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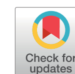
Seawater transfer down-regulates C-type natriuretic peptide-3 expression in prolactin-producing cells of Japanese eel: Negative correlation with plasma chloride concentration



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ABSTRACT

In euryhaline fishes, atrial and B-type natriuretic peptides are important hormones in hypo-osmoregulation, whereas osmoregulatory functions of C-type natriuretic peptides (CNPs) remain to be investigated. Although four CNP isoforms (CNP1-4) are mainly expressed in the brain, multiorgan expression of CNP3 was found in euryhaline Japanese eel, *Anguilla japonica*. Here we identified the CNP3-expressing cells and examined their response to osmotic stress in eel. CNP3 was expressed in several endocrine cells: prolactin-producing cells (pituitary), glucagon-producing cells (pancreas), and cardiomyocytes (heart). Pituitary CNP3 expression was the highest among organs and was decreased following seawater transfer, followed by a decrease in the freshwater-adaptating (hyper-osmoregulatory) hormone prolactin. We also showed the negative correlation between CNP3/prolactin expression in the pituitary and plasma Cl^- concentration, but not for plasma Na^+ concentration. These results suggest that CNP3 in the pituitary (and pancreas) plays a critical role in freshwater adaptation of euryhaline eel together with prolactin.

1. Introduction

Vertebrates including teleosts maintain their body fluid osmolality at approximately one-third that of seawater regardless of whether they inhabit freshwater, seawater, or land. In teleosts that have wide ranges of salinity tolerance (euryhaline) like eels and salmonids, hormones from the anterior pituitary are important for maintenance of water and ion balance by remodeling the functions of osmoregulatory organs. Prolactin is a critical component of freshwater adaptation as it increases active ion uptake through osmoregulatory surfaces and decreases water and ion permeability by thickening the epithelial layer in eels, salmonids, and tilapia (Manzon, 2002) while growth hormone and adrenocorticotropic hormone (ACTH)-cortisol axis enhance branchial and intestinal ion permeability for seawater adaptation in these euryhaline species (McCormick, 2001; Takei et al., 2014). Besides the long-lasting actions by anterior pituitary hormones, short-lived actions by various vasoactive peptides such as angiotensin II, neurohypophysial hormones, and natriuretic peptides (NPs) that alter activities of pre-existing ion transporters and channels are crucial for teleost osmoregulation (Katayama et al., 2018; McCormick, 2001; Takei et al., 2014). Indeed, NPs are linked to the maintenance of cardiovascular and body fluid homeostasis in vertebrates (Potter et al., 2005; Takei and Hirose, 2002; Toop and Donald, 2004). Atrial NP (ANP) and B-type NP (BNP) secreted primarily from the heart to the circulation are natriuretic. Systemic ANP also lowers plasma sodium level by inhibiting salt appetite in terrestrial mammals, and by inhibiting drinking and subsequent intestinal sodium absorption in seawater-acclimated eels (Blackburn et al., 1995; Fitzsimons, 1998; Toop and Donald, 2004; Tsukada et al., 2005). C-type NPs (CNP) are synthesized mainly in the brain as autocrine or paracrine factors in mammals (Chen and Burnett, 1998), although the existence of mammalian CNP (orthologue of teleost CNP4) in the blood implies systemic function as an endocrine factor of CNP (Stingo et al., 1992). CNP knockdown/knockout models have been examined and they displayed severe dwarfism in mouse (Komatsu et al., 2002) and cardiac atrophy in medaka (Miyanishi et al., 2013). Although these findings suggest peripheral paracrine actions of CNP in endochondral bone growth and cardiac development, the central and systemic functions of CNP regarding osmoregulation are not fully

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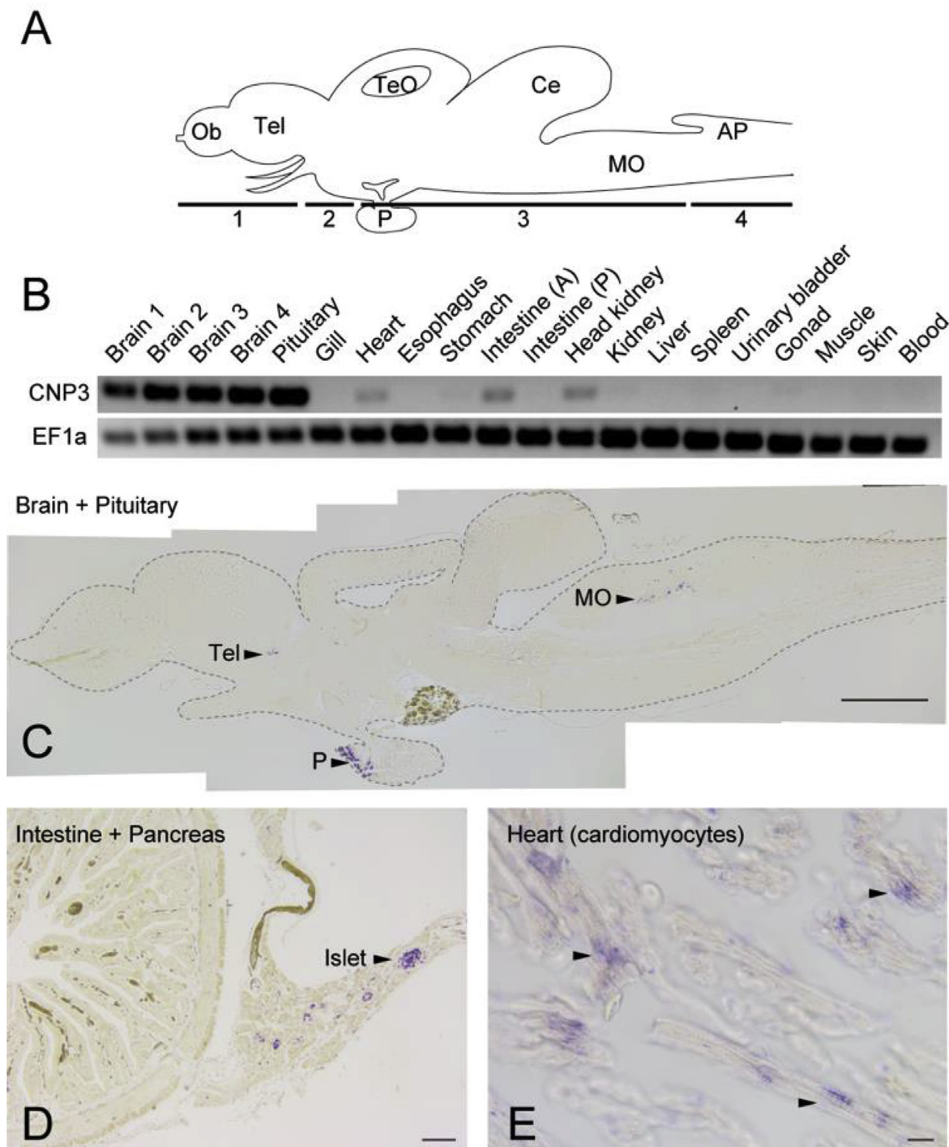

