

Regulation of Intermuscular Electrical Coupling by the *Caenorhabditis elegans* Innexin *inx-6*

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Submitted November 8, 2002; Revised March 13, 2003; Accepted March 18, 2003
Monitoring Editor: Daniel Goodenough

The innexins represent a highly conserved protein family, the members of which make up the structural components of gap junctions in invertebrates. We have isolated and characterized a *Caenorhabditis elegans* gene *inx-6* that encodes a new member of the innexin family required for the electrical coupling of pharyngeal muscles. *inx-6(rr5)* mutants complete embryogenesis without detectable abnormalities at restrictive temperature but fail to initiate postembryonic development after hatching. *inx-6* is expressed in the pharynx at all larval stages, and an *INX-6::GFP* fusion protein showed a punctate expression pattern characteristic of gap junction proteins localized to plasma membrane plaques. Video recording and electropharyngeograms revealed that in *inx-6(rr5)* mutants the anterior pharyngeal (procorpus) muscles were electrically coupled to a lesser degree than the posterior metacarpus muscles, which caused a premature relaxation in the anterior pharynx and interfered with feeding. Dye-coupling experiments indicate that the gap junctions that link the procorpus to the metacarpus are functionally compromised in *inx-6(rr5)* mutants. We also show that another *C. elegans* innexin, EAT-5, can partially substitute for INX-6 function in vivo, underscoring their likely analogous function.

INTRODUCTION

Intercellular communication is essential for coordinating cellular processes such as pattern formation during development, maintenance of metabolic homeostasis, and synchronous muscle contraction (Bruzzone *et al.*, 1996; Phelan and Starich, 2001). This communication is mediated in part via gap junctions, which permit small molecule flow between the cytoplasm of connected cells. Gap junctions between excitable cells, i.e., muscles and neurons, allow these cells to be electrically coupled, facilitating synchronous changes in membrane potential (Bruzzone *et al.*, 1996).

Two protein families can form gap junctions: the vertebrate-specific connexins and the innexins, which are typical to invertebrates. The connexins form integral membrane protein assemblies, called connexons, consisting of six subunits. Connexons in adjacent cells interact in the extracellular space to form the gap junction (Yeager and Nicholson, 1996; Unger *et al.*, 1999).

Although gap junctions were first characterized in invertebrate organisms (Furshpan and Potter, 1957), the genes that encode their structural proteins were only recently characterized, mainly because the innexins have no significant sequence similarity to the connexins (Phelan *et al.*, 1998a).

Nevertheless, innexins are both necessary and sufficient to form gap junctional channels. Innexin mutants lack dye coupling between muscle and neuronal cells (Starich *et al.*, 1996; Todman *et al.*, 1999). Moreover, the innexins *Ce-inx-3*, *Dm-inx-2*, and *shaking-B* can form gap junctional channels when expressed in the paired *Xenopus* oocyte system. (Phelan *et al.*, 1998b; Landesman *et al.*, 1999; Stebbings *et al.*, 2000). Structure predictions based on primary sequence indicate that, like the connexins, innexins possess four transmembrane domains with the same overall topology (Phelan and Starich, 2001). Thus, homology between connexins and innexins may be evident at the structural level.

Both the connexins and innexins constitute large gene families, presumably reflecting their diverse roles in organisms. About 20 connexins have been identified in vertebrates (White and Paul, 1999), 25 innexins in *C. elegans*, and 8 in *Drosophila* (Phelan and Starich, 2001). Because both connexins (Nicholson *et al.*, 1987) and innexins (Stebbing *et al.*, 2000) can form heteromeric channels, the subunit composition of which can have important effects on the properties of the gap junction formed, a large number of distinct gap junctional channels could assemble in vivo.

Several human diseases have been linked to connexin polymorphisms (Bergoffen *et al.*, 1993; Kelsell *et al.*, 1997; Shiels *et al.*, 1998) and directed mutations in mice have revealed diverse roles for connexins in transplacental nutrient transfer (Gabriel *et al.*, 1998), ovulation (Simon *et al.*, 1997), and cardiac development and function (Cx40, Cx43,

Article published online ahead of print. Mol. Biol. Cell 10.1091/mbc.E02-11-0716. Article and publication date are available at www.molbiolcell.org/cgi/doi/10.1091/mbc.E02-11-0716.

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and Cx45) (Kanter *et al.*, 1994; Reaume *et al.*, 1995; Ewart *et al.*, 1997; Simon *et al.*, 1998).

