Gene expression and hormone secretion profile of urotensin I associated with osmotic challenge in caudal neurosecretory system of the euryhaline flounder, *Platichthys flesus*

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ABSTRACT

The caudal neurosecretory system (CNSS) is a part of stress response system, a neuroendocrine structure unique to fish. To gain a better understanding of the physiological roles of CNSS in fluid homeostasis, we characterized the tissue distribution of urotensin I (UI) expression in European flounder (*Platichthys flesus*), analyzed the effect chronic exposure to seawater (SW) or freshwater (FW), transfer from SW to FW, and reverse transfer on mRNA levels of UI, L-type Ca^{2+} channels and Ca-activated K^+ channels transcripts in CNSS. The tissue distribution demonstrated that the CNSS is dominant sites of UI expression, and UI mRNA level in fore brain appeared greater than other non-CNSS tissues. There were no consistent differences in CNSS UI expression or urophysis UI content between SW- and FW-adapted fish in July and September. After transfer from SW to FW, at 8 h CNSS UI expression was significantly increased, but urophysis UI content was no significantly changes. At 24 h transfer from SW to FW, expression of CNSS UI was no apparent change and urophysis UI content was reduced. At 8 h and 24 h after transfer from FW to SW UI expression and urophysis UI content was no significantly effect. The expression of bursting dependent L-type Ca^{2+} channels and Ca-activated K^+ channels in SW-adapted fish significantly decreased compared to those in FW-adapted. However, there were no differences in transfer from SW to FW or from FW to SW at 8 h and 24 h. Thus, these results suggest CNSS UI acts as a modulator in response to osmotic stress and plays important roles in the body fluid homeostasis.

1. Introduction

Urotensin I (UI) is a 41-amino acid neuroendocrine peptide that belongs to the superfamily of corticotropin-releasing factors, isolated first from the urophysis of white sucker *Catostomus commersoni* (Lederis et al., 1982). In mammals, the UI orthologue – urocotrins regulate the stress response and many other vital functions such as regulation of glucocorticoid synthesis (Bernier et al., 1999), cardiovascular homeostasis (Conlon et al., 1996) and inhibition of appetite central nervous system functions (Yasuda et al., 2012), whereas in fish an involvement for UI in osmoregulation has also been suggested (Craig et al., 2005). However, many fundamental questions regarding the physiological roles of the UI in the modulation of osmotic stress remain unanswered (McCrohan et al., 2007).

The UI peptide has been isolated and characterized in several teleosts, mammals and the frog (Lederis et al., 1982; Lu et al., 2004). In euryhaline flounder (*Platichthys flesus*), UI is predominantly expressed and synthesized in the caudal neurosecretory system (CNSS), contributing most circulating UI (Lu et al., 2004). The CNSS of *P. flesus* has suggested roles in osmoregulatory, reproductive and nutritional adaptation, as fish migrate between seawater (winter) and brackish/freshwater (summer) environments (Flik et al., 2006). This neuroendocrine structure unique to fish, which comprised magnocellular neurosecretory Dahlgren cells, project to a discrete neurohaemal organ, urophysis. A number of neuron-peptides such as UI and corticotropin releasing hormone (CRH) (Lu et al., 2004), urotensin II (UII) (Lu et al., 2006), Stanniocalcin (STC) (Greenwood et al., 2009)and parathyroid hormone-related protein (PTHrP) (Lu et al., 2017)are potentially secreted from

the urophysis directly into the caudal vein and renal-portal system, ensuring their rapid delivery to possible peripheral target organs – kidney, intestine, gonad, spleen and liver.

As neuroendocrine cells, the secretory output of the Dahlgren cells depends as much on their electrical activity (i.e., firing frequency) which depends, in turn, on excitatory and inhibitory inputs as their expression of neuropeptides and ion channels. Our previous study has identified that the firing pattern is dependent on intrinsic membrane properties, a long-duration (up to 200 s) burst depends on a post-stimulus after depolarising potential (ADP) in depolarised cells (Brierley et al., 2004). Our studies suggest that ADP is due to an L-type Ca^{2+} conductance. Firing activity within a burst is maintained by short-duration (< 100 ms) depolarising after potentials (DAP), which follow each spike and are again voltage- and L-type Ca^{2+} channel-dependent (Brierley et al., 2001; Brierley et al., 2004). Our findings suggest that voltage-dependent bursting activity in Dahlgren cells is dependent on L-type Ca^{2+} conductance, with some role for Ca^{2+}-activated K^+ conductance in burst termination. Modulators that influence these conductances, or resting membrane potential, could have profound effects on firing pattern and promote more efficient peptide release.

