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Effects of environmental salinity on gill endothelin receptor expression in the killifish, *Fundulus heteroclitus*

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ABSTRACT

We recently determined that rapid changes in environmental salinity alter endothelin-1 (*EDN1*) mRNA levels in the euryhaline killifish, *Fundulus heteroclitus*, so we hypothesized that EDN1 may be a local regulator of gill ion transport in teleost fishes. The purpose of the present study was to examine the effects of changes in environmental salinity on the gill endothelin receptors: EDNRA, EDNRB, and EDNRC. Using quantitative real-time PCR, we determined that after a fresh water (FW) to seawater (SW) transfer, there is a two to threefold increase in gill *EDNRA* and *EDNRB* mRNA levels. Likewise, we found a two to three fold increase in gill EDNRA and EDNRB protein concentration. In addition, killifish that have acclimated to FW for 30 days had significantly lower EDNRA mRNA and protein levels than SW killifish. ENDRA were immunolocalized to the mitochondrion-rich cells of the killifish gill, suggesting that EDN1 signaling cascades may affect MRC function. EDNRB were found throughout the gill vasculature and on lamellar pillar cells. We previously immunolocalized EDN1 to the pillar cell suggesting that EDN1 acts as an autocrine signaling molecule and potentially regulates pillar cell tone and lamellar perfusion. We conclude that EDN1 is physiologically active in the teleost gill, and regulated by environmental salinity. Future functional studies examining the physiological role of this system are necessary to completely understand EDN1 in the fish gill.

1. Introduction

The endothelin (EDN) family of paracrine peptides consists of three isoforms, EDN1, EDN2 and EDN3, (Yanagisawa et al., 1988a; Inoue et al., 1989), and is found only in vertebrates (Hyndman and Evans, 2007). These proteins are involved in diverse physiological functions including: regulation of vascular tone (Yanagisawa et al., 1988a,b; La and Reid, 1995), alteration of ion transport (Zeidel et al., 1989; Garvin and Sanders, 1991; Prasanna et al., 2001; Ahn et al., 2004; Evans et al., 2004; Ge et al., 2006), and migration of neural crest cell during craniofacial development (Kurihara et al., 1994; Clouthier and Schilling, 2004). These physiological functions are mediated via three G-protein-coupled receptors termed: endothelin A receptor (EDNRA) (Arai et al., 1990), endothelin B receptor (EDNRB) (Sakurai et al., 1990), and the endothelin C receptor (EDNRC) (Hyndman, Miyamoto, Evans unpublished)(Karne et al., 1993; Lecoin et al., 1998).

Endothelin receptors (EDNRs) have been characterized in some fishes, but the results are often species specific. For example, pharmacological studies have suggested that the aortic vascular smooth muscle of the dogfish shark has EDNRB-like receptors (Evans et al., 1996), but that eel, lamprey, and hagfish aortic vascular smooth muscle contains EDNRA-like receptors (Evans and Harrie, 2001). In addition, pharmacological studies using receptor binding assays demonstrated EDNRB-like receptors in the dogfish gill (Evans and Gunderson, 1999), but autoradiographic studies showed EDNRA throughout the vasculature of the trout (*Oncorhynchus mykiss*) gill (Lodhi et al., 1995). Recently, EDNRBs were immunolocalized in filamental arteries, lamellar arterioles and pillar cells of the cod (*Gadus morhua*) gill (Stenslokken et al., 2006). In addition, Stenslokken et al. (2006) described EDNRA on branchial nerves throughout the filaments. Finally, EDNRA were immunolocalized to pillar cells in the *Takifugu rubripes* gill (Sultana et al., 2007). The characterization of the EDNRs may be species and protocol specific. We recently sequenced gill cDNA for *EDNRA* (accession EU391601), *EDNRB* (accession EU391602), and *EDRNC* (accession EU391603) from the euryhaline killifish, *Fundulus heteroclitus*, demonstrating that all three EDNRs are present in the fish gill. Thus, by using molecular techniques, the question of EDNR expression and tissue distribution in these fishes can be resolved, and help one to understand the earlier pharmacological, autoradiographical and immunohistochemical experiments (Lodhi et al., 1995; Evans et al., 1996; Evans and Gunderson, 1999; Evans and Harrie, 2001).

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Recently, we described two EDN1s (EDN1A and EDN1B) from the killifish (Hyndman and Evans, 2007). Besides being a cardiovascular peptide, EDN1 has been hypothesized to regulate ion transport in fishes (Evans et al., 2004). In the killifish opercular epithelium (a model for the SW teleost gill) (Karnaky et al., 1977), EDN1 inhibited net chloride transport as measured by a reduction of the short circuit current (Evans et al., 2004). Incubation of killifish proximal tubules with EDN1 led to inhibition of transport by the multidrug resistance protein 2 (Mrp-2) in the tubules (Notenboom et al., 2002). The multidrug resistance protein 2 is found in the luminal membrane of the proximal tubules and transports a wide range of chemicals from lipophilic organic anions to polypeptides (Notenboom et al., 2002). These studies suggest that EDN1 and EDNRs are involved in the regulation of transport. We recently determined that 24 h post-sea to fresh water transfer there is a significant threefold increase in EDN1 expression, suggesting that EDN1 signaling cascades may be involved in the regulation of ion transport in killifish (Hyndman et al., 2007).

