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TTX-sensitive and TTX-insensitive control of spontaneous gut motility in the developing zebrafish (*Danio rerio*) larvae

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Summary

Spontaneous regular gut motility in zebrafish begins around 4 days post fertilisation (d.p.f.) and is modulated by release of acetylcholine and nitric oxide. The role of intrinsic or extrinsic innervation for initiating and propagating the spontaneous contractions, however, is not well understood. By creating spatiotemporal maps, we could examine spontaneous motility patterns in zebrafish larvae *in vivo* at 4 and 7 d.p.f. in more detail. Tetrodotoxin (TTX) was added to elucidate the importance of nervous control. Anterograde and retrograde contraction waves originated in the same region, just posterior to the intestinal bulb. This area correlates well with the distribution of Hu (human neuronal protein C/D)-immunoreactive nerve cell bodies. Whereas numerous immunoreactive nerve cells were present in the mid and distal intestine at both 4 and 7 d.p.f., fewer cells were seen anterior to the origin of contractions. The overall frequency of contractions (1.16±0.15 cycles min^{-1}, *N*=14 at 4 d.p.f.; 1.05±0.09 cycles min^{-1}, *N*=13 at 7 d.p.f.) and the interval between individual anterograde contraction waves (54.8±7.9 s at 4 d.p.f., *N*=14; 56.9±4.4 s, *N*=13 at 7 d.p.f.) did not differ between the two stages but the properties of the contractions were altered. The distance travelled by each wave increased from 591.0±43.8 μm at 4 d.p.f. (*N*=14) to 719.9±33.2 μm at 7 d.p.f. (*N*=13). By contrast, the velocity decreased from 4 d.p.f. (49.5±5.5 μm s^{-1}, *N*=12) to 7 d.p.f. (27.8±3.6 μm s^{-1}, *N*=13). At 4 d.p.f., TTX did not affect any of the parameters whereas at 7 d.p.f. anterograde frequency (control 1.07±0.12 cycles min^{-1}, *N*=8; TTX 0.55±0.13 cycles min^{-1}, *N*=8) and distance travelled (control 685.1±45.9 μm, *N*=8; TTX 318.7±88.7 μm, *N*=6) were decreased. In conclusion, enteric or extrinsic innervation does not seem to be necessary to initiate spontaneous contractions of the gut in zebrafish larvae. However, later in development, nerves have an increasingly important role as modulators of intestinal activity.

Key words: intestine, enteric nervous system, spatiotemporal map, teleost.

Introduction

Rhythmic, coordinated contractions are the foundation for peristaltic movements, involved in transport of food along the gastrointestinal tract. Propagating contractions also occur in situations where there is little or no food present in the gut. In mammals, these contractions are particularly evident during migrating motor complexes (MMCs) that are characterised by periods of regular contractions interrupted by longer periods of irregular or no activity (Husebye, 1999). Similar motility patterns, although not as elaborate, have also been seen in other vertebrates, for example in the intestine of a fasted teleost (*Gadus morhua*) (Karila and Holmgren, 1995).

Various factors including enteric and extrinsic innervation, release of hormones and the endogenous cyclic activity in interstitial cells of Cajal (ICCs) all play important roles in the initiation and maintenance of gut motility. For example, in mammals it is believed that ICCs act as pacemaker cells and are responsible for setting the frequency of contractions (Sanders, 1996; Smith et al., 1987). Nevertheless, in knockout mice lacking pacemaker ICCs, rhythmic contractions are still present (Spencer et al., 2003). In addition, the sodium channel blocker tetrodotoxin (TTX) abolishes gut motility, suggesting that initiation and propagation of MMCs mainly depend on neuronal activity (D'Antona et al., 2001; Sarna et al., 1981; Spencer et al., 2003). The importance of a functional enteric nervous system is further emphasised in conditions such as Hirschsprung's disease, where the absence of neurons in the distal part of the gastrointestinal tract causes severe constipation as result of impaired motility (Gershon, 2002). Whereas the intrinsic enteric nervous system is crucial for the phase containing regular contractions during the MMCs, extrinsic innervation probably is needed to develop all three phases (Chung et al., 1994; Sarna et al., 1981; Spencer et al., 2003; Husebye, 1999).

In parallel with the MMCs found in fasted adult mammals, in human foetuses where no food is present in the gut,

spontaneous gastrointestinal motility has been observed from the 14th week of gestation (Sase et al., 2000). The motility pattern gradually develops and by the 37th week of gestation, clearly defined MMCs are distinguished in preterm infants (Bisset et al., 1988). There are only few studies looking at the mechanisms that trigger the onset of motility or how it is maintained at early (pre-natal) developmental stages. In foetal mice, anterograde propulsion of gut content is observed at embryonic day 17.5 (E17.5) (Anderson et al., 2004). Since mice lacking enteric neurons showed normal propulsion, it has been suggested that enteric neurons are not needed for this activity. On the other hand, ICCs are present in the gut of mice and also in individuals with an impaired enteric nervous system, from the second half of gestation. In these animals slow waves appear shortly before birth (Ward et al., 1997; Ward et al., 1999).

