Atrial natriuretic peptide is eliminated from the brain by natriuretic peptide receptor-C-mediated brain-to-blood efflux transport at the blood–brain barrier

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Cerebral atrial natriuretic peptide (ANP), which is generated in the brain, has functions in the regulation of brain water and electrolyte balance, blood pressure and local cerebral blood flow, as well as in neuroendocrine functions. However, cerebral ANP clearance is still poorly understood. The purpose of this study was to clarify the mechanism of blood–brain barrier (BBB) efflux transport of ANP in mouse. Western blot analysis showed expression of natriuretic peptide receptor (Npr)-A and Npr-C in mouse brain capillaries. The brain efflux index (BEI) method confirmed elimination of [¹²⁵I]human ANP (hANP) from mouse brain across the BBB. Inhibition studies suggested the involvement of Npr-C *in vivo*. Furthermore, rapid internalization of [¹²⁵I]hANP by TM-BBB4 cells (an *in vitro* BBB model) was significantly inhibited by Npr-C inhibitors and by two different Npr-C-targeted short interfering RNAs (siRNAs). Finally, treatment with 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) significantly increased Npr-C expression in TM-BBB4 cells, as determined by liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based targeted absolute proteomics. Our results indicate that Npr-C mediates brain-to-blood efflux transport of ANP at the mouse BBB as a pathway of cerebral ANP clearance. It seems likely that levels of natriuretic peptides in the brain are modulated by 1,25(OH)₂D₃ through upregulation of Npr-C expression at the BBB.

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Introduction

Natriuretic peptides in the central nervous system have been proposed to be involved in the central control of cardiovascular and neuroendocrine functions (Imura *et al*, 1992). Atrial natriuretic peptide (ANP), originally identified in the heart and peripheral tissues, has been detected in rodent brain and human brain by radioimmunoassay and immunohistochemistry (McKenzie *et al*, 1994; Morii *et al*, 1985). Accumulating evidence indicates that cerebral ANP

has important functions in the regulation of brain water and electrolyte balance, blood pressure and local cerebral blood flow (Imura *et al*, 1992; Levin *et al*, 1998; Nakao *et al*, 1992), as well as in neuroendocrine functions (Itoh *et al*, 1986; Yamada *et al*, 1986). Therefore, changes in cerebral ANP levels may affect central nervous system homeostasis.

The cerebral level of ANP is thought to be controlled not only by production, but also by clearance, as peripheral clearance regulates the ANP level in circulating blood (Chiu *et al*, 1991). We have reported that the blood–brain barrier (BBB) possesses a brain-to-blood efflux transport system for neuromodulators and their metabolites as a part of the cerebral clearance system (Terasaki and Ohtsuki, 2005). As regards peptides, it was reported that a somatostatin analogue was bound to and internalized into isolated brain microvessels, and this internalization was proposed to be one mechanism of inactivation of neuropeptides (Pardridge *et al*, 1985). [¹²⁵I]Human ANP (hANP) binding studies

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indicated the existence of an ANP binding receptor in brain capillaries (Chabrier *et al*, 1987; Steardo and Nathanson, 1987). These reports raised the possibility that the BBB possesses a receptor-mediated brain-to-blood efflux transport system for ANP, although there is as yet no report demonstrating the elimination of ANP across the BBB.

The ANP exerts its biological effects by binding to guanylyl-cyclase (GC)-linked natriuretic peptide receptor (Npr)-A and non-GC-linked Npr-C with similar affinity (Potter *et al*, 2006). The Npr-A is involved in signal transduction through elevation of intracellular cyclic guanosine monophosphate, whereas Npr-C acts as a clearance receptor to modulate extracellular ANP levels through internalization and degradation. Interestingly, [¹²⁵I]hANP was cross-linked to a 60- to 70-kDa protein in bovine brain capillary endothelial cells (Whitson *et al*, 1991), and the size of Npr-C is almost the same as this (Fuller *et al*, 1988), whereas that of Npr-A is different (~135 kDa) (Oliver *et al*, 1997). Therefore, if the BBB possesses an efflux transport system for cerebral clearance of ANP, Npr-C seems to be a good candidate. However, it is still not known whether Npr-C protein is expressed in brain capillaries.

The purpose of this study was to identify the mediator of ANP clearance across the BBB by application of the brain efflux index (BEI) method (Kakee *et al*, 1996; Nishida *et al*, 2009) and to investigate the protein expression of Npr-A and Npr-C in brain capillaries by Western blot analysis. Further, the transport mechanism of ANP at the BBB was investigated by means of cellular uptake experiments, and the regulatory mechanism of Npr-C at the BBB was examined by highly selective quantification with liquid chromatography–tandem mass spectrometry (LC-MS/MS)-based targeted absolute proteomics (Kamiie *et al*, 2008) in conditionally immortalized mouse brain capillary endothelial cells, TM-BBB4 (Hosoya *et al*, 2000).

Materials and methods

Reagents

[¹²⁵I]Human ANP (2000 Ci/mmol) radioiodinated with sodium [¹²⁵I]iodide and lactoperoxidase was purchased

from GE Healthcare (Buckinghamshire, UK) and Institute of Isotopes Co., Ltd (Budapest, Hungary). [³H]Dextran (100 mCi/mg) was obtained from American Radiolabeled Chemicals, Inc. (St Louis, MO, USA). Unlabeled hANP and human C-type natriuretic peptide (hCNP) were purchased from Peptide Institute (Osaka, Japan). cANP(4-23) was obtained from Phoenix Pharmaceuticals, Inc. (Burlingame, CA, USA). 1 α ,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) was purchased from Biomol Research Laboratories (Plymouth Meeting, PA, USA). Xylazine hydrochloride was obtained from Sigma-Aldrich (St Louis, MO, USA). Ketalar 50 (ketamine hydrochloride) was purchased from Sankyo Co. (Tokyo, Japan). All peptides (purity >95%) listed in Table 1 were synthesized by Thermolectron Corporation (Sedanstrabe, Germany). All other chemicals were analytical grade commercial products.

Animals

Male C57BL/6 mice (8 to 10 weeks old) were purchased from Japan SLC (Hamamatsu, Japan). All experiments were approved by the Animal Care Committee of the Graduate School of Pharmaceutical Sciences, Tohoku University.

TM-BBB4 Cell Culture

The TM-BBB4 cell line established from transgenic mice harboring the temperature-sensitive SV40 large T-antigen gene was used in this study (Hosoya *et al*, 2000). TM-BBB4 cells were cultured at 33°C in Dulbecco's modified Eagle's medium (DMEM; Nissui Pharmaceutical Co., Tokyo, Japan), supplemented with 20 mmol/L NaHCO₃, 2 mmol/L L-glutamine, 15 ng/mL endothelial cell growth factor, 100 U/mL benzyl penicillin, 100 mg/mL streptomycin sulfate, and 10% fetal bovine serum (Moregate, Bulimba, Australia) in an atmosphere of 95% air and 5% CO₂.

