Ontogeny of excitatory and inhibitory control of gastrointestinal motility in the African clawed frog, *Xenopus laevis*

Monika Sundqvist and Susanne Holmgren

*Department of Zoophysiology, Göteborg University, Göteborg, Sweden*

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Sundqvist, Monika, and Susanne Holmgren. Ontogeny of excitatory and inhibitory control of gastrointestinal motility in the African clawed frog, *Xenopus laevis*. *Am J Physiol Regul Integr Comp Physiol* 291: R1138–R1144, 2006.—The transparent body wall of *Xenopus laevis* larvae during the first developmental stages allows in vivo studies of gastrointestinal tract activity. The purpose of this study was to chart the ontogeny of gut motility in *Xenopus* larvae and to identify the most important control systems during the first developmental stages. Coordinated descending contraction waves first occurred in the gut at Nieuwkoop and Faber stage 43 [0.8 ± 0.1 contractions/min (cpm)] and increased to 4.9 ± 0.1 cpm at stage 47. The cholinergic receptor agonist carbachol (5–10 μM) increased contraction frequency already at stage 43, as did neuretinin A (NKA, 0.3–1 μM). The muscarinic antagonist atropine (100 μM) first affected contraction frequency at stage 45, which coincides with the onset of feeding. The tachykinin antagonist MEN-10,376 (6 μM) blocked NKA-induced contractions but not spontaneous motility. Both sodium nitroprusside [nitric oxide (NO) donor, 1–10 μM] and vasoactive intestinal peptide (VIP, 0.1–1 μM) inhibited contractions from the earliest stage onward. Blocking NO synthesis using *N*^o^-nitro-L-arginine methyl ester (100 μM) had no effect per se, but antagonized VIP evoked inhibition at stage 47. We conclude that gastrointestinal motility is well developed in the *Xenopus laevis* larvae before the onset of feeding. Functional muscarinic and tachykinin receptors are present already at the onset of motility, whereas a cholinergic tone develops around the onset of feeding. Endogenous tachykinin tone was found. Functional VIP receptors mediate inhibition at the onset of motility. NO seems to mediate the VIP effect at later stages.

An emerging motility pattern. The development of the enteric nervous system is of great interest in the study of gastrointestinal motility during development. However, the functionality of these agents in regard to gastrointestinal motility has not been studied. Therefore, the purpose of this study was to follow the time course of motility development in the amphibian gut. Furthermore, the contribution of various neurotransmitters and neuromodulators to the control of gut motility during development was assessed.

MATERIALS AND METHODS

Adult *X. laevis* were induced to breed by injection of human chorionic gonadotropin (200 U/female and 150 U/male in the morning and 400 U/female and 300 U/male in the afternoon). They were kept overnight in a semidark aquarium. The adult frogs were then removed...

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and the fertilized eggs were left to develop into larvae. The breeding procedure and all experiments were approved by the animal ethics committee of the administrative court of appeal of Göteborg.

Larvae were anesthetized in 0.01% MS-222 (3-aminobenzoic acid ethyl ester; Sigma), and their developmental stage was determined according to Nieuwkoop and Faber (24). Animals from stages 40–47 were transferred to an experimental chamber with 50 ml of circulating, aerated modified McKenzie’s amphibian Ringer solution (in mM: 115 NaCl, 20 NaHCO₃, 5.0 HEPES, 3.2 KCl, 1.4 MgSO₄, and 1.3 CaCl₂) containing 0.01% MS-222 and kept at 22°C. They were placed on their back, kept in position by insect pins placed as supports around the animal, and viewed in a microscope (model SMZ-10A; Nikon) with a digital camera (model WV-CL350S/G; Panasonic) attached. The camera was connected to a computer with the software program Optimas 6.0 (Media Cybernetics) for image analysis. The recording speed was one picture per second. To reduce biased analysis, the experiments were saved under a randomly allocated file number and later analyzed blindly. The frequency of contractions was counted visually from the video recording, whereas velocity was determined by measuring the length of a visible segment of gut in one frame using Optimas 6.0 and counting the number of 1-s frames during which the contraction swept through that segment. This was repeated for four independent contraction waves per animal.

To investigate whether the obtained effects of drugs could be the result of or influenced by changes in the circulatory system, the heat rate was analyzed. Using a stopwatch, beats were counted directly from the video sequences during 1 min for each of the recordings. Heart rate is presented as beats per minute. Atropine, N⁶-nitro-L-arginine methyl ester (l-NAME), VIP, neurokinin A (NKA), and MEN-10,376 did not affect heat rate significantly in the experiments.

