compared gene expression levels in the hypothalamus of the tickled rats and control rats using the microarray technique. After 4 weeks of stimulation, the expression levels of 321 of the 41,012 genes (including transcripts) were changed; 136 genes were up-regulated (>1.5-fold) and 185 were down-regulated (<0.67-fold) in the tickled rat group. Upon ontology analysis, the up-regulated genes were assigned to the following Gene Ontology (GO) terms: feeding behavior, neuropeptide signaling pathway, biogenic amine biosynthesis and catecholamine biosynthesis. Down-regulated genes were not assigned to any GO term categorized as a biological process. In conclusion, repeated tickling stimulation with positive emotion affected neuronal circuitry directly and/or indirectly, and altered the expression of genes related to the regulation of feeding in the adolescent rat hypothalamus.

Introduction

The mind and genes are considered to interact with each other (1,2). We have demonstrated that laughter, an expression of positive emotion, influences the mind and body at a molecular-biological level (3-6). Studies concerning the physiological effect of laughter were triggered by Cousins (7), who reported on his own recovery from disease through laughter. Since then, most studies have focused on the immunological aspects of laughter (8-10). We recently found that laughter suppressed an increase in the postprandial blood glucose level in patients with type 2 diabetes (3), and caused specific changes in gene expression in peripheral blood leucocytes (4). Laughter up-regulated the expression of genes ameliorating the progression of diabetic microvascular complications (5) and improving glucose intolerance by modulating NK cell activity (6). Positive emotional expressions such as laughter are the result of neurotransmission, but how this process is related to glucose metabolism has not been clarified. Therefore, we focused on changes in gene expression initiated by positive emotion in the hypothalamus as the regulatory center of blood glucose level.

Rats exhibit specific ultrasonic vocalizations (USVs) in response to various social interactions and stimulation, with 50-kHz USVs reflecting positive emotional states, proposed as an evolutionary antecedent to human joy (11). This 50-kHz USV index of positive emotion was identified by Panksepp, who also established a positive stimulus administration method that mimicks the rough-and-tumble play of rats (12,13). In the present study, we analyzed changes in gene expression after tactile stimulation (tickling) accompanied by positive emotion using the microarray technique. This is the first study to demonstrate that repeated tactile stimulation affects neuronal circuitry and alters the expression of many genes in the rat hypothalamus.

Materials and methods

Subjects. Twenty-one day old post-weaning male Wistar rats (Japan SLC Inc., Shizuoka, Japan) were used. To avoid ludic behavior, such as rough-and-tumble play, animals were individually housed in standard polycarbonate cages (W270 x L440 x H187 mm) with wood chip bedding. Rats were allowed *ad libitum* access to water and food and maintained under specific pathogen-free conditions at a room temperature of 21±1˚C, 50-60% humidity, and a 12:12 light/dark cycle (lights on at 7:00) during the study period. After 1 week of acclimatization, the rats were weighed and then divided into two groups: the tickled group and, as the control, the light-

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touched group (n=4 each). The body weights of rats in the tickled and light-touched groups were 85.6±4.8 and 86.0±2.1 g (means ± SD), respectively. Animals received stimulation once a day for 5 consecutive days (Monday-Friday) for 4 weeks. The experimental procedures were approved by the Institutional Animal Experiment Committee of the University of Tsukuba.

Stimulation. A modified version of the Panksepp method (12) was used for tickling stimulation. Rats were individually transferred to a test box (W270 x L440 x H187 mm, with floor and four sides covered with black felt). After a 15-sec stimulation-free period, tickling stimulation was administered for 15 sec. This procedure was repeated four times (one tickling session). This method mimics the dorsal contact and pinning behavior of the rough-and-tumble play of rats (14). The rat was grasped on the dorsal side and tickled on the posterior neck with fingers, rapidly overturned and vigorously tickled over the abdomen while being pushed onto the floor in a supine position, and then released. After the first tickling session, there was a 1-min rest period, followed by the second session (5 min in total).