Western Blot Analysis

Under deep anesthesia induced with pentobarbital, mice were transcardially perfused with phosphate-buffered saline to remove blood, and then tissues were collected. The brain capillary fraction was separated from mouse cerebrum by the glass bead column method using the reported procedure (Ohtsuki *et al*, 2007). The crude

Table 1 Peptide probe sequences and selected ions for quantification of each protein with LC-MS/MS in the MRM mode

Protein	Accession no.	Probe sequence	MRM channel (m/z)				
			Q1	Q3-1	Q3-2	Q3-3	Q3-4
Npr-C	P70180	ALFSLVDR	460.8	736.4	589.3	502.3	—
		ALFSL*VDR	464.3	743.4	596.3	509.3	—
Na ⁺ /K ⁺ ATPase	Q64436	VDNSSLTGESEPPQTR	810.4	501.3	717.4	630.3	846.4
		VDNSSLTGESEPP*QTR	813.4	507.3	723.4	636.3	852.4

Conditions of MRM analysis optimized for high signal intensity after direct injection of peptide solution into mass spectrometer through turbo ion spray source. Theoretical *m/z* values of doubly charged ions of intact peptides (Q1) assumed as precursor ions. Three of four singly charged fragment ions derived from precursor ion indicated as Q3-1, -2, -3, and -4. Bold letters with asterisks indicate amino-acid residues labeled with stable isotope (¹³C and ¹⁵N).

membrane protein fraction of mouse kidney, brain capillary-rich fraction and TM-BBB4 cells were prepared using nitrogen cavitation. Briefly, the cells were washed with 1 mL phosphate-buffered saline twice and then scraped in 10 mmol/L Tris-HCl (pH 7.4) containing 250 mmol/L sucrose and protease-inhibitor cocktail (Sigma-Aldrich) and collected in a precooled cavitation chamber. The cell suspension was stirred under a pressure of 450 p.s.i. for 15 minutes. The crude membrane protein fraction of TM-BBB4 cells treated with or without 10 or 100 nmol/L $1,25(\text{OH})_2\text{D}_3$ was isolated using the reported procedure (Kamiie *et al*, 2008). TM-BBB4 cells were homogenized in buffer containing 10 mmol/L Tris-HCl (pH 7.4), 10 mmol/L NaCl, 1.5 mmol/L MgCl_2 , 1 mmol/L and a protease-inhibitor cocktail (Sigma-Aldrich). These homogenized samples were centrifuged at 10,000g for 10 minutes and the supernatants were collected. These supernatants were centrifuged at 100,000g for 30 minutes. The crude membrane fraction was obtained from the resulting pellet, which was suspended in 10 mmol/L Tris-HCl (pH 7.4) containing 250 mmol/L sucrose. Protein concentrations were measured by the Lowry method using the DC protein assay reagent (Bio-Rad, Hercules, CA, USA). The crude membrane fraction of proteins was boiled for 5 minutes and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1 to 4.7 μg per lane). The separated proteins were electrotransferred to polyvinylidene difluoride membranes (GE Healthcare). After incubation with 4% skim milk (Meiji Seika, Osaka, Japan) for 1 hour at room temperature, the membranes were incubated with goat anti-Npr-A (0.2 $\mu\text{g}/\text{mL}$, R&D system, Minneapolis, MN, USA) or goat anti-Npr-C antibody (1 $\mu\text{g}/\text{mL}$, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight. Membranes were washed four times with 0.1% Tween 20/phosphate-buffered saline and then incubated with horseradish peroxidase-conjugated donkey anti-goat immunoglobulin G (1:1,000; R&D Systems) for 1 hour at room temperature. Immunoreactivity was visualized with an enhanced chemiluminescence kit (ECL Plus, GE Healthcare).

In Vivo Brain Efflux Index Study

The *in vivo* brain elimination experiments were performed using the intracerebral microinjection technique reported earlier (Kakee *et al*, 1996; Nishida *et al*, 2009). Briefly, mice were anesthetized with an intramuscular injection of xylazine (1.22 mg/kg) and ketamine (125 mg/kg), and placed in a stereotaxic frame (SRS-6; Narishige, Tokyo, Japan) that determines the coordinates of the mouse brain coinciding with the secondary somatosensory cortex (S2) region. A small hole was made 3.8 mm lateral to the bregma, and a fine injection needle fitted on a 5.0- μL microsyringe (Hamilton, Reno, NE, USA) was advanced to a depth of 2.5 mm. The applied solution (0.30 μL) containing each [^{125}I]hANP and [^3H]dextran in an extracellular fluid (ECF) buffer (122 mmol/L NaCl, 25 mmol/L NaHCO_3 , 3 mmol/L KCl, 1.4 mmol/L CaCl_2 , 1.2 mmol/L MgSO_4 , 0.4 mmol/L K_2HPO_4 , 10 mmol/L D-glucose, and 10 mmol/L HEPES, pH 7.4) was administered into the S2 region over

a period of 30 seconds. After microinjection, the microsyringe was left in place for 4 minutes to minimize any backflow. At designated times after microinjection, cerebrospinal fluid was collected from the cisterna magna and then ipsilateral (left) and contralateral (right) cerebrum and cerebellum were excised and dissolved in 2.0 mL 2 mol/L NaOH at 60°C for 1 hour. The [^{125}I] and [^3H] radioactivity of the samples were measured in a γ -counter (ART300, Aloka, Tokyo, Japan) for 3 minutes and in a liquid scintillation counter (TRI-CARB2050CA, Packard Instruments, Meriden, CT, USA) for 5 minutes, respectively. The BEI was defined by Eq. 1, and the percentage of substrate remaining in the ipsilateral cerebrum (100-BEI) was determined using Eq. 2:

$$\text{BEI (\%)} = \frac{\text{test substrate undergoing efflux at the BBB}}{\text{test substrate injected into the brain}} \times 100 \quad (1)$$

$$100 - \text{BEI (\%)} = \frac{\frac{\text{amount of test substrate in the brain}}{\text{amount of reference in the brain}}}{\frac{\text{concentration of test substrate injected}}{\text{concentration of test reference injected}}} \times 100 \quad (2)$$

The apparent elimination rate constant (K_{el}) was determined from the slope given by fitting a semilogarithmic plot of (100-BEI) versus time, using the nonlinear least-squares regression analysis program MULTI (Yamaoka *et al*, 1981).