Experimental protocol. Animals between stages 40–47 were used in the experiments. Analysis of gut motility at later stages was impossible because migration of chromatophores over the abdomen of the larvae formed a gold-colored layer obscuring the view of the gut. Removing the body wall disturbed the motility pattern too much to consider the action as justified.

Because the gut shape changes with developmental stage in these animals (see anatomical maps in Ref. 24), we have focused on motility waves sweeping along the whole visible gut (~75% of the total gut). For each animal, the spontaneous activity was recorded for 5 stages (45–47) or 10 stages (40–44) min. These measurements were used to study the development of the spontaneous motility in the larvae. A small hole was then opened in the body wall above one of the aortic arches to facilitate drug diffusion in the animal. The spontaneous activity was recorded for another 10 min to establish that this procedure did not affect the gut motility. If motility was affected by the surgery, the animal was discarded. Compounds were added to the bath, and 5 min later (allowing the compound to diffuse into the animal) the effect of the compound was recorded for another 10 min. The entire 10-min period was subsequently analyzed. A slightly different protocol was used for sodium nitroprusside (SNP). Because of the rapid penetration and effect of this substance, the recording was started immediately after the addition to the bath and continued for 15 min. The resulting recording was divided into 3-min periods, which were analyzed. For all inhibitory substances, animals were only used if the basal spontaneous activity was ≈0.2 contractions/min (cpm), otherwise inhibitory effects might be difficult to perceive.

Drugs. Carbamoylcholine chloride (carbachol), atropine, SNP dehydrate, and l-NAME were all purchased from Sigma-Aldrich Chemical (St. Louis, MO). Human NKA was obtained from Euro-Diagnostica (Malmö, Sweden), and VIP, 2-(carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxide (carboxy-PTIO), and MEN-10,376 were obtained from (Tocris Cookson, Bristol, UK). RP-67580 was a gift from Dr. C. Garret (Rhone-Poulenc, France). All compounds used were dissolved in Millipore water to stock solution and further diluted in amphibian Ringer solution, except for RP-67580, where DMSO was used for the stock solution.

Carbachol and atropine were added at a volume of 0.5 ml to the bath (50 ml). For the other drugs, a plastic ring was coated with silicon grease and placed around the larvae, thus limiting the bath volume to 5 ml. The drugs were then added at a volume of 50 μl. VIP and SNP doses were chosen in accordance to doses effective in adult Xenopus (25); the concentrations of the other drugs were chosen after pilot tests to obtain maximum response.

Data analysis and statistics. In most experiments, the motor activity is presented as cpm. Effects of compounds are statistically analyzed using precompound basal activity as control. However, in graphs showing multiple concentrations, the control value shown reflects the mean value for all animals in the experiments. The motility decreased slightly over time in control experiments (see Table 1). This decrease is corrected for when analyzing effects of compounds. In experiments at stage 47, when combinations of compounds are used, each individual is related to its precompound control (set to 100%) and then compared with vehicle experiments, thus allowing easier overview of results.

Spontaneous activity at different stages was shown to follow a Gaussian distribution by using a D’Agostino and Pearson omnibus normality test. Experiments were statistically compared using one-way ANOVA followed by Bonferroni’s post hoc test. Results are presented as means ± SE and are considered significant at P < 0.05. All statistical analyses were performed using GraphPad Prism 4.0 (GraphPad Software, San Diego, CA).

RESULTS

Development of spontaneous motility. At stage 41 and more regularly at stage 42, uncoordinated, segmenting contractions began to occur in the gut (Fig. 1A). These contractions appeared at different sites in the gut, both rostrally and caudally, and did not seem to conform to any pattern. At stages 42–43, coordinated contractions arose, and a pattern of rhythmic descending contraction waves emerged. These waves swept along the entire visible gut. Some ascending contraction waves could also be seen at this stage, but were not present at later stages. The uncoordinated contractions and the propagating waves occurred together during stages 42–44. At stage 47 the uncoordinated contractions disappeared altogether and only the wave pattern persisted. The frequency of the descending motor activity patterns was 0.8 ± 0.1 cpm at stage 43 (n = 57) and increased in a stage-dependent manner, reaching approximately a sixfold higher frequency at stages 46–47 (4.5 ± 0.1 and 4.9 ± 0.1 cpm, respectively, n = 57; Fig. 1A).

The velocity of the contractions also increased with developmental stage and did not seem to have reached a plateau at stage 47 (Fig. 1B). Hence, there is a positive correlation between both velocity and frequency with developmental stage during the investigated stages.

