Comparative Biochemistry and Physiology, Part A 164 (2013) 17–20

Contents lists available at SciVerse ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Evidence for an ontogenetic change from pre-programmed to meal-responsive *cck* production in Atlantic herring, *Clupea harengus* L.

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ARTICLE INFO

Article history:
Received 23 April 2012
Received in revised form 5 October 2012
Accepted 5 October 2012
Available online 11 October 2012

Keywords:
Fish larvae
Cholecystokinin
Gut
Yolk
Starvation
Feeding
In situ hybridization
Nutrient sensing

ABSTRACT

The effects of up to three days of food deprivation on the cholecystokinin (CCK)-producing cells in the Atlantic herring gut were assessed by quantifying the number of cells detected by *in situ* hybridization at three ontogenetic stages. In feeding larvae that still possessed yolk-sacs (2 and 8 days after hatch, DAH), intestinal *cck* expression appeared to be maintained regardless of external nutritional conditions. In 30 DAH-old herring larvae with well-established exogenous feeding only, very few CCK-producing cells could be identified, indicating that *cck* production in the gut had shut down after three days of starvation. This suggests that *cck* transcription is pre-programmed by a local timer in the midgut during the yolk-sac stage, regardless of the nutritional status and presence of nutrients in the gut lumen; however, it becomes strongly influenced by the external nutritional conditions after the yolk has been completely absorbed. Our results suggest that CCK-producing cells in the gut develop "meal-responsiveness" later in post-hatch development.

1. Introduction

The peptide hormone cholecystokinin (CCK) plays an important role in regulating the digestive function in vertebrates, including fish (Liddle, 1995; de Pedro and Björnsson, 2001; Polakof et al., 2011). CCK also plays an important role in the gut–brain communication axis, where the signals include nutrients, hormones and neural pathways, and where CCK released from the digestive tract acts as a satiety signal to the brain (Polakof et al., 2011). Most gastrointestinal hormone-producing cells, including CCK, are receptosecretoric, that is, they are sensitive to chemical changes in their external environment in the apical region (facing the gut lumen) and respond by releasing their messenger substances to the internal basolateral environment (Fujita et al., 1981). Stimulation of CCK secretion from these enteroendocrine cells is associated with an increase in intestinal CCK mRNA content (*cck*), resulting from an increase in CCK gene transcription (Liddle et al., 1988). It is therefore of interest to investigate how CCK-producing cells are influenced by the presence or lack of a food stimulus. Studies on rats have shown that CCK plasma levels, tissue concentration, and number of CCK-producing cells fell during a few days of fasting and rose again after refeeding (Koop et al., 1987; Kanayama and Liddle, 1991). In Atlantic salmon, *Salmo salar* L. postsmolts, *cck* levels decreased in both brain and pyloric caeca after six days of starvation (Murashita et al., 2009). The role of CCK in the larval stages of teleosts has started to be explored (Kurokawa et al., 2000; Kamisaka et al., 2001, 2002, 2003, 2005; Rojas-García et al., 2001, 2011; Koven et al., 2002; Rojas-García and Rønnestad, 2002; Drossou, 2006; Rønnestad et al., 2007; Hama et al., 2009; Hartviksen et al., 2009; Webb et al., 2010; Webb and Rønnestad, 2011); however, we still lack knowledge of how starvation affects *cck* production, including differences in the response in early and late larval stages.

Many fish larvae have a "first feeding window" which is in between the first possible onset of exogenous feeding based on their morpho-physiological and behavioral status and the "point of no return" when the larvae have exhausted their yolk reserves and tissues have been irreversibly degraded (Rust, 2002). During this period of possible mixed feeding, deprivation of exogenous food may affect the gut differently than in later stages, when they rely exclusively on ingesting feed and do not have a supply of nutrients originating from the yolk. We are aware of no studies that have examined the possible ontogenetic differences in the receptosecretoric function of enteroendocrine cells and their sensitivity to nutrients in the gut.

We have earlier cloned Atlantic herring, *Clupea harengus* L., CCK and established an *in situ* hybridization method for detecting the expression of mRNA (Kamisaka et al., 2005). Here, we report the impact of up to three days of food deprivation on the CCK-producing system and assess

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http://dx.doi.org/10.1016/j.cbpa.2012.10.006

the quantity and distribution pattern of CCK-producing cells by *in situ* hybridization in the gut at three ontogenetic stages from the mixed feeding window to larvae with well-established exogenous feeding only.

2. Materials and methods

Atlantic herring (*C. harengus* L., Clupeidae) were sampled from the same batch of larvae that were used by Kamisaka et al. (2005). In this study, three ages were tested for the effects of one to three days of food deprivation: 2 DAH (the day for onset of exogenous feeding in most studies of herring), 8 DAH (the end of the yolk-sac stage), and 30 DAH (exogenous feeding only). At each of these ages, around 150 larvae were carefully transferred from the main rearing tank to a 5 L white bucket of clean seawater, whereafter 15 larvae that displayed normal behavior and morphology were sampled every day after transfer. These samples were identified as follows; 2-1 (transferred at 2 DAH — 1 day starvation), 2-2, 2-3, 8-1, 8-2, 8-3, 30-1, 30-2, and 30-3. The larvae were fixed in Bouin's solution for 20 h, embedded in paraffin, and sagittally sectioned at 5 μm.