Fig. 1. C-type natriuretic peptide 3 (CNP3) was highly expressed in the brain and pituitary, and to a much lesser extent in the heart, anterior intestine, and head kidney.

understood (Moyes and Hobbs, 2019; Tsukada, 2016) with an exception that plasma CNP level is higher in freshwater-acclimated eels than seawater-acclimated eels (Takei et al., 2001).

NP family peptides including ANP and BNP were evolutionarily generated from an ancestral CNP3 gene by tandem duplications (Inoue et al., 2005). CNP3 is the sole NP expressed in both the heart and brain in elasmobranchs, which are a basal vertebrate group (Kawakoshi et al., 2001). In teleosts, on the other hand, CNP has been diversified into four types; namely, CNPs 1–4 (Inoue et al., 2003a, 2005). Eel CNP1 and 4 isoforms are expressed only in the brain like mammalian CNP, while CNP3 is distributed in peripheral organs including pituitary and intestine (Nobata et al., 2010). However, it has not been studied what types of cells produce CNP3 and whether the CNP3 expression is regulated. Here, we aimed to characterize CNP3-expressing cells and examine the response of CNP3 to osmotic stress in the Japanese eel, *Anguilla japonica*, as a model species of euryhaline teleost (Wong and Takei, 2012). Pituitary CNP3 expression in prolactin-producing cells was down-regulated in seawater and high NaCl environments, suggesting novel functions of CNP in fish osmoregulation. We also evaluated the correlation between pituitary CNP3 expression and plasma ion levels to examine whether distinct ion(s) in plasma is (are) linked to pituitary CNP3 expression.

2. Methods

2.1. Animals

Cultured immature Japanese eels (*Anguilla japonica*, ca. 200 g) were purchased from a local dealer (Chuhei, Chiba, Japan). Eels were kept in freshwater tanks containing 30 L freshwater at least two days for acclimatization (approximate freshwater ion composition: 0 ppt salinity, 1.07 mM Na^{+}, 0.48 mM Ca^{2+}, and 0.27 mM Mg^{2+}; maximum 3 eels/tank). Cl^{-} concentration in freshwater was under the detection limit (less than 1 mM) of a chloride meter (Labconc, Thermo Fisher Scientific, Waltham, MA, USA). To minimize the culturing stress, PVC pipes were placed in the tanks to provide hiding places for eels and the tanks were kept on a shelf which is surrounded with a lightproof curtain such that light only enters through the roof of the tank as in the natural habitat. Indeed, culturing eels in freshwater tanks did not cause stress-induced cortisol production (Li et al., 2003). After the acclimation, eels

were processed for each experiment. Some eels were transferred from freshwater to seawater (approximately, 35 ppt, 520 mM Na^+, 567 mM Cl^-, 14 mM Ca^{2+}, 1019 mOsml/kg) for 3 h, 12 h, 1 day, 3 days, and 7 days. Eels kept in seawater for a minimum of a week were used as seawater-acclimated eels. Some eels were transferred to artificially-modified medium containing 250 mM NaCl solution (approximately half-salinity of seawater) or 14 mM $CaCl_2$ solution (equivalent to the Ca^{2+} concentration of seawater) for a week. All specimens were maintained in the same manner at room temperature of 22–25 °C under a daily photoperiod cycle of 12-h light/12-h dark (lights on at 7:00 a.m.) and were unfed during the experiment. Fish were deeply anesthetized for handling with 0.1% ethyl m-aminobenzoate methanesulfonate (Wako Pure Chemical, Osaka, Japan) neutralized with sodium bicarbonate. All experiments were approved by the Animal Experiment Committee of Toho University, Shizuoka University, and the University of Tokyo, and performed in accordance with the manuals prepared by the committees.

2.2. Measurement of plasma ions and osmolality

Blood was collected from caudal vessel of anesthetized eel individuals with a 2.5 ml syringe and then 0.1 M EDTA-2Na (Dojindo laboratories, Kumamoto, Japan) was added to the blood (at 10 μl/ml blood). After centrifugation at 15,000 rpm for 5 min at room temperature, plasma was obtained and was kept in −20 °C freezer until measurements. Plasma Na^+, Ca^{2+}, and Mg^{2+} concentrations were measured by using an atomic absorption spectrophotometer (Hitachi ZA3300, Hitachi High-Tech Science, Tokyo, Japan). A digital chloride meter and a vapor pressure osmometer (Wescor Vapro, Logan, UT, USA) were used for plasma Cl^- concentration and osmolality, respectively.

2.3. RNA extraction and subsequent cDNA synthesis

After blood sampling, the brain was divided into 4 segments as shown in Fig. 1A and other tissues (pituitary, gill, heart, esophagus, stomach, intestine, head kidney, kidney, liver, spleen, urinary bladder, gonad, muscle, and skin) were dissected from eels. The samples were then quickly frozen in liquid nitrogen. For samples obtained from seawater transfer experiments (Fig. 3A and B), total RNAs were extracted using ISOGEN (Nippongene, Toyama, Japan) with DNase I (Thermo Fisher Scientific, Waltham, MA, USA) to remove genomic DNA. cDNA was synthesized by iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) or superscript III (Thermo Fisher Scientific). For samples obtained from NaCl and $CaCl_2$ exposure experiments (Fig. 4), total RNAs were extracted using a RNeasy mini-kit and an RNase-free DNase set (for the pituitary; Qiagen, Hilden, Germany) or a Trizol reagent (for other tissues; Thermo Fisher Scientific) with DNase treatment (Promega, Madison, WI, USA). cDNA was synthesized using the PrimeScript RT reagent kit (Takara Bio, Otsu, Japan).

