

Chemical Deafferentation of the Nasal Neuroepithelium Profoundly Reduces Cholecystokinin Gene Expression in the Mouse Olfactory Bulb

Ikuo KOZAKAI¹, Yasuhiro ITOH², Ryozo KUWANO², Yutaka AOYAGI¹ and Hitoshi ASAKURA¹

¹Department of Internal Medicine (III), Niigata University School of Medicine, ²Research Laboratory for Molecular Genetics, Niigata University, Niigata, Japan

Received December 10 2001; accepted January 15 2002

Summary. A denervation study was carried out to determine whether damage to the neuronal perikarya in the primary olfactory pathway alters the transneuronal level of cholecystokinin (CCK) mRNA expression. The time course study of denervation-induced change in CCK mRNA expression was done using northern blot and *in situ* hybridization methods. Northern blot analysis revealed a significant reduction in CCK mRNA expression. The quantified radioactive orbital densities in the olfactory bulb in the control at 48 hours, 7 days, and 14 days after the denervation were 1025 (100%), 743 (72%)/513 (50%), 358 (35%)/498 (49%) and 325 (32%)/338 (33%) photo-stimulated luminescence, respectively. *In situ* hybridization indicated that CCK expression in external tufted cells and middle tufted cells decreased time-dependently, reaching 76% of the control levels at 48 hours, 45% at 7 days, 26% at 14 days, and 15% at 30 days, respectively. CCK mRNA was expressed also in the deep tufted cells and mitral cells. Low-power darkfield photomicrography revealed that deep tufted cells and mitral cells did not undergo a profound decrease as seen in the external and middle tufted cells. These data suggest that CCK mRNA expression is differentially controlled by cell subpopulations in the olfactory bulb after deafferentation.

Key words—chemical deafferentation, olfactory bulb, CCK mRNA, *in situ* hybridization, tufted cell, mitral cell.

INTRODUCTION

Cholecystokinin (CCK) is a brain-gut peptide that is widely distributed not only in the intestinal tract but also throughout the central nervous system, particularly in the olfactory bulb, the cerebral cortex, basal ganglia, putamen, hypothalamus, and mesencephalon. The important function of CCK lies its diverse effects on satiety, pain, perception, fear and anxiety as well as construction of the gall bladder. In addition to these roles, several studies have revealed that CCK might further play an important part in a meningo-cortical injury or peripheral nerve injury. Injury to the meninges and the underlying cortex is known to enhance the expression of the procholecystokinin gene in the whole ipsilateral hemisphere. By way of example, 1-3 days after the lesion, its respective immunoreactivity and mRNA expression increase.^{1,2)}

In contrast, an examination by X. J. Xu³⁾ of the distribution of CCK mRNA with *in situ* hybridization in the rat lumbar dorsal root ganglia following sectioning of the sciatic nerve showed a dramatic increase in the number of neurons in the dorsal root ganglia synthesizing CCK 14 days after sciatic nerve sectioning. These results indicate that a marked up-regulation of CCK mRNA occurred not only after the direct cortical injury, but also after deleterious effects on the neuron, due to the transectioning of its axon. Therefore, the present study undertook a denervation of the primary olfactory pathway to determine whether damaging the neuronal perikarya would alter the transneuronal level of CCK mRNA expression with northern blot and *in situ* hybridization.

Correspondence: Ikuo Kozakai, The Third Department of Internal Medicine, Niigata University Graduate School of Medical and Dental Sciences, 1-757 Asahimachi-dori, Niigata 951-8510, Japan.

The olfactory bulb is the only region of the central nervous system in which total degeneration of the primary sensory fibers can be experimentally induced. This pathway is suitable for such a study since the perikarya lie outside the central nervous system and are fairly accessible in the nasal epithelium while the synaptic endings are within the cranium itself, in the olfactory bulb (Fig. 1).

