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Ontogeny of the gut motility control system in zebrafish *Danio rerio* embryos and larvae

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Summary

Using digital motion analysis, the ontogeny of the cholinergic, tachykinin and pituitary adenylate cyclase-activating polypeptide (PACAP) control systems was studied in zebrafish *Danio rerio* larvae, *in vivo*. For the first time we show that the regular propagating anterograde waves that occur in the zebrafish larval gut before and around the onset [at 5–6 days post fertilization (d.p.f.)] of feeding are modulated by acetylcholine or atropine, PACAP and NKA (neurokinin A). At 3 d.p.f., when no spontaneous motility has developed, application of acetylcholine did not affect the gut. However, at 4 d.p.f., acetylcholine increased and atropine reduced the frequency of propagating anterograde waves. At 5 d.p.f., NKA increased and PACAP reduced the wave frequency. This suggests that both excitatory and inhibitory pathways develop at an early stage in the gut, independent of exogenous feeding. Immunohistochemistry established the presence of gut neurons expressing PACAP and NKA in the proximal part of the developing gut from the first stage investigated (2 d.p.f.) and before regular motility was observed. 1 d.p.f. (PACAP) or 2 d.p.f. (NKA) stages later the whole gut was innervated. This supports physiological results that gut motility is under neuronal control during the period when regular motility patterns develop.

Key words: enteric nervous system, development, PACAP, tachykinin, acetylcholine, zebrafish, *Danio rerio.*

Introduction

Zebrafish *Danio rerio* is increasingly important as a model animal, particularly for studies of early development, and its genome will soon be fully sequenced. We have previously shown that the developing zebrafish is an excellent experimental animal in which to study the development of gut motility (Holmberg et al., 2003). In its earliest stages the animal is transparent (Fig. 1), allowing us to use a video technique to study the gut motility *in vivo* and thereby add significant functional data to existing molecular and morphological information on the development of the gut.

To our knowledge, information about the development of gut motility in vertebrates is sparse. In mammals, absorptive functions and gut motility are developed during fetal life, but the first stools are not passed until after birth and first feeding (e.g. Neu, 1989; Meetze et al., 1993; Sherman et al., 1996). In contrast, fish and amphibians theoretically may deposit stools (from slayed off intestinal material) into the surrounding water at an earlier stage without risk of infection. Consequently, they may benefit from a propagating motility already functioning between hatching [at 2–3 days post fertilization (d.p.f.)] and the first feeding (at 5–6 d.p.f.). In agreement with this, in our previous study we observed spontaneous (i.e. not induced by drugs, food or mechanical stress) motility in the zebrafish gut before 5 d.p.f. (Holmberg et al., 2003). Sporadic activity was present from the first stage investigated (3 d.p.f.), and a regular motility pattern was present at one stage later (4 d.p.f.).

Spontaneous motility of the gut is proposed to have a housekeeper function and is known to occur in between meals in vertebrates. It is believed that the enteric nervous system (ENS) is central for the coordination of such spontaneous activity, as well as for mixing and propulsive contractions after feeding (Kunze and Furness, 1999; Olsson and Holmgren, 2001). Other factors, such as interstitial cells of Cajal (ICCs) and hormones, are also known to affect spontaneous motility. However, the ENS may be particularly important for the control of spontaneous activity at the earliest stages, suggested from studies in the mouse, where the ENS and gut are developed before the ICCs (Wu et al., 2000).

The development of the ENS has been studied in many vertebrate species, including the zebrafish. Enteric neurons as such have been detected in the zebrafish embryo before the onset of feeding (Bisgrove et al., 1997; Holmberg et al., 2003), but so far there are no reports on the expression of different transmitters in these nerves. Earlier studies have shown the presence of regulatory neuropeptides in neurons in the developing gut of another teleost (Reinecke et al., 1997), as well as in amphibians (Holmberg et al., 2001; Maake et al., 2001), birds (Epstein et al., 1981, 1983; Epstein and Poulsen,

1991) and mammals (Larsson et al., 1987; Pham et al., 1991; Van Ginneken et al., 1998).

The observed parallel development of enteric neurons and regular propagating contractions of the gut in zebrafish larvae (Holmberg et al., 2003) suggests that the ENS could affect this motility. However, in humans and mice, rhythmic propagating activity can develop in the absence of functional enteric neurons (Ward et al., 1999; Huizinga et al., 2001) and furthermore, even if they are present, the enteric neurons might not be functional, i.e. the transmitters might not be released or the smooth muscle cells might not express the proper receptors. Therefore, we find it of interest to investigate the development and interaction of functioning control systems in the gut of zebrafish. As a first step, the present study aims to investigate the ontogeny of the cholinergic, tachykinin and pituitary adenylate cyclase-activating polypeptide (PACAP) control systems of the gut. Acetylcholine and substance P/neurokinin A (NKA) are major excitatory transmitters in the ENS in adult animals, and the vasoactive intestinal polypeptide (VIP)-related peptide PACAP often participates in inhibitory control (Costa et al., 2000; Matsuda et al., 2000; Olsson and Holmgren, 2001). Furthermore, SP/NKA and VIP/PACAP are neuropeptides that have been detected in larval gut neurons before the onset of feeding in some other fish and amphibian species (turbot *Scophthalmus maximus*, Reinecke et al., 1997; axolotl *Ambystoma mexicanum*, Maake et al., 2001; *Xenopus laevis*, Holmberg et al., 2001). In the present study, gut motility *in vivo* in zebrafish embryos and larvae is investigated following microinjections of acetylcholine, atropine, NKA and PACAP, in combination with video recordings of the frequency of anterior anterograde motility waves in the developing gut with subsequent motion analysis.