Light-touch stimulation, which serves as a discernible stimulation (12), was adopted as the control in this study. Rats were gently touched on the dorsal region every 3 sec instead of undergoing the 15-sec tickling stimulation. The timing of stimulation was the same as that outlined above.

Ultrasonic vocalization recording and fast Fourier transform analysis. To analyze the index of positive emotion in rats, 50-kHz USVs were recorded for each rat during stimulation and resting using a high-frequency microphone (MI-3140, Ono Sokki Co., Kanagawa, Japan) suspended 25 cm above the floor of the test box. Frequency component analysis (hanning window; frequency range, 10-100 kHz; sampling frame length, 4096 points; average, power spectrum peak hold) was performed using an ultra high-band acoustic analysis system (DS-2100, Ono Sokki Co.). After Fourier transformation of vocal sound, the peak sound pressure levels (dB) of the frequency components in every 15-sec period were plotted on a graph for analysis.

Approach latency. To analyze the index of positive behavior reinforcement, the rats were tested for approach latency to tactile stimulation. After two tickling (or light-touch) sessions, rats were placed in a corner of the test box and the period until the rat approached and touched the experimenter's hand to receive tickling (or light-touch) was recorded. The maximum 30-sec latency was recorded.

Sample collection and preparation. On the day following the final stimulation session at the end of the 4-week period, rats aged 8 weeks were perfused with saline and decapitated under ether anesthesia, and their brains were excised by posterior craniotomy. Each excised brain was rapidly dissected according to the method of Glowinski and Iversen (15), and the hypothalamus was obtained. Hypothalamic tissues were suspended in RNAlater RNA Stabilization Reagent (Qiagen GmbH, Hilden, Germany) and stored at -80˚C. Total RNA was prepared from stored tissue specimens using an RNeasy Mini Kit (Qiagen GmbH) following the manufacturer's protocol.

Microarray hybridization and data acquisition. Equal amounts of total RNA preparations from the four rats were mixed in each group and comprehensively analyzed by the microarray technique. cDNA was synthesized using 500 ng of total RNA and T7 RNA promoter sequence-bound Oligo (dT)24 as the primer. Subsequently, aminoallyl nucleotide-incorporated cRNA was prepared by *in vitro* transcription. This cRNA was labeled with a fluorescent dye, cyanine (Cy)3 or Cy5 (16,17). Equal amounts of Cy3-labeled cRNA derived from the light-touched group and Cy5-labeled cRNA derived from the tickled group were mixed. The mixture was applied to a Whole Rat Genome Oligo Microarray (G4131A, Agilent Technologies, CA, USA) on which 41,012 genes (including transcripts) were spotted, and hybridization was allowed to proceed for 17 h at 65˚C as described by the manufacturer. After hybridization, the arrays were washed and scanned using a confocal laser scanner (Agilent G2565BA). The fluorescence intensities on the scanned images were quantified. Background correction and normalization were performed as previously reported (8). Data were registered in the Gene Expression Omnibus (National Center for Biotechnology Information) with accession no. GSE11267. Genes with 1.5- and 0.67-fold differences in expression level in the tickled group compared to the light-touched group were discriminated.

Gene ontology analysis. The discriminated genes were examined for their biological meaning based on gene ontology (18). Gene ID and GO were collated using the Biological Network Gene Ontology tool, and GO hierarchies were drawn using Cytoscape (19). Each GO term was analyzed to determine whether the frequency in the discriminated gene group was elevated compared to that in all genes on the microarray using the hypergeometric test at a false discovery rate (FDR) of <0.1. GO terms judged significant by this examination were regarded as specific terms for discriminated genes.

Results

Behavioral analysis. All rats in the tickled group emitted a sign of positive emotion at a frequency of ~50 kHz during the tickling sessions from the initial session on the first day, and did not emit ~20 kHz USVs, an aversive sign of stress or negative emotion, at any time during the experimental period (Fig. 1A and B). This is consistent with the previous study (12). In contrast, no such 50-kHz USVs were noted in the light-touched group throughout the experimental period, though the light-touched rats emitted 20-kHz USVs on only the first day (Fig. 1C and D). The approach latencies on the first day of the tickled and the light-touched groups were 5.5±5.75 sec (mean ± SD) and >30 sec, respectively. These data are consistent with the previous study, demonstrating that tickling positively reinforces tactile stimulation (12).

