# Expression of Secretin in Porcine and Rat Central Nervous System

## Kazuaki Iguchi, Hiroyuki Yamamoto, Masaki Ogata, Tohru Mochizuki,<sup>1</sup> and Minoru Hoshino\*

Laboratory of Bioorganic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan

(Received August 23, 2004; Accepted September 6, 2004)

Many intestinal hormones are also distributed throughout the central nervous system (CNS). However, the existence of secretin, a typical intestinal hormone, has not been clearly shown in the brain. There have been conflicting data concerning the expression of secretin in the CNS. The aim of this study was to confirm the existence of secretin in the CNS immunochemically and histochemically. Proforms of secretin were detected in porcine cortex extracts by gel filtration chromatography and reverse-phase HPLC with a radioimmunoassay using different specific antibodies raised against secretin and prosecretin (1-41), indicating the production and processing of secretin precursor in the CNS. An immunocytochemical search in several regions of the rat CNS with the two antibodies revealed that secretin-positive neurons were concentrated in a limited area ranging from the corner of the dentate gyrus to the molecular layer in the hippocampus. *In situ* hybridization also supported this finding. The limited location of secretin-positive cells shown here caused difficulty in detecting secretin in the CNS. The hippocampus is thought to contribute to aging, and secretinergic neurons in this region might play an important role. This study suggests that secretin is produced in the CNS and may act as a neurotransmitter or neuromodulator in the hippocampus.

Key words — secretin, central nervous system, hippocampus, proform, immunoreactivity, histochemistry

#### INTRODUCTION

Secretin was discovered by Bayliss and Starling<sup>1</sup>) and was first isolated and sequenced from the porcine upper intestine as a 27-amino acid peptide with a C-terminal amide.<sup>2,3</sup> Rat secretin was also isolated, showing a high similarity in the primary structure to porcine secretin with only one residue substitution at position 14.<sup>4</sup> The structure of the secretin precursor has been deduced by cloning cDNA for rats<sup>5,6</sup> and pigs.<sup>5</sup> Rat prosecretin consists of 113 amino acid residues: N-terminal peptide (10 residues), secretin peptide, Gly-Lys-Arg (processing and amidating sequence) and C-terminal peptide (72 residues). Rat secretin corresponds to prosecretin (11-38) amide. Four bioactive proforms of secretin have been isolated from the porcine intestine: secretin-Gly,<sup>7)</sup> secretin-Gly-Lys-Arg,<sup>8)</sup> a C-terminally extended form (secretin-Gly-Lys-Arg followed by 41 amino acid residues)<sup>9)</sup> and an N-terminally extended form (secretin-(–9 to 27) amide).<sup>10)</sup>

The distribution of secretin-producing cells is well established in the intestinal tract.<sup>11-15)</sup> On the other hand, there has been controversy about the distribution of secretin-like immunoreactivity (LI) in the central nervous system (CNS). Secretin-LI was detected in the rat and porcine brain and the secretin content per tissue weight for pituitary and pineal glands was reportedly higher than in the duodenum.16) Secretin-LI was also reported in extracts of rat brain, most notably in the pineal and septum.<sup>17)</sup> The molecular heterogeneity of secretin-LI from rat and canine brain extracts was shown by gel filtration and ion exchange chromatography.<sup>18)</sup> Secretin-LI was detected at a low level only in the rat cerebral cortex and pituitary by an antibody against prosecretin (1-41), but was not detected in any re-

<sup>&</sup>lt;sup>1</sup>Present address: Advanced Genome Informatics Division, Shizuoka Cancer Center Hospital and Research Institute, 1007 Shimonagakubo, Nagaizumi Sunto, Shizuoka 411–8777, Japan \*To whom correspondence should be addressed: Laboratory of Bioorganic Chemistry, School of Pharmaceutical Sciences, University of Shizuoka, 52–1 Yada, Shizuoka 422–8526, Japan. Tel.: +81-54-264-5733; Fax: +81-54-264-5909; E-mail: hoshino @smail.u-shizuoka-ken.ac.jp

gion of the CNS by an antibody against secretin.<sup>19)</sup>

In terms of secretin cDNA, Northern blot hybridization with high stringency showed only trace levels of secretin mRNA in the rat hypothalamus, brainstem and cortex, although secretin-LI was detected in several regions of the CNS.<sup>16)</sup> In contrast, polymerase chain reaction showed a wide distribution of secretin mRNA in the rat CNS,<sup>6,20)</sup> whereas secretin-LI could not be detected in the rat brain.<sup>20)</sup> By Northern blot analysis, secretin receptor was not detected in the rat<sup>21)</sup> or human<sup>22)</sup> brain, although a trace level of secretin receptor in the human brain was reported.<sup>23)</sup> On the other hand, specific binding to <sup>125</sup>I-secretin was demonstrated in the rat brain membrane.<sup>24)</sup>