The classical transmitter acetylcholine exhibits an excitatory effect on gut motility in fish (e.g. Nilsson and Fänge, 1969; Edwards, 1972; Thorndyke and Holmgren, 1990; Burka et al., 1996; Aronsson and Holmgren, 2000). Atropine is a general muscarinic receptor antagonist and blocks the effect of acetylcholine. It is hypothesised that if atropine blocks spontaneous motility, then endogenous acetylcholine is released and functioning in the fish. In addition, an effect of acetylcholine applied to animals not expressing spontaneous activity would suggest that functional muscarinic receptors are present, even though there may be no endogenous release of acetylcholine. Furthermore, if functional NKA and PACAP receptors are present, then exposure to NKA and PACAP, respectively, will affect the gut motility.

Materials and methods

Adult and developing zebrafish *Danio rerio* Hamilton were used for this study. Embryos and larvae were collected from laboratory breeding stocks. The day of fertilization was termed day zero (0 d.p.f.). At 0 d.p.f. fertilized eggs were collected from the breeding aquarium and transferred to small containers (beakers) floating in a larger aquarium with a water temperature of 28°C. Embryos (3 and 4 d.p.f.) and larvae (5 d.p.f. and older) of the desired developmental stages were collected from the containers (N=7–13 for each d.p.f. stage). For a detailed description of breeding and holding of larvae, see The Zebrafish Book (Westerfield, 2000). The embryos and larvae were not fed during the experimental period (active feeding at temperature 28°C usually starts around 5–6 d.p.f., and at 6 d.p.f. most of the yolk is depleted).

The study was performed at the University of Göteborg, Sweden and at the University of Innsbruck, Austria. The Swedish National Board for Laboratory Animals' guidelines were followed.

Motion analysis in zebrafish embryos and larvae

Mounting of embryos and larvae

For the experiments, 70 embryos and larvae from several batches were used. Each animal was anaesthetized in phosphate-buffered MS222 (3-aminobenzoic acid ethyl ester, 75–100 mg l^{-1}, pH 7; Sigma Chemical Co., St Louis, MO, USA) and embedded on its side in a liquid agarose solution (1%, Sea Plaque; gelling point 26–30°C, dissolved in phosphate-buffered MS222). The agarose was allowed to settle and was covered with MS222 (75–100 mg l^{-1}, phosphate-buffered) in order to maintain the anaesthetic condition. The animals are transparent at early stages (Fig. 1), and the gut and its movements could be observed *in vivo* using an inverted microscope (10× magnification).

Imaging system and image analysis

For a detailed description of the imaging system and the image analysis used see Schwerte and Pelster (2000) and Holmberg et al. (2003). Still images were extracted (every second) from video recordings of the gut, using the Optimas program package (Media Cybernetics, Silver Spring, MD, USA). Using the Optimas software, a film sequence could be created from the still images. From this film sequence the frequencies of propagating anterograde contraction waves in the anterior intestine were determined and the average frequency per minute (cycles min^{-1}) was calculated. The data were exported to Excel for further analysis.

Application of the drug outside the body wall

The tip of a syringe was placed immediately outside the animal, and a bolus of between 50 and 100 nl of drug solution was applied next to the abdomen. For control purposes, the effects of the application *per se* were studied by adding the same volume of saline (NaCl 0.9%). The following drugs (dissolved in saline) were used: atropine (Sigma, A-0257), acetylcholine (Sigma), neurokinin A (NKA 7359; Diagnostika, Falkenberg, Sweden) and pituitary adenylate cyclase-activating peptide (PACAP-27, 4031084.0500; Bachem, Weil an Rein, Germany).

Animals showing spontaneous activity during the control period were treated with atropine and NKA. The following experimental protocol was used (9 min per treatment): control period; saline; atropine (10^{-6} mol l^{-1}); saline; NKA (10^{-6} mol l^{-1}). The effect of acetylcholine was studied in

specimens showing no spontaneous activity during the control period, using the protocol (9 min per treatment): control period; saline; acetylcholine (10^{-5} mol l^{-1}).

Application of drug inside the body cavity

PACAP is a fairly large polypeptide that does not easily penetrate the body wall, and injections were made intraperitoneally. The needle tip of the syringe was placed just inside the abdominal wall and the peptide (dissolved in 20 nl saline) was injected into the body cavity using a micromanipulator/microinjection apparatus. For control purposes, 20 nl of saline were injected into the animal. The following experimental protocol was used: control (3 min); injection of saline or PACAP 10^{-6} mol l^{-1} (6 min).

Statistical analysis

Animals that were unaffected by the drug were not included in the statistical analysis, and are reported separately. The frequency (cycles min^{-1}) data were pooled and averaged for each d.p.f. group, and mean values were calculated and presented as the mean of one d.p.f. group ± S.E.M. For control and saline application/injection the full experimental period was used to analyse the average frequency. The effects of PACAP, acetylcholine and NKA appeared soon after application/injection and therefore the first 6 min were used for analysis. Atropine had a slower effect and the last 6 min of the experimental period were used for the analysis. Statistical analyses were performed using Wilcoxon–Mann–Whitney test (SPSS 10.0 for Windows); repetitive use of experimental groups were taken into account. Differences in mean values were regarded as significant at $P<0.05$.

In vitro *preparations of adult zebrafish intestine*

For comparison, the effects of acetylcholine and PACAP were studied *in vitro* on strip preparations of the gut from adult zebrafish.