Like unborn mammals, fish larvae rely on internal food sources (the yolk sac) immediately after hatching. Zebrafish *Danio rerio* usually start to eat at around 5–6 d.p.f. (days post fertilisation) but the first propagating gut contractions are detected from 4 d.p.f. (Holmberg et al., 2003). Acetylcholine and nitric oxide already affect the spontaneous gut activity at this stage (4 d.p.f.), and it has been postulated that there is a cholinergic and a nitrergic tone present, both of which modulate muscle activity, either directly or indirectly (Holmberg et al., 2004; Holmberg et al., 2006). In addition, regulatory neuropeptides such as NKA (neurokinin A) and PACAP (pituitary adenylate cyclase activating polypeptide) have effects on contraction frequency from 5 d.p.f. (Holmberg et al., 2004). The presence of enteric neurons at 2 d.p.f. and the expression of nitric oxide synthase (NOS), indicative of nitrergic neurons, as well as NKA and PACAP from 2–3 d.p.f. further support the theory that intrinsic enteric innervation is involved in the control of gut motility before the onset of exogenous feeding (Bisgrove et al., 1997; Holmberg et al., 2003; Holmberg et al., 2004; Holmberg et al., 2006; Holmqvist et al., 2004; Poon et al., 2003; Wallace et al., 2005).

To investigate the possible mechanisms behind these spontaneous propagating contractions in zebrafish gut and how the control mechanisms may develop around the onset of exogenous feeding, TTX was used to block neurotransmission in unfed anesthetized larvae at 4 and 7 d.p.f. Gut motility patterns were studied using spatiotemporal maps of gut flow in control and treated animals. In addition, the distribution of enteric neurons during development was studied in intact zebrafish embryos and larvae.

Materials and methods

Zebrafish *Danio rerio* Hamilton larvae were bred and raised at the Department of Zoophysiology, Göteborg, Sweden. Adult zebrafish were allowed to breed spontaneously in an aquarium with a breeding box. Eggs were collected on the day of fertilization (0 d.p.f.) and transferred to small beakers of water kept at a constant temperature of 28°C and a 12 h:12 h light:dark cycle. The larvae were not fed during the experimental period. At 28°C, active feeding usually starts around 5–6 d.p.f., and at 6 d.p.f. most of the yolk is depleted, however the animals will survive for up to a week and a half without food. The study protocol was approved by the animal ethics committee of Göteborg, and followed the guidelines of the Swedish National Board for Laboratory Animals.

In vivo recordings of gut motility

Larvae from 4 and 7 d.p.f. (N=15 and 14) were studied using *in vivo* video recording of spontaneous gut movements. Only animals that had hatched naturally at 3 d.p.f. were included in the study. The larvae were anaesthetized in phosphate-buffered MS222 (3-aminobenzoic acid ethyl ester, 75–100 mg l^{-1}, pH 7; Sigma, St Louis, MO, USA) and embedded in 1% agarose (gelling point 26-30°C; Sea Plaque, Sigma) dissolved in phosphate-buffered MS222. After the agarose had settled, the entire gastrointestinal tract of the larvae was monitored *in vivo* and gut movements were recorded onto videotape, using an inverted microscope (Nikon, ×4 magnification) equipped with a Panasonic WV-350 camera.

After an initial 5 min control period, 10 μl of tetrodotoxin (TTX, 1 mmol l^{-1}; Tocris, Bristol, UK) or NaCl (0.9%) were applied to the experimental chamber and gut activity was recorded for a further 30 min. The effects of TTX or saline applications were calculated over the last 5–10 min.

Data analysis

The video sequences (5 min) were digitised at 4 frames s^{-1} using the Optimas program package (Media Cybernetics, Germany). Spatiotemporal maps (STMaps) (Hennig et al., 1999) of the movement of luminal content and gut walls were created by averaging the intensity of the darker coloured luminal content across the diameter of the gut from a point midway along the swim bladder to the anus. Contractions could be inferred from STMaps, as a narrowing of the diameter decreased the overall amount of opaque material at the point of contraction and perturbed luminal contents, appearing as a coloured band (see Fig. 1). The average background intensity was subtracted, revealing only dynamic changes in gut opacity.