In rainbow trout, studies indicate that CNSS UI neurons responded to the stress is stressor-, time-, and region-specific (Bernier et al., 2008). Physical restraint, subordination, and hypoxia elicited no marked changes of UI mRNA expression in neurons (Bernier et al., 2008; Lu et al., 2004). However, osmolality and hyperammonemia stressor are associated with increase in UI gene expression (Bernier et al., 2008; Craig et al., 2005). Isolation caused a decrease in the expression of UI transcripts (Bernier et al., 2008). These results suggest that the regulation of UI expression in teleosts maybe stressor-specific and species-specific, and the potential physiological roles of UI in different fish species are still a matter of discussion.

Thus, in order to assess the potential roles played by CNSS in the acclimation to low salinity stress in euryhaline flounder (*P. flesus*), we first characterized the mRNA levels of UI in different tissues by real-time PCR to assess the relative importance of the CNSS as sites of UI expression in this species, and investigated the impacts of hyposmosis on the CNSS UI, L-type Ca^{2+} channels and Ca-activated K^+ channels mRNA levels and the urophysis UI content.

2. Materials and methods

2.1. Animals

The flounder (*Platichthys flesus*, 360–500 g) were collected from Morecambe Bay (Cumbria, UK) and transported to aquarium facilities at the University of Manchester. They were then maintained in re-circulating, filtered 100% SW at 10–12 °C under 12 h:12 h light: dark photoperiod, for at least 2 weeks prior to experimentation. Fish were not fed during this time. All experiments were performed in accordance with United Kingdom Home Office Regulatory requirements and local Ethics Committee approval.

2.2. Experimental protocol

The salinity challenge experiment was performed as previous described by Lu et al. (2006). In brief, fish were held for at least 2 weeks in either medium prior to sampling. In order to examine the effect of osmotic challenge and control for the handling and disturbance of fish, SW-adapted fish were removed from 100% SW tanks and transferred directly to equivalent FW tanks (experimental transfer) or new 100% SW tanks (time-matched control). This experiment was carried out in July. A second group of fish were fully adapted to FW (at least 2 weeks) and then removed from 100% FW tanks and transferred directly to equivalent SW tanks (experimental transfer) or new 100% FW tanks (time-matched control). This experiment was carried out in September. Groups of 8 fish were sampled 8 and 24 h after transfer. Blood samples (3–5 ml) were

taken within 90 s by direct needle puncture of caudal blood vessels. Fish were humanely killed using Schedule One procedure detailed under UK Home Office licence procedures, and tissues were removed and snap frozen in liquid nitrogen. All samples were taken during the daytime.

2.3. RNA preparation

Different tissues were dissected out from 20 SW-adapted fish. These included the CNSS (the caudal 8 segments of the spinal cord) and separate urophysis. Brain tissue was further separated into 5 regions (forebrain, midbrain, hindbrain, hypothalamus and pituitary). Tissues were homogenized in 4 M guanidium thiocyanate buffer (pH 7.5) containing 1% β-mercaptoethanol. Total RNA was extracted by ultracentrifugation at 27,000 g for 20 h on a bed of 5.7 M CsTFA (Amersham-Phamacia Biotech, UK). RNA for distribution RT-PCR was treated with DNase (Roche, UK).

For chronically SW or FW adapted groups and SW or FW transfer experiment samples, total RNA was extracted from individual fish CNSS by Trizol™ (Invitrogen, UK).

2.4. Plasma osmolality and ion measurements

Plasma osmolality was measured by freezing point depression (Roebing Osmometer, Berlin, Germany), sodium concentration were determined by flame photometry (Corning 480, Corning Ltd, Essex, UK) and chloride concentrations were analyzed by electrometric titration (Chloride Analyser 925, Corning Ltd., Essex, UK).

2.5. Hormone measurements

UI content of isolated urophysis were determined in triplicate by RIA (Lu et al., 2013), using anti-serum which was raised in rabbit against the last 18 amino acid residues at the C terminus of flounder UI by BioCarta, UK. The affinity purified antibody was used at a final dilution of 1:600. The iodination of flounder 0Tyr-UI peptide was performed using the Iodogen method. The ^{0}Tyr-UI-^{125}I was then purified by reverse-phase high performance liquid chromatography (HPLC, Vista 5500, Varian UK) using C5-column (Phenomenex, UK). The large single peak eluted after 42 min, corresponding with an acetonitrile concentration of 42%, was found to be ^{0}Tyr-UI-^{125}I, and this was confirmed following successful binding (typically 81%) with an excess of the UI antibody (final dilution 1/50). Radioimmunoassay for ^{0}Tyr-UI-^{125}I, antibody, and standards (synthetic full length UI peptide from BioCarta, UK) were all prepared in assay buffer to final dilutions of 2500 cpm/tube, 1:600, and 5.29×10^{-8}–1.03×10^{-10} mol/L M UI/tube, respectively. Intra- and inter-assay coefficients of variation were 6.1% and 12.3% (n = 10 for both). There was no displacement of UI radiolabel with any of flounder CRH, UII, PTHrP and AVT peptides tested in standard concentration range indicating that there was no cross reactivity with the antibody and the specificity of the assay for UI. The UI RIA has an approximate working range of 1×10^{-8}–1×10^{-10} mol/L.