Killifish are euryhaline fish, distributed in the coastal waters from Florida to Newfoundland (Bigelow and Schroeder, 2002). They live in a harsh environment, where there are daily changes in environmental salinity and temperature (Marshall, 2003). They are a commonly used model species to test a variety of ecological, epidemiological, and physiological questions (Burnett et al., 2007). They have been instrumental in understanding the effects of changing environmental salinity on fish physiology because killifish can tolerate direct transfers between fresh and seawater. The purpose of this study was to determine the effects of environmental salinity on gill EDNR expression in an excellent osmoregulator, the killifish, with the aim to elucidate the putative role of EDN1 signaling in the fish gill.

2. Materials and methods

2.1. Fish maintenance

The University of Florida and Mount Desert Island Biological Laboratory (MDIBL) Institutional Animal Care and Use Committee approved all protocols. Killifish (*F. heteroclitus*) were trapped in the brackish waters of Northeast Creek, Mount Desert Island, ME, and maintained in free-flowing, 15 °C, 31 ppt seawater (SW) for 3 months before being transported to the University of Florida. There they were maintain in 150 gal stock tanks (Rubbermaid, Winchester, VA, USA) in 20 °C, 32 ppt SW or dechlorinated Gainesville Fl tap water for 30 days before experimentation. Fish were fed commercial pellets to satiation every other day, and ammonia, nitrites, and nitrates were below 0.1 ppt and pH was maintained between 7.8 and 8.0.

2.2. Salinity challenges

The killifish salinity challenge experimental design was previously described by Hyndman and Evans (2007). In short, killifish were subjected to one of four treatments: 1) SW to FW; 2) SW sham (SW to SW); 3) FW to SW; or 4) FW sham (FW to FW). At 0, 3, 8, 24 h (acute acclimation) and 30 days (chronic acclimation) after transfer, 8 or 9 killifish were sacrificed by decapitation, and the gills from the right side snap frozen for RNA analysis, and the left side snap frozen for Western blotting. In addition, from 3 killifish, gill arch 2 from both sides was fixed in 4% paraformaldehyde (in 10 mM phosphate buffered saline, PBS) for 24 h at 4 °C for immunohistochemical analysis.

2.3. Quantitative real-time PCR

We previously sequenced killifish cDNA for *EDNRA*, *EDNRB*, and *EDNRC* (Hyndman, Miyamoto and Evans, unpublished). From these sequences, we designed non-degenerate primers for quantitative real-time PCR (qRT-PCR) (Table 1). These primers were designed to amplify a product of 50–100 bp, across a predicted exon–exon boundary (if possible) to prevent amplification of genomic DNA. All reactions were run in duplicate, and all values were normalized to L8 mRNA values (Choe et al., 2005) and standardized to a cDNA standard curve as previously described by Hyndman and Evans (2007). With each reaction a melting curve analysis was completed to ensure only one product was amplified. In addition, we sequenced samples and confirmed that we had amplified the target of interest. Finally, qRT-PCRs were run using RNA instead of cDNA as a negative control, and confirmed that there was no genomic contamination.

Table 1
Non-degenerate primers used in the quantitative real-time PCR (q) experiments

Primer	5′ to 3′ orientation
EDNRAqF1	GCA TCA ACC TGG CGA CAA T
EDNRAqR1	CAG CAG CAC AAA CAC GAC TTG
EDNRBqF1	CTG ATG ACC TGC GAG ATG CTA A
EDNRBqR1	TCC CGC CGC TGC TTA ATA T
EDNRCqF1	CCT GCG AGA TGC TGA GTC G
EDNRCqR1	TTC CCT CCG CTG TTT CAT GT

2.4. Immunohistochemistry and immunoblotting

The immunohistochemical methods of Piermarini et al. (2002) and Hyndman and Evans (2007) were used. Five slides per animal (each slide was about 100 μm deeper into the tissue) were analyzed. In addition, Western blots were made from gill samples using the protocols of Piermarini et al. (2002) and Hyndman et al. (2006). Finally, to accurately quantify protein level differences, we made immunoblots following the methods of Joyner-Matos et al. (2006). In all of these protocols, we used the following antibodies: anti-EDNRA (1/500, Alomone Laboratories, Jerusalem); anti-EDNRB/C (1/1000, Alomone Laboratories). Currently a commercial antibody that can discriminate between EDNRB and EDNRC is not available. Anti-EDNRA was a rabbit polyclonal, affinity purified antibody made against rat EDNRA (amino acids 413–426) that shared 58% homology with killifish EDNRA. Anti EDNRB/C was also a rabbit polyclonal, affinity purified antibody made against rat EDNRB (amino acids 298–314), and shared 88% identity with killifish EDNRB and EDNRC. Negative controls were run using peptide-absorbed antibodies (1 μg peptide/1 μg antibody, mixed overnight, shaking, 4 °C following manufacturer's instructions). All of the protocols were run using the preabsorbed antibody to determine any non-specific binding. Slides were double-labeled with anti-chicken Na^+, K^+-ATPase as previously described (Hyndman et al., 2006). Westerns and dot blots were digitized using a flat bed scanner, and analyzed using Biorad's Quantity One software (Hercules, CA). The brightness and contrast of each slide image were adjusted with Photoshop CS3 (Adobe, San Jose, CA).