By analysing the STMaps a number of parameters such as frequency of contraction waves (cycles min^{-1}), interval between contractions (Δt; s), velocity (v; μm s^{-1}) and distance (d; μm) travelled by individual contraction waves were calculated. A line of best fit was manually drawn over a propagating contraction, from which the slope ($v=\Delta d/\Delta t$) and distance (d) were calculated. The intervals (Δt) between two consecutive contraction waves were determined for each pair of contractions initiated within the experimental period (5 min). By contrast, contraction frequency was calculated as the total number of contractions over the whole 5 min period (see Fig. 1). Hence, a change in frequency is not necessarily followed by a change in interval time and this could be reflected in the total time of activity, expressed as the time

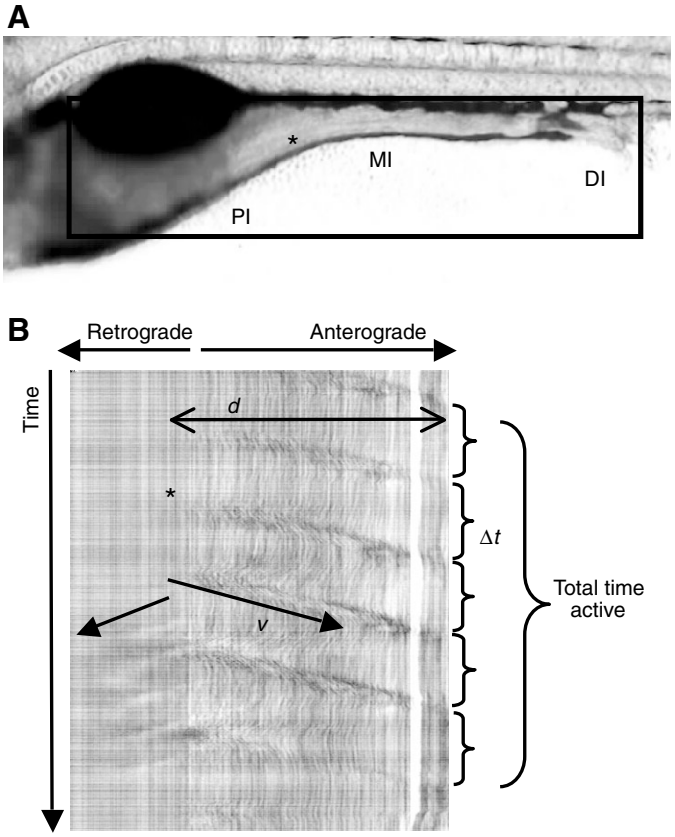

Fig. 1. Spatiotemporal maps (STMap) of the luminal flow were constructed from unfed larvae at 4 and 7 d.p.f. (A) A box was drawn around the area of interest, covering most of the gut (~900 μm). PI, proximal intestine (intestinal bulb); MI, middle intestine; DI, distal intestine. (B) STMaps, over the area within the box in A, were created based on changes in gut opacity as the gut content was propelled in a retrograde or anterograde direction. Individual contractions are identified as darker bands, travelling in either direction over time (downwards in map, ~3.8 min). In this STMap, six anterograde contractions waves are present. Velocity (v, the slope of the line), interval between consecutive contractions (Δt), distance (d) travelled by the contractions and the total time of activity were calculated. Asterisks indicate the region where the contraction waves originated.

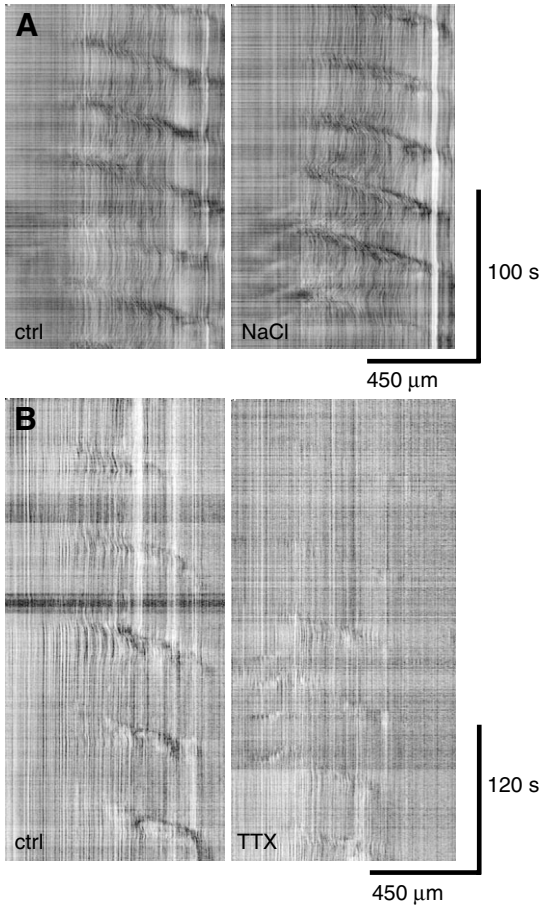

Fig. 2. Spatiotemporal maps (STMap; ~900 μm) showing the effect of NaCl (A; ~3.6 min) and tetrodotoxin (TTX; B; ~3.8 min) on anterograde contraction waves at 7 d.p.f. (A) Anterograde gut activity was unaffected by 0.9% NaCl in comparison with the control period (ctrl). (B) TTX decreased the frequency and distance of anterograde contraction waves in comparison with the control period (ctrl).

between the onset of the first contraction and the end of the last contraction, in proportion to the total experimental period. Results are presented as mean ± s.e.m.; only animals showing some activity during the control period were included. Student's t-test (two-sample assuming equal variances) was used for statistical evaluation of the results, with $P<0.05$ regarded as significant.