Three randomly selected larvae from each age group (*i.e.* a total of 27 larvae for the study) were processed for *in situ* hybridization as described in Kamisaka et al. (2005). In brief, the tissues were dewaxed, rehydrated, and treated with proteinase K (10 μg/mL) for 10 min at room temperature, followed by post-fixation in 4% paraformaldehyde in PBS, treated with acetic anhydride, and dehydrated. A 100 μL hybridization mix containing about 100 ng DIG-labeled RNA probes corresponding to amino acids 6–128 of herring CCK was applied directly to each air-dried slide and incubated in a humidity chamber with hybridization slips (Sigma, Steinheim, Germany) for 16 h at 60 °C. The probes were detected with anti-DIG alkaline phosphatase Fab fragment (Roche, Basel, Switzerland). Labeling was visualized with chromogen substrate (NBT and BCIP). The CCK gene sense probe was used as a control to monitor nonspecific staining. Since the larval herring gut is straight, the longitudinal distribution of CCK-positive cells was quantified directly (for example of a visible staining of a CCK-producing cell; see Fig 1).

After *in situ* hybridization, serial sections were used to reconstruct the gut of each larva. CCK-positive cells were plotted on the reconstructed gut figures and the distribution pattern was shown as a histogram. All serial sections were used to count CCK-producing cells, except for the 30-1, 30-2, and 30-3 larvae. Because of the large size of the sections, only every second section was used and the numbers of CCK-producing cells actually counted were doubled to estimate the number of CCK-producing cells. The data of Kamisaka et al. (2005) on feeding larvae aged 2, 8 and 30 DAH from the same rearing tank were used as fed controls. All animal treatments observed Norwegian guidelines for the use of animals for experimental purposes.

3. Results

In situ hybridization revealed the CCK-producing cells located individually between the enterocytes and showed a typical pear-like enteroendocrine cell shape (Fig. 1). All the larvae examined at 2 DAH had a large yolk-sac. At 8 DAH, the larvae still possessed a small yolk-sac, but the yolk had been almost completely consumed by the end of the food deprivation period (8-3, data not shown).

The CCK-producing cells were widely distributed in the midgut at all stages (Fig. 2). In 2 DAH larvae, the distribution patterns and the signal indicating the *cck* expression were still maintained despite food deprivation for another three days. One to three days of starvation for 8 DAH larvae (8-1, 8-2 and 8-3) apparently led to a reduced number of CCK-producing cells compared to that of the control larva (8 DAH fed), but the number of detected cells was similar to that in the 2 DAH larvae. In 30 DAH larvae, the CCK-producing cells were identified throughout much of the midgut on 30-1 and 30-2, but almost no positive CCK-producing cells were detected in the digestive tract after three days of starvation (30-3).

4. Discussion

At all stages, the distribution pattern of the CCK-producing cells, which were scattered throughout the whole of the midgut, was unaltered by food deprivation. The level of *cck* was apparently maintained during starvation in the 2 DAH and to a large extent also in the 8 DAH group. This indicates that food deprivation had little or no effect on *cck* production in yolk-sac larvae, and that nutrient sensing in the gut lumen is less important for the maintenance of *cck* level in enteroendocrine cells, as long as there is a supply of nutrients to the tissues (including the gut) from the yolk-sac. After three days of starvation in the 30 DAH group (30-3), however, almost no intestinal CCK-producing cells could be detected in any of the three larvae examined. The starvation groups of herring from 30 DAH depended entirely on exogenous feeding as an energy source, and were less tolerant of food deprivation than younger larvae that still possessed a yolk-sac.

After the exhaustion of the yolk, Atlantic herring larvae can survive without external sources of food for 5 to 9 days at 8–12 °C, which is a similar period to larvae of Pacific herring, *C. harengus pallasi*, which can survive for 6 to 8.5 days at 6°–10 °C (Blaxter and Hempel, 1963; McGurk, 1984). Our three days of starvation should therefore not be critical for survival. However, Ueberschär and Clemmesen (1992) showed that the lowest trypsin values in wild-caught herring larvae were obtained after 3–4 days of starvation. Even though a few days of starvation are not critical for herring survival, enzyme production and hormonal control seem to respond quickly to changes in dietary supply.

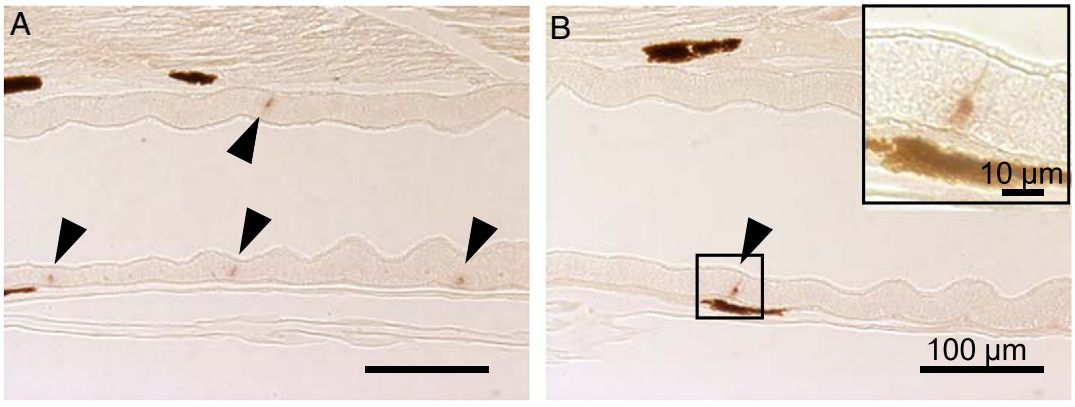

Fig. 1. CCK-producing cells in the gut of Atlantic herring larvae at 31 days after hatching (DAH) after one day of starvation (group 30-1) detected by *in situ* hybridization. A: Representative distribution of CCK-producing cells in a longitudinal section. B: An example of the typical pyramidal shape of a CCK-producing cell.