2.5. Statistics

All values were tested for normality and equal variance and if these were not met, the values were log-transformed for statistical analysis. Quantitative real-time PCR values were tested using two-factor ANOVA (treatment and time), and when significance was found specific differences between sham and treatment were determined using unpaired, two-tailed, *T*-test (α=0.05). Protein level differences were analyzed with one-factor ANOVAs and Dunnett's post hoc test. Chronic qRT-PCR and protein level differences were analyzed using unpaired, two-tailed, *T*-tests to test for differences between the SW and FW treatments.

3. Results

3.1. Endothelin receptor mRNA levels

In killifish transferred from FW to SW, there was a significant threefold increase in gill *EDNRA* mRNA levels 24 h post-transfer

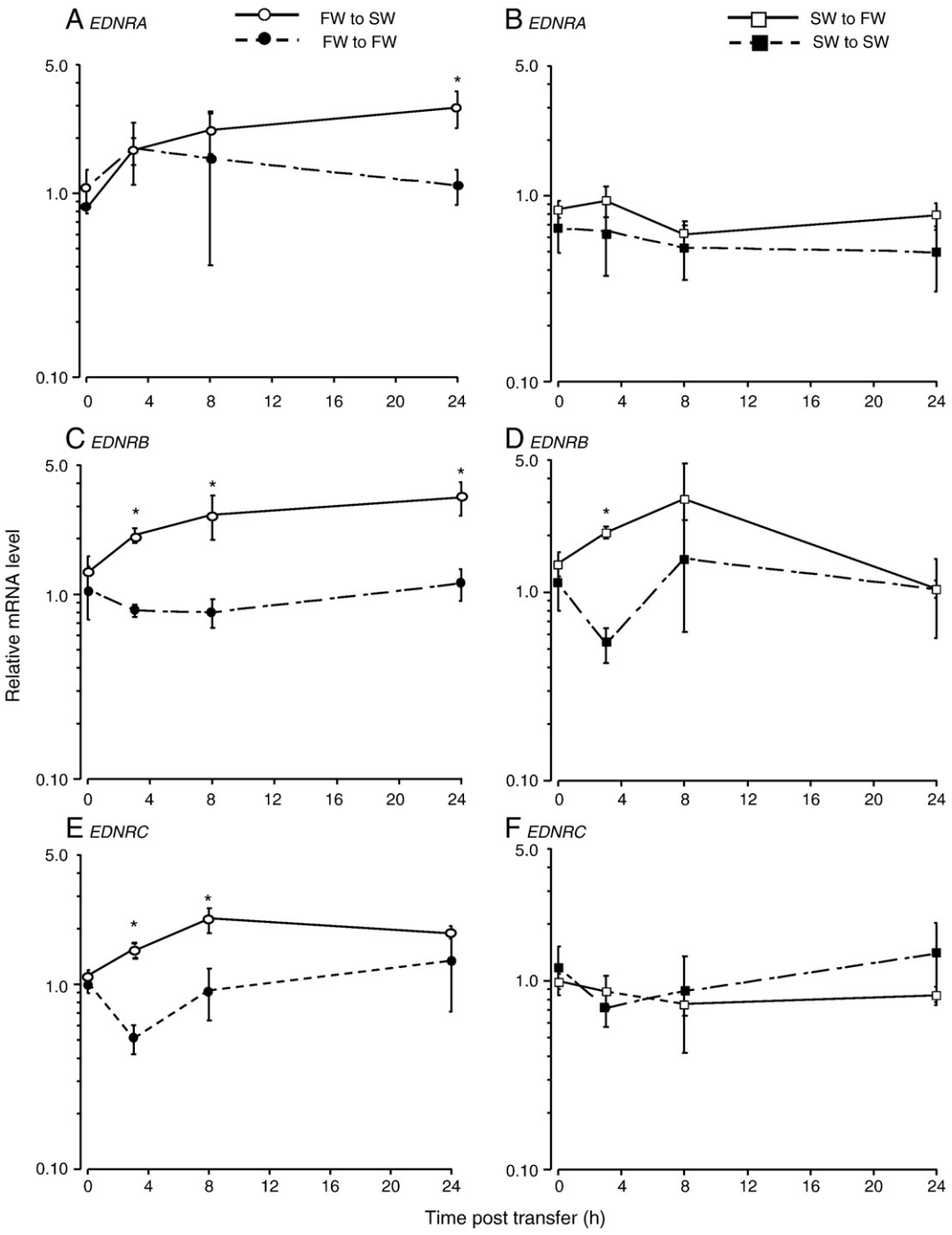

Fig. 1. Relative gill mRNA levels for endothelin receptors from killifish acclimating to seawater (SW) or fresh water (FW) over a 24 h period (n=5–6 fish/treatment). Dotted lines and closed symbols represent shams, and solid lines and open symbols represent treatments. A, C, E) fish transferred from FW to SW (○) or maintained in FW (sham, ●), and B, D, F) fish transferred from SW to FW (□), or maintained in SW (sham, ■). A, B) *EDNRA*, C, D) *EDNRB1*, and E, F) *EDNRB2*. All values are normalized to L8 mRNA levels and are made relative to chronic SW levels (Fig. 2). Asterisks (*) represents statistically significant differences compared to sham determined by 2-Factor ANOVA and unpaired, two-tailed, *T*-tests (compared to sham). Mean±s.e.m. Note that the scale is logarithmic.