Mutations in four innexins have been characterized in *Drosophila* (Curtin *et al.*, 1999; Bauer *et al.*, 2002; Tazuke *et al.*, 2002). The *shaking-B* (lethal) mutation causes animals to die early, possibly due to feeding defects (Crompton *et al.*, 1995), whereas the *shaking-B* (neural) mutation disrupts the gap junctions between giant fibers and its postsynaptic motorneuron partners (Thomas and Wyman, 1984; Krishnan *et al.*, 1993; Phelan *et al.*, 1996; Sun and Wyman, 1996). *Ogre* mutations lead to defects in optic lobe development and abnormal electrical activity in the eye (Lipshitz and Kankel, 1985; Watanabe and Kankel, 1990).

UNC-7 was the first innexin identified in *C. elegans*, and mutations in *unc-7* cause impaired forward locomotion and ivermectin resistance. The uncoordinated phenotype could result from the aberrant formation of an UNC-7-dependent channel or may reflect ectopic electrical junctions between motorneurons and interneurons in *unc-7* mutants (Starich *et al.*, 1993; Dent *et al.*, 2000). The mutant phenotype of another innexin gene, *unc-9*, is very similar to that of *unc-7*, indicating that UNC-9 subunit may partner with UNC-7 to form the functional gap junction (Barnes and Hekimi, 1997).

Several *C. elegans* innexins are expressed in the pharynx (Phelan and Starich, 2001), probably because the control of current flow through coupled muscles requires gap junctions with diverse properties in this organ. The pharynx is a neuromuscular pump that has some developmental and functional similarities to the heart (Haun *et al.*, 1998; Maduro *et al.*, 2001) and is required for ingesting food (Figure 4, A and B). The pharyngeal muscles are extensively gap junctioned so that a pump results in a compound action potential and simultaneous contraction of most of the pharyngeal muscles. This compound action potential can be measured by a simple electrophysiological technique, the electropharyngeogram (EPG) (Avery and Thomas, 1997).

One explanation for the large number of innexins in the pharynx is that the control of current flow through coupled muscles requires gap junctions with diverse properties. *eat-5*, for instance, is expressed only in the muscles of the metacarpus and in the muscles of the isthmus, which connects the metacarpus with the terminal bulb of the pharynx (Avery, 1993). A mutation in *eat-5* uncouples the muscles of the terminal bulb from those of the metacarpus but leaves intermuscular junctions within each bulb intact. In *eat-5* mutants the metacarpus muscles contract in synchrony and the terminal bulb muscles contract in synchrony but, unlike in wild-type animals, contraction of the anterior and posterior pharynx is asynchronous (Starich *et al.*, 1996).

To further understand the functions of innexins in *C. elegans* behavior and development, we characterized a temperature-sensitive mutation of the innexin family member *inx-6*. *inx-6(rr5)* mutants are unable to initiate development after hatching at restrictive temperature due to defects in pharyngeal pumping. Our characterization of this mutant suggests that *inx-6* is required to couple muscle cells of the anterior pharynx.

MATERIALS AND METHODS

C. elegans Strains and Culture

C. elegans strains were cultured using standard techniques described by Brenner (1974). All experiments were performed at 20°C unless otherwise noted.

The Bristol strain N2 was used as the wild-type throughout. The following strains were also used: Bergerac strain RW7000, DA465 [*eat-2(ad465) II*], DA491 [*dpy-20(e1282) unc-30(e191) IV*], DR107 [*unc-26(e205) dpy-4(e1166) IV*], DR282 [*dpy-13(e184) unc-31(e169) IV*], JD118 [*inx-6(rr5) IV; avr-15(ad1051) V*], JD125 [*inx-6(rr5) IV; exp-2(sa26ad1426) avr-15(ad1051) V*], MT2115 (nDf27/nT1 IV; +/nT1 V), PD4792 (*mIs11 IV*), and VT765 [*unc-36(e251); mals103 [rnr::GFP unc-36(+)] X*].

A Genetic Screen for Temperature-sensitive Mutants Defective in the Initiation of Postembryonic Development

The strain VT765 [*unc-36(e251); mals103 [rnr::GFP unc-36(+)]*] (Hong *et al.*, 1998) was mutagenized with 47 mM ethylmethanesulfonate as described previously (Brenner, 1974). To select mutants efficiently, we used a potent inhibitor of DNA replication, hydroxyurea (HU), as a tool to select against animals that initiate postembryonic development at 25°C. HU-affected adult animals are uncoordinated and sterile due to effects on the development of the neuroblast lineage and germline. However, HU has no effect if worms do not initiate the cell divisions typical of early postembryonic development. Arrested L1s were transferred to individual NGM plates without HU and allowed to recover at 15°C for further analysis.

