Chronic Stress Induces Neurotrophin-3 in Rat Submandibular Gland

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Purpose: Plasma neurotrophin-3 (NT-3) levels are associated with several neural disorders. We previously reported that neurotrophins were released from salivary glands following acute immobilization stress. While the salivary glands were the source of plasma neurotrophins in that situation, the association between the expression of neurotrophins and the salivary gland under chronic stress conditions is not well understood. In the present study, we investigated whether NT-3 levels in the salivary gland and plasma were influenced by chronic stress.

Materials and Methods: Expressions of NT-3 mRNA and protein were characterized, using real-time polymerase chain reactions, enzyme-linked immunosorbent assay, and immunohistochemistry, in the submandibular glands of male rats exposed to chronic stress (12 h daily for 22 days).

Results: Plasma NT-3 levels were significantly increased by chronic stress \( (p<0.05) \), and remained elevated in bilaterally sialoadenectomized rats under the same condition. Since chronic stress increases plasma NT-3 levels in the sialoadenectomized rat model, plasma NT-3 levels were not exclusively dependent on salivary glands. Conclusion: While the salivary gland was identified in our previous study as the source of plasma neurotrophins during acute stress, the exposure to long-term stress likely affects a variety of organs capable of releasing NT-3 into the bloodstream. In addition, the elevation of plasma NT-3 levels may play important roles in homeostasis under stress conditions.

Key Words: Chronic stress, NT-3, sialoadenectomized rat, submandibular gland

INTRODUCTION

The salivary gland system consists of the major salivary glands, including the parotid, submandibular, and sublingual glands, and numerous minor salivary glands scattered throughout the oral cavity. The main role of the salivary glands is to secrete saliva for assisting in food digestion, as well as to promote mastication and...
antimicrobial activities. They are predicted to have other important roles, as the salivary glands produce a variety of substances.

Salivary glands produce several cell growth factors and play an important role in human health. Cell growth factors such as epidermal growth factor (EGF) and nerve growth factor (NGF), in particular, are found in the rat submandibular gland, leading to the recognition of new functions for the salivary glands. Mouse salivary gland tissue expresses a high level of NGF, which is released in abundance from salivary glands into the bloodstream during fighting, and plasma EGF levels are reduced after damage to the major salivary glands. Hence, the salivary glands may play an important role in systemic health.

The neurotrophins consists of NGF, brain-derived neurotrophic factor (BDNF), and neurotrophins (NT)-3, -4/5, -6, and -7. They play key roles in neuronal survival, differentiation, connectivity, and plasticity. NT-3 expression is particularly high during embryogenesis, and may influence the development of the hippocampus. Neurotrophins interact with the tyrosine receptor kinase (Trk) family of high-affinity protein kinase receptors. Specifically, BDNF interacts with the TrkB receptor, and NT-3 binds mainly to TrkC with weak binding to TrkB as well. It is possible that NT-3 and BDNF may have related effects in other organs. In the hippocampus in particular, the expression of NT-3 and BDNF varies depending on stress, exercise, and learning. NT-3 also plays an important role in facilitating the formation of neural networks. NT-3 is also found outside the central nervous system, such as in pancreatic beta cells, lymphocytes, and skeletal muscle.

Rat salivary glands have been shown to increase their expression of BDNF under acute stress conditions. Furthermore, BDNF plasma levels are increased by acute immobilization stress with the submandibular glands contributing to this. Indeed, the salivary glands are considered to be a primary source of plasma BDNF, the concentration of which is greater in intact rats than in sialoadenectomized rats. These results suggest that BDNF produced by the salivary glands may affect various organs during acute stress. However, the effects of chronic stress on salivary gland NT-3 levels have not yet been reported, even though the role of NT-3 in chronic stress has been the subject of several studies.

Decreased circulating NT-3 levels have been found in hypochondriasis and schizophrenia. Furthermore, alteration in NT-3 content in the central nervous system has been reported to play an important role in the pathogenesis of neural diseases related to chronic stress. Because blood NT-3 levels increase with the use of psychotropic agents, the NT-3 content in serum and plasma may have clinical significance in the evaluation of mental disorders. Furthermore, chronic stress, an important onset factor for mental disorders, decreases NT-3 content in the rat hippocampus, however few studies have measured blood NT-3 levels during chronic stress.

The aim of this study was to determine whether chronic stress affects the expression of NT-3 in rat salivary glands. In addition, we sought to identify an association between alterations in plasma NT-3 levels during chronic stress and the salivary glands.