To characterize the efflux transport system at the BBB, the percentage of [^{125}I]hANP remaining in the brain at 30 minutes was determined in the presence or absence of preadministration of inhibitors. When the inhibitor concentration at the microinjection site could not be maintained high enough by coadministration, or to avoid possible physicochemical interactions, a sufficient volume (30 μL) of the inhibitor solution was preinjected 5 minutes before at the microinjection site of [^{125}I]hANP to minimize the dilution effect. The percentage of [^{125}I]hANP remaining in the brain at 30 minutes when 30 μL of ECF buffer was preinjected was not significantly different from that of the coadministered control ($P = 0.492$) (Figure 2).

In Vitro Internalization Study in TM-BBB4 Cells

The internalization of [^{125}I]hANP into TM-BBB4 cells was examined as described earlier (Ito *et al*, 2007). Briefly, TM-BBB4 cells were seeded on a collagen-coated 24-well plate at a density of 1.0×10^5 cells/well (Becton Dickinson, Bedford, MA, USA) and cultured for 48 hours. The extent of TM-BBB4 cell-[^{125}I]hANP (0.015 $\mu\text{Ci}/200 \mu\text{L}$ in ECF buffer) binding was measured at 37°C. After the indicated times, the ECF buffer was removed, and the cells were washed three times with 1 mL ice-cold ECF buffer. The cells were then incubated with 1 mL ice-cold acetate-barbital buffer (28 mmol/L CH_3COONa , 120 mmol/L NaCl, 20 mmol/L barbital sodium (pH 3.0) and 360 mOsm/kg) for 20 minutes at 4°C to remove [^{125}I]hANP bound to the cell surface. After incubation, the buffer was recovered (fraction of acid-soluble binding) and the cells were washed three times with ice-cold acetate-barbital buffer.

Acid-resistant binding represents the amount of internalized [^{125}I]hANP in the TM-BBB4 cells. The cells were solubilized with 200 μL of 5 mol/L NaOH overnight and

neutralized with 200 μ L of 5 mol/L HCl. [125 I]Radioactivity was measured using a γ -counter (ART310, Aloka). The protein content of the cultured cells was measured using a DC protein assay kit (Bio-Rad) with bovine serum albumin as a standard. Total binding was obtained as the sum of acid-resistant binding and acid-soluble binding.

For kinetic studies, the Michaelis–Menten constant (K_m) and the maximum rate (V_{max}) of acid-resistant binding of hANP, which reflected internalization into cells, were calculated from Eq. 3 using the nonlinear least-squares regression analysis program, MULTI (Yamaoka *et al*, 1981).

$$V = V_{max1} \times C / (K_{m1} + C) + V_{max2} \times C / (K_{m2} + C) \quad (3)$$

where V is the uptake velocity of the substrate, C is the substrate concentration of the medium, K_m is the Michaelis constant, and V_{max} is the maximal uptake velocity.

The half-saturation constant (K_d) for the acid-soluble binding of hANP was calculated from Eq. 3 by replacing K_m , V , and V_{max} with K_d , the binding of the substrate (B), and the maximal binding capacity (B_{max}), respectively.

Transfection of Short Interfering RNA into TM-BBB4 Cells

Short interfering RNA (siRNA) knockdown of mNpr-C was accomplished using the following sequences: siRNA #1 sense-5'-ACACGACUCUGAAGCUAAA-3' and antisense-5'-UUUAGC UUCAGAGUCGUGU-3'; siRNA #2 sense-5'-GGU CAUUGGUGAUUACUUU-3' and antisense-5'-AAAGUAA UCACCAAUGACC-3' and negative control siRNA (Ambion/Applied Biosystems, Foster City, CA, USA). Because of low transfection efficiency and toxicity of the transfection reagent to TM-BBB4 cells, we examined four different siRNAs and various transfection conditions, including time of incubation, siRNA concentration, medium volume, chemically modified siRNA, and lipofection reagents, to optimize the conditions of siRNA treatment of TM-BBB4 cells. Briefly, TM-BBB4 cells were seeded into collagen-coated 24-well plates at 0.75×10^5 cells/well, and grown for 24 hours in DMEM supplemented with 10% FBS. TM-BBB4 cells were transfected with 5 nmol/L mouse Npr-C siRNA-#1, #2 or negative control siRNA using Lipofectamine RNAiMAX (1 μ L/200 μ L/well; Invitrogen, Carlsbad, CA, USA) and OPTI-MEM I reduced serum medium (Invitrogen). Five hours after transfection, 200 μ L DMEM supplemented with 20% FBS and 600 μ L DMEM supplemented with 10% FBS were added to each well, and incubation was continued for 43 hours. The mRNA expression level and the transport activity were examined at 48 hours after the transfection.

Quantitative Real-Time Polymerase Chain Reaction Analysis

Total RNA was extracted from TM-BBB4 cells with an RNeasy kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol. RNA integrity was checked by electrophoresis on agarose gel. Single-stranded cDNA was

prepared from 1 μ g total RNA by reverse transcription (ReverTraAce, Toyobo, Osaka, Japan) using oligo (dT) primer. The sequences of the primers were as follows: sense primer 5'-TCT GCC TAC AAT TTC GAC GAG-3' and antisense primer 5'-CAC AGA GAA GTC CCC ATA CCG-3' for Npr-C (GenBank accession number; NM_008728); sense primer 5'-TTT GAG ACC TTC AAC ACC CC-3' and antisense primer 5'-ATA GCT CTT CTC CAG GGA GG-3' for β -actin (GenBank accession number; NM_031144). Quantitative real-time PCR analysis was performed using an ABI PRISM 7700 sequence detector system (Applied Biosystems) with $2 \times$ SYBR Green PCR Master Mix (Applied Biosystems) according to the manufacturer's protocol. To quantify the amount of specific mRNA in the samples, a standard curve was generated for each run using pGEM-T Easy vector (Promega, Madison, WI, USA) containing Npr-C or β -actin (dilution ranging from 0.1 fg/ μ L to 1 ng/ μ L). This enabled standardization of the initial mRNA content of cells relative to the amount of β -actin.

Quantitation of Proteins Using LC-MS/MS System

Quantitation of crude membrane fraction of proteins in TM-BBB4 cells was performed using a nano-LC-MS/MS system as described earlier (Kamiie *et al*, 2008; Niessen *et al*, 2009). Tryptic peptides were chosen for synthesis based on in-silico selection criteria as described earlier (Kamiie *et al*, 2008). The amino-acid sequences and m/z values of the precursor ion and four product ions for each protein are given in Table 1.

The proteins of the crude membrane fraction were suspended in 100 mmol/L Tris-HCl (pH 8.5) containing 8 mol/L urea. The proteins were S-carbamoylmethylated as described (Mawuenyega *et al*, 2003). The alkylated proteins were fourfold diluted with 100 mmol/L Tris-HCl (pH 8.5) and digested with TPCK-treated trypsin (Promega) at an enzyme/substrate ratio of 1:100 at 37°C for 16 hours.