Gene expression analysis. Repeated stimulation with tickling altered the expression of many genes in the hypothalamus of the adolescent rats. After the 4-week stimulation, the expression levels of 321 of the 41,012 genes (including transcripts) were altered in the tickled group compared to the light-touched groups; 136 genes were up-regulated (>1.5-fold) and 185 were down-regulated (<0.67-fold).

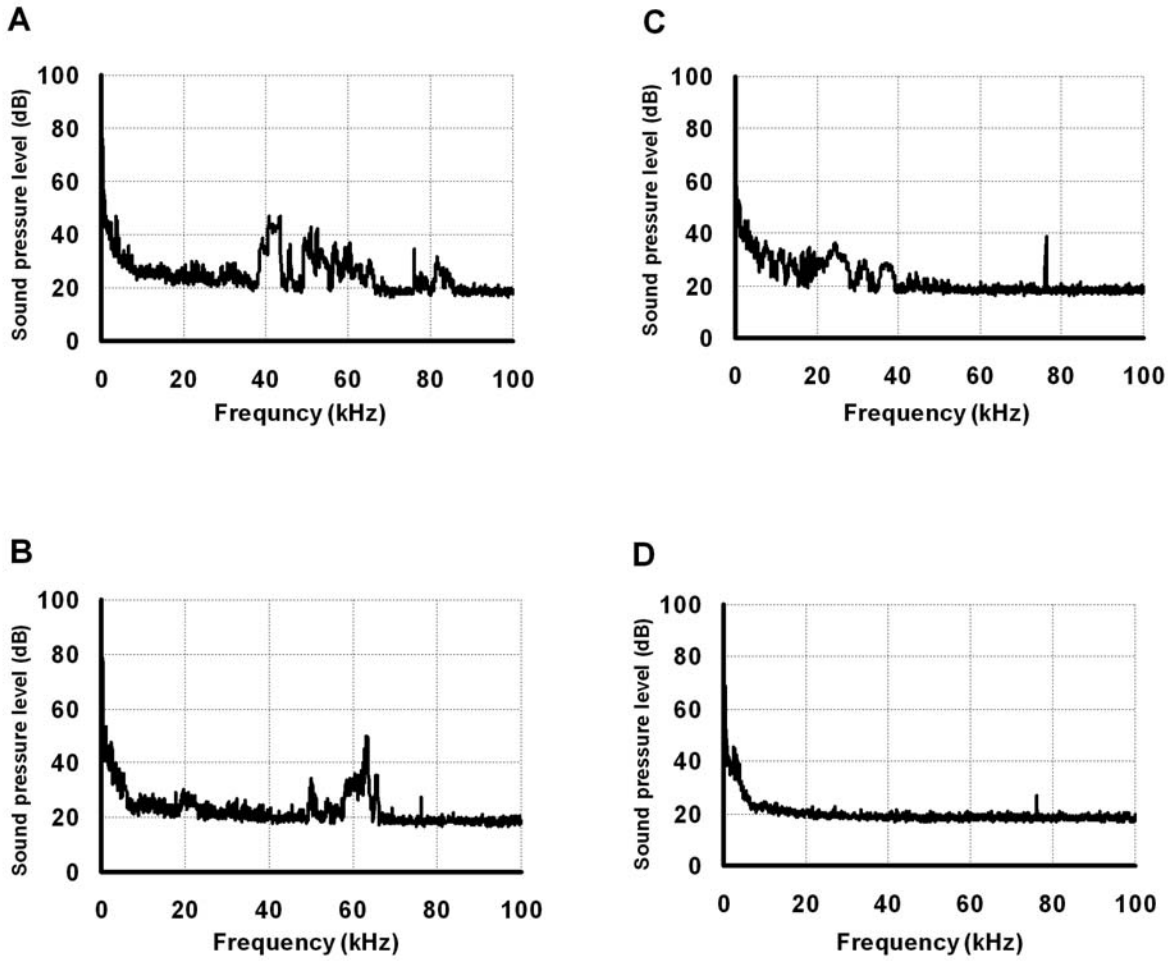

Figure 1. Power spectra of the ultrasonic vocalizations emitted by rats. After FFT analysis, data were plotted as the peak of sound pressure levels during 15 sec of stimulation in the tickled group on the first day (A) and after 4 weeks (B), and in the light-touched group on the first day (C) and after 4 weeks (D).