Immunohistochemistry

Embryos and larvae from 2, 3, 4 and 7 d.p.f. (N=4 from each stage) were anaesthetized in 0.01% MS222 and fixed in 4% formaldehyde (pH 7.3) for 2 h at room temperature. The embryos and larvae were permeabilized for 3 h in distilled water, before being incubated with 10% normal donkey serum (Jackson ImmunoResearch, West Grove, PA, USA) for 1 h in order to reduce non-specific staining. The preparations were incubated overnight with primary antisera consisting of a mixture of anti-acetylated tubulin (AcT, diluted 1:1000; T-6793, Sigma) and anti-human neuronal protein C/D (Hu, 1:100-1:200; A21271, Molecular Probes, Eugene, OR, USA), both raised in mice. Primary antibodies were subsequently detected by a common FITC (fluorescein isothiocyanate)-conjugated secondary antibody (1:100; Jackson ImmunoResearch) following a further overnight incubation. All sera and antisera were diluted with phosphate-buffered saline (PBS, 0.9% NaCl) containing 1% bovine serum albumin (BSA), 1% dimethylsulfoxide (DMSO), 0.1% Triton X-100 and 0.2% sodium azide (Ungos et al., 2003) and the preparations were rinsed in PBS. Preparations were mounted in Vector H-1000 medium and examined with a Nikon Eclipse E1000 digital fluorescence microscope equipped with a Nikon Digital Camera DXM1200 and the Nikon software, ACT1. Contrast and brightness were adjusted and montages were made using Microsoft PowerPoint.

Table 1. *Comparison between gut motility of zebrafish larvae at 4 and 7 d.p.f. with regard to frequency of anterograde contraction waves, interval between contractions, distance travelled and velocity*

	Frequency (cycles min^{-1})	Interval (s)	Distance (μm)	Velocity (μm s^{-1})
4 d.p.f.	1.16±0.15 (14)	54.8±7.9 (14)	591.0±43.8 (14)	49.5±5.5 (12)
7 d.p.f.	1.05±0.09 (13)	56.9±4.4 (13)	719.9±33.2* (13)	27.8±3.6* (13)

d.p.f., days post fertilisation.
Values are means ± s.e.m. (number of animals in parentheses); *P<0.05 compared with 4 d.p.f.

Results

In vivo recordings of gut motility

Most animals at 4 d.p.f. (14 out of 17) and 7 d.p.f. (13 out of 15) showed spontaneous anterograde (oral-to-anal direction) contractions during the 5 min control period (see Table 1). In agreement with previous studies (Holmberg et al., 2003; Holmberg et al., 2004; Holmberg et al., 2006), the contractions originated in the anterior part of the middle intestine (see Fig. 1). By analysing the STMaps, it was possible to discern periods of regular activity interspersed with longer or shorter periods of low activity (Fig. 2). In general, the time including contractions at any point along the gut was approximately 80–95% of the total time. Contractions propagated in a linear fashion from the site of initiation as shown by the steady slope (see Figs 1, 2). The activity was not affected by application of NaCl (Table 2).

However, when comparing the two developmental stages, it was obvious the motility patterns were gradually changing. At 7 d.p.f., the distance each wave travelled along the gut was increased compared with 4 d.p.f. larvae (Fig. 3; see Table 1 for a summary of the results). By contrast, there was an age-dependent decrease in velocity of almost 50% from 4 to 7 d.p.f. (Fig. 3, Table 1). The frequency and the interval between contraction waves were not different between the two age groups.

In addition, retrograde contractions were seen in 7 out of 17 animals, originating in the same area as the anterograde contraction waves (Fig. 2). At 4 d.p.f., the retrograde frequency was higher (1.93±0.41 cycles min^{-1}, N=7) than the anterograde (1.16±0.15 cycles min^{-1}, N=14) (cf. Holmberg et al., 2003; Holmberg et al., 2006). Consequently, it appears that contractions in either direction can begin independently of each other. At 7 d.p.f., retrograde contractions were more difficult to trace and are hence not included in the results.

Application of TTX did not affect any of the observed parameters (frequency, interval, velocity or distance) of the anterograde contractions at 4 d.p.f., when compared to the control period (Table 2). By contrast, at 7 d.p.f., TTX reduced the distance the contractions travelled by 55±11% compared with the control period (Figs 2, 3). Likewise, the frequency of contractions was reduced by 49±14% as was the proportion of the time the gut was active (from 94±1% to 44±15%, N=8). However, TTX did not affect the interval time or velocity of the anterograde contraction waves at 7 d.p.f. (see Table 2 for a summary of the results).