Smooth muscle preparations

The middle intestine was dissected out and placed in cold zebrafish Ringer's solution (composition in mmol l^{-1}: NaCl 116, KCl 2.9, $CaCl_2$ 1.8, Hepes 5, glucose 11, pH 7.2). Ring preparations (3–4 mm wide; circular muscle preparation) or the whole middle intestine cut open longitudinally (longitudinal muscle preparations) were mounted in organ baths containing zebrafish Ringer's solution (22°C, bubbled with 0.3% CO_2 in oxygen). The force developed by the smooth muscle was recorded using a force displacement transducer (model FT03, Grass Instruments; West Warwick, RI, USA) connected to a polygraph (model 7, Grass). An initial tension (circular muscle preparation, 0.2 mN; longitudinal muscle preparation, 1 mN) was applied and the strip preparations were left for 1–2 h to develop a steady baseline (resting) tension and spontaneous rhythmic contractions. PACAP-27 (longitudinal muscle preparations only) and acetylcholine were applied to the organ bath at increasing concentrations in a cumulative fashion, starting at 10^{-10} or 10^{-9} mol l^{-1}, and allowing maximal response to be obtained before addition of a higher concentration. To study the effects of PACAP, circular preparations of the middle intestine were pre-treated with L-NAME (N^G-nitro-L-arginine methyl ester; Sigma, 26521), and PACAP-27 was subsequently added to the organ bath in single doses.

Statistical analysis

Alterations in force developed by the strip preparations were recorded on the Grass polygraph, and at the same time collected on a computer (Labview Instruments; Austin, Texas, USA; acquisition software). A control period of 1 min was recorded before the addition of drug. The response of each drug concentration added was calculated as the mean force during the minute of peak response to the drug, minus mean force during the control minute. Concentration–response curves were constructed for PACAP (longitudinal muscle preparation) and acetylcholine and the mean EC_{50} value ± S.E.M. was calculated. The effects of L-NAME and PACAP (single exposures) were calculated relative to the control (spontaneous) activity, which was set to 100%. The inhibitory effect of PACAP was calculated relative to the increased tonus obtained after exposure to L-NAME. Differences in mean values were regarded as significant at $P<0.05$.

Immunohistochemistry

Embryos and larvae from 2, 3, 5 and 7 d.p.f. (N=4 from each stage) were anaesthetized in 0.01% MS222 and fixed whole. For control purposes adult animals were collected and anaesthetized with 0.1% MS222 and decapitated. Tissues from the intestine of adult zebrafish were dissected out and fixed. All tissues were fixed for 24 h in Zambonis' fixative [15% picric acid, 2% formaldehyde in phosphate buffer (PB) with 2% NaCl, pH 7.2], and then repeatedly rinsed in 80% ethanol until all fixative was removed. This was followed by dehydration in 95 and 99.5% ethanol, xylene treatment, and rehydration in an ethanol series (99.5%, 95%, 80%, 50%) to phosphate-buffered saline (PBS, 0.9% NaCl) (30 min for each step). The fixed embryo and larvae were stored in PBS–sucrose solution (30% sucrose) at least overnight.

The animals were placed in a fluid agarose–sucrose solution (1.5% agarose, 5% sucrose), which was left (at room temperature) to solidify (approximately at 26°C). The agarose blocks were placed at 4°C in PBS–sucrose solution until they had sunk to the bottom and were then frozen in isopentane chilled by liquid nitrogen. Sections (16 μm) were cut on a cryostat (Zeiss Micron International GmbH, Walldorf, Germany), picked up on gelatine-coated slides, left to dry in darkness overnight and then stored at –20°C until used for immunohistochemistry.

From adult intestines, tissue pieces of approximately 10 mm×5 mm were cut out, embedded in OCT (Sakura, Zoeterwoude, The Netherlands) and frozen in isopentane chilled by liquid nitrogen. Sections (4 μm) were cut on a cryostat (Zeiss) and picked up on gelatine-coated slides. The slides were kept overnight to dry in the dark and then stored at –20°C until used.

To avoid unspecific staining, normal serum was applied on sections (10% normal donkey serum, 017-000-1; Jackson ImmunoResearch Laboratories, West Grove, PA, USA) for 30 min in a moist chamber. The sections were then incubated with the primary antibody for 48 h in a moist chamber at room temperature. Excess antibodies were washed away with PB with 2,0% NaCl (3×) and incubated with secondary antibody for 1 h in the moist chamber (at room temperature). The sections were washed, mounted in Vectashield-mounting medium (H-1000, Vector, Burlingame, CA, USA) and examined using a digital fluorescence microscope (Nikon Eclipse E1000, Nikon Digital Camera DXM1200, Nikon, Tokyo, Japan) and the Easy Image package (Nikon's software). Pictures were further processed using Adobe PhotoShop 6.0.

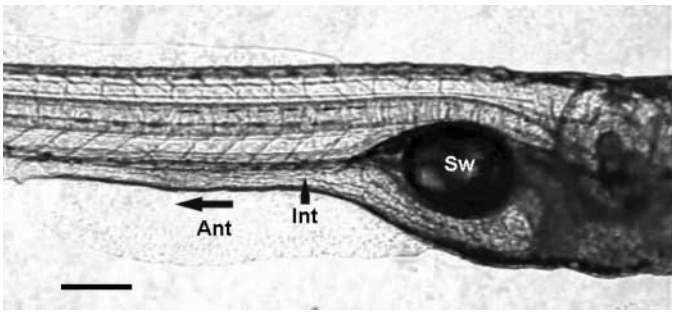

Fig. 1. Zebrafish at 7 d.p.f. stage. Int, intestine; Sw, swimbladder. The arrow indicates the direction of anterograde contraction waves (Ant). Bar, 0.2 mm.