The tryptic digests were acidified with formic acid and 50 fmol of the stable isotope-labeled peptides were spiked into the digest to provide internal references. The mixtures were then analyzed with a nano-LC system (LC-assist, Tokyo, Japan) connected to an ESI-triple quadrupole mass spectrometer (4000Q trap, Applied Biosystems). Nano-LC was performed with a direct nano flow spray tip reversed-phase column (150- μ m inner diameter $\times$ 50 mm) of Mightysil-C18 (3- μ m particles; Kanto Chemicals, Tokyo, Japan) connected in tandem through an automated solvent desalting device and nano ion source (Nanospray, Applied Biosystems). Linear gradients of 1% to 35% acetonitrile in 0.1% formic acid were applied to elute the peptides at a flow rate of 100 nL/min for 35 minutes. Four products derived from single peptides are monitored in specific m/z channels. Individual signal peaks are identified on the basis of equal retention times in each channel of three to four product ions (Table 1). The ion counts in the chromatograms were determined by using the quantitation procedures in Analyst software version 1.4.2 (Applied Biosystems). To obtain the amounts of target proteins, the peptides were quantified by calculating the ratios of the

peak areas to those of isotope-labeled peptides as described earlier (Kamiie *et al*, 2008).

Data Analysis

Unless otherwise indicated, all data represent the mean $\pm$ s.e.m. Unpaired two-tailed Student's *t*-tests were used to determine the significance of differences between the means of two groups. One-way analysis of variance followed by Dunnett's test was used to assess the statistical significance of differences among the means of more than two groups.

Results

Expression of Natriuretic Peptide Receptor-A and Natriuretic Peptide Receptor-C in Mouse Isolated Brain Capillaries and TM-BBB4 Cells

Protein expression of Npr-A and Npr-C in mouse isolated brain capillaries and TM-BBB4 cells, which are conditionally immortalized mouse brain capillary endothelial cells, was determined by Western blot analysis. As shown in Figure 1A, bands reacting with anti-Npr-A antibody were observed at ~ 130 kDa in mouse isolated brain capillaries, but not in TM-BBB4 cells. A band of the same size was detected in the kidney, which has been reported to express Npr-A and Npr-C (Fuller *et al*, 1988; Oliver *et al*, 1997). As shown in Figure 1B, bands of ~ 65 kDa were detected with anti-Npr-C antibody in mouse isolated brain capillaries and TM-BBB4 cells, as well as in the kidney, and the band intensity in TM-BBB4 cells was much greater than that in isolated brain capillaries. These results indicate that Npr-A and Npr-C are expressed in mouse brain capillary endothelial cells, and Npr-C is highly expressed in TM-BBB4 cells.

Figure 1 Expression of natriuretic peptide receptor (Npr)-A and Npr-C proteins in mouse brain capillaries, TM-BBB4 cells, and kidney. The crude membrane fractions (1 to 4.7 μ g) were subjected to Western blot analysis. The bands at 130 kDa (**A**) and 65 kDa (**B**) are indicated by arrows on the right-hand side. BCAP, brain capillaries.

[125 I]Human Atrial Natriuretic Peptide Elimination from the Brain After Intracerebral Microinjection

The *in vivo* elimination of hANP from the brain across the BBB was examined by means of the BEI method. As shown in Figure 2A, the percentage of [125 I]hANP remaining in the brain decreased time dependently with an K_{el} of $3.64 \times 10^{-2} \pm 0.35 \times 10^{-2}/\text{min}$ (mean $\pm$ s.d.) and a half-life of 19.1 minutes after intracerebral microinjection into the S2 region of mouse brain. No significant radioactivity associated with this elimination was detected in the contralateral cerebrum, cerebellum, or cerebrospinal fluid (data not shown).

To characterize the hANP elimination process at the BBB *in vivo*, unlabeled excess amounts of hANP and hCNP were preadministered with [125 I]hANP to examine the inhibitory effect (Figure 2B). Preadministration prevents a dilution effect of unlabeled hANP and hCNP, so that a high enough concentration is maintained at the microinjection site. The values of percentage of [125 I]hANP remaining in the mouse brain after 30 minutes were 78.2% and 70.2% after preadministration of 100 $\mu\text{mol/L}$ unlabeled hANP and hCNP, respectively, being significantly increased compared with the control (44.8%).

Characteristics of [125 I]Human Atrial Natriuretic Peptide Internalization into TM-BBB4 Cells

To characterize ANP transport at the BBB, TM-BBB4 cells were used as an *in vitro* BBB model (Hosoya

Figure 2 Elimination of [125 I]hANP from mouse brain after microinjection. (**A**) Time courses of [125 I]hANP remaining in the ipsilateral cerebrum after intracerebral microinjection. A solution of [125 I]hANP (0.012 μCi , 20 nmol/L) and [^3H]dextran (0.12 μCi) in 0.30 μL of extracellular fluid (ECF) buffer was injected into the S2 region of mouse brain. The solid line was fitted using a nonlinear least-squares regression analysis program. Each point represents the mean $\pm$ s.e.m. ($n = 3$ to 4). (**B**) Inhibitory effect of unlabeled NPs on [125 I]hANP elimination from mouse brain. A measure of 30 μL inhibitor solution (100 $\mu\text{mol/L}$) was preinjected 5 minutes before administration of [125 I]hANP (0.012 μCi , 20 nmol/L) and [^3H]dextran (0.12 μCi) into the same brain region. The column was determined 30 minutes after intracerebral administration. Each value represents the mean $\pm$ s.e.m. ($n = 3$ to 4). *** $P < 0.001$, significantly different from control. BEI, brain efflux index; hANP, human atrial natriuretic peptide; hCNP, human C-type natriuretic peptide.

et al, 2000). Total binding of [125 I]hANP by TM-BBB4 cells was time dependent and increased linearly for at least 10 minutes (Figure 3A). The amount of internalized [125 I]hANP in the TM-BBB4 cells was evaluated by removing the labeled peptide bound to the cell surface with an acid wash solution. Acid-resistant binding of [125 I]hANP by TM-BBB4 cells was time dependent and increased linearly for at least 5 minutes (Figure 3B). The acid-resistant binding of [125 I]hANP was concentration dependent, and the Eadie-Scatchard plot indicated that two saturable processes are involved in [125 I]hANP internalization by TM-BBB4 cells (Figure 3C). Nonlinear least-squares regression analysis of the data gave the following parameters: high-affinity site: $K_{m1} = 0.163 \pm 0.106$ nmol/L; V_{max1} of 4.75 ± 1.99 fmol/(min mg protein), low-affinity site: $K_{m2} = 5.22 \pm 2.28$ nmol/L; V_{max2} of 25.1 ± 2.6 fmol/(min mg protein) (mean $\pm$ s.d.).