Each snap frozen urophysis was homogenised in 1 ml of ice cold 0.01 M HCl for 10 min and then centrifuged for 10 min at 4 °C and 3000g. The supernatant was frozen in liquid nitrogen, and stored at −80 °C until use in the RIA. This homogenate was used for the subsequent UI RIA's. The urophysial extract was diluted in assay buffer to 1 in 1000 for UI.

2.6. Relative quantitative RT-PCR

The quantitative real-time PCR reaction was carried out in 96-well qPCR plates on an ABI PRISM 7000 detector (Applied Biosystems, Foster City, CA). UI, L-type Ca^{2+} channel, Ca-activated K^+ channel and beta-actin primers were previously described by Lu et al. (2004, 2007). The optimization and validation of primers and probes were performed using standard ABI protocols. PCRs were performed in triplicate as described by Lu et al. (2013).

1 μg total RNA of fish CNSS tissues from the salinity transfer experiments were treated with DNase (Invitrogen, UK), and then first strand cDNA was synthesised (SuperScript™ II cDNA kit, Invitrogen, UK) according to the manufacturer's instructions using random primers. The real-time PCR was performed in a final volume of 25 μl consisting of optimal concentration (12.5 ng) of reverse transcribed cDNA mixed with optimal concentrations of primers (300 nM for UI and beta-actin; 600 nM for ion channels) and Taqman™ probe (100 nM for UI and beta-actin; 200 nM for ion channels) and qPCR™ Master mix plus kit (Eurogentec, Belgium), using a standard amplification profile (2 min at 50 °C, 10 min at 95 °C and then 40 cycles of the following: 15 s at 95 °C and 1 min at 60 °C).

Flounder β-actin was used as reference genes. Relative quantization values were expressed using the $2^{-\Delta\Delta Ct}$ method as fold changes in the target gene normalized to the reference gene and related to the expression of a control sample (Lu et al., 2004; Lu et al., 2006).

2.7. Statistical analysis

Results from measurements of plasma osmolality, electrolytes, urophysial UI content and relative mRNA levels of UI, L-type Ca^{2+} channel, Ca-activated K^+ channel are expressed as means ± SE. Differences between groups were analyzed by Student's *t* test. Significance level were set at $p < 0.05$.

3. Results

3.1. Tissue distribution of flounder UI mRNA

The relative mRNA expression levels in different tissues for UI were determined by real-time PCR (Fig. 1). The results indicated that UI mRNA was predominantly expressed in CNSS, the relative UI mRNA levels in CNSS was 2226 times higher than the second most expressed
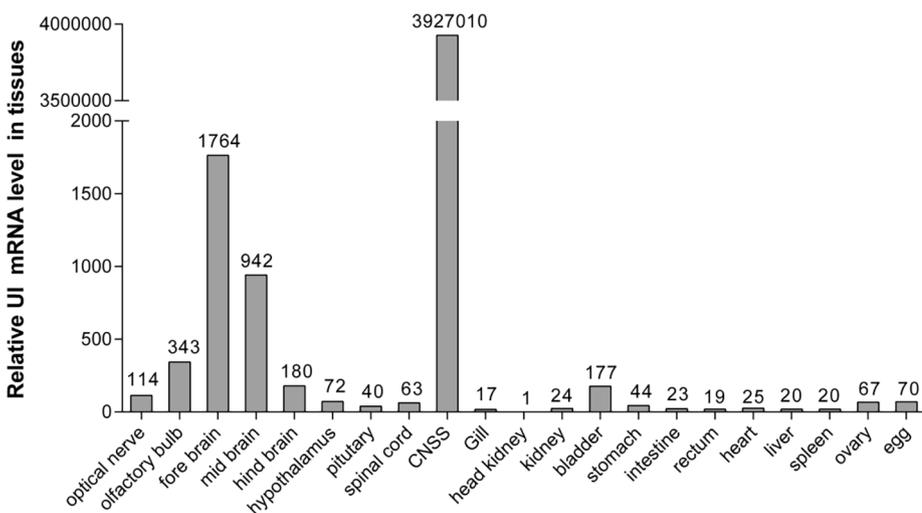