MATERIALS AND METHODS

Male ICR mice, aged 9 months (35–40 g; Charles River Breeding Laboratories, Kanagawa), were used as subjects. Deafferentation was induced according to the procedure by F. L. Margolis⁴⁾. The mice were briefly anesthetized with ether anesthesia and a 26-gauge needle 20 mm in length with a rounded tip was

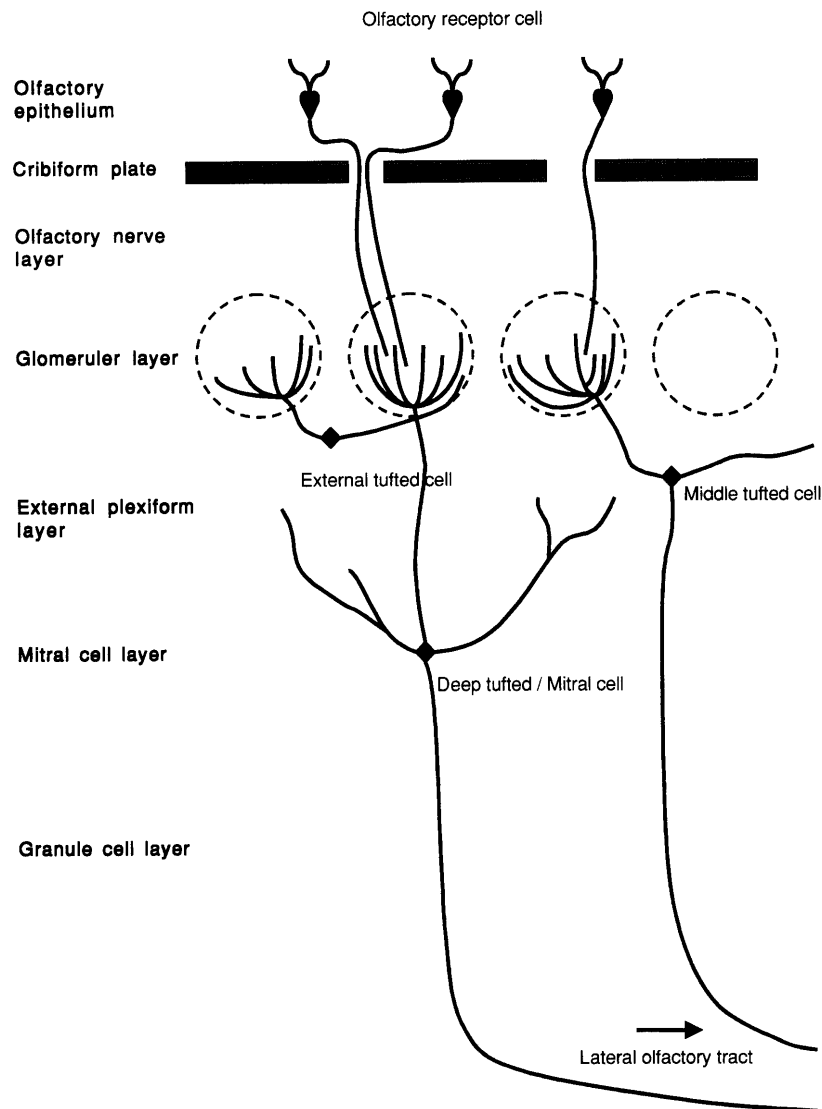


Fig. 1. Laminar distribution of the principal cells in the olfactory bulb. Sensory neurons in the olfactory epithelium project axons to spherical regions of the neuropil in the olfactory bulb called glomeruli. Each glomerulus represents a discrete unit of synaptic contact comprising the terminals of sensory axons and the dendrites of external, middle, and deep tufted cells and mitral cells.

inserted about 2 mm into one nostril and 0.1 ml of 0.17 M zinc sulfate solution was injected. Controls were treated in the same way, using physiological saline instead of zinc sulfate solution. The excess solution was drained from the nasal passages by gently shaking the mouse. The survival rate was over 80%. At 2 days, 7 days, 14 days, and 30 days post deafferentation, the mice ($n=4$ at 2 days, 7 days and 14 days. $n=2$ at 30 days.) were killed by decapitation under an overdose of ethyl ether for either northern blot or *in situ* hybridization analysis.

Northern blot analysis

Total RNA was isolated from the olfactory bulb ipsilateral to the zinc sulfate irrigation and the RNA (10 μ g) was denatured with glyoxal and 50% dimethyl sulfoxide, electrophoresed in a 1% agarose gel containing 7% formaldehyde, and transferred onto a nitrocellulose membrane filter. The filter was hybridized with [32 P] labeled cDNA fragment from exon III of rat CCK in a solution containing 50% formamide, $5\times$ SSC (20 \times SSC stock solution: 3 M sodium chloride and 0.3 M sodium citrate, pH 7.0), 50 mM sodium phosphate (pH 6.5), Denhardt's solution, and 250 μ g/ml heat-denatured salmon sperm DNA at 42°C according to the method described by P. S. Thomas⁵⁾.