Results

Motion analysis in embryos and larvae

In general, a regular spontaneous motility pattern of anterior anterograde waves was observed from 4 d.p.f., but in some animals no activity was expressed at this stage. This is probably due to normal differences in individual development, and agrees with our earlier study (Holmberg et al., 2003).

Effects of acetylcholine and atropine

The first or second application of saline did not affect the frequency of spontaneous activity compared to the control period (Table 1, Fig. 2).

Acetylcholine (10^{-5} mol l^{-1}) was applied to animals that did not show spontaneous gut motility during the control period. Acetylcholine did not initiate gut motility at 3 d.p.f., while at 4 d.p.f., 38% of the animals showed contractile activity after the application (Table 1, Fig. 2). After an initial peak in frequency, the frequency decreased, probably due to desensitisation. From 5 d.p.f. most observed animals expressed spontaneous gut motility and therefore no experiments were performed.

Atropine (10^{-6} mol l^{-1}) was applied to animals that expressed spontaneous gut motility, in order to determine if endogenous acetylcholine was released in the animal. Atropine reduced the frequency of anterior anterograde waves, in an increasing number of animals, from the first stage investigated (4 d.p.f.) (Table 1, Fig. 2). The effect of atropine persisted after the second application of NaCl (Table 1, Fig. 2). The motility was never completely quenched by atropine (Fig. 2).

Neurokinin A

After reduction of spontaneous contractile activity by atropine, application of NKA (10^{-6} mol l^{-1}) had no significant

Table 1. *Inhibitory effect of atropine and PACAP, and excitatory effect of acetylcholine and NKA, on anterior anterograde contraction waves in developing zebrafish gut*

Treatment	Gut contraction frequency (cycles min^{-1})		
Acetylcholine	3 d.p.f. (0%), *N*=9	4 d.p.f. (38%), *N*=13	
Control	0	0	–
NaCl	0	0.22±0.22	–
Acetylcholine	0	1.00±0.19	–
Atropine and NKA	4 d.p.f. (77%), *N*=9	5 d.p.f. (88%), *N*=9	6–8 d.p.f. (100%), *N*=8
Control	1.02±0.24	0.90±0.22	0.97±0.19
NaCl (first)	1.13±0.27	1.06±0.24	1.22±0.22
Atropine	0.67±0.31	0.40±0.20	0.54±0.21
NaCl (second)	0.42±0.25	0.14±0.06	0.35±0.16
NKA	0.53±0.27	0.76±0.24	0.19±0.88
PACAP	4 d.p.f. (60%), *N*=5	5 d.p.f. (100%), *N*=6	5–6 d.p.f. (100%), *N*=11
Control	0.89±0.11	0.89±0.19	0.91±0.23
NaCl	–	–	0.83±0.24
PACAP	0.11±0.06	0.06±0.06	–

Values are means ± S.E.M.

Percentage values indicate the proportion of animals affected by the treatment. Mean values different from zero are calculated from responding animals only. *N*, total number of animals used.

d.p.f., days post fertilization; PACAP, pituitary adenylate cyclase-activating polypeptide; NKA, neurokinin A.

effect at 4 d.p.f., but increased the frequency of the contraction waves from 5 d.p.f. (Table 1, Fig. 2).

PACAP

The injection of saline into the body cavity in control experiments did not affect the frequency of anterior anterograde waves (Table 1, Fig. 3).

After injection of PACAP-27 (20 nl of 10^{-6} mol l^{-1}) into 4 d.p.f. animals, however, there was a tendency of a decreased frequency of anterior anterograde waves for a short period of time (3–6 min after injection) but the effect did not persist throughout the whole experimental period. One stage later, at 5 d.p.f., the frequency of contraction waves decreased during the whole experimental set-up after exposure to PACAP (Table 1, Fig. 3). However, motility was still present after injection of PACAP (Fig. 3).

Strip preparations

Acetylcholine increased the mean force of contractions in both circular (pD_2: 5.3±0.1, N=6; where pD_2 is the negative log of EC50) and longitudinal (pD_2: 7.0±0.1, N=5) smooth muscle strip preparations from the middle intestine (Fig. 4).

