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Secretin receptor involvement in prion-infected cells and animals

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ABSTRACT

The cellular mechanisms behind prion biosynthesis and metabolism remain unclear. Here we show that secretin signaling via the secretin receptor regulates abnormal prion protein formation in prion-infected cells. Animal studies demonstrate that secretin receptor deficiency slightly, but significantly, prolongs incubation time in female but not male mice. This gender-specificity is consistent with our finding that prion-infected cells are derived from females. Therefore, our results provide initial insights into the reasons why age of disease onset in certain prion diseases is reported to occur slightly earlier in females than males.
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1. Introduction

Transmissible spongiform encephalopathies or prion diseases are fatal neurodegenerative disorders that include Creuzfeldt–Jakob disease in humans, and bovine spongiform encephalopathy and scrapie in animals. These diseases are characterized by deposition of a partially protease-resistant abnormal prion protein isoform (PrPres), which is produced from the normal cellular isoform (PrPc) in the central nervous system and lymphoreticular system [1]. Biosynthesis and metabolism of PrPc and PrPres have been eagerly investigated at the cellular level using persistent prion-infected cells [2,3], but the cellular mechanism of the PrPc-to-PrPres conversion remains enigmatic [4–6].

In our efforts examining endogenous factors that affect PrPres formation in prion-infected cells [7], we identified secretin (Sct) signaling as one such factor. Sct is a 27-amino acid peptide released by endocrine S cells in the duodenum. Subsequently, it acts on the pancreas to stimulate bicarbonate and water secretion [8]. Sct is not only capable of crossing the blood–brain barrier but is also synthesized within the brain [9,10]. Moreover, Sct function in the central nervous system is a focus of constant attention in psychiatric disease and autistic spectrum disorder research [11–13]. The Sct receptor (SctR) is a G-protein coupled receptor expressed in the brain [10,14] and pancreas [15]. SctR is involved in neural plasticity and neural networks. Indeed, *SctR* knockout mice exhibit impaired synaptic plasticity and social behavior [16], indicating that SctR-mediated signaling is important for maintaining brain function.

Here, we report on *SctR* and *Sct* gene silencing effects, and also *Sct* gene overexpression or exogenous Sct peptide supplementation on PrPres formation in prion-infected cells. In addition, we examined disease progression in prion-infected mice deficient in either *SctR* or *Sct*. Finally, we discuss the significance of gender-dependent involvement of SctR-mediated signaling in prion disease.

Abbreviations: PrPc, normal cellular isoform of prion protein; PrPres, abnormal isoform of prion protein; Sct, secretin; SctR, secretin receptor

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2. Materials and methods

2.1. siRNAs and compounds

Double-stranded small interfering RNAs (siRNAs) for *SctR* (Sctr-MSS282665) and *Sct* (Sct-MSS276963) were purchased from Invitrogen Corp. (Carlsbad, CA, USA). Mouse or rat Sct peptides were purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA) and Tocris Bioscience (Ellisville, MO, USA), respectively.

2.2. Expression vectors

Mouse *SctR* and *Sct* were cloned by PCR with KOD-plus DNA polymerase (Toyobo Co. Ltd., Osaka, Japan) from oligo(dT) primed-cDNA synthesized from mouse neuroblastoma N2a cell total RNA and ready mouse brain cDNA library (Marathon; Takara Bio Inc.), respectively. PCR products were inserted into the pcDNA3.1 Myc/His expression vector (Invitrogen Corp.). Ligated vectors were introduced into *Escherichia coli*, and plasmids of interest were obtained and sequenced. Mutated *SctR* for rescue experiments was obtained from cloned *SctR* by site-directed mutagenesis using PCR, as described previously [7].

2.3. Gene silencing and overexpression

Gene silencing experiments were performed in N2a cells infected persistently with 22L (N167 cells) or RML prion strain (ScN2a cells), as described previously [7,17]. These two scrapie-derived prion strains have distinct differences in both the phenotype of diseased animals and the responsiveness to anti-prion compounds [18–20]. In brief, the cells were seeded onto 6-well plates at a 10% confluence density in 2.4 ml. Transfections were performed the day after seeding. For gene silencing, siLentFect (3.0 μl/well, Bio-Rad Laboratories, Inc.) was used to transfect double-stranded siRNAs. SctR and Sct siRNAs were used at 10 and 20 nM, respectively. Transfection reagent without siRNA was used as the negative control for gene silencing, because in our experience even commercially available, universal, non-targeting siRNAs produce off-target phenomena [17]. For gene overexpression, TransFectin (3.0 l/well; Bio-Rad Laboratories, Inc.) was used to transfect expression vectors (0.4 μg per well). Three days after transfection, cells were harvested after washing in PBS.