In situ hybridization

In situ hybridization was carried out by a modification of the method as described previously⁶⁾. Frozen tissue sections (10 μ m) on the horizontal plane were prepared on a cryostat microtome, thaw-mounted on silicon coated glass slides, and dried at room temperature for 30 min. The section was fixed in a 4% paraformaldehyde/phosphate buffer (pH 7.2) solution and immersed in 0.25% acetic anhydride/0.1 M Tris-HCl (pH 8.0) for 10 min. After prehybridization with a solution composed of 50% formamide, $5\times$ SSC, 50 mM sodium phosphate (pH 6.5), 2% Sarcosyl, 1 \times Denhardt's solution, 250 μ g/ml heat-denatured salmon sperm DNA and 10 mM-mercaptoethanol, the 260 bp cDNA fragment from CCK exon III (362–620) labeled with [35 S] dATP and 10% dextran sulfate was added to the prehybridization buffer and incubated in a humidified chamber at 42°C for 12–16 hrs. The sections were sequentially rinsed in three time changes of $0.1\times$ SSC/0.1% Sarkosyl at 42°C for 40 min and then dehydrated in 70%, 95%, and 100% ethanol. After air drying, the sections were exposed to an Imaging Plate (Fuji Photo Film, Tokyo) for 3 days. Then the slides were dipped in NTB-2 nuclear track emulsion (Kodak, Rochester), exposed for 4 weeks

with dessicant at 4°C, developed and counterstained with methyl-green pironic acid.

Quantitative autoradiographic analysis by the imaging plate system

We used the computer-assisted image-processing system, BAS 5000 (Fuji Photo Film, Tokyo). This system consists of an image reader, and image processor (a 128-bit workstation), and a high-quality hard-copy printer (Pictography, Fuji Photo Film, Tokyo). The exposed imaging plate was scanned, emitted, and stored energy quantified as photo-stimulated luminescence (PSL) under specific reading conditions: gradation, 65536 resolution, 25 μ m; sensitivity, 30000; latitude, 5. The PSL were collected and digitized to allow a measurement of the relative radioactivity of the section. The image data was recorded as the digitized values (PSL) of each pixel (25 μ m \times 25 μ m) in the analyzing unit of this system. Quantification of radioactive density was performed by calculation of the mean gradation per mm² (PSL/mm²). The autoradiographic technique provides quantitative data because the film intensities observed are proportional to the time of exposure and tissue content of the radiography. We made use of phosphor-imaging plates for quantitative evaluation of radioactive energy. This newly-developed radioimmunographic technique has a wide linear dynamic range as well as a high sensitivity in ensuring a good quantitative accuracy and great reduction in exposure time.

Light microscopic image analysis

Low-power darkfield photomicrographs of horizontally sectioned olfactory bulb were scanned by an Epson scanner (GT9500) and processed and analyzed by NIH images.

RESULTS

Northern blot hybridization

The time course study of the denervation-induced change in CCK mRNA expression was carried out first by northern blot hybridization. Deafferentation elicited a significant decrease in CCK mRNA expression. Treatment with 0.17 M ZnSO₄ resulted in a denervated lesion that was essentially irreversible. The reduction of CCK mRNA content also occurred in a time-dependent manner. Quantified radioactive densities in the olfactory bulb in the control, at 48 hours, 7 days, and 14 days after the denervation were 1025 (100%), 743 (72%)/513 (50%), 358 (35%)/498

(49%), and 325 (32%)/338 (33%) PSL, respectively (Fig. 2). In contrast, the expression of 28S and 18S rRNA was nearly equal in all the lanes.

In situ hybridization

In order to verify the reduction of CCK mRNA expression at the single cell level, we employed the *in situ* hybridization method. CCK mRNA were widely distributed in the mouse brain, with especially high levels in the neocortex, olfactory bulb, claustrum, amygdala, the dentate gyrus, hippocampus proper, and several subnuclei of the thalamus and hypothalamus. The main olfactory bulb contained a large number of CCK-labeled neurons throughout the external plexiform layer (EPL). The highest labeling density was found in the most superficial portion of the external plexiform layer in the neurons classified as external tufted cells and middle tufted cells. Deep tufted cells and mitral cells were sporadically labeled along the base of the external plexiform layer (Fig. 3).