Fig. 1. Distribution of UI mRNA in different tissues of European flounder. The gene expression of each tissue relative to head kidney analyzed by real-time RT-PCR with β-actin as reference gene. Values are estimates for pooled samples from 20 fishes. The relative mRNA expression level of UI (CNSS > fore brain > mid brain > olfactory bulb > hind brain > bladder > optical nerve > hypothalamus > egg).
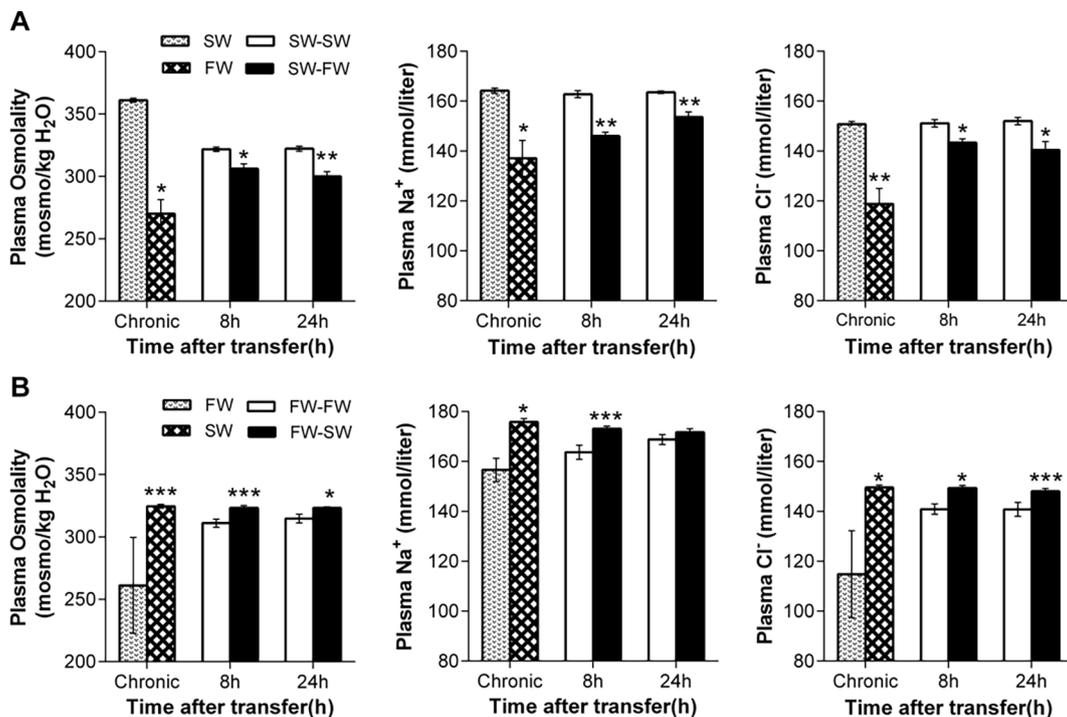

Fig. 2. Effect of transfer between SW and FW on plasma osmolality, sodium, and chloride (A, July; B, September). Values plotted are means ± SE, n = 7–8 per group, $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.0005$. An independent-samples *t* test was used to assess differences between chronically adapted FW and SW flounder and between experimental and time-matched controls at each time point.

tissue – fore brain. The UI mRNA level in fore brain, mid brain, olfactory bulb, hind brain, and bladder appeared greater than other non-CNSS tissues, whereas the fore brain appeared to express more UI than other regions of the brain.

3.2. Plasma composition of flounder

Osmolality, sodium, and chloride of plasma were significantly higher in SW- than FW-adapted fish in both of experimental series (Fig. 2). At 8 h and 24 h after transfer fish from SW to FW, plasma osmolality, sodium, and chloride were significantly lower compared with time-matched SW-maintained controls (Fig. 2A). Transfer fish from FW to SW caused increase in plasma osmolality, sodium, and chloride at 8 h and 24 h (Fig. 2B).

3.3. UI mRNA levels and urophysis UI content

No differences in relative CNSS UI mRNA expression levels were evident in SW- and FW-adapted flounder in both of experimental series (Fig. 3A and D). There were no statistically significant changes in urophysial stored UI, while UI content was higher in FW than SW fish in July but higher in SW than in FW-adapted fish in September (Fig. 3E and G). At 8 h after transfer fish from SW to FW, UI mRNA expression were significantly higher compared with time-matched SW maintained controls, while at 24 h no significant changes were observed in July (Fig. 3B; July). Urophysial stored UI showed a decrease trend in FW transferred fish relative to time-matched SW controls (Fig. 3C; July). At 8 h and 24 h after the reverse transfer from FW to SW, UI mRNA and urophysis UI was no apparent changes (Fig. 3 E and F; September).

3.4. L-type Ca^{2+} channels and Ca-activated K^+ channels mRNA levels

Expression of mRNA for L-type Ca^{2+} channels was significantly higher in SW- than in FW-adapted fish (Fig. 4A and C). Similarly a higher mRNA expression level for Ca-activated K^+ channels was seen in SW-compared to FW-adapted fish, but there was no significant change in July (Fig. 5 A and C). Transfer fish from SW to FW or reverse transfer had no impact on expression level of L-type Ca^{2+} and Ca-activated K^+ mRNA expression (Fig. 4B and D; Fig. 5B and D).