The acid-soluble binding reflects the amount of surface binding of [125 I]hANP not internalized into the cells. The acid-soluble binding showed similar concentration dependency to the acid-resistant binding (Figures 3C and 3D). Analysis of the acid-soluble binding based on two saturable components indicated an K_{d1} value at the high-affinity site of 0.0900 ± 0.0646 nmol/L (mean $\pm$ s.d.), which is similar to K_{m1} of acid-resistant binding. The K_{d2} value was estimated to be 22.8 ± 30.4 nmol/L (mean $\pm$ s.d.). However, this value is greater than the highest concentration of hANP examined in Figure 3D, so it remains possible that the lower-affinity site(s) has a greater K_d value than estimated and/or involves a nonsaturable component. These results suggest that at least two different binding sites exist on the surface of TM-BBB4 cells.

To investigate the substrate selectivity of the hANP internalization system in TM-BBB4 cells, the inhibitory effects of several natriuretic peptides on [125 I]hANP uptake by TM-BBB4 cells were examined (Figure 3D). Unlabeled hANP and hCNP inhibited [125 I]hANP internalization by 86.2% and 80.8%, respectively. cANP(4-23), a selective ligand for Npr-C, also reduced [125 I]hANP internalization by 85.7%. This result suggests that Npr-C is involved in [125 I]hANP internalization into TM-BBB4 cells.

Effect of Natriuretic Peptide Receptor-C Knockdown on [125 I]Human Atrial Natriuretic Peptide Internalization into TM-BBB4 Cells

To clarify the involvement of Npr-C in hANP internalization into TM-BBB4 cells, we examined the effect of siRNA knockdown of Npr-C. The Npr-C mRNA expression and [125 I]hANP internalization were determined 48 hours after siRNA treatment. The expression of Npr-C mRNA in TM-BBB4 cells was significantly decreased by Npr-C siRNA treatment by 53.2% (siRNA-1) and 28.1% (siRNA-2) compared with that in the case of negative control siRNA treatment (Figure 4A). The [125 I]hANP

Figure 3 Internalization of [125 I]hANP in TM-BBB4 cells. (A) Time courses of total binding and (B) acid-resistant binding of [125 I]hANP to TM-BBB4 cells. TM-BBB4 cells were incubated with [125 I]hANP (34 pM) at 37°C for the indicated times. The cells were washed with ice-cold extracellular fluid (ECF) buffer and then washed with acetate-barbital buffer at 4°C for 20 minutes to remove cell-surface-bound [125 I]hANP. Total binding was obtained as the sum of acid-resistant binding and acid-soluble binding. Each point represents the mean $\pm$ s.e.m. ($n = 3$). (C,D) Concentration dependence of acid-resistant binding (C) and acid-soluble binding (D) of [125 I]hANP to TM-BBB4 cells. The binding of [125 I]hANP by TM-BBB4 cells was measured at 37°C for 1 minute in the presence of various concentrations of unlabeled hANP. The cells were washed with ice-cold ECF buffer and then washed with acetate-barbital buffer at 4°C for 20 minutes. Inset; Eadie-Hofstee plot of the same data. Each point represents the mean $\pm$ s.e.m. ($n = 4$). (E) Inhibitory effect of NPs on acid-resistant [125 I]hANP binding to TM-BBB4 cells. The binding of [125 I]hANP (34 pM) was measured at 3 minutes in the absence or presence of several inhibitors at 100 nmol/L. The cells were washed with ice-cold ECF buffer and then washed with acetate-barbital buffer at 4°C for 20 minutes. Each column represents the mean $\pm$ s.e.m. ($n = 3$ to 4). *** $P < 0.001$, significantly different from control. hANP, human atrial natriuretic peptide; hCNP, human C-type natriuretic peptide.

internalization into TM-BBB4 cells was also reduced by Npr-C siRNA treatment, by 22.8% (siRNA-1) and 12.2% (siRNA-2) compared with that in the case of negative control siRNA treatment (Figure 4B).

Figure 4 Effect of natriuretic peptide receptor (Npr)-C short interfering RNAs (siRNAs) on Npr-C mRNA expression and the acid-resistant [125 I]hANP binding to TM-BBB4 cells. **(A)** TM-BBB4 cells were treated with 5 nmol/L several siRNAs for 48 hours as described in the Materials and methods section. The Npr-C and β -actin mRNA levels were determined by quantitative real-time PCR analysis. Each mRNA level was normalized with respect to β -actin mRNA. Each column represents the mean $\pm$ s.e.m. ($n = 4$). * $P < 0.05$, ** $P < 0.01$, significantly different from negative control siRNA. **(B)** The acid-resistant binding of [125 I]hANP (34 pM) by siRNA-transfected TM-BBB4 cells was measured at 37°C for 3 minutes. The cells were washed with ice-cold extracellular fluid (ECF) buffer and then washed with acetate-barbital buffer at 4°C for 20 minutes. Each column represents the mean $\pm$ s.e.m. ($n = 4$). * $P < 0.05$, ** $P < 0.01$, significantly different from negative control siRNA treatment. hANP, human atrial natriuretic peptide.

Effect of 1 α ,25-Dihydroxyvitamin D $_3$ on the Expression Level of Natriuretic Peptide Receptor-C in TM-BBB4 Cells

Protein expression of Npr-C is upregulated by 1,25(OH) $_2$ D $_3$, a key regulator of mineral metabolism, in osteoblasts (Yanaka *et al*, 1998). To investigate the regulation mechanism of Npr-C in brain capillary endothelial cells, we examined the effect of 1,25(OH) $_2$ D $_3$ treatment on protein expression of Npr-C in TM-BBB4 cells. After treatment of TM-BBB4 cells with 10 and 100 nmol/L 1,25(OH) $_2$ D $_3$ for 24 hours, an increase of Npr-C expression was observed by Western blot analysis (Figure 5A). To quantify Npr-C protein expression, multiplexed multiple reaction monitoring (MRM) analysis was performed with nano-LC/MS/MS, which measures the absolute amount of a tryptic peptide specific for the target molecule in trypsin-digested protein samples. The expression level of Npr-C protein in TM-BBB4 cells was increased to 1.23 ± 0.06 and 1.55 ± 0.18 fmol/ μ g protein after treatment of 10 and 100 nmol/L 1,25(OH) $_2$ D $_3$, respectively, compared with that in the control (0.57 ± 0.05 fmol/ μ g protein) (Figure 5B). The protein expression of Na $^+$ /K $^+$ ATPase was not significantly affected by 1,25(OH) $_2$ D $_3$.