Table 1. Decrease in spontaneous contraction frequency of the intestine with experimental time (5–15 min after start of recording) in Xenopus laevis larvae in vivo at different developmental (NF) stages

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|-----------------|-----------------|
| NF Stage        | 5–15 min, %decrease |
| 43              | 16.9±8.3        |
| 44              | 17.7±8.7        |
| 45              | 10.2±1.1        |
| 46              | 6.6±2.8         |
| 47              | 3.5±2.5         |

Data are presented as mean values ± SE; n = 8–10 experiments. NF stage, Nieuwkoop and Faber stage.

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Cholinergic muscarinic regulation. The cholinergic receptor agonist carbachol (5 and 10 μM) increased contraction frequency at stage 43 in a concentration-dependent manner, with 10 μM causing a fivefold increase (Fig. 2A). Heart rate was dose dependently decreased in stage 43 larvae (control 108.5 ± 2.5 beats/min vs. 5 μM carbachol 101.5 ± 2.1 beats/min and 10 μM carbachol 82.75 ± 1.8 beats/min, n = 4, P < 0.05). The effect of carbachol on the gut was blocked by atropine (100 μM, Fig. 2A), suggesting the presence of functional muscarinic receptors. At one stage earlier, stage 42, only one animal of six responded to carbachol at a high concentration (100 μM) by developing descending contractions (data not shown). Administration of carbachol (1 or 10 μM) at stage 47 did not visibly affect the gut; however, the higher concentration severely decreased the heart rate of the animal, thereby making this result somewhat difficult to interpret.

Basal motor activity at stages 43 and 44 was not significantly affected by atropine (100 μM; Fig. 2B), whereas atropine attenuated the frequency of intestinal motor activity by ~30% at stages 45–47 (Fig. 2B). A higher concentration of atropine (1,000 μM) did not result in further reduction of contraction frequency (data not shown).

Tachykinin regulation. The tachykinin NKA (0.3 and 1 μM) increased the frequency of contractions at stage 43 in a concentration-dependent manner (Fig. 3A). NKA (1 μM) also generated descending contraction waves in some animals at stage 42 although they were very weak and difficult to follow along the gut. At stage 47, NKA increased the contraction frequency by 23% compared with vehicle. The tachykinin NK2 receptor antagonist MEN-10,376 (6 μM) had no effect per se on spontaneous motility; however, the antagonist blocked NKA-induced contractions at stage 47 (Fig. 3B). The NK1 receptor antagonist RP-67580 (10 μM) had no effect discernible from the effect of the vehicle, DMSO (0.17%), which made the results difficult to interpret (data not shown).

Nitrergic regulation. The effect of the NO donor SNP was transient, and analysis of the response was made over a 3-min period when the maximal effect was seen (i.e., 3–6 min after administration). SNP (1 μM) abolished contractions at stages 43 and 44 and reduced the frequency at stages 45–47. Increasing the concentration of SNP 10-fold abolished contractions at stages 45–47 as well (Fig. 4A). SNP (1 μM) had no effect on the heart rate [control 128 ± 2.3 beats/min vs. SNP 132.4 ± 1.2 beats/min, n = 5, not significant (NS)] in any stages investigated. SNP (10 μM) decreased heart rate in stage 43 larvae (control 128 ± 2.3 beats/min vs. SNP 111 ± 4.1 beats/min, n = 5, P < 0.05) although not in stage 47 larvae (control 136.8 ± 2.6 beats/min vs. SNP 132.8 ± 2.6 beats/min, n = 5, NS).

The NOS synthase inhibitor L-NAME (100 μM; Fig. 4B) did not affect spontaneous motility at stages 44 or 47. In addition, the NO scavenger carboxy-PTIO (100 μM) had no effect on spontaneous motility at stage 47 (Fig. 4C).
VIPergic regulation. The neuropeptide VIP inhibited motor activity at stage 43 in a concentration-dependent manner (Fig. 5A). Maximal effect was seen at 0.3 μM, which inhibited contraction frequency by 87%. Increasing the concentration of VIP to 1 μM did not cause further inhibition. At stage 47, 0.3 μM VIP was less potent than at stage 43 (27% inhibition), whereas 1 μM attenuated the frequency of contractions by 68% compared with control. At this stage, the VIP-evoked inhibition could be blocked partially by adding L-NAME (0.1 mM) to the bath 5 min preceding VIP administration (Fig. 5B). Adding L-NAME before VIP had no effect at earlier stages (data not shown).