4. Discussion

4.1. Tissue distribution of UI mRNA

The real-time expression data in this study confirmed the CNSS as the primary site of UI gene expression. This corroborate the results of our previous results in flounder and Beriner et al. in the rainbow trout (Bernier et al., 2008; Craig et al., 2005; Lu et al., 2004). In the brain, the detection of UI transcripts in the telecephalon-preoptic region, optic tectum-thalmus, posterior brain and hypothalamaic region in flounder is consistent with results from goldfish (Bernier and Craig, 2005). In rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*), the majority of UI mRNA expression is also found in the CNSS relative to the brain (Barsyte et al., 1999; Ishida et al., 1986). These suggest that this hormone plays its greatest role in peripheral endocrine actions from CNSS and has a comparatively small role from the brain. The highest expression of UI in non-neural tissue is in the urinary bladder of flounder. The regulation of osmotic pressure is related to the urinary system. Bladder is the final place to reabsorption water from urine. Previous studies show that urotensin I and II, will stimulate electrogenic Na^+ reabsorption across the urinary bladders (Loretz and Bern, 1981). The highest expression of UI in non-neural tissue is in the urinary bladder indicted that UI may also directly produce in the bladder associated with osmoregulation locally.
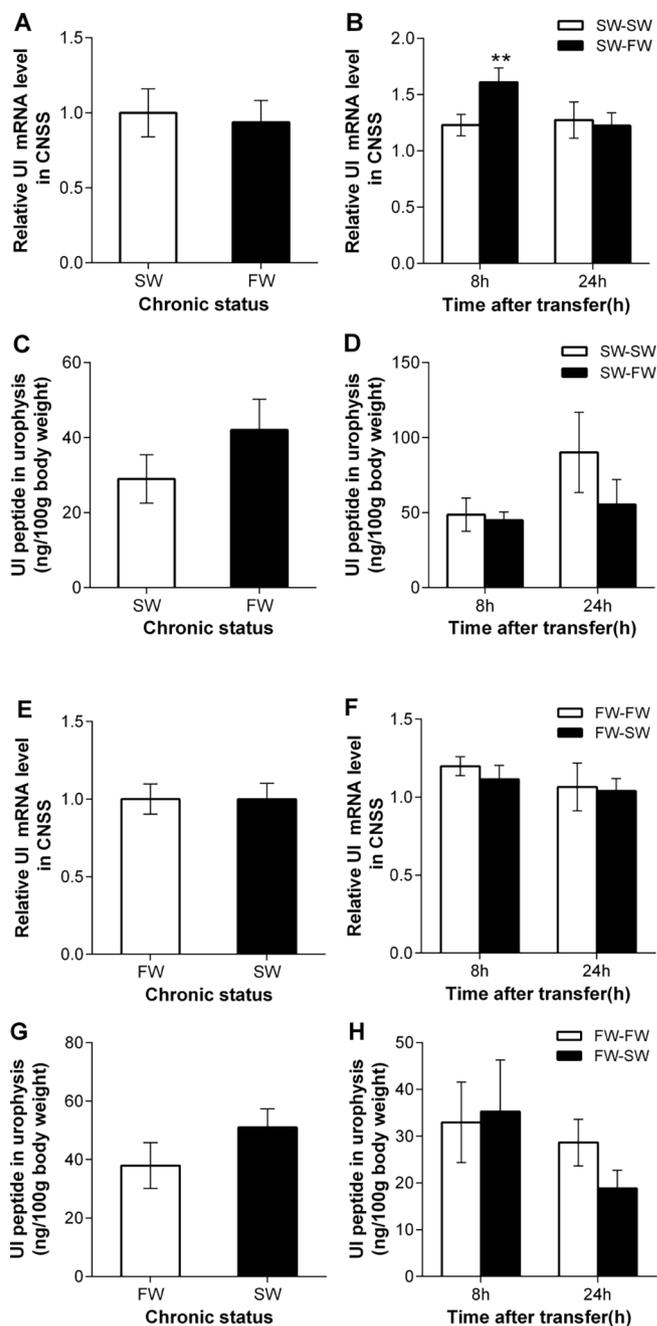

Fig. 3. UI mRNA levels and urophysis UI content in CNSS from flounder chronically adapted to SW or FW (A and C, July; E and G, September), and at 8 h and 24 h following experimental transfer from SW to FW (B and D, control SW to FW, July), and from FW to SW (F and H, control FW to FW, September). Values plotted are means + SE, n = 7–8 per group, **$P < 0.005$, compared to time-matched control.