(p=0.03) (Fig. 1A). In addition, there was a significant twofold increase in gill *EDNRB* mRNA compared to sham at 3 h (p<0.001), 8 h (p=0.003), and 24 h (p=0.016) post-transfer (Fig. 1C). Gill *EDNRC* mRNA levels increased 3 h and 8 h post-transfer (p<0.001), but *EDNRC* mRNA levels were not different from sham by 24 h (Fig. 1E). In killifish transferred from SW to FW, there were no significant changes in gill *EDNRA* or *EDNRC* mRNA levels compared to sham over the 24 h acclimation period (Fig. 1B, F). There was a significant increase in gill *EDNRB* mRNA 3 h post-transfer (p<0.001); however, *EDNRB* mRNA levels were not different from sham at 8 or 24 h post-transfer (Fig. 1D). After chronic acclimation (30 days) to SW or FW, there were no differences in *EDNRB*, or *EDNRC* mRNA levels; however gill *EDNRA* mRNA were 55% lower in the FW killifish compared to SW killifish (p=0.022) (Fig. 2).

3.2. Endothelin receptor protein concentrations

Using Western blots made from SW killifish gills, we found a single ~37-kDa band with the anti-EDNRA, and a single band of ~40-kDa with anti-EDNRB/C (Fig. 3). In the killifish gill, there was a threefold

increase in the total EDNRA protein level compared to control (time zero), 24 h post-FW to SW transfer ($p=0.014$) (Fig. 4A). Likewise, there was a twofold increase in EDNRB/C in the killifish gill compared to control over this 24 h period ($p=0.002$) (Fig. 4C). Post-SW to FW transfer, there was a significant decrease in EDNRA protein level (3 and 8 h) ($p<0.001$); however, by 24 h EDNRA protein levels return to control values (Fig. 4B). There was no significant change in EDNRB/C level post-SW to FW transfer (Fig. 4D). After chronic acclimation to SW or FW, there was a significant 60% decrease in gill EDNRA protein levels in the FW killifish compared to the SW killifish ($p=0.004$) (Fig. 4E). There were no statistical differences between SW and FW chronic acclimated killifish gill EDNRB/C levels (Fig. 4E).

3.3. Immunohistochemistry

In the gill, epithelial cells in the interlamellar region were immunopositive for EDNRA (Fig. 5). In addition, on the afferent side of the filament where there are no lamellae, there were many, large ovoid, cells immunopositive for EDNRA (Fig. 5D, E, H, I). All of the EDNRA-immunoreactive cells (-IR) were also immunopositive for NKA (Fig. 5C, E, G, I), suggesting that EDNRA is expressed in the MRC. There were no immunolocalization differences between SW control gills (Fig. 5A–C) and gills from killifish acclimated to SW for 24 h (Fig. 5D, E); however, 24 h post-SW to FW transfer, the EDNRA immunoreactivity became punctate, and less diffuse throughout the cell compared to the other treatments (Fig. 5H, I). In addition, gills from killifish acclimated to FW for 30 days, had a shift in EDNRA distribution. Compared to SW gills where the EDNRA-IR took up the whole cell (Fig. 5A, D), in FW gills there was a shift to only EDNRA along the bottom of the cell (Fig. 5F, G). Negative controls using a peptide-absorbed antibody were double-labeled with anti-NKA, and showed no non-specific binding of the EDNRA antibody (Fig. 5B).

Throughout the gill vasculature there was EDNRB/C-IR (Fig. 6A, C–H), including the prelamellar arterioles (Fig. 6E, F arrows). In addition, EDNRB/C-IR was found on lamellar pillar cells (Fig. 6D). Unlike EDNRA, the EDNRB/C-IR did not colocalize to the same cell as the NKA-IR (Fig. 6C, E, F, H). There were no obvious immunolocalization differences between the SW (Fig. 6A, C) and FW control samples (Fig. 6G, H). Also, there was no obvious difference between the 24 h fish acclimated to SW or FW (Fig. 6E, F). A negative control using peptide-absorbed EDNRB antibody displayed no immunoreactivity (Fig. 6B).

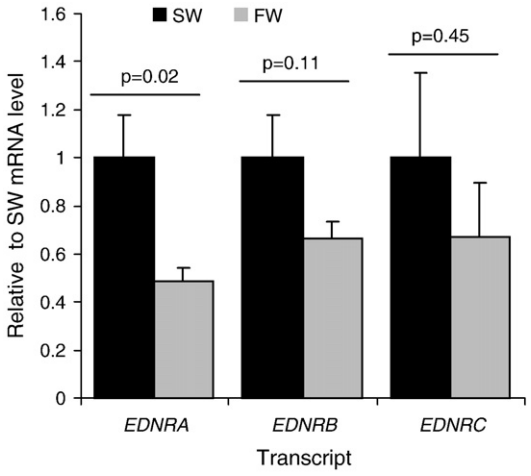

Fig. 2. Relative gill mRNA levels for the endothelin receptors from the killifish ($n=5$–6) acclimated for 30 days to either seawater (SW, black bars) or fresh water (FW, grey bars). All values relative to SW. Statistical significance was determined using unpaired, two-tailed, *T*-tests comparing SW to FW treatments (*p* values listed on figure). Mean ± s.e.m.