Figure 5 Effect of 1 α ,25-dihydroxyvitamin D $_3$ (1,25(OH) $_2$ D $_3$) on the expression of natriuretic peptide receptor (Npr)-C in TM-BBB4 cells. **(A)** TM-BBB4 cells were treated with 10 or 100 nmol/L 1,25(OH) $_2$ D $_3$ for 24 hours. Crude membrane fraction proteins (3 μ g) were subjected to Western blot analysis using anti-Npr-C and anti-Na $^+$ -K $^+$ ATPase antibodies. **(B)** Quantitation of Npr-C and Na $^+$ -K $^+$ ATPase proteins in crude membrane fraction of TM-BBB4 cells, based on the peak area ratio of the analyte and stable isotope-labeled peptide in each 3 to 4 multiple reaction monitoring (MRM) channel as determined by nano-liquid chromatography-tandem mass spectrometer (LC-MS/MS) under optimized conditions. Each column represents the mean $\pm$ s.e.m. ($n = 3$ to 4). ** $P < 0.01$, significantly different from control.

Discussion

This study has provided evidence that ANP is eliminated from the brain across the BBB after intracerebral administration of [125 I]hANP into mouse cerebral cortex *in vivo* (Figure 2A). The [125 I]hANP was eliminated across the BBB with a half-life of 19.1 minutes. The [125 I]hANP elimination process was significantly inhibited by preadministration of unlabeled hANP (Figure 2B). These results suggest that a saturable brain-to-blood efflux transport system contributes to the cerebral ANP clearance. In central nervous system, both ANP and CNP are present (Imura *et al*, 1992). The inhibitory effect of preadministration of hCNP, shown in Figure 2B, suggests that the brain-to-blood efflux transporter for ANP recognizes CNP as well. Therefore, the BBB efflux transport system could be responsible for the removal of NPs from the ISF to maintain functional homeostasis of NPs in the central nervous system.

It has been reported that ANP primarily binds Npr-A and Npr-C, whereas CNP preferentially binds Npr-B and Npr-C (Potter *et al*, 2006). The

Npr-C is known to bind ANP, Npr-B and CNP with similar affinity (He *et al*, 2001). The [125 I]hANP elimination process was significantly inhibited by preadministration of unlabeled hANP and hCNP (Figure 2B). Western blot analysis using anti-Npr-A and anti-Npr-C antibodies showed that Npr-A and Npr-C proteins were expressed in mouse isolated brain capillaries, as well as kidney (Figure 1). These results suggest the involvement of Npr-A and/or Npr-C in the clearance of ANP from mouse brain across the BBB.

Cells of the conditionally immortalized mouse brain capillary endothelial cell line TM-BBB4 expressed Npr-C protein and exhibited internalization of [125 I]hANP (Figures 1 and 3). As internalization of [125 I]hANP into TM-BBB4 cells was inhibited by hANP and hCNP, in accordance with the *in vivo* observations, the molecules involved in [125 I]hANP internalization into brain capillary endothelial cells were analyzed using TM-BBB4 cells. The [125 I]hANP internalization by TM-BBB4 cells was markedly inhibited by cANP(4-23), which is a ligand for Npr-C, but not for Npr-A or Npr-B (Figure 3D). Furthermore, treatment with Npr-C-targeted siRNA suppressed the internalization of [125 I]hANP, as well as the expression of Npr-C mRNA in TM-BBB4 cells (Figure 4). In addition, siRNA #1 exhibited greater suppression of Npr-C mRNA expression than did siRNA #2. The internalization of [125 I]hANP was also suppressed by greater extent by siRNA #1 than by siRNA#2. These results suggest that Npr-C has an important function in the internalization of [125 I]hANP into TM-BBB4 cells.

This study in mice was performed with hANP. The amino-acid sequence of hANP differs by only one amino acid from that of mouse; methionine at position 12 in hANP is isoleucine in mouse ANP. We confirmed that the acid-resistant binding of [125 I]hANP in HEK293 cells transfected with mouse Npr-C isolated from TM-BBB4 cells was significantly greater than that in mock HEK293 cells; the values were $20.1 \pm 0.5 \mu\text{L}/\text{mg}$ protein and $4.77 \pm 0.15 \mu\text{L}/\text{mg}$ protein, respectively, at 30 minutes (mean $\pm$ s.e.m., $n = 4$). This result indicates that mouse Npr-C is able to internalize iodinated hANP.

In contrast to Npr-C, the expression of Npr-A was suppressed in TM-BBB4 cells. Therefore, involvement of not only Npr-C, but also Npr-A, in the efflux transport of [125 I]hANP at the BBB *in vivo* cannot be ruled out. Indeed, it was reported that ANP binding triggered Npr-A internalization into cells (Pandey *et al*, 2005). If Npr-A has a significant function in the elimination of [125 I]hANP from the brain, a marked inhibitory effect of ANP, compared with CNP, would have been expected in the BEI study. However, no significant difference in inhibitory effect was detected between ANP and CNP (Figure 2B). Furthermore, [125 I]hANP was cross-linked to a protein with a molecular weight of 60 to 70 kDa in brain capillary endothelial cells (Whitson *et al*, 1991), and this molecular weight matches that of Npr-C, but not

Npr-A. These observations suggest that Npr-A does not have a significant function in the efflux transport of [125 I]hANP from the brain, although further study is necessary to clarify in detail the contributions of Npr receptors to the efflux transport of hANP at the BBB.

Cerebral ANP has been reported to participate in the regulation of brain volume (Doczi *et al*, 1987; Vajda *et al*, 2001). Intracerebral injection of ANP decreased brain sodium levels and attenuated brain edema in rat models of cerebral ischemia (Doczi *et al*, 1987; Nakao *et al*, 1990; Naruse *et al*, 1991). Therefore, the regulation of ANP efflux transport at the BBB through Npr-C is expected to be important to regulate brain water content and brain volume. The protein expression of Npr-C was reported to be upregulated by $1,25(\text{OH})_2\text{D}_3$ in osteoblasts (Yanaka *et al*, 1998), and our results show that protein expression of Npr-C was also induced by $1,25(\text{OH})_2\text{D}_3$ in TM-BBB4 cells (Figure 5), suggesting that ANP efflux transport at the BBB through Npr-C is regulated by $1,25(\text{OH})_2\text{D}_3$. Spontaneously hypertensive rats, an animal model of essential hypertension, were resistant to stimulation with $1,25(\text{OH})_2\text{D}_3$ (Toraason and Wright, 1981) and the number of [125 I]ANP binding sites in the brain capillaries was significantly lower than that in the case of Wistar-Kyoto rat brain capillaries (Ibaragi and Niwa, 1989; Okazaki *et al*, 1990). This could be explained by regulation of Npr-C expression of brain capillaries by $1,25(\text{OH})_2\text{D}_3$. The effect of $1,25(\text{OH})_2\text{D}_3$ on the efflux transport of ANP and cerebral ANP level should be examined in further studies.