4.2. Effect of transfer between SW and FW on plasma osmolality, sodium and chloride

Flounders are able to survive in both FW and SW but, in common with other euryhaline fish species, maintain a lower blood tonicity in FW. In both of the July and September experimental series, osmolality, sodium, and chloride of plasma were significantly higher in SW- than FW-adapted flounder. The same effect of seawater to freshwater transfer on plasma osmolality and inorganic ion concentrations has previously been studied in the same species (Bond et al., 2002). It is notably that plasma osmolality in SW is higher than that in SW-SW in
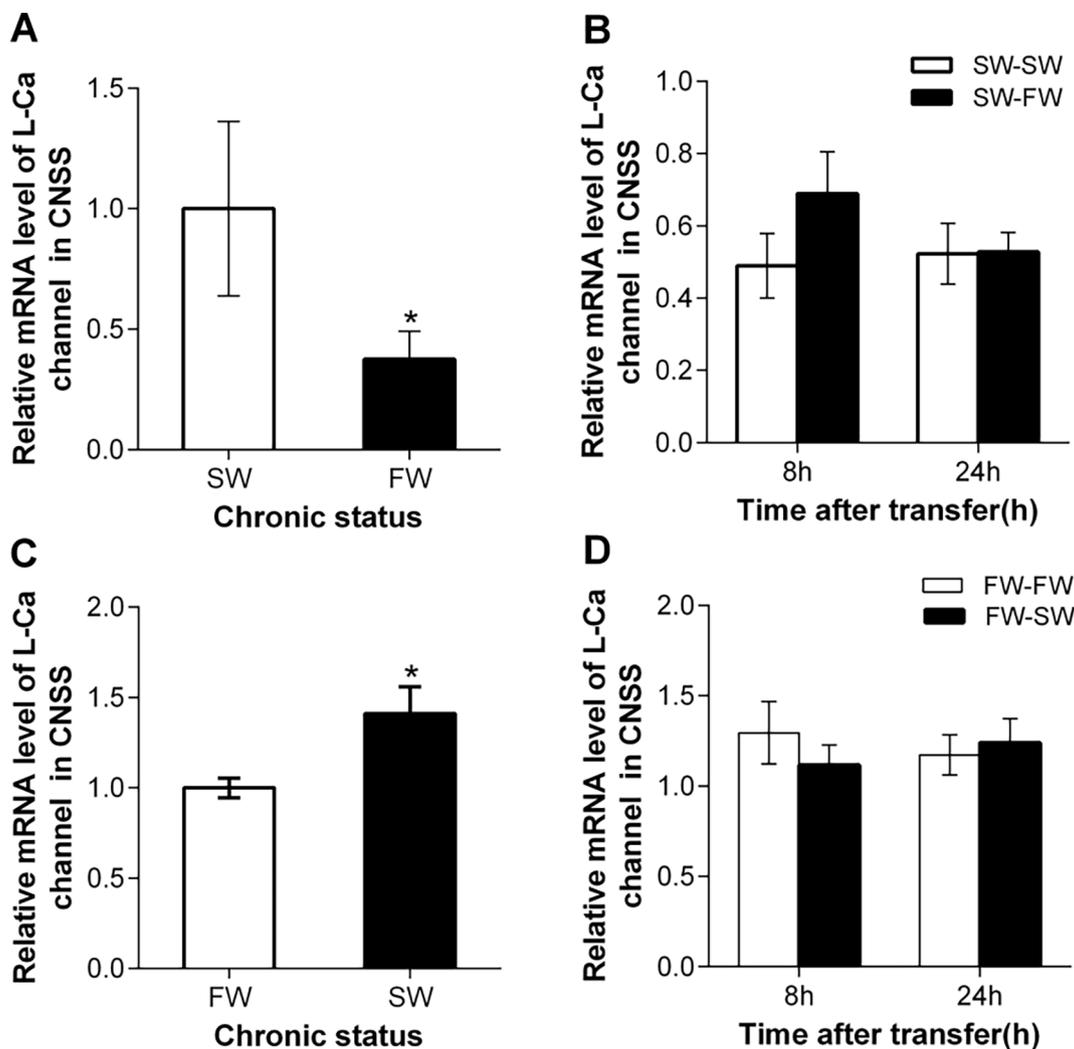
Fig. 4. L-type Ca^{2+} channels mRNA levels in CNSS from flounder chronically adapted to SW or FW (A, July; C, September), and at 8 h and 24 h following experimental transfer from SW to FW (B, control SW to FW), and from FW to SW (D, control FW to FW, September). Values plotted are means ± SE, n = 7–8 per group, [*]$p < 0.05$, compared to time-matched control.

July experimental series, that is not mirrored in the plasma [Na$^+$] or [Cl$^-$]. On the contrary in September, plasma osmolality in FW is lower than that in FW-FW. Such effect may be due to combination of transfer handling stress and salinity changes, transit elevated cortisol or other stress hormone could alter plasma osmolality and plasma ions after transfer (Lu et al., 2013; Miguel Mancera et al., 2002), stress caused by transfer can cause changes in plasma osmolality but does not necessarily cause changes in plasma [Na$^+$] or [Cl$^-$], which may cause changes in other component in plasma. Such stress and elevated cortisol level will return to normal levels after two days (Bolasina, 2011; Hiroi et al., 1997).