2.4. Tissue distribution of CNP3 mRNAs by reverse-transcription PCR

cDNA was mixed with gene-specific primers (Table 1), Blend Taq DNA polymerase, buffer, and dNTPs according to the manufacturer's instructions (TOYOBO, Osaka, Japan), and then subjected to PCR as follows: 2 min at 94 °C, 30–35 cycles of 30 s at 94 °C, 30 s at 64 °C for CNP3 or at 58 °C for EF1α, 30 s at 72 °C, and an additional 7 min at 72 °C using a GeneAmp PCR System 9700 (Applied Biosystems, Foster City, CA, USA). The products were run on 1.0% agarose gels and visualized with ethidium bromide. Gel images were taken by an ImageQuant LAS4000 imager (GE Healthcare, Chicago, IL, USA).

PRL, prolactin; EF1α, elongation factor 1α; ISH, *in situ* hybridization; qPCR, real-time quantitative PCR. Accession numbers: CNP3 (AB513621), PRL (AY158009), EF1α (AB593812). Primers for eel glucagon and insulin were designed based on the predicted sequences obtained from the eel genome database provided by Dr. Wataru Iwasaki of the University of Tokyo.

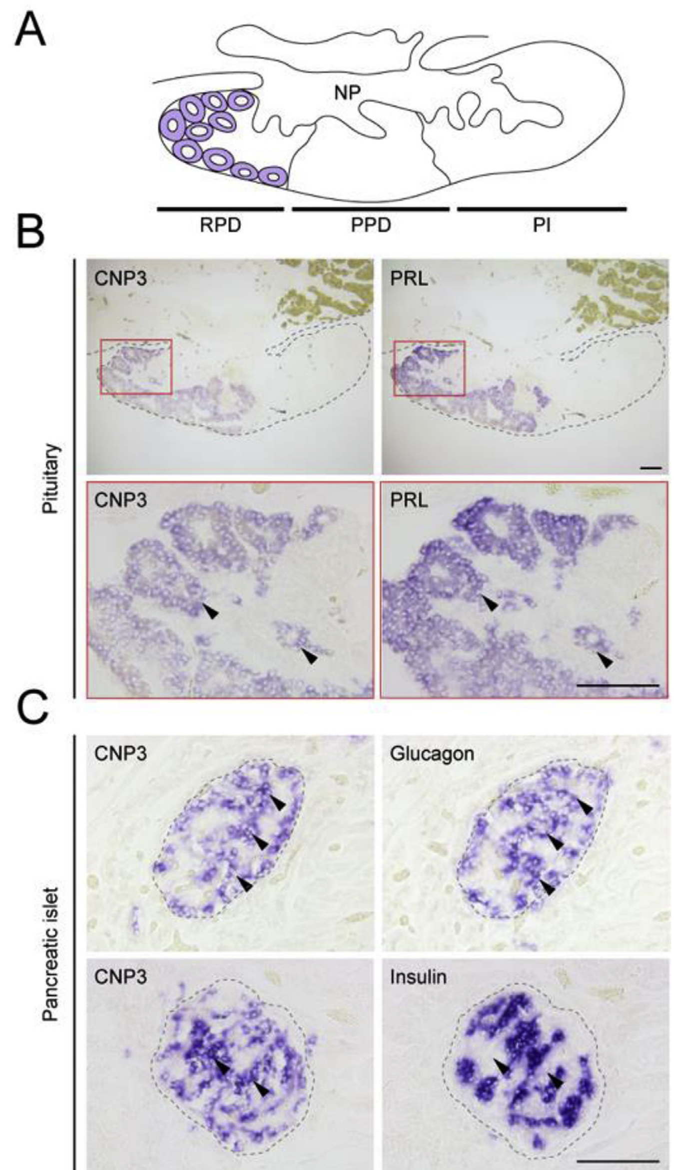

Fig. 2. CNP3 was synthesized in the prolactin-secreting cells (anterior pituitary) and the glucagon-producing cells (pancreatic islets).

2.5. Real-time quantitative PCR

The amount of CNP3 and prolactin mRNAs were determined by real-time quantitative PCR (qPCR) method using AriaMx Real-Time PCR G8830A (Agilent Technologies, Santa Clara, CA, USA) with SYBR Green Real-time PCR Master Mix Plus (Toyobo, Osaka, Japan) or ABI 7900HT Fast Real Time PCR System (Life Technologies, CA, USA) with KAPA SYBR Fast qPCR kit (Kapa Biosystems, Boston, MA, USA), as previously described in detail (Takei et al., 2016). cDNA template was added to reaction mixture containing specific primer sets for each target gene (Table 1). The Primer3 Input (http://bioinfo.ut.ee/primer3-0.4.0/) was used for the design of the primers. Specificity of primer was confirmed by analyzing the melting curve after cycling and only single amplicon was detected. All measurements were done in duplicate. To generate a standard curve, plasmids containing partial cDNA fragments of target genes of known concentration were serially diluted and used as the standard templates. Total copy numbers of mRNAs in the