MATERIALS AND METHODS

Animals
Sprague-Dawley (SD) male rats, 5 weeks of age, and weighing 150-170 g (Japan SLC, Shizuoka, Japan), were used in this study. They were housed in groups of six rats per cage in a room maintained under standardized 12 hours light/dark cycle (lights on at 07:00) and temperature (22±3°C). Rats had free access to food pellets and tap water. Body weights were recorded every 2 days in the morning after being taken out of restraining cages, for the duration of the experimental period.

Restraint and sialoadenectomy procedure
Restraint stress was employed and involved enclosing each rat in a flexible wire mesh (5×5 mm) shaped to fit its body. Restrained rats were able to move a little and had no access to food or water during the restraint; control rats also had no access to food or water during this time. Because rats are nocturnal, they were restrained from 19:00 to 07:00 (12 hours) every 24 hours for the 22-day experimental period. After release from the restrainer, rats were returned to their home cages each morning and had access to food and water ad libitum, as did the controls.

The major salivary glands were removed bilaterally (sialoadenectomy), or a sham operation was performed, in 3-week-old male rats under sodium pentobarbital anesthesia (65 mg/kg, i.p.). A 2-cm skin incision in the neck was made, the major salivary glands were isolated from the surrounding subcutaneous fat tissue, a ligature was placed around the supplying vessels, and the salivary glands were then removed bilaterally. Two weeks after surgery, the re-
peated restraint stress and handling procedure began. All experiments were performed with 12 rats per experimental group. At the end of the experiment, all rats were deeply anaesthetized with sodium pentobarbital (65 mg/kg, i.p.) for blood and tissue collection. To minimize diurnal variations in NT-3 expression, all rats were sacrificed between 07:00 and 11:00; the stressed rats were anaesthetized immediately following the last restraint session. The experimental protocol used in this study was reviewed and approved by the Ethics Committee on Animal Experiments of Kanagawa Dental College, and was carried out with adherence to the Guidelines for Animal Experimentation of Kanagawa Dental College.

**Blood sampling**
At the end of the experiment on the 23rd day, all rats were deeply anaesthetized with sodium pentobarbital as above, and blood samples were collected between 07:00 and 11:00 by cardiac puncture into Venoject® tubes containing EDTA (Terumo, Tokyo, Japan). The tubes were immediately placed on ice and then centrifuged (2000 rpm, 15 minutes, 4°C). Plasma was stored at -20°C prior to radioimmunoassay.

**RNA extraction and cDNA synthesis**
After blood sampling, the anaesthetized intact rats were sacrificed by decapitation, and submandibular glands were removed. Total RNA isolation from the submandibular glands was performed using the ISOGEN reagent (Nippon Gene, Toyama, Japan) in accordance with the manufacturer’s instructions. The RNA was resuspended in 20 µL of diethyl pyrocarbonate treated water. RNA quality was judged from the pattern of ribosomal RNA after electrophoresis through a 1.5% agarose gel containing ethidium bromide and visualization by UV illumination. RNA concentrations were determined by absorbance readings at 260 nm with a SmartSpec Plus spectrophotometer (Bio-Rad, Tokyo, Japan). RNA was stored at -80°C until use. Total RNA was reverse transcribed at 50°C for 30 minutes, 99°C for 5 minutes, and 5°C for 5 minutes using a single-strand cDNA synthesis kit (Roche Diagnostics Ltd., Lewes, UK) according to the manufacturer’s instructions. Following the reverse transcription reaction, the cDNA was stored at -20°C until use.

**Real-time PCR analysis**
Real-time polymerase chain reactions (PCR) was performed using a LightCycler (Roche) according to the manufacturer’s instructions. Reactions were performed in a 20 µL volume (NT-3: 0.5 µM each primer and 3 mM MgCl2). Reactions with Taq DNA polymerase, nucleotides, and buffer for NT-3 were performed with LightCycler-DNA Master Hybridization Probe mix (Roche). Oligonucleotide primers were designed to amplify rat NT-3. The NT-3-specific primers were 5'-TGTTGGGAGCCCCAACGTC-3' (forward) and 5'-GAGTTCCAGTTTTGTAC-3' (reverse; NT-3 PCR product: 175 bp) as designed and synthesized by Nippon Gene Laboratory.21 Real-time PCR for amplification of the rat β-actin housekeeping gene was performed using a Lightcycler Primer/Probe set, 5'- CCTGTATGCTCTG-GTTCGA-3' (forward) and 5' - CCACTCTTTGCTC-GAAGTCT-3' (reverse; β-actin PCR product: 260 bp),22 following the manufacturer’s instructions (Nihon Gene Research Labs Inc., Sendai, Japan). Denaturation was performed at 95°C for 10 minutes, after which Segment 1 (95°C for 10 seconds), Segment 2 (60°C for 10 seconds), and Segment 3 (72°C for 10 seconds) were repeated for 40 cycles. We performed melting analysis and agarose gel electrophoresis to confirm the specificity of the PCR products obtained using each primer pair. Gene expression is stated in terms of the copy number ratio of NT-3 mRNA to β-actin mRNA for each sample.