DISCUSSION

The present study demonstrates that random gastrointestinal contractions first occur at stage 41 during X. laevis development. Coordinated propagating motor activity is not present until stage 43 in most animals. The increase in motor frequency and velocity with increasing developmental stage implies a maturation of motility starting before the onset of exogenous feeding (i.e., stage 45) and continuing during the subsequent stages. The enhanced contraction frequency from stage 43, after administration of carbachol or NKA to the animal, and the inhibition in frequency from stage 43 by administration of SNP or VIP suggests the expression of the respective receptors in the gastrointestinal tract well before the onset of feeding. The inhibitory effect by atropine on motility at stage 45 implies that cholinergic neurotransmission has developed. In contrast, constitutive NO probably plays a minor role if any in the control of intestinal contractions during the development of gut motility, since L-NAME per se did not affect motility at any stage. An overview including the time scale of larval gut development, immunohistological findings (13), and the results from the present study is shown in Fig. 6.

Nerve fibers have been found in the esophagus of X. laevis from stage 38–39, in the stomach from stage 40, and in the intestine from stage 41 (8, 13). The development of circular smooth muscle also occurs during these stages, with actin fibers appearing in the gut at stage 41 (13, 18). Thus the gastrointestinal motility monitored in the current study appears after the development of intestinal nerve fibers (and muscle), suggesting that nerves can influence motility even at the...
earliest stages of development. Of course, part of the spontaneous activity seen at earlier stages (stages 41–44) and indeed also some of the activity at later stages is probably independent of neuronal activity and instead depends on intrinsic pacemaker activity. Interestingly, the propagating movements do not seem to require two muscle layers, since the longitudinal muscle layer does not develop until stage 50 (18). The uncoordinated, segmenting contractions seen only during stages 41–44 may serve to mix the intestinal contents when the larvae receive nutrition from the yolk in the intestinal cells. These uncoordinated contractions are still present when the coordinated contraction waves start to appear (stages 42–44). Subsequently, once nutrients are mostly obtained from exogenous food sources, the coordinated propagating contractions take over.

In humans, fetal gastrointestinal motility starts during the third trimester with a random pattern at first, which develops into a phasic activity, and finally into migrating motor complexes (MMC) shortly before birth (2). Similar patterns have been shown in other mammalian species (6, 33) although whether adult-like rhythmicity appears before or shortly after birth seems to depend on the developmental stage the animal is born at. Because birth coincides with the onset of exogenous feeding in mammals, it can in some aspects be compared with the onset of exogenous feeding in Xenopus larvae.

It has been suggested that the spontaneously occurring contractions in the teleost species Gadus morhua could be the equivalent of MMCs (17). The present study shows that the speed of the propagating motor activity seen in the Xenopus larvae at the later stages (0.5–0.7 mm/s at stages 46–47) lies in the same range as the proposed MMCs in G. morhua (0.58 ± 0.2 mm/s) or in mammalian species [0.5–1.6 mm/s (guinea pig), 0.8 mm/s (cat), 0.6–0.7 mm/s (dog); see Refs. 7, 29, and 30]. In Xenopus, velocity continued to increase at stage 47; therefore, it cannot be excluded that velocity increases even further at subsequent stages. However, one study performed on the amphibian Rhacophorus viridis using tadpoles corresponding to around Nieuwkoop and Faber stage 53 (Gosner stage 33) showed a similar speed of peristaltic waves (0.56 mm/s at 20°C; see Ref. 23), as demonstrated in the current study.

The atropine-sensitive increase in contraction frequency evoked by administration of carbachol at stage 43 shows that functional muscarinic receptors are present from the onset of contraction cycles. However, at stages 43–44, cholinergic neurotransmission seemed to be absent given that atropine per se did not affect motility. A cholinergic tone, suggested by the

Fig. 5. Effects of vasoactive intestinal peptide (VIP) on gastrointestinal contraction frequency in X. laevis larvae in vivo. A: dose-dependent inhibitory effect of VIP at stage 43 (n = 7–8). B: at stage 47, the dose-dependent effect of VIP on spontaneous motility could be partially blocked by the NO synthase inhibitor L-NAME (n = 7). Data are presented as mean values ± SE. *Statistical significance (P < 0.05).

Fig. 6. Schematic overview of developmental events in the X. laevis larval gut around the onset of feeding. Open bars, immunoreactivity only present in the esophagus; filled bars, immunoreactivity present all through the gastrointestinal tract. A, current study; B, Ref. 13; C, Ref. 18; D, Ref. 24.
ability of atropine to attenuate the spontaneous contraction frequency, was first seen at stage 45. The early appearance of a cholinergic tone agrees well with the results of Holmberg et al. (14), who found an endogenous cholinergic tone present even shortly before the onset of exogenous feeding in the zebrafish. Similarly, studies in mammals have found cholinergic regulation before birth in several species (11, 12, 27, 28). The remaining noncholinergic motor activity seen at stages 45–47 after atropine administration could be dependent on the release of other transmitters or to intrinsic pacemaker cells (i.e., the ICCs).