Temperature-Shift Experiments

Mutant embryos were isolated with alkaline/hypochlorite and hatched in M9 buffer (20 mM KH₂PO₄, 40 mM Na₂HPO₄, 85 mM NaCl, 85, 1 mM MgSO₄). The hatched L1 larvae were cultured at 15°C and after every 8 h, 50 worms were transferred to plates that were pre-equilibrated at 25°C. Animals that grew to reproductive maturity were scored as a percentage of the total population present on the plate after 48 h.

Plasmid Constructs

pMR341 contains a 8.2-kb *SalI* fragment from the cosmid T23F6, which was subcloned into vector p-Bluescript (Stratagene, La Jolla, CA). pMR342 contains a 6.2-kb *SacII*/*Small* fragment from the cosmid T23F6 that was subcloned into p-Bluescript (Stratagene). pMR346 (*inx-6::GFP*) is a transcriptional green fluorescent protein (GFP) fusion containing 3 kb of sequence 5' to the *XbaI* site upstream of the *inx-6* translation start site. pMR347 (*inx-6::INX-6::GFP*) is a translational GFP fusion containing the same upstream sequence described above, driving an *inx-6* cDNA amplified from a cDNA library (a gift from Dr. A. La Volpe, International Institute of Genetics and Biophysics, Napoli, Italy) by using the primers 5' catgctagaatggcgtcgcaagttggag3' (upstream) and 5' atgggatccagtatgctaatcgattgacaaatg3' (downstream) and inserted in frame into the Fire Lab vector pPD95.77. In pMR348, the *inx-6* promoter of pMR342 was removed by digestion with *SacII*/*XbaI* and replaced with a *SacII*/*XbaI* fragment of the *mjy-2* promoter amplified from the Fire Lab vector pPD30.69 by using primers 5' catgcatcagaaccttgggtctcttggc3' (upstream) and 5' ataccgaggagatccccagcttgat3' (downstream). In pMR350 (*inx-6::EAT-5*), the coding sequence of *inx-6* in pMR342 was replaced with a *XbaI*/*PstI* polymerase chain reaction fragment containing *eat-5* coding sequence.

Germline Transformation

Germline transformation was performed as described previously (Mello *et al.*, 1991). Cosmids and plasmids for rescue experiments

were injected at a concentration of 20 $\mu\text{g}/\text{ml}$, whereas the cotransformation marker pRF4 (*rol-6 D*) was injected at a concentration of 100 $\mu\text{g}/\text{ml}$. For rescue experiments, mutant animals were injected and maintained at 15°C. Adult F2 animals exhibiting a Rol phenotype were transferred to 25°C, and rescue of L1 arrest of their progeny (F3) was scored.

RNA Interference

inx-6 double-stranded RNA (dsRNA) was produced and injected according to Fire *et al.* (1998). The gel-purified template (1 μg) was used for in vitro transcription reactions and the RNA was then phenol/chloroform extracted, ethanol precipitated, and annealed. *inx-6* dsRNA was injected into N2 or MR127 animals at a concentration of 1 mg/ml. The injected animals were transferred daily to new plates, and development of F1 progeny was monitored.

Video Recording and Electropharyngeogram

inx-6(rr5) mutant worms grown at 25°C were incubated in 1 mM levamisole (to induce paralysis) and 10 mM serotonin (to induce pumping) in M9 buffer for 30 min (both drugs from Sigma-Aldrich, St. Louis, MO). Worms were mounted in M9 buffer on 35 \times 60-mm coverslips. A recording chamber was created by applying a ring of gasket sealant to the surface of the coverslip and allowing it to cure overnight. Worms were viewed on an Olympus IX-70 inverted microscope with Nomarski optics and a 40 \times lens. EPGs were recorded using 1.2 OD borosilicate suction pipettes pulled on a Sutter P-97 pipette puller (Sutter Instrument, Novato, CA). Recording of EPGs was essentially as described in Raizen *et al.* (1994) by using a Warner Instrument (Hamden, CT), patch-clamp PC-501A with a 1-G Ω headstage but without filtering in the amplifier. EPGs were digitized using a Digidata 1322A and recorded using Clampex 8.1 software (Axon Instruments, Union City, CA). Recordings were formatted and digitally filtered using a 1-kHz Gaussian filter with Clampfit 8.1 software (Axon Instruments).