Moreover, the intravenous administration of secretin has been found to improve social and language skills in autistic patients.<sup>25)</sup> Dysfunction of the mindbrain-body stress axis such as the limbic lobe and hippocampus might be included in autism. Secretin in systemic circulation might reach the CNS across the blood-brain barrier, suggesting the existence of secretinergic neurons and the role of secretin as a neurotransmitter or neuromodulator in the CNS.

From these conflicting papers, we developed the hypothesis that secretin is expressed not widely but in very restricted areas in the CNS, causing different results in immunoreactivity and mRNA levels between investigators. This study aimed to confirm the production of secretin in the CNS by the chromatographic analysis of proforms and histochemical observation of secretin-producing neurons with two types of antibodies and cDNA probe.

### MATERIALS AND METHODS

Animals ——— Sprague-Dawley male rats (9 weeks old, Nihon SLC Co., Hamamatsu, Japan) were used. Prior to experiments, the rats were housed under conditions of controlled temperature, humidity, and illumination (12 : 12 light-dark cycle) in large cages with wire mesh bottoms to prevent coprophagy. They were fed laboratory chow and water freely.

Fresh porcine brains were obtained from a local slaughterhouse.

**Radioimmunoassay** — Rabbit antibodies, RY28 against rat secretin or prosecretin (12-38) amide, and R0311 against rat prosecrein (1-41), were used for radioimmunoassay (RIA).<sup>19)</sup> Rat secretin and antibody RY28 were generously provided by Dr. N. Yanaihara. Antibody R0311 recognized both secretin and N-terminally extended proforms on an equimolar basis. Antibody RY28 strongly detected the C-terminal amide of secretin and showed only 0.03% crossreactivity with rat prosecretin (1-41) that included the whole sequence of secretin but lacked the C-terminal amide. RIA for rat prosecretin (1-41) and secretin showed crossreactivy of about 84.4% and 76.3% with porcine secretin, respectively. Prosecretin-related peptides were synthesized by solid-phase methodology and purified by reverse-phase HPLC. Porcine secretin was purchased from Peptide Institute Inc. (Osaka, Japan).

**Tissue Extraction** — Fresh porcine brains were immediately dissected on ice and the dura was removed carefully. Regions were dissected and frozen at -30°C until use. Frozen cerebral cortex (about 200 g) was cut into small blocks and heated in boiling water (four-fold volume) for 15 min. After cooling, 1 M acetic acid and 0.02% (v/v) 2-mercaptoethanol were added at a final concentration, respectively. The cortex was homogenized with a Polytron homogenizer (KINEMATICA AG, Lucerne, Switzerland). The homogenate was centrifuged, and the supernatant was incubated in octadecyl silica (ODS) gel (100 g gel, ODS-A 120-230/70, YMC Co., Kyoto, Japan) at 4°C overnight. The gel suspension was washed on a glass filter with 0.01 M HCl to remove salts and proteins, and then peptides were eluted with 70% (v/v) acetonitrile in 0.01 M HCl. The eluate was lyophilized and dissolved in 0.1 M potassium phosphate-buffered saline (PBS), followed by centrifugation. The supernatant was applied on an antibody-linked Sepharose 4B column (8 ml), eluted with 1 M acetic acid and lyophilized. Antibody R0311 was coupled to CNBr-activated Sepharose 4B (Amersham Biosciences, Piscataway, NJ, U.S.A.) according to the manufacturer's instruction. The cortex extracts were used for gel filtration chromatography and reverse-phase HPLC.

Gel Filtration Chromatography — The cortex extracts were eluted on a Sephacryl S-100 column ( $10 \times 60$  cm, Amersham Biosciences) with 1 M acetic acid as an eluent. Each fraction (1.6 ml) was lyophilized for RIA. Synthetic porcine secretin was eluted as a marker.

**Reverse-Phase HPLC** — The cortex extracts were separated by an ODS column ( $4 \times 250$  mm, Wakosil-II 5C18 AR, Wako Chemical Co., Osaka, Japan) with a linear gradient of 20–40% (v/v) acetonitrile in 0.01 M HCl over 40 min at a flow rate of 1 ml/min. The fractions (1 ml per each) were collected and lyophilized for RIA. Synthetic porcine secretin was eluted as a marker.