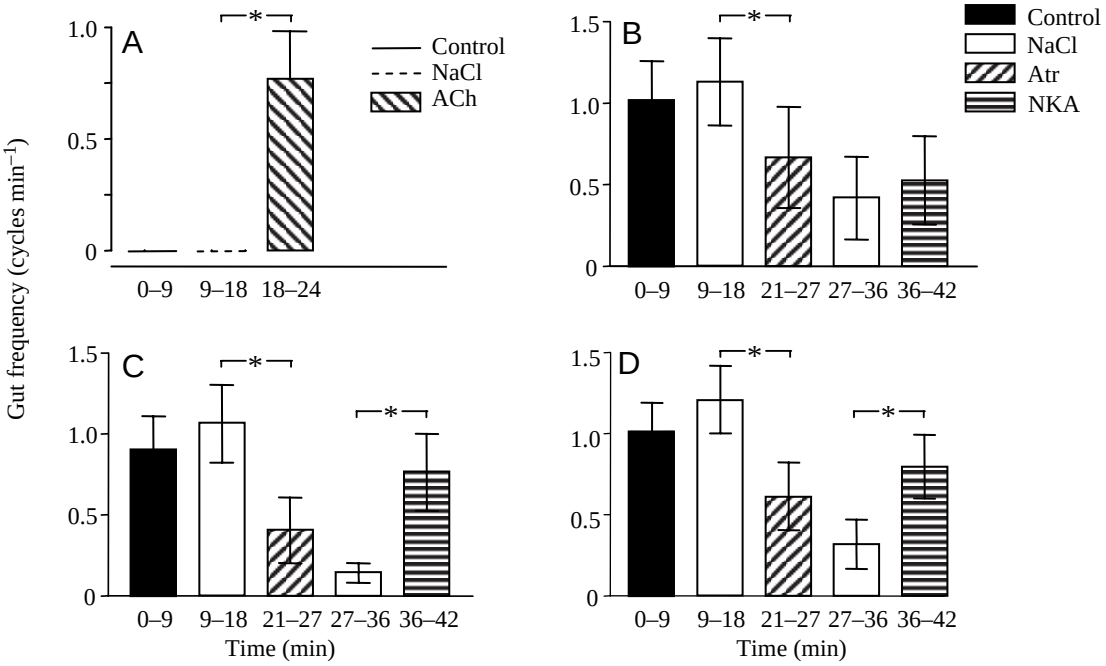

Fig. 2. The effects of acetylcholine (ACh), atropine (Atr) and neurokinin A (NKA) on gut motility in zebrafish larvae over successive 9 min periods (*in vivo* experiments). (A) ACh was applied to animals expressing no spontaneous motility (4 d.p.f.). (B–D) Atropine and NKA were applied to animals showing spontaneous motility (B, 4 d.p.f.; C, 5 d.p.f.; D, 6 d.p.f.). Application of saline (NaCl; A–D) did not affect the frequency of anterior anterograde waves (cycles min^{-1}) compared to the control period. (A) ACh (10^{-5} mol l^{-1}) increased the frequency at 4 d.p.f., indicating the presence of functional muscarinic receptors, but was without effect one stage earlier (not shown). (B–D) Atropine (10^{-6} mol l^{-1}) reduced the frequency of the spontaneous motility from 4 d.p.f. (B), indicating a release of endogenous acetylcholine in the animal. NKA (10^{-6} mol l^{-1}) increased the frequency from 5 (C) to 6–8 d.p.f. (D) after block by atropine, but had no visible effect if applied before atropine (not shown).

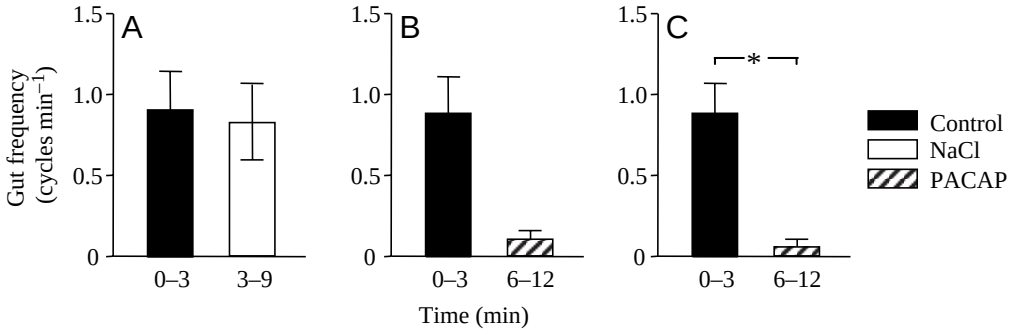

Fig. 3. The effects of PACAP (pituitary adenylate cyclase-activating polypeptide) on gut motility in zebrafish larvae over successive time periods (*in vivo* experiments). In the control experiment (A) the injection of saline (NaCl) did not affect the frequency of anterior anterograde waves (cycles min^{-1}). At 5 (C) but not 4 d.p.f. (B) the injection of PACAP (20 nl, 10^{-6} mol l^{-1}) reduced the frequency of the anterior anterograde waves, indicating the presence of functional PACAP receptors.

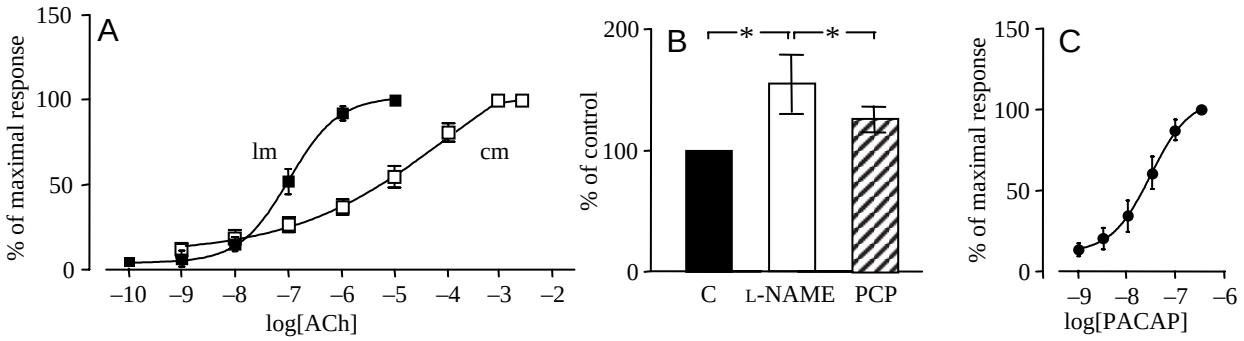

Fig. 4. The effects of acetylcholine and pituitary adenylate cyclase-activating polypeptide (PACAP) on smooth muscle preparations from adult zebrafish intestine. (A) Acetylcholine increased mean force and amplitude of contractions in both longitudinal (filled squares) and circular (open squares) preparations of the middle intestine. (B) PACAP-27 decreased the mean force and amplitude in circular strip preparations of middle intestine, after blocking nitric oxide synthase formation by L-NAME (which increased the mean force and amplitude). (C) PACAP-27 increased mean force in longitudinal preparations of the middle intestine. cm, circular muscle layer; lm, longitudinal muscle layer.