Video images were recorded using a Hitachi KP-M1U charge-coupled device camera and frames were captured using a Matrox Meteor-II frame grabber (Matrox Electronic Systems, Dorval, QC, Canada). The EPG signal was used to trigger the frame grabber to collect 15 frames. To do this, the EPG signal was sent to the a Digitimer D.130 spike processor (Medical Systems, Great Neck, NY) which, upon encountering an E-spike, simultaneously sent a signal to the Digidata 1322A and to the frame grabber. The signal to the Digidata initiated the recording of the EPG and the signal to the frame grabber initiated image acquisition (acquisition software, written by J.A. Dent with MIL-lite, available on request). Grabbed images were deinterlaced and digitally condensed in the *x*-axis to restore the aspect ratio yielding an image every \sim 17 ms. By simultaneously recording the EPG in one channel and the camera synchronization signal in a second channel, it was possible to correlate in time both the images and the EPG.

Dye Diffusion Experiment

Newly hatched L1 larvae were washed with M9 buffer and soaked in saturated carboxyfluorescein (Sigma-Aldrich) solution (\sim 20 mM) for at least 3 h. Due to the pharyngeal abnormalities in *inx-6(rr5)* animals, *inx-6(rr5)* larvae were initially soaked in the carboxyfluorescein solution at permissive temperature (15°C). Animals were then transferred to 25°C for 5 h to allow worms to eliminate the wild-type *inx-6* gene product before laser treatment. After thoroughly washing with M9 buffer, animals were mounted on a 2% agarose pad as described with 1 mM levamisole and 10 mM serotonin. Dye was introduced into the pharyngeal muscles by focusing a low-intensity laser pulse at the posterior edge of the grinder. Images of the diffusion of the fluorescent dye were captured at 10-s intervals by using identical exposure time. The dye diffusion pattern

was verified 10 min after the laser treatment to confirm that the dye distribution remained unchanged.

Microscopy and Image Processing

Light microscopy was performed using a Leica DMR compound microscope with Nomarski optics. Images were captured with a Hamamatsu C4742-95 digital camera. Image processing, analysis and computational deconvolution were performed using Openlab 3.07 software (Improvision, Lexington, MA) and Adobe Photoshop.

RESULTS

rr5 Is a Temperature-sensitive Mutation that Blocks the Initiation of Postembryonic Development

In a screen to isolate temperature-sensitive mutants that are unable to initiate postembryonic development, we isolated a mutant (*rr5*) that could not initiate postembryonic development at restrictive temperature (25°C). Growth at restrictive temperature does not affect embryogenesis in *rr5* animals because they show no embryonic lethality and their morphology at hatching seems identical to wild-type animals after culture at 25°C. However, newly hatched *rr5* larvae are unable to initiate postembryonic development at restrictive temperature, and worms seem starved and remain at the L1 stage for several days before dying. After 5–6 d at 25°C, developmental events typical of L1 stage development, including cell divisions or P-neuroblast nuclear migrations, were undetectable in *rr5*-arrested animals. After transfer from restrictive temperature to permissive temperature (15°C), animals recover from their arrested state and grow into normal adults without obvious developmental or reproductive consequence.

Therefore, *rr5* seems to be absolutely required to initiate postembryonic development. Because this allele is temperature sensitive, we sought to determine when *rr5* activity was required during development. By down-shifting to permissive or up-shifting to restrictive temperature, respectively, an approximate temporal window can be obtained to describe when a gene product is required for wild-type function. Normally this would be performed by executing an up-shift and a down-shift time course; however, due to the highly penetrant developmental arrest phenotype of *rr5* animals at 25°C, only up-shift experiments could be performed (Table 1). By shifting *rr5* animals to restrictive temperature at various stages during development, we found that *rr5* disrupts a gene that is necessary during the L1 and L2 stages for correct larval development. All post-L2 stage up-shifted animals grew to become fertile at restrictive temperature and produce viable progeny that arrested as hatchlings.

rr5 Is a Temperature-sensitive Allele of an *unc-7*-like Protein *inx-6*

To understand how *rr5* functions at the molecular level, we mapped *rr5* initially using a sequence-tagged site mapping approach (Williams *et al.*, 1992). Our results indicated that the mutation is on LG IV and to the left of *dpy-20*. Further three-point recombination mapping showed that *rr5* is between *dpy-20* and *unc-31* and close to *unc-31* (\sim 0.03 map units) (Figure 1A).