Immunohistochemistry — Sprague-Dawley male rats were decapitated and the brains were dissected immediately. Brains were fixed with 4% (w/v) paraformaldehyde in 0.1 M phosphate buffered saline (pH 7.4) at 4°C for 2 hr, immersed sequentially in 5% (30 min), 15% (2 hr) and 30% (overnight) sucrose in phosphate buffer at 4°C, and embedded in OTC compound (Sakura Seiki, Tokyo, Japan). Frozen tissues were sectioned at 10  $\mu$ m thickness on a cryostat (model 1720, Leitz, CA, U.S.A.) and mounted on poly-L-lysine-coated slides. After blocking with normal goat serum (Vector Laboratories, Burlingame, CA, U.S.A.) and 4% (w/v) BlockAce (Dainihon-Seiyaku Co., Osaka, Japan), the sections were incubated at 4°C overnight with antibodies RY28 against rat secretin and R0311 against rat prosecretin (1-41), respectively. Normal rabbit serum was used as a background control. The sections were then sequentially incubated with biotinylated goat anti-rabbit immunoglobulin (Vector Laboratories) as the secondary antibody and fluorescein isothiocianate (FITC)-conjugated avidin (Vector Laboratories). Nuclei were stained with 4', 6diamidino-2-phenylindole (DAPI, Sigma-Aldrich Co., St. Louis, MO, U.S.A.) at 37°C for 1 hr. The sections were coverslipped with glycerol-PBS (3:1, v/v) and examined by a laser scanning confocal microscope (LSM510, Carl Zeiss Co. Ltd., Oberkochen, Germany). FITC and DAPI were excited at 488 and 350 nm of Argon laser, respectively. At the same time, the sections were examined under a differential interference (Nomarski) system.

In situ Hybridization —— RT-PCR for secretin mRNA was performed using total RNA in rat jejunum. Primers were as follows: forward 5'TAT CAC ACC CAG AAC CCG ACC AT 3', reverse 5'ACT CCC CTT CCT CCC TCA TCT3', product size 443 bp. RT-PCR and extraction of the PCR product from agarose gel were performed with a GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences). The biotinylated DNA probe was made with a Detector Random Primer DNA Biotinylation Kit (Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) using random hexamer primers and biotin-N4-dCTP. Rat brain was fixed with 4% (w/v) paraformaldehyde in PBS at 4°C overnight, exchanged with 5% (30 min), 15% (2 hr) and 30% sucrose (overnight), and embedded in OTC compound. Thin sections were made at 5  $\mu$ m thickness in a cryostat and dried at 37°C overnight. Sections were incubated sequentially with 0.1 M triethanolamine in 0.25% (v/v) acetic acid, 4% (w/v) paraformaldehyde, 1 mg/ml priteinase K and the probe. After blocking with BlockAce, FITC-avidin D (Vector Laboratories) was added to hybridized sections. Sections were then stained with DAPI. From fixation to hybridization, diethyl pyrocarbonate (DEPC) was added to the solutions. Sections were observed with a laserscanning confocal microscope. Serial sections were stained with hematoxylin and eosin Y and observed with a light microscope.

### RESULTS

# Secretin and Proforms in the Porcine Cerebral Cortex

Figure 1 shows the gel filtration chromatography profiles of secretin-LI in a porcine cerebral cortex extract assayed with antibodies RY28 (Fig. 1a) and R0311 (Fig. 1b), respectively. In RIA with antibody RY28 specific to the C-terminal amide, the major peak corresponded to secretin (Fig. 1a). An immunoreactive peak with a slightly smaller size than secretin was also detected, and might have been produced by cleavage in the N-terminal region. With antibody R0311 recognizing both the C-terminal amide and non-amidated form, larger molecular forms of low secretin-LI were detected in addition to the secretin peak (Fig. 1b). As these peaks were not detected by antibody RY28, they must be C-terminally extended forms or processing intermediates, strongly indicating that the porcine cortex contained secretin precursor and produced secretin.

Reverse-phase HPLC revealed several forms of secretin-LI in porcine cortex extracts (Fig. 2). Both antibodies RY28 (Fig. 2a) and R0311 (Fig. 2b) detected three major immunoreactive peaks in a similar manner. The highest peak eluted at the same retention time as synthetic porcine secretin. The other peaks eluted earlier or later than secretin, respectively. From the cross-reactivity of the antibodies, these two peaks were C-terminal amide forms, which were not detected in gel filtration. As the HPLC only separated peptides of relatively low molecular weight, these two C-terminal amide forms were particularly large. Combined with a gel filtration profile and HPLC, several secretin-LI such as secretin, C-terminally extended forms and C-terminal amide forms were produced in the porcine cortex, indicating the occurrence of processing and the production of secretin from the precursor in the porcine brain.