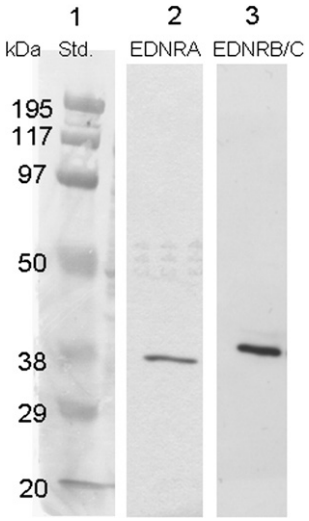

Fig. 3. Western blots from seawater killifish gills. Lane 1 is a Coomassie blue stained ladder. Lane 2 is a representative blot stained with anti-EDNRA. Lane 3 is a representative blot stained with anti-EDNRB/C.

4. Discussion

Endothelin signaling is a regulator of kidney salt transport in mammals (Pollock and Pollock, 2008) and hypothesized to play a similar role in the teleost gill (Evans et al., 2004). We previously reported that EDN1 is produced in the killifish gill, and that EDN1 levels are regulated by environmental salinity (Hyndman et al., 2007). In this study we present data that show that the three EDNRs are also expressed in the killifish gill and are regulated by rapid changes in environmental salinity, lending further support that EDN1 is a regulator of ion transport.

In the killifish, EDNRA are expressed on the mitochondrion-rich cell (MRC) of the interlamellar region of the filament. We previously reported that EDN1 is produced in a cell adjacent to the MRC (Hyndman et al., 2007) suggesting that EDN1 acts a paracrine signaling molecule and potentially modulates MRC function via the EDNRA. The MRC is the main site of ion transport in the teleost gill and Evans et al. (2004) determined that EDN1 inhibits net chloride transport by MRCs (as determined by a change in short circuit current across the opercular epithelium, a model for the SW teleost gill (Karnaky et al., 1977). Thus, our current model is that EDN1 can alter MRC function via EDNRA found on these cells.

In contrast, EDNRB/C was expressed throughout the gill vasculature, prelamellar arterioles, and lamellar pillar cells. Given the epithelial cell localization of EDN1 (Hyndman et al., 2007), it may function as a paracrine regulator of gill vascular tone, and perfusion of lamellae through control of the tone of the prelamellar arteriole; however, evidence suggests that EDN1 does not contract filamental arteries or lamellar arterioles (Stenslokken et al., 1999, 2006). In mammals, EDNRB function as clearance receptors in the pulmonary circuit (see La and Reid, 1995). In fishes, 55% of an EDN1 bolus was removed during a single pass through the gills (Olson, 1998). Thus, we postulate that gill vascular EDNRB/C are the clearance receptor in the killifish. There is not a commercial antibody that can differentiate between EDNRB and EDNRC. In the future, determining the specific localization of these two receptors will be beneficial in furthering our understanding of this system in the fish gill.

Given that EDN1 and EDNRB/C were both found on the lamellar pillar cells, it seems plausible that EDN1 is acting as an autocrine on these cells, potentially regulating pillar cell contractility. It was