Table 1. *rr5* disrupts a gene required during early larval development

Time (h)	Stage	Adult animals after up-shift (%)
8	L1	0.0 ± 0.0
16	L1	0.6 ± 1.1
24	L1	2.1 ± 1.0
32	L2	8.7 ± 1.3
40	L2	40.1 ± 0.5
48	L3	47.1 ± 5.8
56	L3	75.4 ± 3.1
64	L3	96.6 ± 0.5
72	L4	100 ± 0.0

rr5 mutant embryos maintained at 15°C were hatched in M9 buffer and then cultured at 15°C. At 8-h intervals, 50 worms were upshifted to restrictive temperature (25°C), and animals that reached the adult stage after 48 h in culture were scored. Results are expressed as percentage of the total worm population present on the plate. All progeny derived from upshifted *rr5* animals that grew to adulthood at 25°C successfully terminated embryogenesis before recapitulating the L1 arrest phenotype typical of *rr5* at 25°C.

Six cosmids and one YAC that span this interval were chosen by consulting the *C. elegans* physical map (Figure 1A). Two cosmids, T23F6 and C36H8, were found to completely rescue the larval arrest phenotype of *rr5* at 25°C, suggesting that a common region to both cosmids carried the wild-type *rr5* gene product. The 9-kb overlapping region contains two predicted genes that encode a major sperm protein-like product and the innexin gene *inx-6*, an *unc-7* homolog (Figure 1B). These two predicted genes were individually subcloned and injected to test for rescue. Transformation rescue experiments indicated that the *inx-6*-containing construct could fully rescue the mutant phenotype, whereas the second candidate had no effect on the *rr5* phenotype (Figure 1C).

To confirm that *inx-6* is indeed the mutated gene in *rr5*, the *inx-6* coding region was frame shifted, and the mutant variant was assessed for its ability to rescue. The Δ *inx-6* construct could no longer complement the *rr5* mutation, suggesting that *rr5* is an allele of *inx-6*, which we will refer to as *inx-6(rr5)*.

To address whether *inx-6(rr5)* is a weak or strong hypomorphic allele, and/or whether maternal products could rescue embryonic functions in this mutant background we compared the phenotype of *inx-6(rr5)* to that of an *inx-6(RNAi)* animal. Double-stranded RNA to any gene of interest can elicit a potent response in *C. elegans* referred to as RNA-mediated interference that is mediated by degrading targeted transcripts posttranscriptionally (Fire *et al.*, 1998). In the F1 progeny of wild-type animals injected with double-stranded RNA corresponding to the *inx-6* coding region, 5–10% animals displayed a postembryonic arrest phenotype very similar to that observed with *inx-6(rr5)* at 25°C, albeit at lower penetrance. To test whether residual wild-type gene activity may be present in the *inx-6(rr5)* mutant, we performed a similar RNAi experiment as described above in an *inx-6(rr5)* background. The F1 progeny of injected parents show the identical phenotype, in severity and penetrance, to

the F1 progeny from uninjected *inx-6(rr5)* mutant parents kept at 25°C. These data suggest that the *inx-6(rr5)* mutant phenotype at 25°C likely represents a strong hypomorph or the *inx-6* null phenotype. This was further confirmed by placing the *inx-6(rr5)* mutant chromosome in *trans* to a deficiency that uncovers this region (our unpublished data).

inx-6 Is a Member of a Highly Conserved Protein Family

inx-6 encodes a protein that belongs to the innexin family (invertebrate connexin analogs), which encode components of invertebrate gap junctions. The closest homolog to INX-6 is UNC-7 with 33% identity at the amino acid level. The strongest homology is seen among the conserved cysteine residues in the extracellular loops and in the transmembrane domains that play roles in normal channel regulation (Figure 2).