**Tissue preparation for immunohistochemistry**
The rats were sacrificed under deep anesthesia as above, between 07:00 and 11:00; rats in the chronic stress groups were sacrificed immediately after the last restraint session. Resected rat submandibular gland tissue samples were fixed in 10% buffered formaldehyde (pH 7.4) for 24 hours and embedded in paraffin. Next, serial 3-µm sections were cut and stained with hematoxylin and eosin and processed for immunohistochemistry. Immunohistochemical analysis was performed using the simple stain MAX-PO Kit (Nichirei, Tokyo, Japan). Slides were pre-incubated in 3% H2O2 for 5 minutes. Sections were then incubated with anti-NT-3 rabbit polyclonal antibody (sc-547, 1 : 500, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) for 1 hour at room temperature. After washing with PBS, sections were reacted with a secondary antibody, horseradish peroxidase (HRP)-labeled anti-rabbit IgG with amino acid polymer (Nichirei), for 30 minutes at room temperature. Color was developed using 0.02% 3,3′-diaminobenzidine-tetrahydrochloride containing 0.0003% H2O2 in Tris-buffered saline for 5 minutes, and sections were then counterstained with hematoxylin. To provide negative controls, non-immunized rabbit or mouse
IgG was used instead of the primary antibody. To determine the binding specificity, a competitive assay was also carried out using recombinant NT-3 (R&D Systems, Inc., Minneapolis, MN, USA).

Measurement of plasma adrenocorticotropic hormone and corticosterone
Adrenocorticotropic hormone (ACTH) was assayed using a radioimmunoassay (RIA) kit [enzyme-linked immunosorbent assay (ELISA)-ACTH, CIS; Atomic Energy Laboratory of Biochemical Products, Gif-sur-Yvette, France], following the manufacturer’s instructions. ACTH concentrations are reported in pg/mL. A COAT-A-COUNT Rat Corticosterone kit (Siemens Medical Solutions Diagnostics, New York, NY, USA) was used for measurement of plasma corticosterone concentration (ng/mL).

Submandibular gland protein extraction and ELISA analysis
Submandibular gland tissue samples were homogenized in ice-cold lysis buffer, containing 137 mM NaCl, 20 mM Tris-HCl (pH 8.0), 1% NP40, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 10 µg/mL aprotinin, 1 µg/mL leupetin, and 0.5 mM sodium vanadate. The tissue homogenates were centrifuged at 14000 g for 5 minutes at 4°C. The supernatants were collected and used for quantification of total protein and NT levels. Total protein concentrations were determined by the Bradford method using absorbance readings at 595 nm with a SmartSpec Plus spectrophotometer (Bio-Rad).

NT-3 concentrations in the submandibular gland and plasma were assayed using an ELISA kit (Promega, Co., Madison, WI, USA). Briefly, standard 96-well flat-bottom NUNC-immunoMaxisorp ELISA plates were incubated with the corresponding captured antibody, which binds the NT of interest, overnight at 4°C. The plates were blocked by incubation for 1 hour at room temperature with a 1×block and sample buffer. Serial dilutions of a known amount of NT-3, ranging from 0 to 300 pg/mL, were performed in duplicate for standard curve determination. Wells containing the standard curve samples and supernatants of submandibular gland tissue homogenates and plasma were incubated at room temperature for 6 or 2 hours, as specified by the protocol. They were then incubated with a second specific antibody overnight at 4°C or for 2 hours at room temperature. A species-specific antibody conjugated to horseradish peroxidase was used for a tertiary reaction for 2.5 or 1 hour at room temperature following this incubation step. TMB One Solution was used to develop color in the wells. This reaction was terminated with 1M hydrochloric acid after a specific time (10-15 minutes) at room temperature, and absorbance was then recorded at 450 nm in a plate reader within 30 minutes of stopping the reaction. NT-3 values were determined by comparison with the regression line for NT-3 standard. Using these kits, NT-3 can be quantified over a range of 0-300 pg/mL. For the NT-3 assay kit, cross-reactivity with other neurotrophic proteins was <2-3%.