TTX decreased the frequency of retrograde contraction waves at 4 d.p.f. but had no effect on either of the other parameters measured (Table 3).

In addition, TTX did not have a significant effect on heart rate (control: 124.5±7.7, TTX: 125.0±6.3 beats min^{-1}, N=8) indicating that the overall status of the animals was not affected by application of the drug.

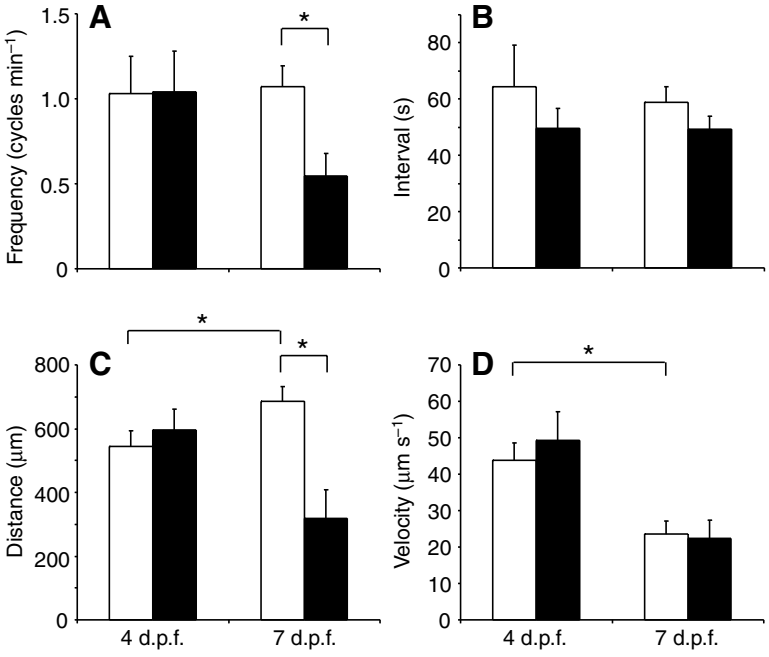

Fig. 3. The effects of tetrodotoxin (TTX; black bars) compared with the control period (white bars) on frequency (A), interval (B), distance (C) or velocity (D) of anterograde contraction waves at 4 and 7 d.p.f. Values are means ± s.e.m.; for N, see Table 2). Neurons do not seem to be necessary for the initiation and progress of anterograde contraction waves before the first feeding as TTX did not affect either of the parameters at 4 d.p.f. At 7 d.p.f., however, after the theoretical time for onset of feeding, TTX reduced frequency and distance (A,C), whereas interval and velocity were unaffected (B,D). Distance (C) increased while velocity (D) decreased when comparing 4 and 7 d.p.f. larvae. *P<0.05.

Table 2. *Comparison between gut motility of zebrafish larvae before and after addition of tetrodotoxin or NaCl, at 4 and 7 d.p.f. with regard to frequency of anterograde contraction waves, interval between contractions, distance travelled and velocity*

	Frequency (cycles min^{-1})	Interval (s)	Distance (μm)	Velocity (μm s^{-1})
4 d.p.f.				
C	1.03±0.22 (7)	64.3±14.7 (7)	543.3±49.6 (7)	43.9±4.7 (6)
TTX	1.04±0.24 (7)	49.6±7.2 (6)	594.9±66.1 (6)	49.3±7.9 (6)
C	1.29±0.19 (7)	45.3±5.0 (7)	638.7±71.3 (7)	55.2±10.0 (6)
NaCl	1.16±0.26 (7)	45.3±5.8 (6)	664.9±46.8 (7)	77.2±16.6 (7)
7 d.p.f.				
C	1.07±0.12 (8)	58.9±5.6 (8)	685.1±45.9 (8)	23.7±3.6 (8)
TTX	0.55±0.13* (8)	49.2±4.7 (6)	318.7±88.7* (6)	22.3±5.1 (2)
C	1.02±0.13 (5)	53.8±7.5 (5)	775.6±37.9 (5)	34.5±6.8 (5)
NaCl	0.80±0.21 (5)	47.4±5.3 (4)	688.8±119.2 (4)	24.8±5.2 (4)

d.p.f., days post fertilisation; C, control (before addition of substances); TTX, tetrodotoxin.
Values are means ± s.e.m. (number of animals in parentheses); *P<0.05 compared with control.