The *inx-6* genomic sequence from *rr5* was sequenced to identify a lesion within this gene that would cause the *inx-6(rr5)* arrest phenotype. A G/C to A/T transition, typical of EMS-induced lesions was identified, and caused a P353L change in the INX-6 protein near the C termini. That a point mutation in the C termini could disrupt the protein function at the restrictive temperature is consistent with a previous study in which the C terminus was shown to be very important for the normal channel function of connexins (Yeager and Nicholson, 1996; Morley *et al.*, 1996; Wang and Peracchia, 1998).

inx-6 Is Expressed Exclusively in Pharyngeal Tissues

To understand more about how *inx-6* affects postembryonic development, we examined its expression pattern by using GFP reporters. Two reporter constructs were generated: a transcriptional fusion that consisted of the *inx-6* promoter driving GFP and a translational fusion that fused the *inx-6* promoter to the *inx-6* cDNA, which in turn was fused in frame to GFP. Results from both GFP-expressing transgenes indicated that *inx-6* is first detected during embryogenesis at the comma stage, in anterior cells that are likely to be the pharyngeal precursors. This pharynx-specific expression expands during the development of the pharynx to the end of embryogenesis, whereas *inx-6* continues to be expressed in the corpus muscles and isthmus marginal cells of the pharynx throughout the larval and adult stages (Figure 3).

The translational GFP fusion protein is expressed in a punctate expression pattern (Figure 3, C and D), which is very different from the transcriptional fusion (Figure 3B). Gap junctional channels often aggregate in plasma membranes to form plaques, an observation that was confirmed using immunocytochemistry in vertebrate cells (Kumar and Gilula, 1996). In invertebrates, this particular pattern has also been shown by the innexin protein wEST01007 (INX-3) in *C. elegans* (Starich *et al.*, 1996). The characteristic expression pattern of INX-6 likely faithfully represents the normal expression of the INX-6 protein because it can fully rescue the mutant phenotype, suggesting that the punctate pattern that we observed for the full-length *INX-6::GFP* fusion may reflect the actual subcellular localization of INX-6. We were unable to determine what membrane surface the plaques associate with. The presence of plaques along the length of the muscle could indicate that gap junctions are being