Quantitative analysis of radioactivity

To confirm denervation-induced changes, the mice were sacrificed at 48 hours, 7 days, 14 days and 30 days, respectively, after the insertion of 0.17 M ZnSO₄ into one nostril. The autoradiographic analysis by the imaging plate system revealed a gradual reduction of CCK mRNA expression at the external tufted cells and middle tufted cell during the above periods (Fig. 4). In spite of the insertion of zinc sulfate into only one nostril, denervation-induced changes were usually observed in the bilateral olfac-

tory bulbs, when concentrations of CCK mRNA were compared with those of the intact mice. The reason why ZnSO₄ in a single nostril damaged the contralateral nasal epithelium was that the right and left nasal cavities are anatomically continuous posteriorly with the nasopharynx through the posterior nasal apertures. As the hybridized signals did not have exactly the same radioactivity among the horizontal tissue sections, the direct measurement of quantified PSL was insufficient for the precise quantification of radioactive analysis. In contrast, no radioactive difference in the cerebral cortex could be detected between the denervation-induced and controlled mice. Accordingly, we determined more accurate autoradiographic intensities of CCK mRNA at the EPL by the following equation, using the density of cerebral cortex for the standard

$$y = [(PSL_{ob} - BG_{ob}) / A_{ob}] \div [(PSL_{cc} - BG_{cc}) / A_{cc}] \quad \text{Eq. 1}$$

where PSL_{ob} was PSL of external tufted cells and middle tufted cells, BG_{ob} was PSL of the background of the bulb, A_{ob} was the area of external tufted cells and middle tufted cells, PSL_{cc} was PSL of the cerebral cortex, BG_{cc} was PSL of the background of the cerebral cortex, and A_{cc} was the area of the cerebral cortex (Fig. 5).

Table 1 shows the results of autoradiographic intensities of these horizontal tissue sections. Following the denervation, there was a marked fall to 76%

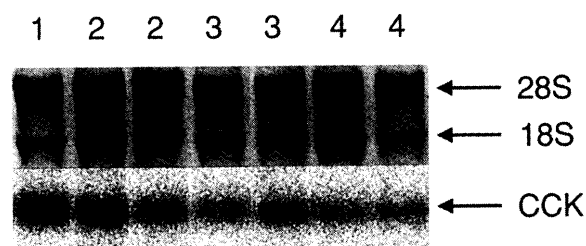


Fig. 2. Northern blot analysis of CCK mRNA in mouse olfactory bulbs at 48 hours, 7 days, and 14 days after deafferentation by intranasal irrigation with 0.17 M ZnSO₄. Control (lane 1) and deafferented mouse olfactory bulbs at 48 hours (lane 2), 7 days (lane 3) and 14 days (lane 4). Deafferentation elicited a significant time-dependent decrease in CCK mRNA. In contrast, the expression 28S and 18S rRNA was nearly equal in all the lanes.

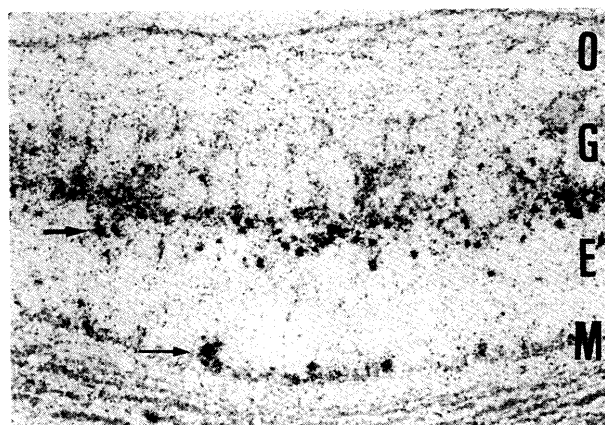


Fig. 3. Photomicrograph taken of the lateral side of the olfactory bulb demonstrates CCK mRNA label in external tufted cells, middle tufted cells, deep tufted cells, and mitral cells. External tufted cells and middle tufted cells (*large arrow*) form a thick band along the base of the glomeruli in the superficial region of the external plexiform layer (EPL). Deep tufted cells and mitral cells (*small arrow*) are scattered along the base of the EPL. O, olfactory nerve layer; G, glomerular cell layer; E, external plexiform layer; M, mitral cell layer.