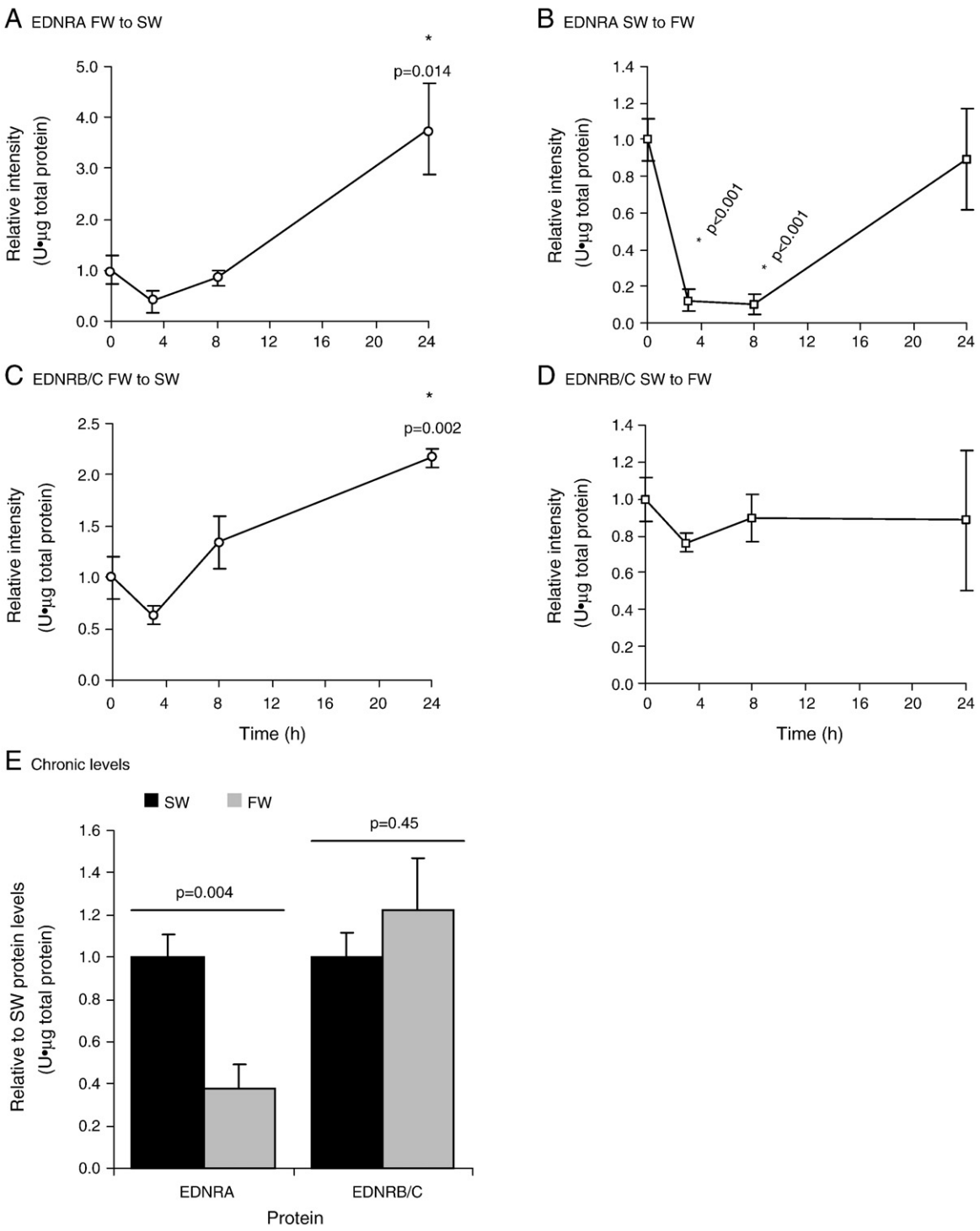

Fig. 4. Killifish gill protein level differences of the endothelin receptors during acclimation to SW or FW (n=5–6/treatment). EDNRA levels during acclimation to SW (A, open circles) or FW (B, open squares). EDNRB/C levels during acclimation to SW (C, open circles) or FW (D, open squares). These means are relative to time zero. Statistical significance was determined using ANOVA and Dunnett's post hoc test (p values listed on figure). E) Chronic (30 days) acclimations to either SW or FW, and all values are relative to SW values and statistical significance was determined using unpaired, two-tailed, T-tests. Mean±s.e.m.

hypothesized that EDN1 can redistribute and regulate lamellar blood flow through the lamellae of the fish gill (Sundin and Nilsson, 1998; Stenslokken et al., 1999). In the trout (*O. mykiss*) and cod, injections of EDN1 into the ventral aorta resulted in constriction of pillar cells, resulting in a shift of intralamellar blood flow to the outer marginal channels (Sundin and Nilsson, 1998; Stenslokken et al., 1999). Recently EDNRB-like receptors were immunolocalized to the cod pillar cells (Stenslokken et al., 2006). Pillar cells contain contractile elements and are not innervated (Bettex-Galland and Hughes, 1973; Mistry et al., 2004); thus paracrine peptides, like EDN1, may cause pillar cells to contract, and our data also support this hypothesis.

Recently, EDNRA were found on the pillar cells of the tiger pufferfish (*T. rubripes*) using a homologous pufferfish EDNRA antibody (Sultana et al., 2007). These receptors were capable of increasing