4.3. Effect of transfer between SW and FW on UI mRNA levels and urophysis UI content

European flounder shows adaptation to both marine and freshwater environments, associated with its annual migratory/reproductive cycle (Marley et al., 2007). Our previous studies have confirmed UI expression peaked in late summer (August), possibly in anticipation of the physiological challenges associated with offshore migration (Lu et al., 2007). In this study, expression of UI in CNSS was no apparently difference between SW- and FW- adapted fish in July and September. Similarly, there appeared to be no consistent difference in the CNSS between chronically SW- and FW-adapted fish in terms of UII mRNA

expression (Lu et al., 2006). It is notably that in July, while no statistically differences, urophysis UI content in FW-adapted fish is higher than that in SW-adapted.

UI is known to be associated with osmoregulatory role and cardiovascular functions in fish (McCrohan et al., 2007; Winter et al., 2000). Previous studies have also reported changes in the immunoreactive pattern of UI and the ultrastructural appearance of the CNSS in response to salinity alternations (Arnold-Reed et al., 1991; Larson and Madani, 1991; Larson and Madani, 1996). Similarly, UI mRNA level in CNSS is shown to be upregulated in rainbow trout when they were transferred from FW to SW (Craig et al., 2005). Together above studies, transfer to a hyperosmotic environment results in an increase in Dahlgren cell UI immunoreactivity and mRNA expression, a decrease in urophysis UI signal, and a reduction in the abundance of urophysis neurosecretory granules. In this study, we observed that UI mRNA expression was significantly upregulated in fish transfer from SW to FW at 8 h, but no differences were seen at 24 h. In parallel, urophysis UI content was appeared no changes at 8 h, but decreased at 24 h in transfer from SW to FW compared with SW time-matched control. In contrast, rainbow trout exposed to SW conditions elicited marked increases in the levels of UI transcripts in the CNSS at 24, 72, and 168 h post transfer (Craig et al., 2005). Compared with rainbow trout, flounder has lower metabolic rate and may response less to the environmental changes (Craig et al., 2005; Lu et al., 2006; Rupia et al.,
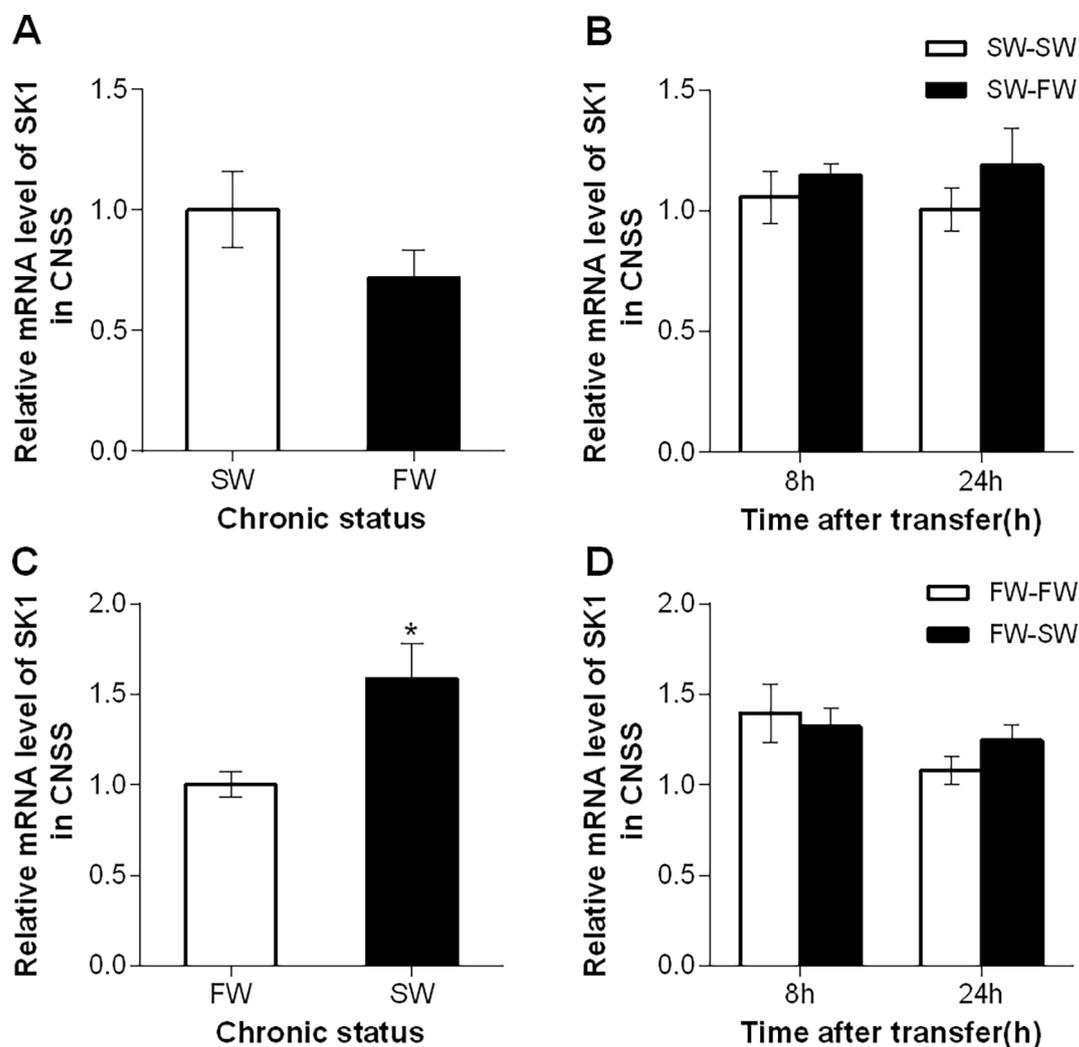
Fig. 5. Effect of salinity changes on mRNA expression for Ca-activated K$^+$ channels in CNSS from flounder chronically adapted to SW or FW (A, July; C, September), and at 8 h and 24 h following experimental transfer from SW to FW (B, control SW to FW), and from FW to SW (D, control FW to FW, September). Values plotted are means ± SE, n = 7–8 per group, $^*p < 0.05$, compared to time-matched control.