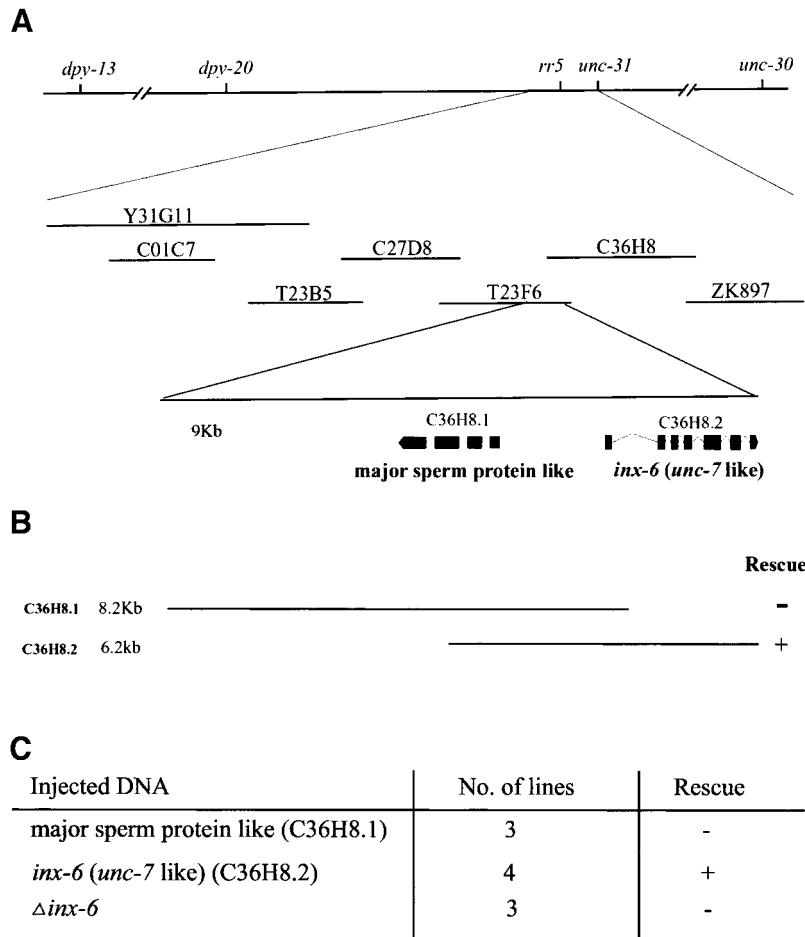


Figure 1. Mutation in the *C. elegans inx-6* gene causes the temperature-sensitive L1 arrest phenotype of *rr5*. (A) Genetic and physical map of the *rr5* region. *rr5* maps between *dpy-20* and *unc-31*, 0.03 map units left of *unc-31*. (B) Two cosmids, T23F6 and C36H8, which share 9 kb of overlapping sequence, were found to completely rescue the larval arrest phenotype at 25°C. (C) Subclones of the overlapping region were used to test for transformation rescue. The C36H8.2 subclone, which contains a predicted gene *inx-6*, could fully rescue the mutant phenotype, whereas a second predicted gene on this cosmid C36H8.1 had no effect. The *inx-6* coding region was mutated by inserting a fragment into one of the exons, which theoretically caused a frame-shift in the predicted *inx-6* coding region. The resulting Δ *inx-6* could no longer rescue the mutant phenotype. All constructs were injected as described in MATERIALS AND METHODS with the dominant coinjection marker pRF4 (*rol-6 D*).

formed between the muscle and the intercalated marginal cells (Albertson and Thomson, 1976). However, we cannot rule out that some of the plaques are aggregated proteins moving between intracellular compartments.

inx-6(rr5) Mutant Pharyngeal Muscles Contract and Respond to Neuronal Stimuli

If *inx-6* is expressed in the pharynx because it is necessary for efficient pharyngeal pumping, then the *inx-6(rr5)* mutant might arrest at the restrictive temperature because it cannot feed. To address this possibility, we scored the rate of pharyngeal pumping in recently hatched L1 stage larvae at both the permissive and restrictive temperatures. Pumping was scored by looking at the rhythmic motion of the grinder in the terminal bulb, the most obvious indicator of muscle contraction. In addition, we scored the worms both in the presence and absence of serotonin. In adult worms, serotonin increases pumping from a basal rate of ~40 pumps/min to a maximal rate of ~250 pumps/min via activation of a pacemaker motor neuron MC. At the permissive temperature, *inx-6(rr5)* showed the same pumping rate as wild-type animals, both in the presence and absence of serotonin, whereas at the restrictive temperature, its pumping rate was

much slower, consistent with an effect of *inx-6(rr5)* on pharyngeal function (Table 2).

To see whether the lower pumping frequency of *inx-6(rr5)* at the restrictive temperature was sufficient to explain the developmental arrest, we compared it with an *eat-2* mutant. *eat-2* encodes a pharyngeal muscle nicotinic acetylcholine receptor that is necessary for neurotransmission by the MC pacemaker motor neuron (Raizen *et al.*, 1995). Because neurotransmission from the MC neuron is absent in the *eat-2* mutant, the worms pump at a slow rate and are severely starved. Nevertheless, *eat-2* mutants complete development and are fertile. Even at the restrictive temperature, *inx-6(rr5)* mutants pump faster than the *eat-2* mutants, indicating that pumping rate alone is not sufficient to explain the developmental arrest of *rr5*. Unlike *eat-2*, the *inx-6(rr5)* mutant still responds to serotonin with an increased pumping rate, indicating that the MC motor neuron is still functional, and the pharyngeal muscle is still regulated by the nervous system (Table 2).

Pharyngeal Muscle Contraction is Unsynchronized in *inx-6(rr5)* Mutants

Because differences in pumping rate alone are not sufficient to explain the developmental arrest of *inx-6(rr5)*, we wanted