The up-regulated genes were assigned 46 GO terms categorized as biological processes (Fig. 2). The GO terms with significant enrichment (FDR <0.01) were feeding behavior (GO ID 7631), neuropeptide signaling pathway (GO ID 7218), biogenic amine biosynthesis (GO ID 42401) and catecholamine biosynthesis (GO ID 42423). The genes encoding galanin-like peptide precursor (Galp 2.14-fold), pro-opiomelanocortin (Pomc, 2.13-fold), pro-melanin-concentrating hormone (Pmch, 1.87-fold), agouti-related protein homolog (Agrp, 1.80-fold), orexin (hypocretin; Hcrt, 1.60-fold) and neuropeptide Y (Npy, 1.59-fold) were assigned feeding behavior terms, and Pomc, Agrp, Npy, Galp, Pmch and cocaine and amphetamine-regulated transcript (Cart, 1.94-fold) were assigned neuropeptide signaling pathway terms. The genes encoding histidine decarboxylase (Hdc, 1.80-fold) and tyrosine hydroxylase (Th, 1.58-fold) were assigned catecholamine biosynthesis terms. Down-regulated genes were not assigned to any GO term categorized as a biological process.

Discussion

The objective of this study was to investigate changes in gene expression precipitated by tactile stimulation accompanied by positive emotion in the brain. Repeated stimulation altered the expression of many genes in the hypothalamus of adolescent rats. Ontology analysis of the genes that underwent a marked increase in expression revealed a significant enrichment in those assigned feeding behavior terms within the GO category of biological processes, namely the genes Agrp, Hcrt, Npy, Galp, Pmch and Pomc. All these genes, with the exception of Hcrt, were also assigned neuropeptide signaling pathway terms. Positive emotion induced the expression of genes encoding feeding behavior-related neuropeptides. The most important brain region related to feeding behavior is the hypothalamus. Within the hypothalmus, the feeding center is located in the lateral hypothalamic area (LHA) and the satiety center is in the ventromedial hypothalamic nucleus (VMH) (20,21). AgRP, HCRT, NPY, GALP, PMCH and POMC are substances which regulate feeding; the first five promote feeding behavior (22-25), while POMC is precursor for α melanocyte-stimulating hormone (αMSH), which suppresses it (26). These substances interact with each other and control feeding and energy expenditure (23,26), suggesting that positive stimulation followed by the process of neurotransmission regulates the function of these genes in the central nervous system. HCRT is also involved in arousal, sleep and emotion (27).

Hdc encodes histidine decarboxylase (HDC), which is involved in histamine synthesis, and Th encodes tyrosine hydroxylase (TH), which is the rate-limiting enzyme of catecholamine synthesis, such as dopamine synthesis. In the