2.4. PrPres and PrPc assay

After rinsing in PBS, cells were lysed in lysis buffer (0.5% sodium deoxycholate and 0.5% Nonidet P-40 in PBS), and debris eliminated by brief centrifugation. The protein content of each sample was measured using the DC protein assay reagent (Bio-Rad Laboratories, Inc.), with bovine serum albumin as the standard. PrPres and PrPc levels in cells were assayed by immunoblotting, as described previously [17,19,21]. In brief, for PrPres, cell lysates containing equivalent amounts of protein were treated with 10 μg/ml proteinase K for 30 min at 37 °C, and PrPres pelleted by centrifugation. For PrPc, an aliquot of cell lysate containing an equivalent amount of protein was used without proteinase K treatment. After denaturation in sample buffer with heating, PrP was separated by SDS–PAGE, and then electrotransfered onto polyvinylidene difluoride membrane. PrP was detected using SAF83 antibody, which recognizes residues 126–164 of mouse PrP (1:5000; SPI-Bio, Massy, France). Immunoreactive signals were densitometrically analyzed using ImageJ (National Institutes of Health, Bethesda, MD, USA).

2.5. Flotation assay

The flotation assay for detergent-insoluble membrane complexes was performed as described previously [17,22]. In brief, cells were washed with ice-cold PBS and then lysed on ice in 550 μl TNET solution (25 mM Tris–HCl (pH 7.5), 150 mM NaCl, 5 mM EDTA, and 1% Triton X-100). Lysates were added to an equal volume of ice-cold 70% Nycodenz™ solution (Cosmo Bio Inc., USA). Using 800 μl of this mixed solution, 200 μl each of 25%, 22.5%, 20%, 18%, 15%, 12%, and 8% Nycodenz™ solution in TNET was overlaid sequentially in TLS-55 ultracentrifuge tubes (Hitachi Ltd., Japan). Ultracentrifugation was performed at 200000×g, 4 °C for 4 h. Fractions (200 μl) were collected from top to bottom and analyzed for PrPc by immunoblotting with SAF83 antibody.

2.6. mRNA quantification

Cells were lysed using RNAiso-plus reagent (Takara Bio Inc.). Total RNA was extracted with FastPure RNA (Takara Bio Inc.), and cDNA synthesized using a first strand cDNA synthesis kit with random 6-mer primers (Takara Bio Inc.). mRNA levels were measured by real-time PCR using SYBR Premix Ex Taq II (Takara Bio Inc.). Gene expression fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method, with *GAPDH* as an internal control.

2.7. Animal experiments

SctR, *Sct* deficient mice, and their wild-type littermates were generated by mating heterozygous mutant parents [16,23]. Eight- to twelve-week-old mice were used for intracerebral or intraperitoneal prion infection, as described previously [18,19,24]. In brief, prion inoculation for intracerebral or intraperitoneal infection was performed using 20 μl or 100 μl, respectively, of 1% (wt/vol) brain homogenate from a RML prion-infected terminally ill mouse. Animals were monitored daily until the disease was terminal. Animal experiments were performed with the approval of the Animal Experiment Ethical Committee of Tohoku University (Permit Number: 2011idou-347), and all efforts were made to minimize animal suffering.

2.8. Immunoblotting and PET-blot analysis of brain tissue

Disease was confirmed by immunoblotting or paraffin-embedded tissue (PET)-blot analysis for PrPres in the brain, as described previously [19,25]. In brief, for immunoblotting, brains were homogenized in 9 volumes of lysis buffer. After low-speed centrifugation, supernatants were treated with 50 μg/ml proteinase K for 1 h at 37 °C. Aliquots were electrophoresed and analyzed by immunoblotting, as described above. For PET-blot analysis, 5 μm paraffin sections were cut and collected onto nitrocellulose membranes, and then dried for overnight at 60 °C. Membranes were dewaxed in xylene, followed by stepwise rehydration. After wetting with TBST (10 mM Tris HCl (pH 7.8), 100 mM NaCl, and 0.05% Tween 20), sections were digested with 250 μg/ml proteinase K (in a buffer: 10 mM Tris HCl (pH 7.8), 100 mM NaCl, and 0.1% Brij 35) for overnight at 55 °C. After washing with TBST, sections were treated for 30 min with 3 M guanidine isothiocyanate. After washing out guanidine using TBST, immunodetection was performed with anti-PrP-C antibody, which recognizes residues 214–228 of mouse PrP (1:1500; Immuno-Biological Laboratories Co. Ltd., Gunma, Japan).

2.9. Gender determination

To determine the gender of mouse neuroblastoma cells, the sex-determining region Y gene (*Sry*) on the Y-chromosome was

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