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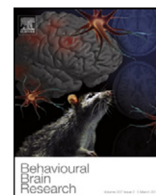

Research report

Tickling stimulation causes the up-regulation of the kallikrein family in the submandibular gland of the rat

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HIGHLIGHTS

- We examine gene expressions in rat salivary gland after tickling stimulation.
- Kallikrein family mRNAs are up-regulated by tickling stimulation accompanied with positive emotion.
- The candidate of biochemical markers indicating positive emotional state is proposed.

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ABSTRACT

We recently showed that tactile stimulation (tickling) accompanied by positive emotion altered the expression of many genes in the rat hypothalamus (Hori et al., 2009 [15]). In this study, the effect of repeated tickling on gene expressions of the rat salivary gland was examined. After 4-week stimulation, several genes of the kallikrein (Klk) family were remarkably up-regulated and the alpha-amylase (amylase) gene was down-regulated in DNA microarray analysis. In quantitative analysis using real-time PCR of the submandibular gland of the rats tickled for 2 weeks, mRNAs of Klk1, Klk2 (Klk1c2, Tonin), Klk7 (Klk1l), Klk1b3 (Nerve growth factor, gamma), Klk1c10, Klks3 (Klk1c9) and GK11 were significantly 2–5-fold increased among 18 members of the Klk gene family examined and the submandibular amylase was decreased compared with the lightly touched and untouched control rats. In immunoblot analysis the increase in Klk7 protein was observed in the whole cell lysate fraction of the submandibular gland. In immunohistochemical analysis with anti-Klk7 polyclonal antibody, the immunostain was increased in duct cells of the submandibular gland of the tickled rat when compared with the lightly touched and untouched control rats. These results suggest that tactile sensory processing in the central nervous system affects the gene expression in the peripheral tissue probably via hormonal and/or autonomic neural activities. Submandibular Klks may be biochemical markers indicating positive emotional states.

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1. Introduction

An event that leads to positive emotion such as laughter is often called eustress, good stress or positive stress [1–3]. The physiological effect of laughter was first reported by Cousins [4], who recovered from ankylosing spondylitis, a collagen disease, with a combination of therapies: massive doses of vitamin C and 10 min of belly laughter a day. Since then, several studies have pursued the immunological effect of laughter [3,5,6]. Mirthful laughter changes the responses of neuroendocrine and stress hormones probably having a role in immunomodulation [1]. We recently found that laughter suppressed an increase in the postprandial blood glucose level in patients with type 2 diabetes [7]. It caused specific changes in gene expression in peripheral blood leucocytes and up-regulated the expression of genes ameliorating the progression of diabetic microvascular complications [8,9]. These results indicate that laughter brings about health outcomes by restoring homeostatic balance.

To elucidate the physiological meaning and mechanisms of the state of joy manifested by laughter, we have done experiments on animals. Rats exhibit specific ultrasonic vocalization (USVs) in

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response to various social interactions and stimulation. Knutson et al. found that USVs at 50 kHz were drastically increased in tactile stimulated (tickled) rats [10]. Tickling stimulation mimics the dorsal contact and pinning behavior of the rough and tumble play of rats [11]. Tickling produces a positive reward state and evokes social preferences in young rats [11,12]. It is also reported that tickling induces many changes in animal's behavior in anxiety and depression researches [13]. The 50 kHz USV indicator reflecting positive emotional states and the 50 kHz-vocalization rat have been used as an animal model for studying some of the fundamental properties of laughter circuitry in humans [14].

We recently reported that repeated tickling stimulation altered the expression of the genes related to feeding regulation in the rat hypothalamus [15] and enhanced neurogenesis in the dentate gyrus of the rat hippocampus [16]. The limbic system containing the hypothalamus controls the salivary gland via the autonomic nervous system [17]. We have found the expression change in many genes in the salivary gland using a DNA microarray technique and especially the up-regulation of several genes of the kallikrein (Klk), which is one of the serine proteases and related to the production of many bioactive peptides. In this paper, we report the changes in the gene and protein expression of the Klk family of the rat submandibular gland after tickling stimulation, and discuss the possibility that some submandibular tissue Klk molecules may be useful as markers indicating positive emotional states.

2. Materials and methods

2.1. Animals

Three-week-old Wistar rats (Japan SLC Inc., Shizuoka, Japan) after weaning were individually housed in standard polycarbonate cages (W270 mm × L440 mm × H187 mm) with woodchip bedding. The cages were shielded with screens to prevent them from seeing each other. All animals were provided with food and water *ad libitum*, and were kept at constant room temperature on a 12:12 light:dark cycle (lights on 7:00), during the study periods. Subjects were weighed and divided into 3 groups (tickled, lightly touched and untouched groups; $n = 24, 23, 23$ each).

All animal experiments were carried out in a humane manner after receiving approval from the Institutional Animal Experiment Committee of the University of Tsukuba, and in accordance with the Regulations for Animal Experiments of the university and the Fundamental Guidelines for Proper Conduct of Animal Experiments and Related Activities in Academic Research Institutions under the jurisdiction of the Japanese Ministry of Education, Culture, Sports, Science and Technology.

2.2. Tickling stimulation

The method of Knutson et al. [10] was slightly modified as described before [15]. A 28-day-old rat was transferred into a test box (W270 mm × L440 mm × H187 mm; the floor and 4 sides of the box covered with soft black cloth) for stimulation. The animal was grasped on the dorsal side and tickled on the posterior neck with the experimenter's fingers, then rapidly turned over and vigorously tickled on the abdomen while being pushed onto the floor in a supine position, and then released. The first tickling session for 2 min consisted of four cycles of 15 s of no stimulation followed by 15 s of tickling stimulation. After the rest of 1 min, the rat had the second tickling session. This stimulation loading for a total of 5 min per day was conducted for 14 or 28 consecutive days.