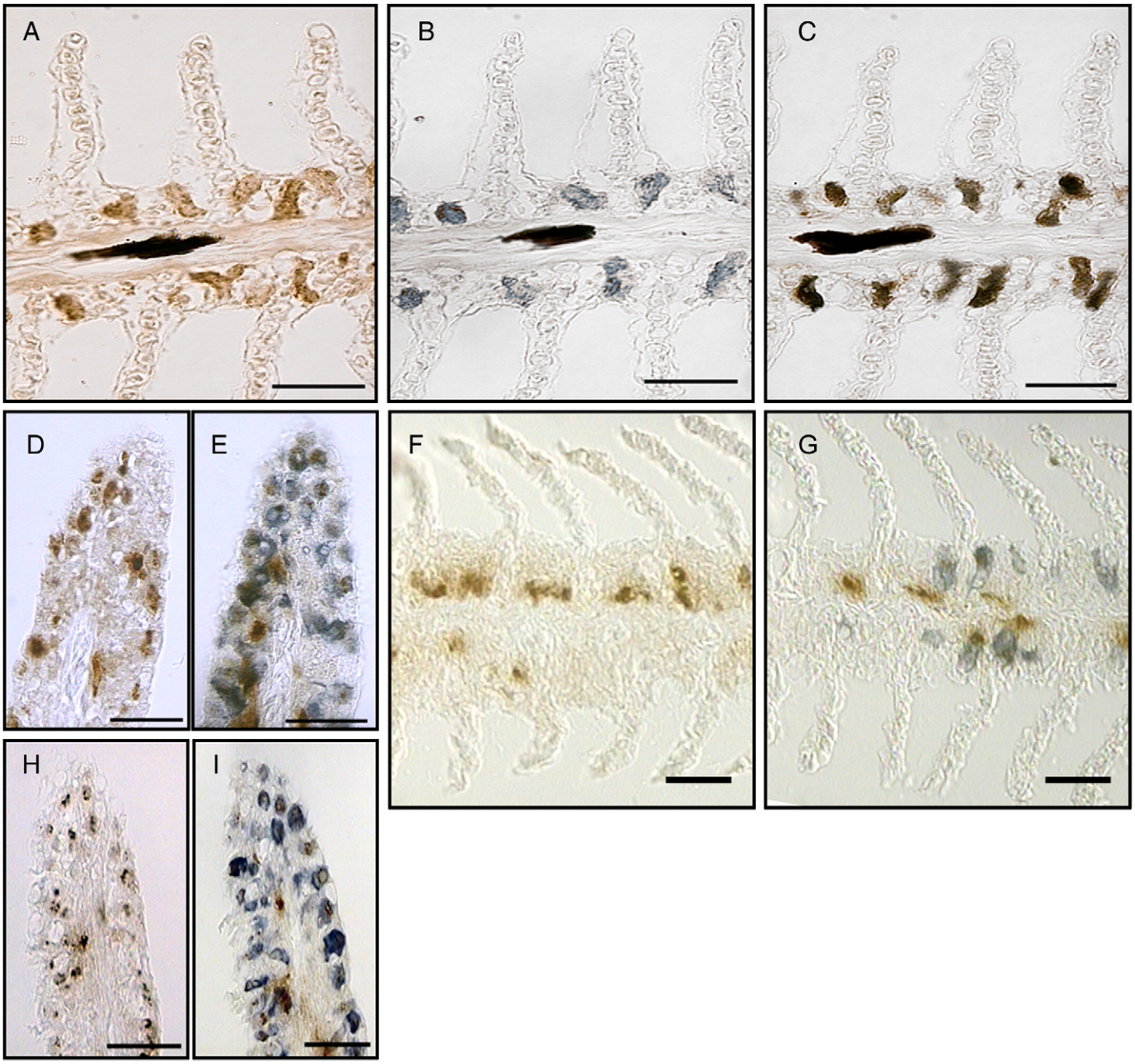

Fig. 5. Representative light micrographs of the immunolocalization of the endothelin A receptor (EDNRA) in the killifish gill. A–C) seawater (SW) chronically (30 days) acclimated fish. D, E) Gills from fish acclimated to SW for 24 h. F, G) Gills from fish chronically acclimated to FW. H, I) Gills from fish acclimated to FW for 24 h. A, D, F, H) EDNRA immunoreactivity (brown color). B) A section of gill 7 μm deeper into the gill from A and C, incubated in peptide-absorbed anti-EDNRA (no staining observed) and double-labeled with anti-Na^+, K^+-ATPase (blue). C, E, G, I) Gill sections double-labeled with anti-EDNRA (brown) and anti-Na^+, K^+-ATPase (blue). The immunoreactivity is in the same cell giving a grey appearance to the cell. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

intracellular calcium *in vitro*, suggesting that they cause contraction of pillar cells (Sultana et al., 2007). Pufferfish are a more derived species, suggesting that there may have been a switch in EDNR distribution from EDNRB/C on the pillar cells of more basal teleosts (i.e. killifish and cod) to EDNRA on the pillar cells in the derived species. Determining the cellular distribution of these receptors in more fishes will elucidate any such patterns.

The euryhaline killifish is an excellent osmoregulator that lives in an environment where environmental salinity and temperature change daily (Marshall, 2003). Thus, these fish are an appropriate model to test questions of the effects of rapidly changing environments on gene and protein expression. Our data demonstrate that rapid changes in environmental salinity lead to changes in killifish gill EDNR mRNA and protein expression. EDNRA mRNA and protein levels increased during 24 h acclimation from FW to SW, while *EDNRA* mRNA levels remained constant during a 24 h acclimation from SW to FW. However, EDNRA protein levels were significantly reduced 3 and 8 h after transfer from SW to FW, even though EDNRA mRNA levels remained constant. Currently the effect of changing salinity on protein and mRNA turnover is undetermined. This finding is intriguing and warrants further investigation.

Along with changes at the mRNA and protein level, we observed changes in EDNRA localization in the killifish gill. In SW, the killifish express EDNRA throughout the MRC, likely on the extensive tubular system of the basolateral membrane (Karnaky et al., 1976). However, during acclimation to FW, there is a shift from EDNRA being expressed