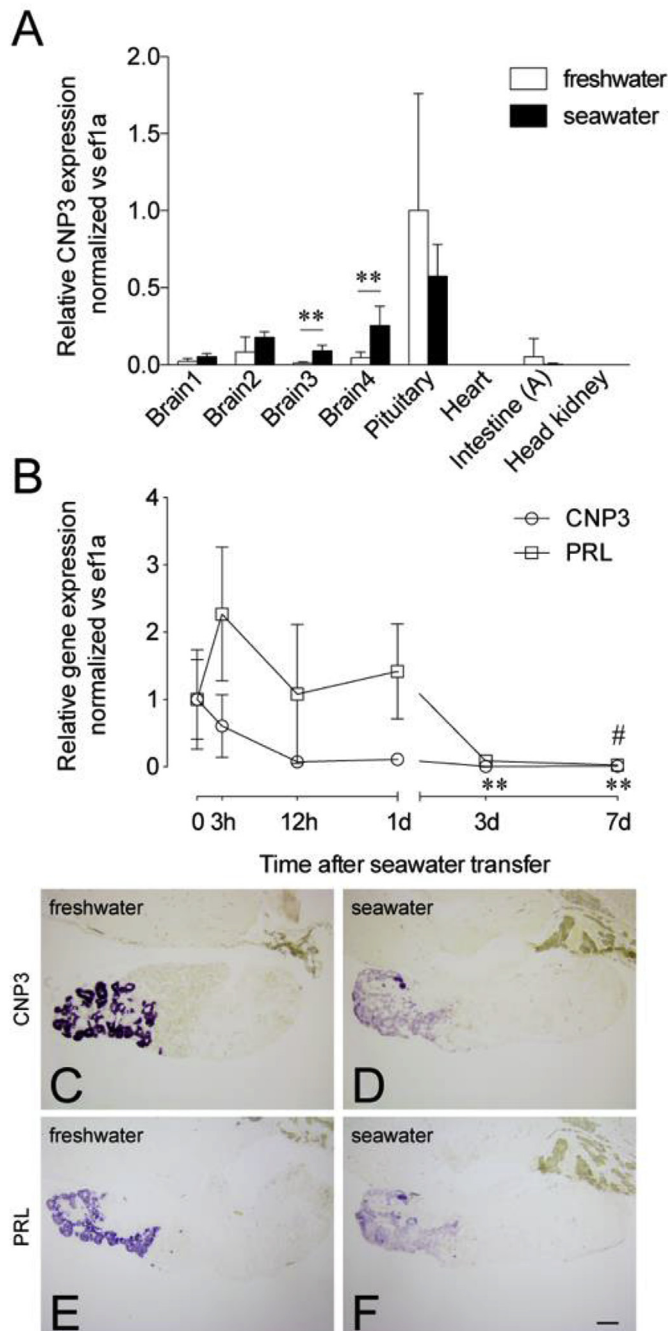

Fig. 3. Seawater exposure reduced pituitary CNP3 expression.

pituitary were then calculated. For normalization, the mRNA of elongation factor 1α (EF1α, AB593812) was quantified. For time-course experiment (Fig. 3B), relative gene expression of CNP3 and prolactin was quantified by the $2^{-\Delta\Delta}$Ct method. Each value was finally divided by mean values of gene expressed in freshwater pituitary. The mean value of CNP3/prolactin in freshwater-acclimated eels was defined as '1' and each data was shown as relative expression.

2.6. In situ hybridization

After deep anesthesia, 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in 100 mM phosphate buffer (PB, pH7.4) was perfused through the ventricle and then brain with pituitary, heart, intestine with pancreas, and head kidney were dissected. The tissues were fixed in same fixative over night at 4 °C. After 2-day incubation with 30% sucrose in 100 mM PB (pH 7.4) at 4 °C, the tissue was embedded in frozen section compound (Leica Microsystems, Wetzlar, Germany) and frozen rapidly. Frozen sections (8 µm) were obtained using a cryostat (CM19500UV, Leica Microsystems). Using the eel specific primers (Table 1), the DNA fragments of CNP3 (501 bp), prolactin (491 bp), glucagon (341 bp), and insulin (308 bp) were amplified from eel brain, pituitary, and pancreas cDNA by the PCR, respectively. Amplified cDNA fragments were ligated into pGEM-T easy vector (Promega) and cloned. Gene-specific antisense or sense digoxigenin (DIG)-labeled cRNA probes were made by using the Roche DIG RNA labeling kit (Roche Diagnostics, Penzberg, Germany). DIG-labeled cRNA probe hybridization was performed in a solution containing 50% formamide (Wako), 3x SSC buffer stock solution and 1x Denhardt solution (Nacalai Tesque, Kyoto, Japan), 120 mM PB (pH 7.4, LSI Medience, Tokyo, Japan), 125 µg/ml tRNA from brewer's yeast (Roche Diagnostics), and 100 µg/ml Deoxyribonucleic acid solution from Calf Thymus (Wako) at 55 °C for 16–18 h. Visualization of each type of mRNA was performed with alkaline-phosphatase-conjugated anti-DIG antibody (Roche Diagnostics) by using 4-nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolyl phosphate (Roche Diagnostics). Control experiments were carried out with DIG-labeled sense cRNA probes. Stained sections were mounted with Aquatex (Merck Millipore, Darmstadt, Germany) and were observed using a microscope (BX53; Olympus, Tokyo, Japan). Images were taken by a CMOS camera with Topeview software (AdvanCam-U3; AdvanVision, Tokyo, Japan). Images were processed for presentation using Photoshop and Illustrator CC (Adobe Systems, San Jose, CA, USA).

2.7. Statistical analysis

Mann-Whitney test was used for two group comparison and Kruskal-Wallis test followed by Dunn's multiple comparison test was applied for multiple comparisons. Pearson correlation test was performed to determine the correlation between plasma CNP/prolactin mRNA levels and plasma ion concentration/plasma osmolality. GraphPad Prism Ver. 6.0 (San Diego, CA, USA) was used for statistical analysis. Significance was determined at $p < 0.05$.

3. Results

3.1. Expression and localization of eel CNP3 mRNA in tissues

Our RT-PCR analysis indicated strong CNP3 expression in the brain and pituitary, and much lesser expression in the heart, anterior intestine, and head kidney (Fig. 1B, Note that brain 1-4 in Fig. 1B are corresponded to the brain segments 1–4 shown in Fig. 1A). We next examined the localization in the CNP3-expressing tissues by *in situ* hybridization (Fig. 1C–E). CNP3 expression in the brain was scattered in the telencephalic area and medulla oblongata (Fig. 1C, for eel brain atlas, see Fig. 1A). Significant expression of CNP3 was observed in the rostral pars distalis of the pituitary (Fig. 1C). Like ANP and BNP (Takei et al., 2018), CNP3 was expressed in the cardiomyocytes (Fig. 1E), whereas no CNP3 signal could be seen in the head kidney.