Prevention of nitric oxide (NO) formation by L-NAME (3×10^{-4} mol l^{-1}, N=5) increased the mean force to 155.6±23.6% of control rhythmic contractions of the circular preparations, and subsequent addition of PACAP-27 (10^{-7} mol l^{-1}, N=5) decreased this activity to 126.5±9.8% of control values (Fig. 4). In contrast, PACAP-27 increased the mean force in the (untreated) longitudinal preparation of the middle intestine (pD_2: 7.5±0.2, N=6, Fig. 4).

Immunohistochemsitry

We have no reliable method for detecting the occurrence of cholinergic neurons by immunohistochemistry. Both NKA-like and PACAP-like immunoreactivity first occurred occasionally in a few weakly stained nerve fibres in 2 d.p.f. embryos, and were found in increasing numbers and staining intensity in 3 d.p.f. and 5–7 d.p.f. specimens (Fig. 5, Table 2). Endocrine cells containing NKA-like immunoreactive material were observed occasionally in the distal intestine at stage 5 d.p.f., and increased to a moderate number at 7 d.p.f. (Table 2). Endocrine cells containing PACAP-like immunoreactive material were observed occasionally in the distal part of the intestine at 7 d.p.f. (Table 2). Both NKA and PACAP-like immunoreactivity was observed in nerve fibre and endocrine cells in adult zebrafish throughout the gut (Table 2).

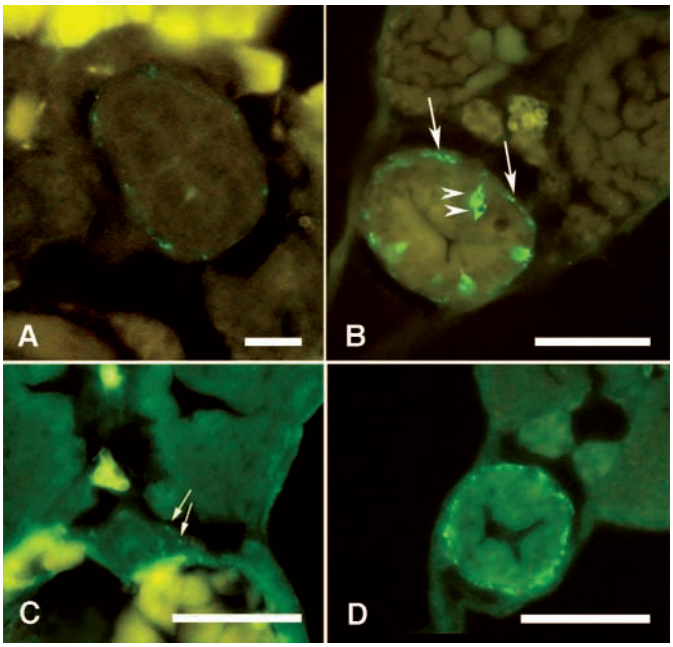

Fig. 5. Immunohistochemical demonstration of the innervation of the gut of developing zebrafish, showing NKA-like immunoreactivity at 3 d.p.f. (A) and 7 d.p.f. (B; arrows indicate nerve fibres and arrowheads indicate endocrine cells), and PACAP-like immunoreactivity in nerves at 2 d.p.f. (C; arrows), and 7 d.p.f. (D). Bars, 50 μm.

Table 2. *The presence of neurotransmitters in the gut of zebrafish at different d.p.f. stages and in adult specimens*

		Intestinal area		
Antiserum	d.p.f.	Proximal	Middle	Distal
PACAP-38	2	+	+	–
	3	+	+	+
	5	+	+	+
	7	+	+	+, o
	Adult	+++, o	+++, o	+++, o
NKA 2809	2	+	–	–
	3	++	++	–
	5	++	++	++, o
	7	++	++	++, oo
	Adult	++, o	++, o	++, o

Proximal (intestinal bulb), middle and distal parts of the gastrointestinal tract of embryo, larvae and adult zebrafish were investigated using immunohistochemistry. The occurrence of nerves (+) and endocrine cells (o) was assessed on a three-step scale (–, none; +, occasional; ++, moderate; +++, abundant).

d.p.f., days post fertilization; PACAP, pituitary adenylate cyclase-activating polypeptide; NKA, neurokinin A.

Discussion

In the present work we used video recordings to study the development of the gut of live (anaesthetized) zebrafish larvae. The great advantage with zebrafish as a model animal for such studies is that the early stages are transparent, allowing recordings of the gut inside the intact animal. Furthermore, the abdominal wall is thin enough to be permeable to drugs during this period, which allowed us to study the development of a functioning control of gut motility *in vivo* before the onset of feeding, which to our knowledge has not been done previously in a vertebrate. The results suggest the presence of functional cholinergic, tachychinergic, and PACAP receptors before or around the time for onset of exogenous feeding. At the same early stages, atropine affected the spontaneous activity, suggesting the presence of a cholinergic tonus. We have also, using immunohistochemistry, observed PACAP- and NKA-like immunoreactivity in nerve fibres of the gut before any spontaneous motility is observed. In adult specimens, we demonstrate an excitatory effect of acetylcholine, an inhibitory NO tonus, and a dual effect of PACAP.