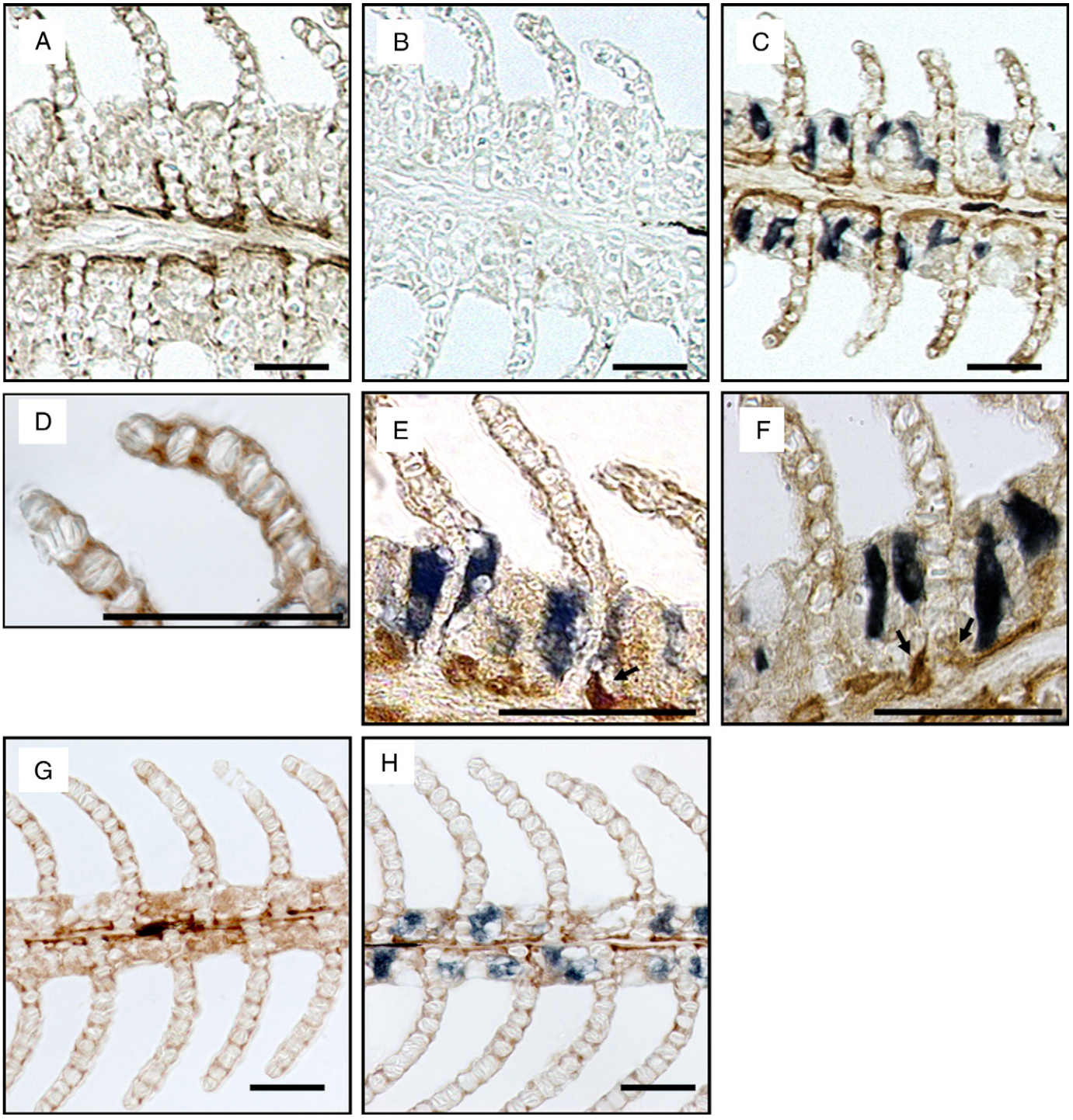

Fig. 6. Representative light micrographs of the immunolocalization of the endothelin B/C receptors (EDNRB/C) in the killifish gill. A–D) Seawater (SW), chronically (30 days) acclimated fish. E) A gill filament from a fish acclimated to SW for 24 h. F) A gill filament from a fish acclimated to fresh water (FW) for 24 h. G, H) FW chronically acclimated fish. A, G) Immunoreactivity for EDNRB/C (brown). B) A section 7 μm away from A or C incubated in peptide-absorbed antibody as a negative control (no staining observed). C, E, F, H) Filaments immunoreactive for EDNRB/C (brown) and Na^+, K^+-ATPase (blue). D) Magnification of lamellar pillar cells immunopositive for EDNRB/C. Arrows are pointing to prelamellar arterioles immunopositive for EDNRB/C. Scale bar = 50 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

throughout the cell to only being found on the basal aspect of the MRC. This localization difference between SW and FW killifish is in agreement with our finding that chronic FW killifish have 60% fewer EDNRA protein than SW killifish. To the best of our knowledge, our study is one of the first to examine mRNA, protein and localization differences for any gene/protein in fishes.

Killifish gill *EDNRB* mRNA levels increased threefold while the fish were acclimating to SW for 24 h, while *EDNRC* mRNA levels also increased significantly 3 and 8 h post-transfer, but were not different from shams at 24 h. EDNRB/C protein levels increased threefold during short term acclimation to SW, but remained unchanged during short and long term acclimation to FW. Given the localization of these receptors to the gill vasculature and our hypothesis that these receptors act as clearance receptors for EDN1, it is interesting to speculate that changes in the EDNRB/C may result in regulation of EDN1 levels by changing clearance rates.

The changes in EDNRB/C expression levels due to changes in environmental salinity may also have an effect on lamellar pillar cell tone, and perhaps be involved in the maintenance of cellular and tissue integrity during volume expansion. Recently, Mistry et al. (2004) sequenced and characterized an actin-binding protein, FHL5, from the pillar cells of the tiger pufferfish. They determined that EDN1 or volume expansion (from isotonic dextran-saline) stimulate FHL5 expression in the lamellar pillar cells. In a comparative study between normal and hypertensive rats, volume expansion stimulated EDN1 production in both groups (Abdel-Sayed et al., 2003). Thus, we hypothesize that during volume expansion (as occurs in fishes during rapid transfer from SW to FW) EDN1 signaling is involved in maintaining pillar cell (lamellae) integrity through the stimulation of an increase in the actin-binding protein FHL5 and regulation of pillar cell tone, thereby preventing lamellar swelling and possible damage.

In conclusion, the EDN1 signaling axis is active in the fish gill. All components of the EDN1 signaling axis (EDN1, ECE, EDNRs) are regulated by changes in environmental salinity (reported here and in Hyndman and Evans (2007)), and the cellular localization of these components have now been determined. Given the complexity of this system, and the potential for EDN1 to affect the multiple functions of the gill, including regulating blood flow or ion transport, future experiments knocking out/antagonizing this system will be integral in our understanding of the physiological function of EDN1 in the fish gill.

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