A Brain atlas of the Japanese eel (Sagittal view). Ob: olfactory bulb, Tel: telencephalon, TeO: optic tectum, Ce: cerebellum, MO: medulla oblongata, AP: area postrema, and P: pituitary. **B** Tissue expression of CNP3 mRNAs evaluated by reverse-transcription PCR. Brain 1-4 correspond to the area numbers given in **A**. Anterior and posterior intestine are shown as Intestine (A) and Intestine (P), respectively, and includes the adjacent pancreas. EF1α mRNA was used as an internal control for each cDNA sample. No band was detected in reverse transcription control without template. **C-E** Tissue localization of CNP3 mRNA evaluated by *in situ* hybridization: **C** A sagittal section of the brain with pituitary (gray dashed line: outline of brain and pituitary), **D** cross section of the intestine with pancreas, and **e** heart. CNP3 signals (purple) in each tissue are indicated by arrowheads. Scale bars, 1 mm

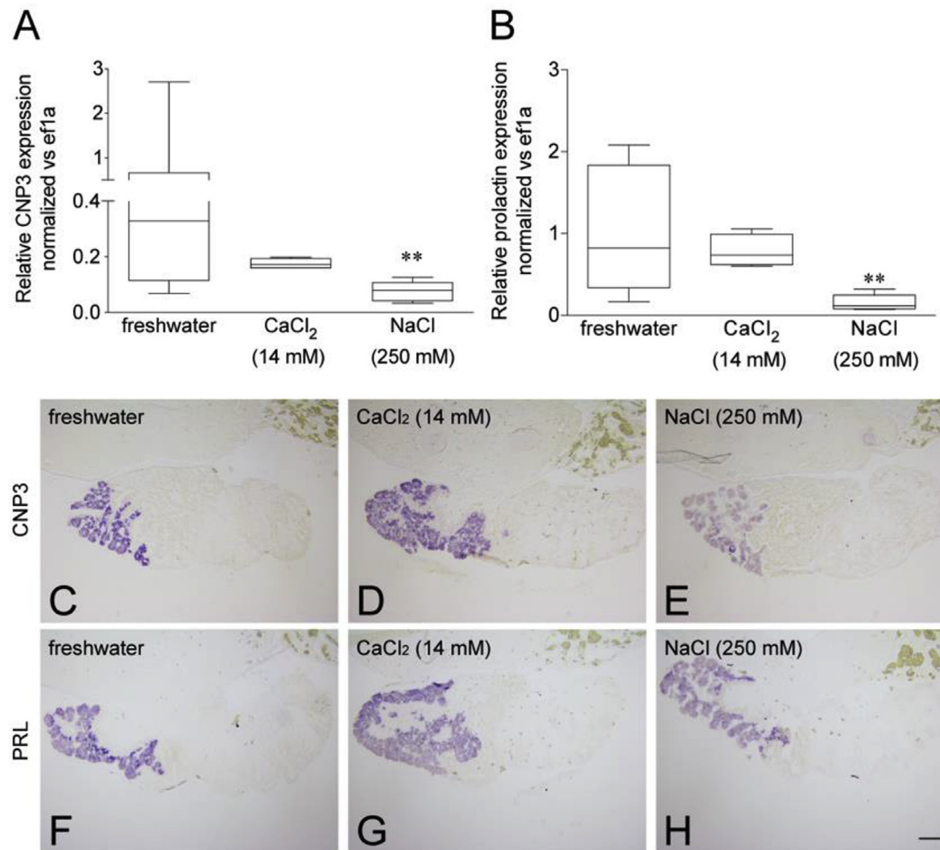

Fig. 4. NaCl exposure, but not $CaCl_2$ exposure, reduced pituitary CNP3 and prolactin mRNA levels.

Table 1
Primers used for molecular analyses.

Gene name_Primer name	For	Sequence (5′- 3′)
CNP3_Forward1	ISH, RT-/q- PCR	AAGCTGAAAACGAACCATC
CNP3_Reverse1	ISH	TTTCTGGGTTGAAGAGGTACG
CNP3_Reverse2	RT-/q- PCR	TGAGACTCTGCAAGCTGGAC
PRL_Forward1	ISH, qPCR	AACGGCAGAAGAACGAAGAC
PRL_Reverse1	ISH	GAGGAGGAGCCAATCTTGTG
PRL_Reverse2	qPCR	GCACAGCAGAGCTGTCAAGA
EF1α_Forward	RT-/q- PCR	CTGAAGCCTGGTATGGTGGT
EF1α_Reverse	RT-/q- PCR	ACGACGGATTTCCTTGACAG
Glucagon_Forward	ISH	GCATTCACTCTTTGGCTGGT
Glucagon_Reverse	ISH	CAGCCAAGAGACGAACTCCT
Insulin_Forward	ISH	TGCCGGTAATCTCCCTACTG
Insulin_Reverse	ISH	GAAGATGTTGCAGGGCTTGT

(**C**), 100 μm (**D**), 10 μm (**E**).

3.2. Identification of CNP3-expressing cells in the pituitary and pancreatic islets

Transcript localization of CNP3, prolactin, insulin, and glucagon were determined by *in situ* hybridization using mirror sections to identify the cell types that expressed CNP3. In pituitary, CNP3 mRNA distribution was almost identical to that of prolactin (Fig. 2B). In the pancreatic islets, CNP3 was expressed in glucagon-producing cells but not insulin-producing cells (Fig. 2C).

A Schematic drawing of eel pituitary (Sagittal view). RPD: rostral pars distalis, PPD: proximal pars distalis, PI: pars intermedia, and NP: pars nervosa. Prolactin-secreting cells form follicles in the RPD (purple). **B**, **C** Identification of CNP3-expressing cells by *in situ* hybridization using mirror sections. Distribution of CNP3 mRNA was identical to that of prolactin and glucagon mRNAs, but not to that of insulin mRNA (**B**, **C**). Magnified views of the RPD region (red box) are shown in the bottom panels (**B**). Gray dashed line shows outline of pituitary (**B**) and pancreatic islets (**C**). Arrowheads indicate areas expressing CNP3 or corresponding areas in each mirror section. Scale bar, 100 μm.

3.3. Higher expression of the pituitary CNP3 in freshwater-acclimated eels than in seawater-acclimated eels

To examine responses of CNP3 expression to different salinity environments, we quantified relative CNP3 mRNA levels in freshwater- and seawater-acclimated eels by real-time qPCR (Fig. 3A). CNP3 expression in the pituitary, especially of freshwater eels, was more than 10-fold higher than those of other CNP-expressing tissues. There was no significant difference in pituitary CNP3 mRNA levels between freshwater- and seawater-acclimated eels due to large variance in freshwater eels. We also examined time course changes in pituitary CNP3 and prolactin mRNA levels after a direct transfer from freshwater to seawater. CNP3 expression was downregulated by seawater transfer after 3 days in seawater, followed by significant decreases in prolactin expression after 7 days (Fig. 3B). The downregulation was consistently observed in the *in situ* hybridization, where the signals for CNP3 and prolactin were lower in seawater than in freshwater given the same color-developing time (Fig. 3C–F).