Fig. 1. Gel Filtration Chromatography of Secretin-Like Immunoreactivity from Porcine Cerebral Cortex Extracts The porcine cerebral cortex was extracted and applied on an ODS column to remove salts and proteins. The extracts were loaded on a Sephacryl S-100 column with 1 M acetic acid as an eluent. Each fraction of 1.6 ml was collected. Synthetic porcine secretin was eluted as a control in a separate run. (a) Assayed with antibody RY28 against rat secretin. (b) Assayed with antibody R0311 against rat prosecretin (1-41). Vo: void volume, Vt: total volume.

## Secretin-Positive Neurons in Rat Hippocampus

Several regions of the rat brain were investigated immunohistochemically to identify secretin-positive cells. Secretinergic neurons were found to be close to the corner of the dentate gyrus in hippocampus (Fig. 3). Abundant secretin-LI neurons were similarly distributed when stained with both antibodies RY28 against secretin (Fig. 3A) and R0311 against prosecretin (1-41) (Fig. 3C). No secretinergic neurons were found in other regions of the hippocampus. Several fibers from secretinergic neurons were observed to elongate toward the dentate gyrus.

*In situ* hybridization confirmed the immunohitochemical results. Fluorescent pictures were arranged side by side to cover a wide area around the corner of the dentate gyrus (Fig. 4A). A serial section was stained with hematoxylin and eosin to identify the areas (Fig. 4B). Secretinergic neurons were mainly observed in an area surrounded by the corner of the dentate gyrus and the molecular layer. The other areas in the hippocampus contained no secretin neu-



Fig. 2. Reverse-Phase HPLC of Secretin-Like Immunoreactivity from Porcine Cerebral Cortex Extracts

The extracts were loaded on an ODS column with a linear gradient system of 20-40% (v/v) acetonitrile in 0.01 M HCl over 40 min. Each fraction of 1 ml was collected. Synthetic porcine secretin was developed as a control. (a) Assayed with antibody RY28 against rat secretin. (b) Assayed with antibody R0311 against rat prosecretin (1-41).

rons.

### DISCUSSION

RIA with antibody R0311 detected secretin-LI in many regions of the rat brain (data not shown), which coincided with the results of previous immunochemical reports<sup>16-18)</sup> and the findings obtained by Northern blot analyses.<sup>6,20)</sup> The content of secretin-LI was relatively high in the hypothalamus and cerebellum. However, antibody RY28 detected high immunoreactivity only in the hypothalamus. The content in the other regions was very low, nevertheless antibodies RY28 and R0311 recognized secretin in a similar manner. Our previous report<sup>19)</sup> failed to detect secretin-LI in the rat brain with antibody RY28, as the quantity of tissue used might have been too small. This lead to the speculation that low production and restricted localization of secretin in the CNS caused apparent inconsistent conclusions be-



100 µm

# Fig. 3. Immunohistochemistry of Secretin-Positive Neurons in Rat Hippocampus

The rat hippocampus was dissected into thin sections with a cryostat and immunostained with antibodies RY28 and R0311 combined with FITC-conjugated goat antibody. The sections were observed with a confocal laser-scanning microscope. (A) Stained with RY28 against rat secretin, (B) Normarsky observation of the same area as (A), (C) Stained with R0311 against rat prosecretin (1-41), (D) Normarsky observation of the same area as (C). Bar: 100  $\mu$ m.

tween investigators.<sup>6,16–20)</sup> In this paper, secretin-LI was extracted and concentrated from a large amount of porcine cortex to investigate the proforms of secretin and to confirm the production of secretin in the CNS. Second, secretin-positive cells were thoroughly searched for in the rat brain histochemically.