To achieve effect with atropine, a comparatively high concentration (100 μM) was used. Lower concentrations were tested, but had no effect. It is common in ectothermic animals that concentrations of both agonists and antagonists need to be higher than in corresponding mammalian studies. The low sensitivity to atropine in early Xenopus larvae also agrees with the low sensitivity to atropine in early developmental stages found in the ovine intestine (28) and might be the result of a lower lever of muscarinic receptor expression in fetal tissue.

It can be argued that the effects of carbachol and NO are caused by changes in blood perfusion of the gut rather than direct effects on gut motility. Indeed, SNP (10 μM) decreased heart rate of stage 43 larvae at the same time as it inhibited gastrointestinal motility. However, a lower concentration of SNP had no effect on heart rate but still inhibited contraction frequency. In contrast, carbachol (10 μM), which also reduced the heart rate of the larvae, stimulated gastrointestinal motility.

Taken together, we find it unlikely that the effects in the present study are not direct on the gut tissues. It is feasible, though, that a reduced perfusion of the gut may reduce the amplitude of the effects of applied carbachol and NO.

The increased contraction frequency from stage 43 after NKA treatment suggests the presence of functional tachykinin receptors from this stage. Using immunohistochemistry, Holmberg et al. (13), demonstrated NKA-positive nerve fibers in the esophagus from stage 42 and in the stomach and intestine from stage 44, corroborating our results and showing development of the tachykinin system around the time when regular contraction cycles appear.

In the toad, Bufo marinus, three isoforms of an NK1 receptor have been cloned (21), showing high conservation of amino acids involved in agonist binding and activation of the receptor but low conservation of amino acids important for antagonist binding. The amphibian NK2 receptor has not been cloned, although functional studies imply the presence of both NK1- and NK2-like receptors in the gut (16, 20). In our study, the peptidergic NK2 receptor antagonist MEN-10,376 had no effect when administered alone; however, NKA-induced contractions were blocked by the antagonist. This suggests that MEN-10,376 binds to a tachykinin receptor in the gut wall, but a tachykinin tone is insignificant or absent. This lack of a tachykinin tone in the larvae is not surprising. Mammalian studies have shown that, under normal physiological conditions in vivo, the NK receptor antagonists have little effect on gut motility, whereas muscarinic antagonists usually have a strong inhibitory effect, implying that the cholinergic tone is much more dominant (10, 15).

Similarly, a nitrergic tone seems to be absent during the investigated stages, since neither block of NO synthesis nor an NO scavenger changes the contraction frequency, although an exogenous NO-mediated inhibition still can be achieved. The fact that L-NAME inhibited VIP-induced inhibition excludes the possibility that the compound did not reach the gastrointestinal tract or that the concentration was too low to cause NOS inhibition. Thus the results suggest that, while tonic NOS activity most likely plays a minor role under normal conditions, the activity can probably be increased by substances such as VIP and thereby contribute to the inhibitory control.

Both NOS- and VIP-positive nerve cells are found in the esophagus and stomach preceding the development of the motility, although NOS was not found in the intestine until stage 46 (13). Indeed, this corroborates the results from the present study where L-NAME blocks the effect of VIP after, but not before, stage 46, suggesting development of NOS activity at this stage. NOS and VIP are partially colocalized in enteric neurons in adult specimens (25). Similarly, in humans, NOS has been shown by immunohistochemistry to appear around 12 wk gestation and around the same time as a cholinergic innervation (5), and in the murine gut, cells showing NAPD diaphorase activity (an early marker for NOS) were first detected on embryonic day 12 (4). In contrast, in another amphibian species, the axolotl, NOS could not be detected until the juvenile stages (1).

Our experiments using VIP showed that the motor activity to a small extent was insensitive to VIP. The VIP inhibition was, however, sensitive to inactivation of NOS at stage 47, although not at earlier stages. The mechanisms involved in the VIP/NOS relationship are not clear, but it has been suggested that receptor binding by VIP triggers NOS activation and NO release both in smooth muscle cells and in neurons, which then elicits relaxation. It is also possible that VIP and NO act in parallel and cause relaxation separately through independent mechanisms (19).

Our results suggest an ongoing development of the nervous control of gastrointestinal motility even after the onset of feeding in X. laevis larvae. In general, the early regulation of motility in amphibians seems to be similar in many aspects to that in mammals, which suggests a strong evolutionary conservation of the important control systems.

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