A Relative tissue expression of CNP3 in freshwater- and seawater-acclimated eels evaluated by real-time qPCR. Brain 1-4 correspond to area numbers given in Fig. 1A. $^{**}p = 0.0043$ (freshwater vs. seawater in Brain 3), $= 0.0087$ (freshwater vs. seawater in Brain 4) < 0.01 with Mann-Whitney test (freshwater, $n = 6$; seawater, $n = 5$). **B** Time-course changes in pituitary CNP3 and prolactin (PRL) expression during seawater transfer determined by real-time qPCR (open circle: CNP3, open

square: PRL, $n = 5$ each). $^{**}p < 0.01$ (CNP3: 0 h vs. seawater 3 d/7 d), $\#p < 0.05$ (PRL: 0 h vs. seawater 7 d) with Kruskal-Wallis test (CNP3, $p = 0.0002$; PRL, $p = 0.0006$) followed by Dunn's multiple comparison test. CNP3 level was reduced at earlier time-point compared with PRL level. Data are shown as mean ± standard deviation (SD); the absence of error bars indicates a small SD. **C–F** Localization of CNP3 (**C**,**D**) and PRL (**E**,**F**) mRNAs in the pituitary of freshwater- and seawater-acclimated eels by *in situ* hybridization, supporting the results in **B**. Scale bar, 100 μm.

3.4. Decrease in the pituitary CNP3/prolactin expression by NaCl transfer

Because the CNP3 and prolactin mRNA levels in the pituitary were considerably decreased 7 days after seawater transfer, we examined effect of environmental composition on pituitary CNP3 and prolactin levels using eels kept in artificially-modified ion environments for 7 days. Since mammalian CNP is involved in calcium homeostasis (Chusho et al., 2001), we examined whether ambient Ca^{2+} whose concentration is equivalent to seawater alters expression levels of eel CNP3. Although slight decrease in CNP3 level was observed by 14 mM $CaCl_2$-transferred eels, there was no significant difference compared with CNP3 level in freshwater-acclimated eels (Fig. 4A). By contrast, CNP3 expression was significantly decreased by 250 mM NaCl transfer (approximately half-salinity of seawater). Similar to CNP3, real-time qPCR showed significant decrease in prolactin level by NaCl transfer, but not by $CaCl_2$ transfer (Fig. 4B). The decreases in CNP3 and prolactin mRNA levels by NaCl transfer were further confirmed by *in situ* hybridization (Fig. 4C–H). These results suggest that CNP3 and prolactin expressions respond to fluctuations of plasma Na^+/Cl^- concentration (s) or osmolality in NaCl-transferred eels.

A, **B** Effect of environmental ions on pituitary CNP3 and prolactin expressions evaluated by real-time qPCR. Eels kept in fresh water ($n = 10$), 250 mM NaCl ($n = 5$), or 14 mM $CaCl_2$ ($n = 5$) for a week were used for this experiment. CNP3 and prolactin expressions were significantly reduced by NaCl transfer. There was no difference in CNP3 and prolactin expressions between freshwater-acclimated eels and $CaCl_2$-transferred eels. $^{**}p < 0.01$ (both CNP3 and prolactin) with Kruskal-Wallis test (CNP3: $p = 0.0064$, prolactin: $p = 0.0042$) followed by Dunn's multiple comparison test (freshwater vs. $CaCl_2$/NaCl treatment). Data are shown as box-and-whisker plot (± SD); the absence of error bars indicates a small SD. **C–H** Localization of CNP3 and prolactin mRNAs in the pituitary of freshwater- and seawater-acclimated eels by *in situ* hybridization, supporting the results in **A** and **B**. Scale bar, 100 μm.

3.5. Strong negative correlations between pituitary CNP3/prolactin expression and plasma Cl^- concentration

To examine for any relationship between pituitary CNP3/prolactin expressions and plasma concentrations of each ion/plasma osmolality, we measured plasma ion concentrations and osmolality in each $CaCl_2$/NaCl-transferred eel. Plasma Na^+ concentration and osmolality were significantly increased by 250 mM NaCl transfer, while there were no significant differences in plasma Cl^- and divalent cations (Fig. 5A, Fig. A1). On the other hand, $CaCl_2$ (14 mM) transfer did not alter plasma ions and osmolality significantly. Since CNP3/prolactin expression decreased only in NaCl-transferred eels (Fig. 4), increases in plasma Na^+ concentration and osmolality may downregulate CNP3/prolactin expression. We also examined the correlation between pituitary CNP3/prolactin expression and plasma concentrations of each ion/plasma osmolality, using freshwater-acclimated eels whose CNP3 expressions were variable (Fig. 4A). Unexpectedly, there were no significant correlations between plasma Na^+ concentration and CNP3/prolactin expression levels ($r^2 = 0.31/r^2 = 0.27$). Rather, strong negative correlations were detected between CNP3 expression level and plasma Cl^- concentration ($r^2 = 0.90$)/osmolality ($r^2 = 0.75$), while there was a relatively weaker relationship between prolactin expression level and plasma Cl^- concentration ($r^2 = 0.53$) (Fig. 5B). There were no significant correlations between concentration of plasma divalent cations and CNP3/prolactin expression levels (Fig. A1).

A Effect of environmental ions on plasma Na^+, Cl^- concentration, and plasma osmolality. Eels kept in freshwater ($n = 10$), 250 mM NaCl (n = 5), or 14 mM $CaCl_2$ (n = 5) for a week were used for this experiment. NaCl transfer significantly increased plasma Na concentration and plasma osmolality. $^*p < 0.05$ (sodium, freshwater vs. NaCl), $^*p < 0.01$ (osmolality, freshwater vs. NaCl) with Kruskal-Wallis test (sodium: $p = 0.024$, osmolality: $p = 0.0033$) followed by Dunn's multiple comparison test (freshwater vs. $CaCl_2$/NaCl treatment). Data are shown as box-and-whisker plot (± SD). **B** Correlation diagram of plasma Na^+, Cl^- concentration/plasma osmolality and CNP3 expression (middle panels) or prolactin expression (bottom panels) in freshwater-acclimated eels. Negative relationship was detected between CNP3 and plasma Cl^- concentration, CNP3 and plasma osmolality, and prolactin and plasma Cl^- concentration (Pearson correlation coefficient test). R square values and p values are indicated in each graph.