Our results show the involvement of Npr-C in the brain-to-blood efflux transport of cerebral ANP at the BBB. However, it is also suggested that other molecules are involved in the BBB efflux transport of ANP, as Eadie-Hofstee plots indicates that internalization of [125 I]hANP into TM-BBB4 cells involved both high- and low-affinity internalization processes with K_m values of 0.163 and 5.22 nmol/L, respectively (Figure 3C). The high-affinity K_m of 0.163 nmol/L is in good agreement with that obtained for hANP binding by hNpr-C-expressing 293 cells (0.675 nmol/L) (Fan *et al*, 2005). However, the molecule mediating the internalization of [125 I]hANP with the higher K_m value remains to be identified. Furthermore, there is no report showing whether ANP is degraded in the brain. The ANP is a substrate of rat insulin-degrading enzyme (Muller *et al*, 1991) and neprilysin (EC3.4.24.11) (Vanneste *et al*, 1988), which contribute to the degradation of amyloid- β peptide (A β) in the brain (Farris *et al*, 2003; Iwata *et al*, 2001). It is likely that a degradation process is also involved in cerebral ANP clearance. As this BEI study did not assess the degradation of [125 I]hANP after administration into the brain, the possibility remains that partially degraded [125 I]hANP is also eliminated from the brain across the BBB. Further work will be needed to clarify the relative contributions of these processes to the cerebral clearance of ANP.

The present results imply that lower levels of serum 25(OH)D₃, a major circulating form of vitamin D, attenuate cerebral clearance of ANP by impairing Npr-C induction at the BBB, leading to elevation of ANP level in the brain. Although the brain level of ANP is unknown, an elevated level of ANP might suppress the insulin-degrading enzyme-mediated A β degradation process, as ANP has 200-fold higher affinity for insulin-degrading enzyme than does A β (60 nmol/L versus 1.2 μ mol/L; Muller *et al*, 1991; Perez *et al*, 2000). Epidemiological study indicated that a low level of serum 25(OH)D₃ may result in an increased risk of Alzheimer's disease (Oudshoorn *et al*, 2008). Therefore, it can be hypothesized that reduced levels of serum 25(OH)D₃ have a part in increasing A β level in the brain as a result of inhibition of A β degradation by attenuation of cerebral clearance of ANP.

In conclusion, our findings indicate that Npr-C is expressed at brain capillary endothelial cells and mediates cerebral clearance of hANP from the brain, at least in part. Therefore, Npr-C may be involved in physiological control of the ANP level in the brain, and thus in brain fluid homeostasis.

Disclosure/conflict of interest

The authors declare no conflict of interest.

References

- Chabrier PE, Roubert P, Braquet P (1987) Specific binding of atrial natriuretic factor in brain microvessels. *Proc Natl Acad Sci USA* 84:2078–81
- Chiu PJ, Tetzloff G, Romano MT, Foster CJ, Sybertz EJ (1991) Influence of C-ANF receptor and neutral endopeptidase on pharmacokinetics of ANF in rats. *Am J Physiol* 260:R208–16
- Doczi T, Joo F, Szerdahelyi P, Bodosi M (1987) Regulation of brain water and electrolyte contents: the possible involvement of central atrial natriuretic factor. *Neurosurgery* 21:454–8
- Fan D, Bryan PM, Antos LK, Potthast RJ, Potter LR (2005) Down-regulation does not mediate natriuretic peptide-dependent desensitization of natriuretic peptide receptor (NPR)-A or NPR-B: guanylyl cyclase-linked natriuretic peptide receptors do not internalize. *Mol Pharmacol* 67:174–83
- Farris W, Mansourian S, Chang Y, Lindsley L, Eckman EA, Frosch MP, Eckman CB, Tanzi RE, Selkoe DJ, Guenette S (2003) Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-protein, and the beta-amyloid precursor protein intracellular domain *in vivo*. *Proc Natl Acad Sci USA* 100:4162–7
- Fuller F, Porter JG, Arfsten AE, Miller J, Schilling JW, Scarborough RM, Lewicki JA, Schenk DB (1988) Atrial natriuretic peptide clearance receptor. Complete sequence and functional expression of cDNA clones. *J Biol Chem* 263:9395–401
- He X, Chow D, Martick MM, Garcia KC (2001) Allosteric activation of a spring-loaded natriuretic peptide receptor dimer by hormone. *Science* 293:1657–62
- Hosoya K, Tetsuka K, Nagase K, Tomi M, Saeki S, Ohtsuki S, Takanaga H, Yanai N, Obinata M, Kikuchi A, Okano T, Terasaki T (2000) Conditionally immortalized brain capillary endothelial cell lines established from a transgenic mouse harboring temperature-sensitive simian virus 40 large T-antigen gene. *AAPS PharmSci* 2:E27
- Ibaragi M, Niwa M (1989) Atrial natriuretic peptide and angiotensin II binding sites in cerebral capillaries of spontaneously hypertensive rats. *Cell Mol Neurobiol* 9:221–31
- Imura H, Nakao K, Itoh H (1992) The natriuretic peptide system in the brain: implications in the central control of cardiovascular and neuroendocrine functions. *Front Neuroendocrinol* 13:217–49
- Ito S, Ohtsuki S, Kamiie J, Nezu Y, Terasaki T (2007) Cerebral clearance of human amyloid-beta peptide (1-40) across the blood-brain barrier is reduced by self-aggregation and formation of low-density lipoprotein receptor-related protein-1 ligand complexes. *J Neurochem* 103:2482–90
- Itoh H, Nakao K, Morii N, Yamada T, Shiono S, Sakamoto M, Sugawara A, Saito Y, Katsuura G, Shiomi T, Eigyo M, Matsushita A, Imura H (1986) Central action of atrial natriuretic polypeptide on blood pressure in conscious rats. *Brain Res Bull* 16:745–9
- Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saido TC (2001) Metabolic regulation of brain Abeta by neprilysin. *Science* 292:1550–2
- Kakee A, Terasaki T, Sugiyama Y (1996) Brain efflux index as a novel method of analyzing efflux transport at the blood-brain barrier. *J Pharmacol Exp Ther* 277:1550–9
- Kamiie J, Ohtsuki S, Iwase R, Ohmine K, Katsukura Y, Yanai K, Sekine Y, Uchida Y, Ito S, Terasaki T (2008) Quantitative atlas of membrane transporter proteins: development and application of a highly sensitive simultaneous LC/MS/MS method combined with novel in-silico peptide selection criteria. *Pharm Res* 25:1469–83
- Levin ER, Gardner DG, Samson WK (1998) Natriuretic peptides. *N Engl J Med* 339:321–8
- Mawuenyega KG, Kaji H, Yamuchi Y, Shinkawa T, Saito H, Taoka M, Takahashi N, Isobe T (2003) Large-scale identification of Caenorhabditis elegans proteins by multidimensional liquid chromatography-tandem mass spectrometry. *J Proteome Res* 2:23–35
- McKenzie JC, Berman NE, Thomas CR, Young JK, Compton LY, Cothran LN, Liu WL, Klein RM (1994) Atrial natriuretic peptide-like (ANP-LIR) and ANP prohormone immunoreactive astrocytes and neurons of human cerebral cortex. *Glia* 12:228–43
- Morii N, Nakao K, Sugawara A, Sakamoto M, Suda M, Shimokura M, Kiso Y, Kihara M, Yamori Y, Imura H (1985) Occurrence of atrial natriuretic polypeptide in brain. *Biochem Biophys Res Commun* 127:413–9
- Muller D, Baumeister H, Buck F, Richter D (1991) Atrial natriuretic peptide (ANP) is a high-affinity substrate for rat insulin-degrading enzyme. *Eur J Biochem* 202:285–92
- Nakao N, Itakura T, Yokote H, Nakai K, Komai N (1990) Effect of atrial natriuretic peptide on ischemic brain edema: changes in brain water and electrolytes. *Neurosurgery* 27:39–43; discussion-4
- Nakao N, Itakura T, Uematsu Y, Yokote H, Nakai K, Komai N (1992) A possible involvement of central atrial natriuretic peptide in cerebral cortical microcirculation. *Neurosurgery* 30:236–40
- Naruse S, Aoki Y, Takei R, Horikawa Y, Ueda S (1991) Effects of atrial natriuretic peptide on ischemic brain