2016; Stiller et al., 2017). Furthermore, previous studies have indicated daily changes in peptide mRNA expression levels may only change the level of cell expression to compensate for loss of stored peptides during higher secretion periods (Lu et al., 2013). Our finding together with the previous results, suggest that the modulation secretion of UI from CNSS may be more important than modification of peptide mRNA expression levels in flounder when response to moderate environmental challenges.

4.4. Effect of transfer between SW and FW on L-type Ca^{2+} channels and Ca-activated K$^+$ channels mRNA level

In mammalian neuroendocrine cells, high frequency, patterned bursts of action potentials have been shown to enhance efficiency of peptide secretion from terminals, when compared to tonic, un-patterned, activity (Bicknell et al., 1988; Cazalis et al., 1985), indicating that bursting provides an important control on secretory output. In founder Dahlgren cells, the L-type Ca^{2+} channels (after-depolarising potential, ADP) and Ca-activated K$^+$ channels (depolarising after potentials, DAP) have been shown to underlie patterned bursting activity, which is thought to promote more efficient peptide release (Brierley et al., 2004). In our study, expression level of L-type Ca^{2+} channel is lower in FW-adapted flounder CNSS than in SW-adapted flounder CNSS in both season. In consistent with our results, electrophysiological

experiments have shown that bursting activity in Dahlgren cells is less robust in FW-adapted compared to SW-adapted fish (Ashworth et al., 2005; Brierley et al., 2003) and this is due, at least in part, to a reduction in Ca^{2+} channel-dependent ADP and Ca-activated K$^+$ channels-dependent DAP (Brierley et al., 2003). In this study, we also examined L-type Ca^{2+} channels or Ca-activated K$^+$ channels gene expression in fish transferred from SW to FW and reverse transfer. The results showed that there was no appeared difference in the CNSS neuroendocrine system between SW- and FW- transfer fish in terms of L-type Ca^{2+} channels or Ca-activated K$^+$ channels mRNA levels.

Our findings, together with the previous results, suggest that the UI in the CNSS of euryhaline flounder play an important role in response to osmotic stress by Dahlgren cells activation and secretion. Further, elevated cortisol level induced by urotensins is also suggested to be involved in maintaining body homeostais during stress due to its gluconeogenic action (Winter et al., 2000). We have shown that UI may modulate cortisol secretion, and suggest that the CNSS affords stress-specific stimulation of cortisol secretion (Kelsall and Balment, 1998) independent of the hypothalamic pituitary input (Winter et al., 2000).

In conclusion, this study provides additional evidence that the CNSS is the major tissue contributing to circulating UI. We were able to confirm that UI mRNAs are widely distributed, and that UI is likely involved in tissue-specific autocrine and paracrine roles in addition to their endocrine effects. The experimental results suggest that the UI in

the CNSS of euryhaline flounder involved in response to osmotic stress by Dahlgren cells activation. Together these findings imply a neuroendocrine role for CNSS-produced UI acts as a modulator response to osmotic stress and plays important roles in the body fluid homeostasis.

5. Authors' contributions

Weiqun Lu designed and carried out experiments, interpreted the results, drafting and finalizing of the manuscript. Gege Zhu and Aqin Chen was responsible for experiments and data analysis. Catherine R. McCrohan and Richard Balment provided scientific concepts and direction, and finalized the manuscript. All authors read and approved the final manuscript.

Acknowledgments

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