Light-touch stimulation which serves as a discernible stimulation [12] was used for the lightly touched group as one of the controls. Each rat was gently touched on the dorsal region every 3 s instead of the 15 s tickling stimulation. An untouched group without the above stimulations in all sessions was used as another control.

2.3. Approach latency

To examine the index of positive behavior reinforcement, rats were tested for approach latency. After the second tickling or control sessions, the rat was placed in a corner of the test box and the time until the rat approached to touch the experimenter's hand in the opposite corner was measured. The maximum recorded latency was 30 s.

2.4. Ultrasonic vocalization

To ascertain the positive emotional state of rats, vocalization of each rat, 50 kHz USVs [11], was recorded during stimulation and resting using a high-frequency microphone (MI-3140, Ono Sokki Co., Japan), and the frequency component (hanning window; frequency range, 10–100 kHz; sampling frame length, 4096 points) was examined using an ultra high-band acoustic analysis system (DS-2100, Ono Sokki Co., Japan). After Fourier transformation of vocal sound, the peak sound pressure levels (dB) of frequency components were recorded for 10 s in every 15 s period. The strongest sound pressure level was regarded as the call's peak frequency in kHz. The call of 40–70 kHz obtained per each 15 s was counted as one point. The counting was carried out 16 times for 5 min. Total points during tickling stimulation were compared between the untouched, lightly touched and tickled groups.

2.5. Total RNA preparation

On the next day after the final stimulation by tickling, rats were decapitated under ether anesthesia and then their submandibular glands were removed. The tissues were suspended in RNAlater RNA Stabilization Reagent (Qiagen GmbH) and stored at −80 °C. Total RNA was prepared from the gland tissue using an RNeasy Mini Kit (Qiagen GmbH) according to the protocol provided by the manufacturer. Absorbance of the purified RNA was measured at 260 nm.

2.6. DNA microarray

Total RNAs obtained from the submandibular glands of the tickled or control rats were mixed and comprehensively analyzed by DNA microarray technique as described before [15]. 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 Cy 5 [18,19]. Equal amounts of Cy 3-labeled cRNA derived from the control group and Cy 5-labeled cRNA derived from the tickled group were mixed. The mixture was applied to a Whole Rat Genome Oligo DNA Microarray (G4131A, Agilent Technologies) 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 that, each array was washed and scanned by a confocal laser scanner (Agilent G2565BA). The fluorescence intensities on the scanned images were quantified. Genes with 1.5- and 0.67-fold differences in the expression level in the tickled group when compared with the control group were identified. The identified genes were examined for their biological meaning based on gene ontology (GO) (The Gene Ontology Consortium, 2000).

2.7. Real-time PCR analysis

Purified RNA was reverse-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) and quantified with TaqMan Gene Expression Assays by using a 7300 real-time PCR system (Applied Biosystems) according to the manufacturer's instructions. The genes examined were as following TaqMan Probes: Klk1 (Assay ID: Rn02345813_g1), Klk2 (Klk1c2, Rn00820615_m1), Klk3 (Klkb1, Rn00562377_m1), Klk4 (Rn01498536_m1), Klk5 (Rn01475996_m1), Klk6 (Rn00569838_m1), Klk7 (Klk1l, Rn00754920_m1), Klk8 (Rn01476995_m1), Klk9 (Rn01771806_m1), Klk10 (Rn01475770_g1), Klk11 (Rn01476753_m1), Klk12 (Rn01476164_m1), Klk13 (Rn01476393_m1), Klk14 (Rn01476389_ml), Klk1b3 (Ngfg, Rn00824646_m1), Klk1c10 (Rn01774875_m1), Klks3 (Klk1c9, Rn01477004_g1), GK11 (Rn02349982_m1) and alpha-amylase (Amy1a, Rn01522160_m1). The relative amounts of their mRNAs were normalized to the level of an internal control, beta-actin (Assay ID: Rn00667869_m1).

2.8. Whole cell lysate fraction and immunoblotting

The submandibular gland was homogenized in lysis buffer (1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 M NaF, 0.5% sodium pyrophosphate, 10% glycerol, 10 mM HEPES, 10 mM NaCl) containing protease inhibitors (Roche Diagnostics) using TissueLyser (Qiagen GmbH). After centrifugation (14,000 rpm, 15 min, 4 °C), the supernatant was recovered as a whole cell lysate fraction. Protein concentrations were measured with BCA Protein Assay Kit (Pierce).

Sixty μg of proteins were size-fractionated by SDS polyacrylamide (15%) gel electrophoresis by the Laemmli system [20] and transferred electrophoretically to a PVDF membrane (Millipore). Electrophoretic blots were incubated with anti-Klk7 rabbit polyclonal antibody (Santa Cruz, sc-20625, 1:500) and then with anti-rabbit antibody HRP-linked IgG (Cell Signaling, 1:2500). They were detected by Western Lightning (Chemiluminescence Reagent Plus-ECL, PerkinElmer). The blot was reprobed with anti-alpha-tubulin antibody (Santa Cruz, sc-8035, 1:5000) as a control for loading.

2.9. Immunofluorescence staining

Rats were anesthetized with diethyl ether and then transcardially perfused with cold saline. Their submandibular glands were removed and embedded in O.T.C. Compound (Sakura Finetek). Sections (6 μm thickness) were made using cryostat, placed on glass slides, and stored at −80 °C after drying. Samples were fixed in the 4% paraformaldehyde/0.1 M PB (77 mM Na_2HPO_4/23 mM NaH_2PO_4, pH 7.3) for 15 min and washed in 0.01 M PBS (phosphate-buffered saline). Nonspecific binding was blocked by incubation with 1% bovine serum albumin/0.3% Triton

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