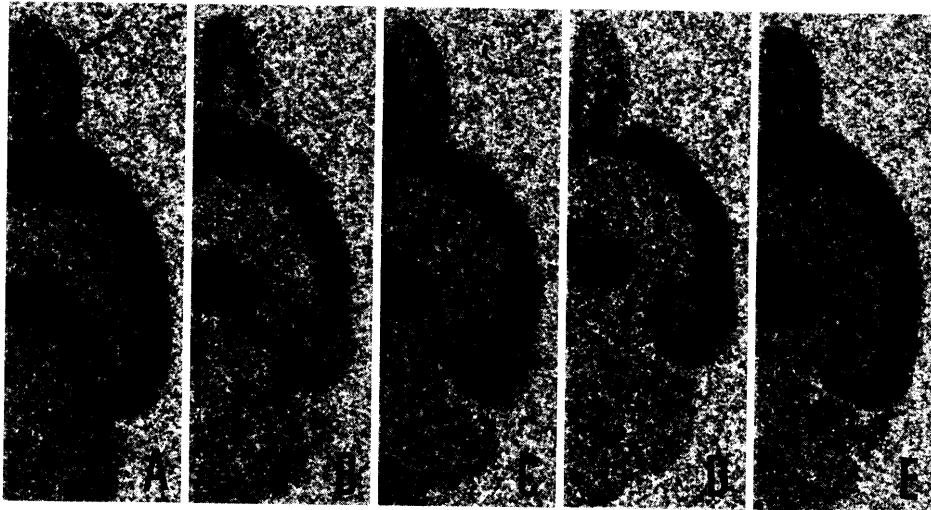


Fig. 4. *In situ* hybridized sections of the mouse brain exposed to an imaging plate for 3 days. The imaging plate was processed by the BAS 5000. Control (A) and deafferented mouse brain at 48 hours (B), 7 days (C), 14 days (D), and 30 days (E). Deafferentation revealed the gradual reduction of CCK mRNA at the external tufted cells and middle tufted cells during the time periods (arrow).

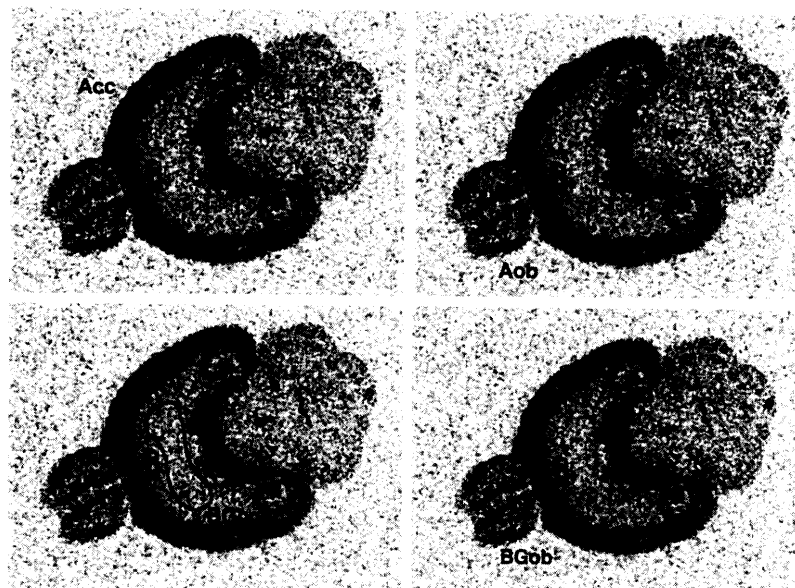


Fig. 5. Resulting images, processed and analyzed from the imaging plate system, BAS 5000. Aob was the area of the external tufted cells and middle tufted cells. BGob was PSL of the background of the bulb. Acc was the area of the cerebral cortex. BGcc was PSL of the background of the cerebral cortex.

of control levels at 48 hours, 45% at 7 days, 26% at 14 days, and 15% at 30 days.