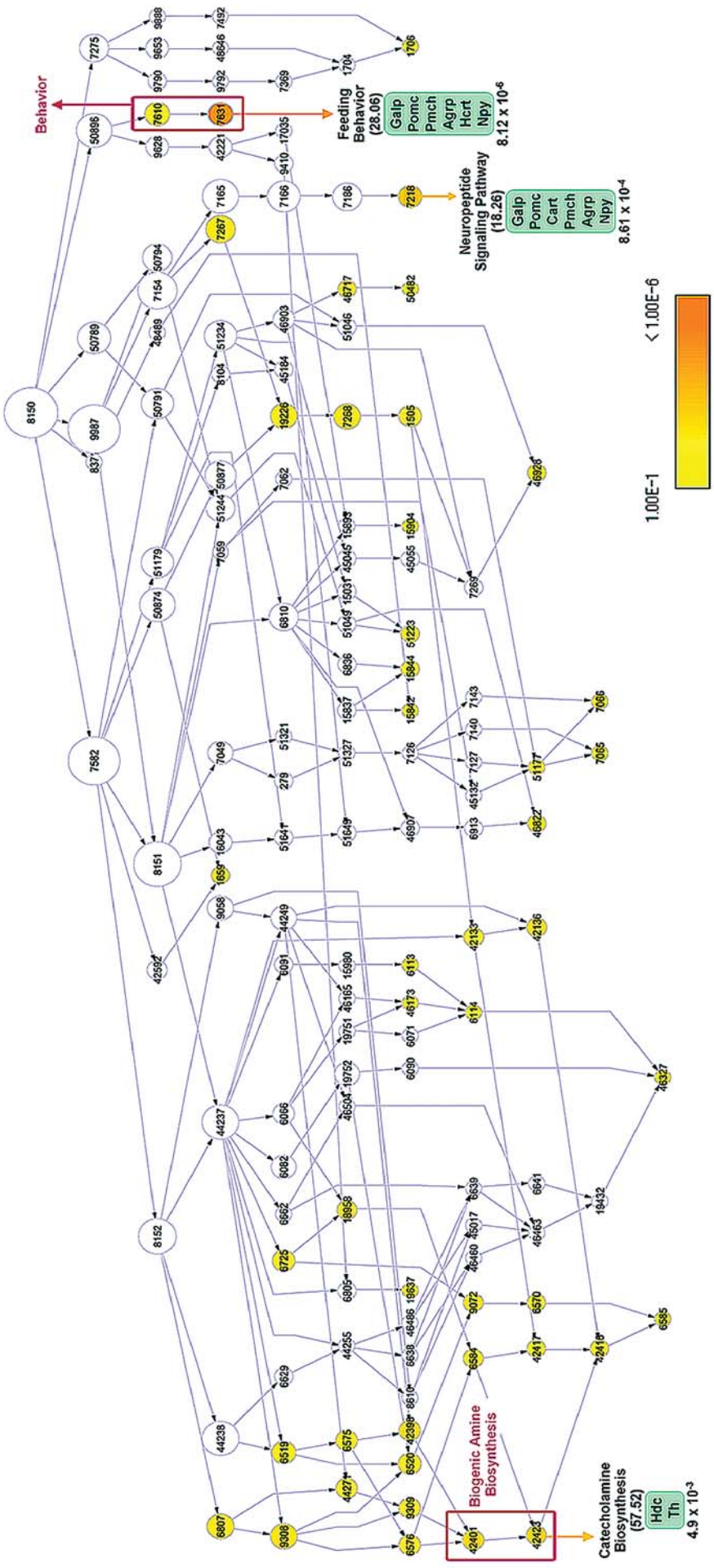

Figure 2. Hierarchy in the GO biological processes category (FDR <0.1), to which genes up-regulated (1.5-fold) in the hypothalamus of the tickled group compared to the light-touched group were assigned. GO IDs are presented as circles in the hierarchy. Their color intensity represents the degree of gene enrichment (refer to the color bar at the bottom). As the color intensity increases, so does the significance. Their size represents the number of genes assigned to the GO term. GO terms with significant enrichment (FDR <0.01) are surrounded by red frames in each category. Gene symbols are presented in the green square presented outside the hierarchy scheme. The frequency and FDR are presented in the upper and lower parentheses of the square, respectively.

various functions of monoamines, the association of dopamine and histamine with feeding behavior, such as the promotion and suppression of feeding, respectively, has also been reported (28,29).

In conclusion, repeated tickling stimulation with positive emotion affected neuronal circuitry directly and/or indirectly, and altered expression of the genes related to feeding regulation in the adolescent rat hypothalamus. It is known that the food intake system in the LHA is driven by a stomach-empty signal (26). Further analysis is necessary to investigate how tickling modulates local expression of feeding-regulation genes and, as a result, the blood glucose level.

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