One major aim of the present study was to determine to what extent the control of gut motility is functional at the onset of exogenous feeding. In the zebrafish embryo, the intestine is formed from a solid endodermal rod (the gut primordium), which develops into a hollow tube around 42 h post fertilization (h.p.f.; Horne-Badovinic et al., 2001; Ober et al., 2003). Around 2 d.p.f., the pharynx, oesophagus, liver and pancreas are joined to the intestine into a functional unit (Wallace and Pack, 2003). Enteric nerve cell precursors entered the anterior gut, spreading backwards while maturing, and neurotransmitters can be observed in nerves at 2 d.p.f. (Bisgrove et al., 1997; Holmberg et al., 2003). At 3 d.p.f. the mouth, and one stage later the anus, open (Wallace and Pack, 2003) and at 4 d.p.f. the gut mucosa, pancreas and liver secrete digestive enzymes and bile (Pack et al., 1996). These studies indicate that the digestive system might be functional around the time for onset of feeding (5–6 d.p.f. at 28°C), even though the gut cells normally are not completely depleted of egg yolk at this stage. However, for an effective digestion and absorption, the food has to be transported along the gut in a controlled manner.

Regular spontaneous propagating contractions (i.e. not induced by food, anticipation of food, or drugs) are present in zebrafish larvae before the onset of feeding (Holmberg et al., 2003). The function of these movements or whether they are induced by fluid or waste products in the gut is not known. Spontaneous motility in gut preparations of adult fish, *in vitro*, has been suggested to be analogous to the migrating motor complexes (MMC, phase III-like; Karila and Holmgren, 1995; Olsson et al., 1999) that occur in between meals in adult mammals (*in vivo*). Phase III MMCs are characterised by rhythmic contractions that travel in an anal direction, and are considered to be important for transportation of waste products and prevention of bacterial overgrowth of the gut in the inter-digestive phase. It can be speculated that motility of the gut is important before the larvae start to feed for a similar reason and that the observed motility in zebrafish larvae is analogous to phase III MMC activity.

Motility of the gut is controlled by the ICCs, the ENS, hormones, and extrinsic sensory and extrinsic autonomic innervation, which may act independently or in interacting systems (e.g. see Kunze and Furness, 1999; Olsson and Holmgren, 2001). The relative importance of the different factors is difficult to assess. For example, ICCs are considered to play a role as pacemakers in the initiation of contractions, and rhythmic propagating activity may occur on the absence of enteric neurons but in the presence of functional ICCs (Ward et al., 1999; Huizinga et al., 2001). On the other hand, in mice, MMCs have been shown also to develop in the absence of slow waves (which originate from the ICCs in the myenteric plexus; Spencer et al., 2003). We have not been successful in determining the presence of ICCs in the developing zebrafish, and it is possible that the development of the ENS preceeds that of the ICCs, as has been found in mice (Wu et al., 2000).

At the onset of feeding, neurons expressing NKA and PACAP are present throughout the gut in the zebrafish larvae, suggesting a possible role in controlling the regular gut motility that was observed by Holmberg et al. (2003). Neurotransmitters are also expressed in other vertebrates at an early stage. A few studies have related the expression of neurotransmitters to the first feeding. In the turbot *Scophthalmus maximus* (Reinecke et al., 1997), the axolotl *Ambystoma mexicanum* (Maake et al., 2001; Badawy and Reinecke, 2003) and chicken (Epstein et al., 1983; Epstein and Poulsen, 1991), vasoactive intestinal polypeptide (VIP)-containing nerve fibres have been observed before or around the time of onset of feeding. In the axolotl, PACAP coexisted with VIP and was found in gut neurons at an early stage (Badawy and Reinecke, 2003). In *Xenopus laevis* larvae, several transmitters, including PACAP and NKA, have been detected by immunohistochemistry in neurons before the onset of feeding (Holmberg et al., 2001). These findings suggest that the neuronal control system, if not already fully functioning, is ready to play a role in the processing of food from the first feed. However, the presence of neuronally contained transmitters does not automatically mean a functional nervous control, which also must include release and inactivation of the transmitter, and the presence and activation of receptors.

Acetylcholine and atropine

Acetylcholine is a common transmitter in the gut, but there is, to our knowledge, little information on the distribution and effect of acetylcholine during gut development in non-mammalian vertebrates. By contrast, a functioning cholinergic regulation of gut motility before birth has been demonstrated in several mammalian species (Gintzler et al., 1980; Rothman and Gershon, 1982; Oyachi et al., 2003a,b).

The excitatory effect of acetylcholine in adult zebrafish (and the inhibitory effect of atropine in embryos and larvae) agrees with a large number of previous reports of a cholinergic gut innervation in vertebrates. Cholinergic excitatory neurons have

been estimated to be the most common nerve population in the adult mammalian gut (e.g. see Kunze and Furness, 1999), and there are also reports on cholinergic mechanisms in the gut of non-mammalian species, including several teleosts (e.g. for references, see Jensen and Holmgren, 1994).

The inhibition of contractions already occurring at 4 d.p.f. by blockade of muscarinic receptors suggests that endogenous acetylcholine is released before the onset of feeding (5–6 d.p.f.) and affects the spontaneous gut activity. Further experiments are needed to determine whether this includes effects directly on the smooth muscle, *via* ICC cells, or *via* interneurons. Our findings also indicate the presence of functional muscarinic receptors from 4 d.p.f., but not one day earlier. It is notable that no effect of acetylcholine was observed at 3 d.p.f., nor was any spontaneous motility, but 1 day later approximately 75% of the animals expressed functional muscarinic receptors as well as spontaneous motility.