Microscopic morphology

At 48 hours post-denervation, a moderate reduction

in CCK mRNA expression was found in external tufted cells and middle tufted cells. Further reductions were observed at 7 days, 14 days, and 30 days post-denervation. Only occasional tufted cells manifested CCK mRNA expression. Meanwhile, deep tufted cells and mitral cells did not show such a profound decrease as seen in external and middle tufted cells (Fig. 6). To quantify the difference in CCK mRNA reduction between the superficial region of EPL (external tufted and middle tufted cells) and the basal region of EPL (deep tufted cells and mitral

cells), low-power darkfield photomicrographs of horizontally sectioned olfactory bulb were analysed by NH images. In this quantification, it was also necessary to consider that the hybridized signals did not have equal radioactivity among the tissue sections. The ratio of the intensity of the superficial region of EPL to the intensity of the basal region of EPL was calculated. The ratio of the controlled mouse was 6.6. After the denervation, there was a gradual reduction; the ratio was 5.0 at 48 hours, 4.0 at 7 days, 3.1 at 14 days, and 1.1 at 30 days (Table 2), indicating that the intensity of the external and middle tufted cells showed more significant reductions than those seen in deep tufted cells and mitral cells.

Next, we counted the cell numbers to confirm whether the transneuronal change caused a substantial loss of tufted cells or mitral cells. Measurements of each of 10 sections showed that the mean numbers of the cell body decreased by 30% for the external tufted and middle tufted cells, and by 25% for the deep tufted cells and mitral cells (Table 3). These almost equivalent cell reductions demonstrated that each external tufted and middle tufted cells was

Table 1. Autoradiographic intensities at the EPL calculated by Eq. 1

Subject	CCK mRNA at the EPL
Control	0.78
48 hours	0.59
7 days	0.35
14 days	0.20
30 days	0.12

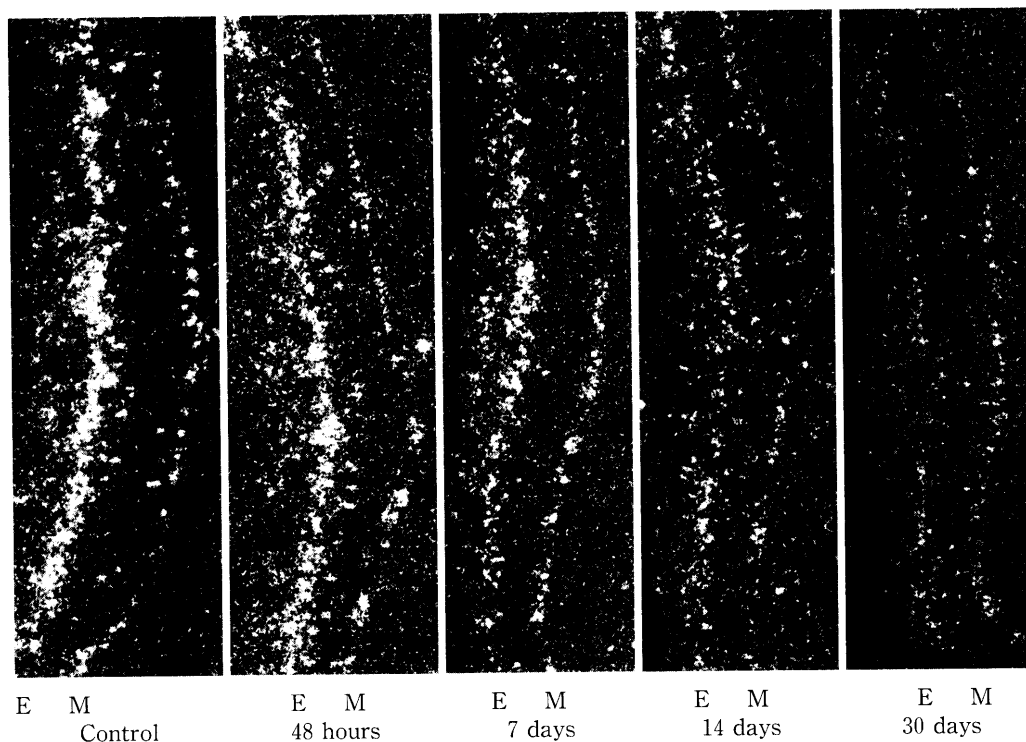


Fig. 6. The time course of the denervation induced down-regulation of CCK mRNA expression in low-power darkfield photomicrographs of the olfactory bulb. CCK mRNA was markedly reduced in external tufted cells and middle tufted cells (E), whereas CCK mRNA was not decreased so profoundly in deep tufted cells and mitral cells (M).