4. Discussion

Here we first demonstrated using eels that CNP3 is a member of pituitary and pancreatic hormone families. Particularly, pituitary CNP3 was localized only in the prolactin-producing cells, and its downregulation preceded that of prolactin (a freshwater-adaptating hormone) after seawater transfer. Furthermore, negative correlations between CNP3 expression level and plasma Cl^- concentration/osmolality in the present study suggest that the CNP3 in prolactin-producing cells may be the product of an early responsive gene related to plasma Cl^- concentration and osmolality.

The previous reports show CNP expression in the pituitary of rodents, chicken, zebrafish, and dogfish (Chand et al., 2010; McArdle et al., 1994; Suzuki et al., 1994), suggesting important function in the pituitary in vertebrates. In rodents, CNP (orthologue of teleost CNP4) is co-localized with luteinizing hormone-producing cells (gonadotrophs) and thought to be an autocrine regulator of gonadotropes (Chand et al., 2010; McArdle et al., 1994; Suzuki et al., 1994). In contrast to mammalian anterior pituitary where 5 types of hormone-producing cells are intermingled, the anterior pituitary in most teleost species is zoned into cell-specific areas producing separately the anterior pituitary hormones, with the prolactin-producing cells specifically distributed in the rostral pars distalis of eels (Clarke and Bern, 2012; Nishioka et al., 1993). The localization of CNP3 mRNA corresponded to that of prolactin mRNA. It is uncertain whether CNP-expressing cells were different between mammalian and teleost pituitaries. Since the CNP isoforms expressed in the pituitary are phylogenetically different (CNP4 in mammals and CNP3 in eels), and the isoforms have different affinity to natriuretic peptide receptor-B in chicken and medaka (Inoue et al., 2003b; Nakamori et al., 2019), CNP3 function in the teleost pituitary could be different from that in mammalian pituitary. We also found CNP3 expression in glucagon-producing cells of the pancreas. Although the present and previous RT-PCR results (Nobata et al., 2010) suggested expression of CNP3 in the intestine, CNP3 signal was undetectable in the intestine. Therefore, the 'intestine' samples used for previous RT-PCR analyses may have included some pancreas tissues adjacent to the intestine. Furthermore, CNP3-producing cells identified in the present study were all endocrine cells, suggesting that CNP3 is secreted into the circulation. The present study showed that noticeably higher expression of CNP3, which is the sole CNP isoform expressed outside of the brain in the eel (Nobata et al., 2010), was found in the pituitary of freshwater-acclimated eels. Intriguingly, the previous radio-immunoassay for CNP1 in eels showed a significantly higher level of plasma CNP1 in the freshwater-acclimated eels than that in seawater-acclimated eels (Takei et al., 2001). However, CNP3 gene was not identified for its time, it is no longer sure whether the antiserum used in the CNP1 radio-

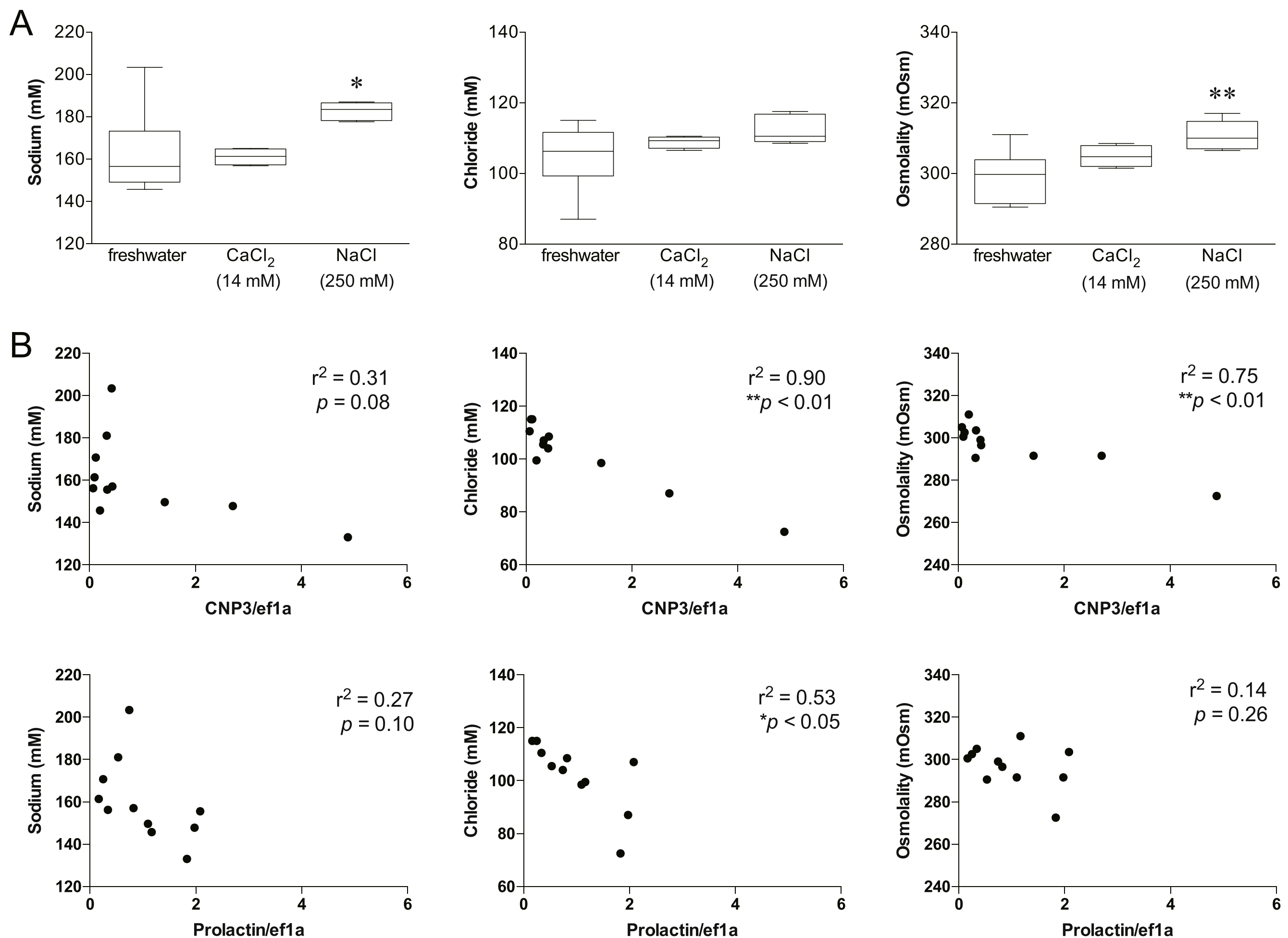

Fig. 5. Pituitary CNP3 and prolactin mRNA expression responded negatively to elevated plasma chloride level, but not to elevated plasma sodium level.