Two region-specific antibodies against secretin and related peptides were used for the detection of secretin-LI in the porcine brain. Antibody RY28 was raised against synthetic rat secretin or prosecretin (12-38) amide and was specific for the C-terminal amide region, showing no cross-reactivity with Cterminally extended forms.<sup>19)</sup> Antibody R0311 was raised against synthetic rat prosecretin (1-41) and equally recognized the C-terminal amide form and the C-terminally extended form of secretin.<sup>19)</sup> Gel filtration chromatography and HPLC of porcine cortex extracts revealed at least four kinds of proforms in addition to secretin. The characteristics of the antibodies described above indicated that these proforms might consist of both C-terminally extended proforms and C-terminal amidated proforms, although the exact structure of these proforms was



200 µm

Fig. 4. In situ Hybridization of Secretin cDNA in the Rat Hippocampus

Rat hippocampus thin sections were hybridized with biotinylated secretin cDNA using FITC-avidin. Serial sections were stained with hematoxylin and eosin (HE). (A) Confocal laser scanning microscopic observaton of *in situ* hybridization (green, FITC; blue, DAPI), (B) Light microscopic observation of HE staining. Bar: 200 µm.

not determined. With respect to the gel filtration profile, the proforms detected in porcine cortex extracts were somewhat similar to those in rat duodenal extracts.<sup>19)</sup> Antibody R0311 detected several immunoreactive peaks larger than secretin in gel filtration.<sup>19)</sup> On the other hand, antibody RY28 detected one peak slightly larger than secretin.<sup>19)</sup> The detection of proforms in the porcine cortex strongly suggested the production and processing of secretin precursor in the CNS as in the intestinal tract. The immunoreactivity of secretin has been previously reported as a single peak in HPLC of rat, porcine and canine brain extracts.<sup>16,18)</sup> These proform results suggested the processing and production of secretin in the CNS.

Our preliminary attempt revealed that a trace of secretin-positive cells was found in the rat cerebral cortex (data not shown). In this study, an immunohisotochemical search revealed a dense area of secretin-positive cells around the corner of the dentate gyrus in the rat hippocampus with both antibodies RY28 and R0311. Secretinergic neurons were not detected in other areas in the hippocampus as far as could be examined. From immunohistochemistry, secretinergic neurons were distributed in limited areas of the hippocampus. In situ hybridization also showed a similar location in the area circled by the corner of the dentate gyrus and the molecular layer. The administration of secretin has been reported to improve social and language skills in autistic patients.<sup>25)</sup> Secretinergic neurons in hippocampus might be undeveloped in autistic children. Moreover, a change in the expression of secretin and receptor mRNA was shown in the hippocampus, amygdala and cerebellum during aging,<sup>26)</sup> although not all areas were presented. Secretinergic neurons in the dentate gyrus might therefore also play an important role in aging and behavior. Independently, the location of secretinergic neurons was shown in the rat hypothalamic paraventricular nucleus (PVN).27)

This study identified the production and processing of secretin and restricted distribution of secretinergic neurons in the CNS. The function of secretin as a neurotransmitter or neuromodulator in the CNS can therefore be investigated.

Acknowledgements The authors greatly appreciate Dr. Noboru Yanaihara for his encouragement and discussion, and also thank E. Arai, T. Yumoto, A. Miyai and F. Kajiyama for experimental support.

### REFERENCES

- Bayliss, W. M. and Starling, E. H. (1902) The mechanism of pancreatic secretion. *J. Physiol.* (London), 28, 325–353.
- Jorpes, E. and Mutt, V. (1961) On the biological activity and amino acid composition of secretin. *Acta Chem. Scand.*, 15, 1790–1791.
- Mutt, V., Jorpes, J. E. and Magnusson, S. (1970) Structure of porcine secretin. The amino acid sequence. *Eur. J. Biochem.*, 15, 513–519.
- 4) Gossen, D., Vandermeers, A., Vandermeers-Piret, M. C., Rathe, J., Cauvin, A., Robberecht, P. and Christophe, J. (1989) Isolation and primary structure of rat secretin. *Biochem. Biophys. Res. Commun.*, 160, 862–867.
- Kopin, A. S., Wheeler, M. B. and Leiter, A. B. (1990) Secretin: Structure of the precursor and tissue distribution of the mRNA. *Proc. Natl. Acad. Sci. U.S.A.*, 87, 2299–2303.
- 6) Itoh, N., Furuya, T., Ozaki, K., Ohta, M. and Kawasaki, T. (1991) The secretin precursor gene: structure of the coding region and expression in the

brain. J. Biol. Chem., 266, 12595–12598.