Statistical analysis
Statistical analyses were carried out using the SPSS (Version 17.0; SPSS Inc., Chicago, IL, USA) statistics program. The Student’s t-test and one-way ANOVA followed by Tukey’s post-test were used for statistical analysis. A probability level of 0.05 or less was accepted as significant.

RESULTS

Effect of chronic stress on body weight
Prior to the experimental period, all rats had similar body weights. Because the rats were in their active growth phase, their body weight increased during the experiment. No significant differences in the mean body weight between the control and chronic stress groups were observed throughout the duration of the experimental period (data not shown).

Effect of chronic stress on plasma concentrations of ACTH and corticosterone
Plasma ACTH levels were significantly elevated in the chronic stress group, with values of 78.24±3.1 pg/mL and 374.67±61.11 pg/mL observed in the control and chronic stress groups, respectively. Similarly, there were also significant differences in ACTH levels between the sham-control group and the sham-chronic stress group. Chronic stress also significantly elevated plasma corticosterone levels from 39.22±5.72 ng/mL to 530.97±49.8 ng/mL in the control and chronic stress groups, respectively. Similarly, significantly elevated corticosterone levels were observed in the sham-chronic stress group compared to the sham-control group.

Quantitative analysis of NT-3 mRNA in submandibular gland following chronic stress
Melting curve analysis demonstrated a single fluorescent peak representing the Tm of NT-3 mRNA in all samples.
except for the negative sample (data not shown). In addition, a single band was observed following agarose gel electrophoresis (data not shown). These findings confirmed that the PCR product was NT-3 mRNA. Expression ratios (NT-3 mRNA/β-actin mRNA) were calculated in submandibular gland of the chronic stress and control groups. The expression ratios in the submandibular gland were 0.0028±0.0017 and 0.0084±0.00467 in the control and chronic stress groups, respectively (Fig. 1). Chronic stress resulted in a significant increase in NT-3 mRNA expression in the submandibular gland (Fig. 1). Similarly, submandibular gland NT-3 mRNA expression was significantly elevated in the sham-chronic stress group as compared to the sham-control group.

Submandibular gland NT-3 content following chronic stress

Exposure to chronic stress significantly increased submandibular gland NT-3 levels from 369.22±49.64 pg/mL to 557.60±244.17 pg/mL (Fig. 2), with a similar, significant increase observed in the sham groups.

Plasma NT-3 concentrations following chronic stress

Plasma NT-3 levels were significantly elevated as the result of chronic stress, increasing from 11.67±6.98 pg/mL to 406.00±322.25 pg/mL (Fig. 3). Similarly, there was also a significant increase in plasma NT-3 levels between the sham-chronic stress and sham-control group.

NT-3 immunohistochemistry in submandibular gland

Rat pancreas sections were used as a positive control for NT-3 immunohistochemistry,19 and showed high levels of NT-3 expression in the islets of Langerhans (data not shown). Non-stressed submandibular gland tissue exhibited a weak positive signal for NT-3 expression in various duct-type cells (Fig. 4A). In comparison, intense NT-3 expression was observed in various duct-type cells of the chronic-stress group; however, NT-3 expression was not consistently observed in acinar cells or myoepithelial cells in the chronic stress group (Fig. 4B). NT-3 expression was not detected in the competitive assay (data not shown).

Plasma concentrations of ACTH and Corticosterone: chronic stress and sialoadenectomy

In sialoadenectomized rats, ACTH levels were 80.02±4.26 pg/mL and 430.00±44.71 pg/mL in the control and chronic stress groups, respectively, representing a significant increase in ACTH levels following chronic stress (Table 1). Similarly, there were also significant differences in ACTH levels between the sham-control group and sham-chronic stress group. Chronic stress also significantly increased corticoste-
sialoadenectomized group, and 292.60±158.30 pg/mL for sham-chronic stress group under the chronic stress condition. There were no significant differences in plasma NT-3 levels among the groups under the chronic stress condition (Table 2).

**DISCUSSION**

Using multiple techniques, we demonstrated increased expression of NT-3 mRNA and protein in rat submandibular gland tissue following repeated restraint stress. Tsukinoki, et al. found that the expression of BDNF in the rat submandibular gland is modulated by acute stress, and NGF levels have also been reported to be altered by acute stress in this gland. The present study indicates that NT-3 expression in the submandibular gland is up-regulated by a chronic stressor.