The early appearance of a functional cholinergic control agrees with findings from several mammals. In mice, acetylcholine immunoreactive neurons have been detected before birth (Rothman and Gershon, 1982), and in guinea pig gut neurons acetylcholine was present at 25 days gestation (guinea pigs have about 114 days of gestation), while an excitatory effect on smooth muscle cells was first established somewhat later, at day 48 of gestation (Gintzler et al., 1980). Functional muscarinic receptors, mainly of the M3 and, to some extent, the M2 types, are present in fetal sheep (Oyachi et al., 2003a). In zebrafish, muscarinic M2-receptor mRNA is expressed in the early embryo at 12 h.p.f. and in neurons of the heart and vagus motor ganglion at 30 and 48 h.p.f. (Hsieh and Liao, 2002), but this study does not include the intestine.

PACAP

In a previous study in a teleost, the stargazer *Uranoscopus japonica*, using both endogenous and mammalian PACAP, Matsuda et al. (2000) found that PACAP relaxed the precontracted rectum, but had no effect on the weak spontaneous activity of (longitudinal preparations) of the intestine and rectum. In the Atlantic cod *Gadus morhua*, spontaneous contractions of both longitudinal and circular preparations of the intestine were reduced by PACAP (Olsson and Holmgren, 2000). The inhibitory effect of PACAP on the zebrafish larval gut, and on circular muscle preparations of the adult gut, agrees with these previous studies in adult teleosts. In contrast, the contraction induced in longitudinal preparations of adult zebrafish gut is opposite to the relaxation obtained in longitudinal preparations of both the stargazer (Matsuda et al., 2000) and the Atlantic cod (Olsson and Holmgren, 2000). Taken together, the results suggest species differences, and that there are several PACAP receptors present in teleosts in general, and in the zebrafish gut in particular. These receptors may have a different expression over time (developing *versus* adult specimen), in different tissues (longitudinal *versus* circular muscle, smooth muscle *versus* interneuron, etc.), and in different species. Indeed, at least two PACAP receptors are expressed in zebrafish at 6 d.p.f., one PACAP type 1 and one PACAP type 2 receptor (Wei et al., 1998). Our results suggest that at least one of these receptors is expressed and already functioning from 5 d.p.f., i.e. around the time for the onset of exogenous feeding. The results also show that the first expression of PACAP in gut nerves occurs even earlier (from the first d.p.f. stage investigated, i.e. 2 d.p.f.). A PACAP–GRF (growth hormone releasing factor) gene is present in zebrafish, and a transient expression in the nervous system during early development (1 d.p.f.) has been described (Fradinger and Sherwood, 2000; Krueckl et al., 2003).

Neurokinin A

NKA, along with substance P, occurs in most excitatory cholinergic motor neurons of the adult vertebrate gut, and acts on tachykinin receptors NK2 and NK1 on smooth muscle cells and enteric neurons, and on NK1 receptors on ICCs (Pennefather et al., 2004).

Available information about the ontogeny of a tachykinin control system in the gut emanates mainly from immunohistochemical studies, and agrees well with the results of the present study. In birds and mammals, occasional nerves (and endocrine cells) showing tachykinin-like immunoreactivity occur early during fetal development, and mature into a more extensive nerve net before birth (human, Paulin et al., 1986; Larsson et al., 1987; sheep, Wathuta and Harrison, 1987; guinea-pig, Saffrey and Burnstock, 1988; chicken, Saffrey et al., 1982; Epstein et al., 1983; duck, Lucini et al., 1993). Similarly, in the amphibian *Xenopus laevis*, both nerves and endocrine cells are present in the gut before the onset of feeding (Holmberg et al., 2001). However, to our knowledge, this is the first demonstration of functional tachykinin receptors during this period. Whether the effect of NKA is direct on the muscle cells or indirect *via* non-cholinergic neurons or ICCs is not known at this stage. It has been shown previously in adult cod *Gadus morhua* and rainbow trout *Oncorhynchus mykiss* that tachykinins may act both directly and indirectly in the fish intestine, involving cholinergic and serotonergic pathways (Jensen et al., 1987; Jensen and Holmgren, 1991).

In conclusion, we present for the first time results suggesting that functional receptors for PACAP, acetylcholine and NKA are present in the fish gut before or around the time for onset of feeding. Furthermore, the effects of atropine show that endogenous acetylcholine is released and acts on muscarinic receptors in the larvae. It seems that both excitatory and inhibitory pathways are well developed when the animal starts to feed. For fish larvae it is of great importance to be able to digest and absorb food early in development in order to survive. In halibut and turbot larvae, for example, the survival chances increase if the larvae start feeding even before the yolk has been completely absorbed (Gadomiski and Petersen, 1988). A corresponding early development of a functional digestive system, including gut motility, as our results imply, would of course further enhance the chances of survival.

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List of abbreviations

d.p.f.	days post fertilization
ENS	enteric nervous system
GRF	growth hormone releasing factor
ICC	interstitial cell of Cajal
L-NAME	N^G-nitro-L-arginine methyl ester
MMC	migrating motor complex
MS222	3-aminobenzoic acid ethyl ester
NO	nitric oxide
NK	neurokinin in NKA, NK1, NK2
PACAP	pituitary adenylate cyclase-activating polypeptide
PB	phosphate buffer
PBS	phosphate-buffered saline
SP	substance P
VIP	vasoactive intestinal polypeptide

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