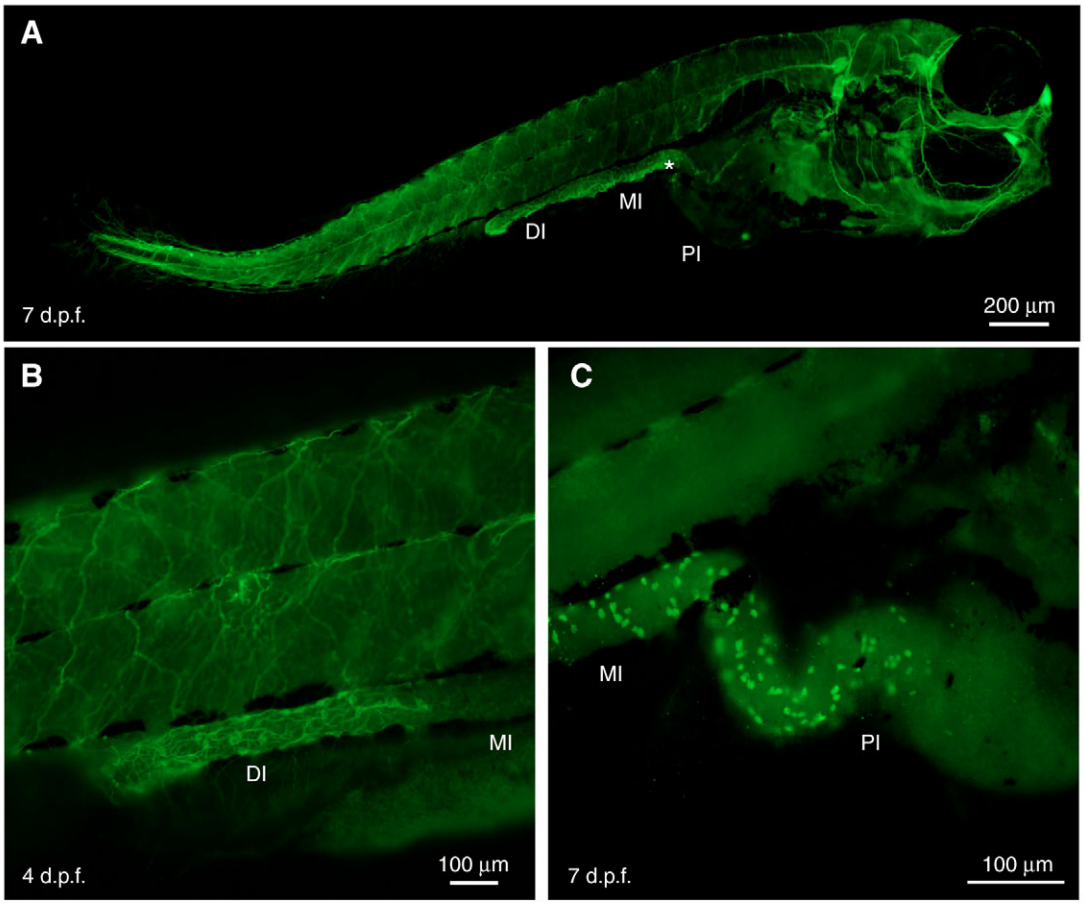

Fig. 4. Whole-mount zebrafish larvae labelled with antibodies against Hu (human neuronal protein) and acetylated tubulin (AcT). (A) Whole larva at 7 d.p.f. The asterisk indicates the region of the gut where both retrograde and anterograde contraction waves originate. (B) At 4 d.p.f. (before onset of exogenous feeding) the distal part of the intestine (DI) is already densely innervated by nerve fibres as well as nerve cell bodies. (C) The number of Hu-positive nerve cell bodies showed a marked decrease between the middle intestine and the posterior part of the intestinal bulb (proximal intestine) compared to the more anterior part of the bulb at the stages investigated. PI, proximal intestine; MI, middle intestine; DI, distal intestine.

Table 3. *Comparison between gut motility of zebrafish larvae before and after addition of tetrodotoxin or NaCl, at 4 days post fertilization with regard to frequency of retrograde contraction waves, interval between contractions, distance travelled and velocity*

	Frequency (cycles min^{-1})	Interval (s)	Distance (μm)	Velocity (μm s^{-1})
4 d.p.f.				
C	2.58±0.35 (4)	17.7±1.6 (4)	300.6±39.1 (4)	17.6±2.9 (4)
TTX	0.52±0.18* (4)	24.2±4.7 (4)	248.4±34.0 (4)	15.9±3.7 (4)
C	1.01±0.40 (4)	34.1±6.2 (4)	246.3±29.0 (4)	50.0±27.2 (2)
NaCl	0.25±0.25 (4)	ND	ND	ND

d.p.f., days post fertilisation; C, control (before addition of substances); TTX, tetrodotoxin.
Values are means ± s.e.m. (number of animals in parentheses); *P<0.05 compared with control.

Immunohistochemistry

Whole intact embryos and larvae showed Hu- and AcT-positive neurons from 3 d.p.f. While Hu mainly labels nerve cell bodies, AcT intensely labels nerve fibres in addition to weak staining of nerve cell bodies. At 3 d.p.f., nerve cells were predominantly seen in the middle and distal intestine whereas the anterior one third of the gut (the part developing into the intestinal bulb) showed no or only occasional immunoreactive cell bodies. At 7 d.p.f., nerve cell bodies occupied a larger area of the gut and there was also a higher density of nerve fibres. Hu-immunoreactive nerve cells were still relatively scarce in the most proximal part of the gut (Fig. 4).