Chronic immobilization stress increases NT-3 mRNA levels in the rat hippocampus and can cause damage and atrophy in this brain region, which expresses high levels of receptors for glucocorticoids (GC). It has been reported that the mechanisms underlying the up-regulation of NT-3 are associated with chronic stress-induced elevation of blood GC content. Furthermore, our results demonstrated increased circulating GC along with increased production of NT-3 in salivary glands during repeated restraint. Nevertheless, atrophy was not evident in the duct-type cells of the salivary glands after chronic stress. The salivary glands are predominantly under the control of autonomic innervation, which regulates secretion of saliva rather than hormones. However, because brain NT-3 levels are markedly affected

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### Table 1. Plasma Concentrations of ACTH and Corticosterone: Control or Chronic Stress (Sialoadenectomy)

|          | ACTH (pg/mL) | Corticosterone (ng/mL) |
|----------|--------------|------------------------|
| Control  | 80.02±4.26   | 35.76±4.91             |
| Chronic stress | 430.00±44.71* | 466.42±43.14*          |

ACTH, adrenocorticotropic hormone.

The effect of chronic stress on plasma concentrations of ACTH and corticosterone in sialoadenectomized. Control: no stress; Chronic stress: after 22 days of daily 12 hours restraint stress. Submandibular glands were removed (sialoadenectomy) prior to the exposure to chronic stress. Values are means±SD; n=12 rats in each group.

*p<0.05, Student’s t-test.

### Table 2. Plasma Concentrations of NT-3: Non-Sialoadenectomy, Sialoadenectomy or Sham Under Chronic Stressed Conditions

|          | NT-3 Plasma (pg/mL) |
|----------|---------------------|
| Non-sialoadenectomy | 406.00±332.25      |
| Sialoadenectomy      | 217.50±187.66      |
| Sham                 | 292.60±158.30      |

NT-3, neurotrophin-3.

NT-3 plasma concentrations in chronically stressed rats. All groups were exposed to chronic stress and received either no sialoadenectomy, sialoadenectomy or sham surgery prior to the exposure to chronic stress. Values are means±SEM; n=12 rats in each group. There were no significant differences among groups, p>0.05, ANOVA/Tukey’s test.
by stressful events,32 stress-induced changes in NT-3 levels also may be due to hormonal influence, as indicated by the present study. A question of whether hormones induced the increased salivary gland expression of NT-3 in the present study, requires further investigation.

In the second part of this study, we examined whether plasma levels of NT-3 following repeated stress is related to salivary gland NT-3 production. When rats were exposed to stress for 3 weeks, plasma NT-3 levels in the chronic stress group were significantly higher than those in the control group. We previously found elevated plasma BDNF levels in rats after acute stress.22 The present study indicates that chronic stress also increases plasma NT-3. To date, there have been no investigations into changes in plasma NT-3 using experimental models that are characterized by physical stress. In clinical studies, blood NT-3 levels have been found to be lower in persons with schizophrenia or hypochondriasis.26,27 Because blood NT-3 levels increase with the use of psychotropic agents,29 the NT-3 content in serum and plasma may reflect the clinical stage of such mental disorders. Interestingly, the elevation of NT-3 levels contributes to the protection of neural cells in the rodent hippocampus.36 Since NT-3 can cross the blood-brain barrier,37 circulating NT-3 is likely to contribute to protecting neural cells and maintaining their function. Hence, an increase in plasma NT-3 levels may be an important neuroprotective response during chronic stress.

Salivary glands have been shown, in studies using sialoadenectomized rats, to be the source of increased plasma BDNF level during acute immobilization stress.23 However, in the present study no significant difference was found in plasma NT-3 levels between sialoadenectomized rats and intact rats following chronic stress. Hence, although NT-3 mRNA and protein levels increased in the submandibular gland during chronic stress, this did not seem to significantly contribute to the increased plasma NT-3 levels. Expression of NT-3 has been investigated in several other organs, including heart, lung, liver, pancreas, and stomach,33,38-41 however, there have been no previous reports that examined the mechanism by which NT-3 increases under stress conditions. Several studies have shown that increased levels of monoaminergic neurotransmitters, including adrenaline, noradrenaline, serotonin, and dopamine, induce BDNF expression in the brain under stress conditions.42-44 Monoaminergic neurotransmitters have also been found to be able to potently and transiently increase BDNF cellular content in rat neonatal astrocytes in vitro.45 In addition, the activation of monoaminergic transmitter receptors, coupled to cAMP production and CREB activation, can induce BDNF gene transcription.46 The foregoing findings suggest the involvement of monoaminergic systems in the regulation of BDNF production.43 NT-3 may also increase under stress conditions by the same mechanism as BDNF.

In conclusion, our present findings together with our previous reports suggest that the contribution to plasma NT-3 levels by certain organs, including salivary glands, may be different during the periods of chronic or acute stress. Further studies are needed to clarify the sources and biological significance of plasma NT-3 during chronic stress.

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