immunoassay cross-reacted with CNP3. It is highly possible that eel pituitary CNP3 is the main source of circulating CNP, although establishment of radio-immunoassay for eel CNP3 is required in future to check the association between pituitary CNP3 expression level and plasma CNP level. On the other hand, CNP3 located in the telencephalic area and medulla oblongata likely act as paracrine/autocrine fashion like other brain CNP isoforms (CNP1 and CNP4 in the case of eels), as these regions are protected by the blood-brain barrier (BBB) in eels (Mukuda et al., 2005). Although a study shows that CNP modulates BBB permeability to regulate transport of small molecules in mammals (Bohara et al., 2014), the functions of CNPs in the specific regions of fish brain remain unclear. Deciphering the novel physiological functions of CNP3 in eel brain will be interesting issues to address in future investigations.

The decrease in pituitary CNP3 and prolactin expression by seawater or hyperosmotic NaCl transfer suggests that eels respond to fluctuations of plasma Na^+ and Cl^- concentration and/or osmolality. The transfer of euryhaline tilapia from freshwater to either brackish water or seawater resulted in a decrease in plasma and pituitary levels of prolactin (Seale et al., 2006). Prolactin is known to reduce ion and water permeability of osmoregulatory surfaces and to increase ion uptake and water excretion in the kidney to facilitate freshwater adaptation in eel and other teleosts (Breves et al., 2014; Cao et al., 2018; Katayama et al., 2018; Manzon, 2002; Watanabe et al., 2016). Because signal intensity of CNP3 mRNAs in pancreatic glucagon-producing cells (Fig. A2), as well as in prolactin-producing cells, were decreased by high salinity stress, endocrine CNP3 derived from the pituitary and/or pancreas may act for freshwater adaptation (i.e., hyper-osmoregulation) in teleosts. A recent study showing that CNP reduces paracellular permeability by altering tight junction protein abundance in a primary cultured gill epithelium derived from rainbow trout (Kolosov and Kelly, 2020), supports a possibility of hyper-osmoregulatory action by CNP3. Similar to rainbow trout, eel CNP3 in freshwater eels might act on the gills to fine-tune branchial ion and water permeabilities. In addition, decrease in CNP3 levels by seawater transfer preceded that in prolactin levels, implying distinct regulation of CNP3 and prolactin gene transcripts. Pituitary CNP3 level, but not prolactin level, was significantly correlated with plasma osmolality. Thus, it is also possible that CNP3 produced by hypo-osmotic stress acts not only as endocrine hormone but also as autocrine hormone to regulate prolactin transcription/secretion in the pituitary, with supportive evidence that ‘fast acting’ NPs regulate mRNA expression and/or release of ‘slow acting’ hormones such as prolactin and growth hormone in teleosts (Fox et al., 2007; McCormick, 2001). Hypothalamic CNP stimulates prolactin secretion in mammals, but similar action has not been demonstrated in the pituitary of rats (Huang et al., 1992; Porzionato et al., 2010). Although CNP is involved in calcium homeostasis in mammals (Bartels et al., 2004; Chusho et al., 2001; Moyes and Hobbs, 2019; Potter et al., 2009), CNP3 expression as well as prolactin did not change significantly in eels kept in $CaCl_2$ medium. In addition, incubation of the eel pituitary in a medium with different concentrations of calcium did not affect the basal release of prolactin (Arakawa et al., 1993; Kaneko and Hirano, 1993). These findings suggest that teleost CNP3 and prolactin may be less important for calcium ion homeostasis, but play critical roles in regulation of univalent ions.

In freshwater-acclimated eels, negative correlation between CNP3

expression levels and plasma osmolality was found. Prolactin-producing cells are responsible for osmoreception via transient receptor potential vanilloid 4 (TRPV4) (Kultz, 2012; Watanabe et al., 2012). It was recently shown that elevation of Na^+ concentration is sensed by a 'NaX' sensor in hypothalamus of mammals, which is a center of osmoregulation (Matsuda et al., 2017; Nomura et al., 2019). Although the present study showed that plasma Na^+ concentration increased as plasma osmolality increased by 250 mM NaCl treatment, the changes in plasma Na^+ concentration were not correlated with expression level of CNP3 or prolactin. Instead, changes in plasma Cl^- concentration was significantly correlated with the expression of CNP3. The previous study shows that chronic exposure of CNP in a primary cultured gill epithelium derived from rainbow trout reduces Cl^- permeability, which results in an increase in branchial Na^+/Cl^- permeability ratio (Kolosov and Kelly, 2020). Negative correlation with plasma Cl^- but not with plasma Na^+ concentration found in the present study may be associated with such different actions of CNP on branchial Na^+ and Cl^- permeabilities. This action may also be related to an elevation of plasma Na^+ concentration but not plasma Cl^- concentration in eels reared in 250 mM NaCl solution.

We have herein analyzed CNP3/prolactin response to osmotic stress, using the euryhaline Japanese eel, and suggested that pituitary CNP3 in prolactin-producing cells might play a role in chloride-dependent hyper-osmoregulation in freshwater acclimation. This stands in contrast to the hyponatremic effect of cardiac NPs (e.g., ANP) in seawater acclimation (Tsukada and Takei, 2006). Further investigation of osmoregulatory functions of peripheral CNP, which is conserved in basal vertebrates (e.g., elasmobranches) (Kawakoshi et al., 2001), will provide a better understanding of ancestral functions of vertebrate NPs.

Disclosure statement

The authors have nothing to disclose.

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Declaration of competing interest

There are no conflicts of interest to declare.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mce.2020.110780.

Author contributions

YK wrote the manuscript and analyzed data; MK performed qPCR for CNP3 in eel tissues; MF performed qPCR for CNP3 and prolactin in eels acclimated in artificially-modified medium and measured plasma ions and osmolality; NT performed *in situ* hybridization for CNP3 in the pancreas; MY performed *in situ* hybridization for CNP3 in the pituitary; MW performed qPCR for CNP3 and prolactin during time-course of seawater transfer; TT coordinated the project together with MW. All authors read, edited, and approved the final manuscript.

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