Discussion

In this study we show the presence of TTX-sensitive, as well as TTX-insensitive, control mechanisms involved in the spontaneous contractions in non-feeding zebrafish larvae. Furthermore, by using spatiotemporal maps of gut flow we could make a detailed analysis of the motility patterns in larvae, demonstrating how it develops with time during the period when the larvae normally start to feed. During control conditions, most values were in the same range as has previously been observed in zebrafish larvae (Holmberg et al., 2003).

Development of spontaneous gut activity

When comparing the control periods of 4 and 7 d.p.f. larvae, it was noted that similar to previous studies, the distance travelled by the anterograde contraction waves increased while the overall frequency did not change. The increased distance could not be related to an increase in gut size since there was no obvious difference in the length of the middle intestine between 4 and 7 d.p.f. The refined method used in the present study showed that the distance that each contraction wave travelled was even longer than previously reported (Holmberg et al., 2003). However, unlike previous studies, we could also see a decrease in velocity between 4 and 7 d.p.f. This was mainly because the contraction waves at 4 d.p.f. travelled at a higher speed (approximately twice as fast) than has previously been observed (Holmberg et al., 2003). The reduced velocity at 7 d.p.f. could be due to several factors. The intestinal smooth muscle cells are differentiated around 4 d.p.f. and a longitudinal and a circular layer can be observed (Wallace et al., 2005). It is likely however, that the muscle layers continue to thicken between 4 and 7 d.p.f. This may shorten the distance the electrical current will spread and result in reduced excitability that will affect the contraction velocity. It could also be suggested that the muscle cells are slightly hyperpolarised, possibly due to endogenous release of nitric oxide (Holmberg et al., 2006). As a consequence, it will take longer until depolarisation reaches the muscle action potential threshold and, hence, the velocity will decrease. There is also a possibility that velocity changes along the gut. If there is a decrease in velocity along the gut, the longer distance travelled by each wave at 7 d.p.f. compared to at 4 d.p.f. would lead to a lower mean velocity. However, when looking at the STMaps, there were no obvious deviations in the slopes of individual contractions, suggesting a fairly consistent velocity. The change in velocity might also be related to an increase in viscosity of the intestinal contents (see Larson and Schulze, 2002).

Usually, an individual wave travels the full distance and dies out before the next wave is initiated. However, given the increase in distance in combination with the decrease in velocity between 4 and 7 d.p.f., the total time every individual wave travels along the gut increases. Although the frequency and interval time were unchanged, suggesting that the clock responsible for the timing of contractions is already well-developed at 4 d.p.f., the variance in interval times at this stage is comparatively larger than at 7 d.p.f. This could indicate that the activity becomes more regular with shorter periods of low activity, as the larvae grow older. Since development is a dynamic process, some individuals will have reached a more developed pattern at 4 d.p.f., while others lag behind.

TTX-sensitive neuronal control

It has previously been suggested that the spontaneous contraction waves seen in the zebrafish larvae might have similar housekeeping functions as the interdigestive mammalian migrating motor complexes (MMCs) (Holmberg et al., 2003; Holmberg et al., 2004; Holmberg et al., 2006). TTX inhibits propagating contractions in mammals as well as in fish (D'Antona et al., 2001; Karila and Holmgren, 1995; Spencer

et al., 2003). Further, it has been suggested that this neuronal input, at least in mammals, depends mainly on the enteric nervous system (Sarna et al., 1981). In the 7 d.p.f. zebrafish, TTX reduced the distance travelled by the anterograde contraction waves as well as the overall frequency but did not affect interval time or velocity. Hence, by blocking the neurotransmission, each contraction wave travels a shorter distance, indicating that neurons are needed for propagation of the wave over the last part of the middle intestine at 7 d.p.f. The distance is even shorter than during control conditions at 4 d.p.f. At the same time, neurons are involved in the initiation of the contractions, indicated by the decrease in frequency after TTX is applied. Although the overall frequency of anterograde contraction waves was reduced compared to the control period, the velocity and interval time were not affected by TTX at 7 d.p.f. This was mainly because the total time the gut is active is reduced by TTX whereas when it is active, the contraction waves occur with the same interval and velocity as before TTX.

At 4 d.p.f., the only parameter that was affected by TTX was the frequency of retrograde contractions. However, this group comprised relatively few individuals and one has to be a bit cautious when drawing any conclusions from these data. It is possible that the retrograde contraction waves are more irregular than the anterograde, as suggested by the fact that only 7 of 17 animals showed any retrograde contractions during the control period (c.f. 14 of 17 that showed anterograde contractions). This could also be reflected in the fact that three of the four control animals were inactive after addition of NaCl. However, it is possible that the initiation of retrograde contraction waves, in contrast to anterograde, is under TTX-sensitive control already at 4 d.p.f.