Table 2. Differences in CCK mRNA reduction between the superficial region of EPL (external tufted cells and middle tufted cells) and the basal region of EPL (deep tufted cells and mitral cells)

	External tufted cell and middle tufted cell	Deep tufted cell and mitral cell	A/B
Control	86.3	13.0	6.6
48 hours	87.0	17.4	5.0
7 days	59.8	14.9	4.0
14 days	37.3	12.0	3.1
30 days	5.4	4.9	1.1

The measures are computed and analyzed by NIH images.

Table 3. Average cell numbers of tufted cells and mitral cells in each of 10 horizontal sections of olfactory bulb

	External tufted cell and middle tufted cell	Deep tufted cell and mitral cell
Control	311 ± 17.9	205 ± 8.5
48 hours	293 ± 17.2	155 ± 14.6
7 days	301 ± 29.7	187 ± 27.9
14 days	235 ± 32.5	137 ± 15.2
30 days	220 ± 32.0	154 ± 12.3

The measures are means ± SE.

more severely affected than were the deep tufted cells and mitral cells in response to deafferentation.

DISCUSSION

In this study, transneuronal changes in CCK mRNA expression was studied by northern blot and *in situ* hybridization. We found that the steady-state level of CCK mRNA gradually decreased after the irreversible denervation of olfactory neuroepithelium. *In situ* hybridization observations showed that the expression of CCK in tufted and mitral cells was altered. The present results provided the first demonstration of the transneuronal modulation of CCK mRNA expression after the deafferentation of the olfactory bulb.

The most important finding was that the expression of CCK mRNA decreased markedly in external and middle tufted cells in the EPL, but was reduced only moderately in deep tufted cells and mitral cells. The number of cells per section in both the superficial region of EPL (external tufted and middle tufted cells) and the basal region of EPL (deep tufted cells and mitral cells) showed no significant discrepancies in cell reduction. We thus concluded that external and middle tufted cells are more sensitive to

deafferentation than deep tufted cells and mitral cells.

The previous study reported another finding that the expression of Na α II and Na β 1 mRNA completely disappeared in tufted cells, whereas a slight decrease in the number of cells expressing Na α II and Na β 1 mRNA was recognized in mitral cells after 5 days of denervation⁷. Tufted cells were long regarded as small mitral cells because they share many of the morphological features of mitral cells and make similar synaptic connections with the granule cells and olfactory receptor axons⁸. However, immunohistochemical studies suggested that some tufted cells did not utilize the same neurotransmitters as mitral cells. Some external tufted cells contained dopamine or substance P, something which mitral cells and deep tufted cells have not been shown to contain^{9,10}. Morphologically, the main and accessory dendrites of the tufted cells assumed a largely tangential or horizontal orientation in the outer part of the EPL, while those of the mitral cells had a more oblique and radial orientation in the inner part of the EPL¹¹. According to electrophysiological experiments, tufted cells can almost always be activated by single-pulse afferent stimulation of the olfactory nerve layer, but only 59% of mitral cells can be activated by this stimulus¹². Taking these differences

into consideration, it is possible that the tufted cells might be more severely affected than the mitral cells¹¹⁾. This different response of CCK mRNA between mitral and tufted cells suggests that tufted cells are more vulnerable to transneuronal changes than are mitral cells.

In the meanwhile, olfactory mechanisms also take part in controlling appetite. For example, during lactation the female rat is hyperphagic. Surgical removal of the olfactory epithelium of the nasal cavity significantly reduces food intake following a 20-hr fast and decreased body weight of the mother rats¹³⁾. Food pellets may be less savory to the anosmic mothers because a impaired olfactory function diminishes the flavor of food. The increased nutritional demands of lactation are partly supported by altered hormonal secretion¹⁴⁾, vagal activated release of gastrointestinal peptides¹⁵⁾, and suckling stimulus on hypothalamic 'appetite centers'¹⁶⁾. The sensory denervation might influence any of these systems. Though the significance of CCK in the brain is not so clear, it could modulate satiety. The satiety produced by food-passage into the intestine also could be brought about by injection of CCK into the hypothalamus and the hindbrain sites¹⁷⁾. A 42-hr fasting in mice significantly increased CCK binding due to an increased number of CCK receptors in the olfactory bulb and hypothalamus, but not in other brain regions¹⁸⁾. The decrease in CCK expression after the denervation of olfactory epithelium shown here suggests that CCK in the olfactory bulb might participate in controlling appetite and satiety.