- edema in rats evaluated by proton magnetic resonance method. *Stroke* 22:61–5
- Niessen J, Jedlitschky G, Grube M, Bien S, Schwartz H, Ohtsuki S, Kawakami H, Kamiie J, Oswald S, Starke K, Strobel U, Siegmund W, Rosskopf D, Greinacher A, Terasaki T, Kroemer HK (2009) Human platelets express organic anion-transporting peptide 2B1, an uptake transporter for atorvastatin. *Drug Metab Dispos* 37:1129–37
- Nishida Y, Ito S, Ohtsuki S, Yamamoto N, Takahashi T, Iwata N, Jishage K, Yamada H, Sasaguri H, Yokota S, Piao W, Tomimitsu H, Saido TC, Yanagisawa K, Terasaki T, Mizusawa H, Yokota T (2009) Depletion of vitamin E increases amyloid beta accumulation by decreasing its clearances from brain and blood in a mouse model of Alzheimer disease. *J Biol Chem* 284:33400–8
- Ohtsuki S, Yamaguchi H, Asashima T, Terasaki T (2007) Establishing a method to isolate rat brain capillary endothelial cells by magnetic cell sorting and dominant mRNA expression of multidrug resistance-associated protein 1 and 4 in highly purified rat brain capillary endothelial cells. *Pharm Res* 24:688–94
- Okazaki M, Kobayashi H, Kuroiwa A, Izumi F (1990) Atrial natriuretic peptide receptors in cerebral microvessels and choroid plexus of spontaneously hypertensive rats. *Brain Res* 518:292–4
- Oliver PM, Fox JE, Kim R, Rockman HA, Kim HS, Reddick RL, Pandey KN, Milgram SL, Smithies O, Maeda N (1997) Hypertension, cardiac hypertrophy, and sudden death in mice lacking natriuretic peptide receptor A. *Proc Natl Acad Sci USA* 94:14730–5
- Oudshoorn C, Mattace-Raso FUS, van der Velde N, Colin EM, van der Cammen TJM (2008) Higher serum vitamin D-3 levels are associated with better cognitive test performance in patients with Alzheimer's disease. *Dement Geriatr Cogn Disord* 25:539–43
- Pandey KN, Nguyen HT, Garg R, Khurana ML, Fink J (2005) Internalization and trafficking of guanylyl (guanylate) cyclase/natriuretic peptide receptor A is regulated by an acidic tyrosine-based cytoplasmic motif GDAY. *Biochem J* 388:103–13
- Pardridge WM, Eisenberg J, Yamada T (1985) Rapid sequestration and degradation of somatostatin analogues by isolated brain microvessels. *J Neurochem* 44:1178–84
- Perez A, Morelli L, Cresto JC, Castano EM (2000) Degradation of soluble amyloid beta-peptides 1-40, 1-42, and the Dutch variant 1-40Q by insulin degrading enzyme from Alzheimer disease and control brains. *Neurochem Res* 25:247–55
- Potter LR, Abbey-Hosch S, Dickey DM (2006) Natriuretic peptides, their receptors, and cyclic guanosine monophosphate-dependent signaling functions. *Endocr Rev* 27:47–72
- Steardo L, Nathanson JA (1987) Brain barrier tissues: end organs for atriopeptins. *Science* 235:470–3
- Terasaki T, Ohtsuki S (2005) Brain-to-blood transporters for endogenous substrates and xenobiotics at the blood-brain barrier: an overview of biology and methodology. *NeuroRx* 2:63–72
- Toraason MA, Wright GL (1981) Transport of calcium by duodenum of spontaneously hypertensive rat. *Am J Physiol* 241:G344–7
- Vajda Z, Pedersen M, Doczi T, Sulyok E, Stodkilde-Jorgensen H, Frokiaer J, Nielsen S (2001) Effects of centrally administered arginine vasopressin and atrial natriuretic peptide on the development of brain edema in hyponatremic rats. *Neurosurgery* 49:697–704; discussion -5
- Vanneste Y, Michel A, Dimaline R, Najdovski T, Deschodt-Lanckman M (1988) Hydrolysis of alpha-human atrial natriuretic peptide *in vitro* by human kidney membranes and purified endopeptidase-24.11. Evidence for a novel cleavage site. *Biochem J* 254:531–7
- Whitson PA, Huls MH, Sams CF (1991) Characterization of atrial natriuretic peptide receptors in brain microvessel endothelial cells. *J Cell Physiol* 146:43–51
- Yamada T, Nakano K, Morii N, Itoh H, Shiono S, Sakamoto M, Sugawara A, Saito Y, Ohno H, Kanai A, Katsuura G, Eigyo M, Matsushita A, Imura H (1986) Central effect of atrial natriuretic polypeptide on angiotensin II-stimulated vasopressin secretion in conscious rats. *Eur J Pharmacol* 125:453–6
- Yamaoka K, Tanigawara Y, Nakagawa T, Uno T (1981) A pharmacokinetic analysis program (multi) for micro-computer. *J Pharmacobiodyn* 4:879–85
- Yanaka N, Akatsuka H, Kawai E, Omori K (1998) 1,25-Dihydroxyvitamin D3 upregulates natriuretic peptide receptor-C expression in mouse osteoblasts. *Am J Physiol* 275:E965–73