- Carlquist, M. and Rokaeus, A. (1984) Isolation of a proform of porcine secretin by ion-exchange and reversed-phase high-performance liquid chromatography. *J. Chromatogr.*, **296**, 143–151.
- Gafvelin, G., Carlquist, M. and Mutt, V. (1985) A proform of secretin with high secretin-like bioactivity. *FEBS Lett.*, **184**, 347–352.
- Gafvelin, G., Jornvall, H. and Mutt, V. (1990) Processing of prosecretin: Isolation of a secretin precursor from porcine intestine. *Proc. Natl. Acad. Sci.* U.S.A., 87, 6781–6785.
- Bonetto, V., Jornvall, H., Mutt, V. and Sillard, R. (1995) Two alternative processing pathways for a preprohormone: A bioactive form of secretin. *Proc. Natl. Acad. Sci. U.S.A.*, **92**, 11985–11989.
- 11) Bloom, S. R. (1974) Hormones of the gastrointestinal tract. *Br. Med. Bull.*, **30**, 62–67.
- Yanaihara, N., Sakagami, M., Sato, H., Yamamoto, K. and Hashimoto, T. (1977) Immunological aspects of secretin, substance P, and VIP. *Gastroenterology*, 72, 803–810.
- 13) Miller, T. A., Llanos, O. L., Swierczek, J. S., Rayford, P. L. and Thompson, J. C. (1978) Concentrations of gastrin and secretin in the alimentary tract of the cat. *Surgery*, **83**, 90–93.
- Straus, E. and Yalow, R. S. (1978) Immunoreactive secretin in gastrointestinal mucosa of several mammalian species. *Gastroenterology*, 75, 401–404.
- Paquette, T. L., Shulman, D. F., Alpers, D. H. and Jaffe, B. M. (1982) Postnatal development of intestinal secretin in rats and guinea pigs. *Am. J. Physiol.*, 243, G511–G517.
- 16) O'Donohue, T. L., Charlton, C. G., Miller, R. L., Boden, G. and Jacobowitz, D. M. (1981) Identification characterization, and distribution of secretin immunoreactivity in rat and pig brain. *Proc. Natl. Acad. Sci. U.S.A.*, 78, 5221–5224.
- Samson, W. K., Lumpkin, M. D. and McCann, S. M. (1984) Presence and possible site of action of secretin in the rat pituitary and hypothalamus. *Life Sci.*, 34, 155–163.
- 18) Chang, T., Berger-Ornstein, L. and Chey, W. Y. (1985) Presence of biologically and immunologically active secretin-like substance in the mammalian brain. *Peptides*, 6, 193–198.
- 19) Yu, X., Kajiyama, F., Iguchi, K., Mochizuki, T., Hoshino, M., Jun, L., Luo, W. Q., Yanaihara, N., Winarto, A. and Iwanaga, T. (1999) Development of region-specific radioimmunoassays against rat prosecretin or secretin and their characterization. *Biomed. Res.*, **20**, 15–26.
- Ohta, M., Funakoshi, S., Kawasaki, T. and Itoh, N. (1992) Tissue-specific expression of the rat secretin

precursor gene. *Biochem. Biophys. Res. Commun.*, **183**, 390–395.

- 21) Ishihara, T., Nakamura, S., Kaziro, Y., Takahashi, T., Takahashi, K. and Nagata, S. (1991) Molecular cloning and expression of a cDNA encoding the secretin receptor. *EMBO J.*, **10**, 1635–1641.
- 22) Chow, B. K. (1995) Molecular cloning and functional characterization of a human secretin receptor. *Biochem. Biophys. Res. Commun.*, 212, 204–211.
- 23) Patel, D. R., Kong, Y. and Sreedharan, S. P. (1995) Molecular cloning and expression of a human secretin receptor. *Mol. Pharmacol.*, 47, 467–473.
- 24) Fremeau, R. T., Jr., Jensen, R. T., Charlton, C. G., Miller, R. L., O'Donohue, T. L. and Moody, T. W.

(1983) Secretin: specific binding to rat brain membranes. *J. Neurosci.*, **3**, 1620–1625.

- 25) Robinson, T. W. (2001) Homeopathic secretin in autism: a clinical pilot study. *Br. Homeopath. J.*, **90**, 86–91.
- 26) Tay, J., Goulet, M., Rushce, J. and Boismenu, R. (2004) Age-related and regional differences in secretin and secretin receptor mRNA levels in the rat brain. *Neurosci. Lett.*, **366**, 176–181.
- 27) Welch, M. G., Keune, J. D., Welch-Horan, T. B., Anwar, N., Anwar, M., Ludwig, R. J. and Ruggiero, D. A. (2004) Secretin: Hypothalamic distribution and hypothesized neuroregulatory role in autism. *Cell. Mol. Neurobiol.*, 24, 219–241.