Acknowledgments. We thank Professor Toshiro Kumanishi for technical and valuable advice, and Kazuo Kobayashi for assistance with *in situ* hybridization.

REFERENCES

- Olenik C, Meyer DK: Effect of partial removal of frontal or parietal bone on concentrations of mRNA coding for preprocholecystokinin and preprosomatostatin in rat neocortex. *Neuropeptides* **15**: 115-121, 1990.
- Olenik C, Lais A, Meyer DK: Effects of unilateral cortex lesions on gene expression of rat cortical cholecystokinin neurons. *Mol Brain Res* **10**: 259-265, 1991.
- Xu XJ, Puke MJC, Verge VMK, Wiesenfeld-Hallin Z, Hughes J, Hökfelt T: Upregulation of cholecystokinin in primary sensory neurons is associated with morphine insensitivity in experimental neuropathic pain in the rat. *Neurosci Letters* **152**: 129-132, 1993.
- Margolis FL, Roberts N, Ferriero D, Feldman J: Denervation in the primary olfactory pathway of mice: Biochemical and morphological effects. *Brain Res* **81**: 469-483, 1974.
- Thomas PS: Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc Natl Acad Sci USA* **77**(9): 5201-5205, 1980.
- Usui H, Katagiri T, Yoshida Y, Nishiyama A, Ichikawa T, Kuwano R, Takahashi Y, Kumanishi T: *In situ* hybridization histochemistry of Spot 35 protein, a calcium-binding protein, in the rat brain. *Mol Chem Neuropathol* **15** (3): 207-216, 1991.
- Sashihara S, Greer CA, Oh Y, Waxman SG: Cell-specific differential expression of Na (+)-channel beta 1-subunit mRNA in the olfactory system during postnatal development and after denervation. *J Neurosci* **16** (2): 702-713, 1996.
- Pinching AJ, Powell TPS: The neuron types of the glomerular layer of the olfactory bulb. *J Cell Sci* **9**: 305-345, 1971.
- Halasz N, Johansson O, Hökfelt T, Ljungdahl A, Goldstein M: Immunohistochemical identification of two types of dopamine neurons in the rat olfactory bulb as seen by serial sectioning. *J Neurocytol* **10**: 251-259, 1981.
- Davis BJ, Burd GD, Macrides F: Localization of methionin-enkephalin, substance P and somatostatin immunoreactive neurons in the main olfactory bulb of hamster. *J Comp Neurol* **204**: 377-383, 1982.
- Matthews MR, Powell TPS: Some observations on transneuronal cell degeneration in the olfactory bulb of the rabbit. *J Anat* **96**: 89-102, 1962.
- Schneider SP, Scott JW: Orthodromic response of rat olfactory bulb mitral and tufted cells correlate with their projection patterns. *J Neurophysiol* **50**: 358-378, 1983.
- Ferreira A, Dahlof LG, Stefan H: Olfactory mechanism in the control of maternal aggression, appetite, and fearfulness: Effects of lesions to olfactory receptors, mediodorsal thalamic nucleus, and insular prefrontal cortex. *Behav Neurosci* **101**: 709-717, 1987.
- Fleming AS: Control of food-intake in the lactating rat: Role of suckling and hormones. *Physiol Behav* **17**: 841-848, 1976.
- Uvnäs-Moberg K: Release of gastrointestinal peptides in response to vagal activation induced by electrical stimulation, feeding and suckling. *J Auton Nerv Syst* **9**: 141-155, 1983.
- Cotes PM, Cross BA: The influence of suckling on food intake and growth of adult female rats. *J Endocrinol* **220**: 168-190, 1954.
- Blevins JE, Stanley BG, Reidelberger RD: Brain regions where cholecystokinin suppresses feeding in rats. *Brain Res* **860**: 1-10, 2000.
- Saito A, Williams JA, Goldfine ID: Alterations in brain cholecystokinin receptors after fasting. *Nature* **289**: 599-600, 1981.