TTX-insensitive control

The difference in response to TTX between 4 and 7 d.p.f. suggests the development of a TTX-sensitive neuronal component during this time. It indicates that before the normal time for onset of feeding, the initiation and propagation of anterograde contraction waves are not under neuronal control, or at least not under TTX-sensitive control. This is similar to the situation in foetal mice, where the absence of enteric neurons does not affect the propulsion of gut content compared with control mice (Anderson et al., 2004). However, whereas TTX abolishes migrating contractions in adult mammals (D'Antona et al., 2001; Spencer et al., 2003), in zebrafish larvae there seem to be a TTX-insensitive part that remains, at least at 7 d.p.f. Whether this mechanism is present in older larvae or adult zebrafish has so far not been investigated. Since velocity was not affected by TTX at 7 d.p.f., but differed between 4 and 7 d.p.f. in control conditions, it can be suggested that development of this TTX-insensitive control mechanism also occurs during this period.

The TTX-insensitive component could be either neurons or putative interstitial cells of Cajal (ICCs). TTX-insensitive sodium channels are expressed on a subpopulation of enteric neurons in, e.g. guinea pig and rat (Rugiero et al., 2003). Further, although lack of myenteric (pacemaker) ICCs in the small intestine of mice did not abolish MMCs, some parameters of the migrating contractions were affected (Spencer et al., 2003). Whereas the interval between each complex was not affected *in vitro,* the contraction frequency within each MMC was reduced in the absence of myenteric ICCs (Spencer et al., 2003).

So far very little is known about the possible presence of ICCs in teleosts. ICC-like cells have been reported in the myenteric plexus of some teleost species, using Methylene Blue (Kirtisinghe, 1940), whereas later studies, using Kit as a marker to detect ICCs in zebrafish using either immunohistochemistry or *in situ* hybridization, have failed (Mellgren and Johnson, 2005; Parichy et al., 1999; Wallace et al., 2005) (C.O. and A.H., unpublished results). Furthermore, mutants lacking one of the two known zebrafish *ckit* orthologues showed no obvious defects in intestinal functions, either in larvae or in adults (Parichy et al., 1999). This suggests that if ICCs are present in fish, they probably do not depend on the Kit receptor for their development, or at least Kit is different from the mammalian counterpart. However, there is one recent report indicating Kit-positive cells in the gut of zebrafish larvae (Rich et al., 2006). In mammals, Kit-positive ICCs have been detected before birth (Torihashi et al., 1997; Wester et al., 1999). Slow waves also occur during foetal life but are preceded by ICCs (Ward, 1996; Ward et al., 1997). The present data indicate the presence of some sort of pacemaker cells in the gut around the time the zebrafish larvae start to feed.

Anterograde and retrograde contractions originate in a distinct area of the intestine, just distal to the developing intestinal bulb (present study) (Holmberg et al., 2003; Holmberg et al., 2006). This region of origin was not affected by TTX. Further, although the motility pattern is less well coordinated very early in development (3 d.p.f.), most contraction waves still originate in this area (Holmberg et al., 2003). This is in contrast to the mammalian small intestine where propagating contractions may develop in different regions (e.g. Furness and Costa, 1987). The area in the zebrafish gut where the contractions originate correlates with an area of dense innervation.

However, the neuronal network gradually decreases in the proximal part of the intestinal bulb both at 4 and 7 d.p.f. This probably explains why gut motility was not observed in this part of the bulb in the present or earlier studies (Holmberg et al., 2003). The immunohistochemical data suggest that enteric neurons are needed for the propagation of a contraction wave along the intestinal bulb. Although contractions can travel for a shorter distance, neurons are needed to maintain the propagating contraction as was indicated by the decreased distance travelled by the anterograde contractions after TTX at 7 d.p.f. During development, the number of nerve cells as well as the length and number of their processes increase at least until 13 d.p.f. (C.O., A.H. and S.H., unpublished results). Retrograde activity was not examined at 7 d.p.f., and hence the full importance of the posterior development of enteric neurons could not be established.

In conclusion, the zebrafish gut motility shows an increasing degree of control when comparing the time before and after the larvae normally start to feed. The control mechanisms include both neuronal and non-neuronal pathways as indicated by the effects of TTX treatment on different parameters of the activity. Although propagating contractions probably serve a housekeeping function in the non-feeding larvae, when the fish start to feed the need for a more controlled motility pattern increases.

List of abbreviations

AcT	acetylated tubulin
Hu	human neuronal protein C/D
ICC	interstitial cell of Cajal
MMC	migrating motor complex
NKA	neurokinin A
NOS	nitric oxide synthase
PACAP	pituitary adenylate cyclase activating polypeptide
STMap	spatiotemporal map
TTX	tetrodotoxin

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