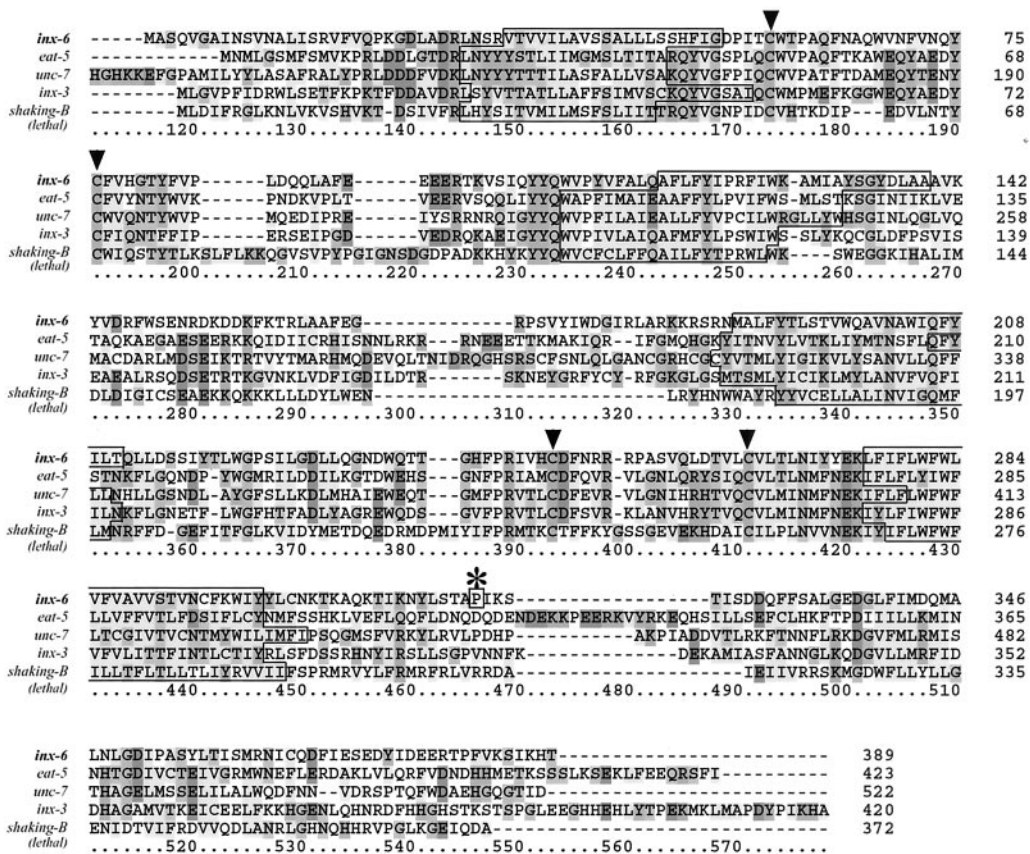


Figure 2. INX-6 shares substantial homology with other *C. elegans* and *Drosophila* innexin family members. The pileup alignment of INX-6, *Drosophila melanogaster shaking-B (lethal)* and the other close homologs in *C. elegans* was done using Clustal method. Four predicted transmembrane regions typical of INNEXINS are outlined, and the conserved cysteine residues presumably involved in maintaining the connections between hemichannels in the extracellular loops are indicated by the arrowheads. * indicates the point mutation identified in *rr5*.

to know whether there was a defect in muscle coordination. On closer examination of L1 arrested *inx-6(rr5)* larvae, we found that >90% (136/150) of the worms lacked procorpus contraction when the terminal bulb was contracted compared with <2% (2/150) of wild-type starvation-arrested L1 worms (Figure 4). Without procorpus contraction, no food can enter the pharynx and worms therefore arrest development as a result of starvation.

The lack of muscle contraction could result from defects in muscle contraction or from defects in electrical excitability. If there were a defect in electrical activity, we should be able to see this in the EPG. Specifically, we hypothesized that *inx-6(rr5)* affected the flow of excitation from the metacarpus to the procorpus. To test this, we simultaneously recorded video and the EPG of mutant L3-L4 stage larvae that escaped arrest at the restrictive temperature (currents generated by L1 larvae are too small to measure by EPG).

We first examined wild-type L3 stage larvae grown at 25°C, which showed the muscle motions typical of wild-type adults (Figure 5, A and B). In wild-type worms, the radially oriented muscles of the corpus contract slowly over a period of ~200 ms (Avery and Thomas, 1997). The contraction pulls the walls of the pharyngeal lumen open and the opening of the lumen is the most obvious visual manifestation of mus-

cle contraction. This contraction is terminated by a very rapid (<20 ms) relaxation of the muscles and closing of the lumen. The terminal bulb muscles are fully contracted for most of this time, and their relaxation is delayed slightly relative to corpus relaxation.

The timing of muscle relaxation is precisely coordinated with the electrical activity of the pharynx as revealed by the EPG. The EPG is an extracellular recording technique whose trace reflects the time derivative of the muscle membrane potential. In the EPG trace, one or two E spikes correspond to the depolarization of the pharyngeal muscles and one or two R spikes represent the repolarization and return to resting potential. Inhibitory postsynaptic potentials (IPSPs) of the motor neuron M3 usually occur as downward spikes during the plateau phase, which is the period of muscle depolarization delimited by the E and R spikes. Corpus and terminal bulb contraction begin immediately after the E spikes. The corpus muscles are maximally contracted just before the first R spike (R1) and relax immediately after. There is often a much smaller R spike (R2) that follows R1 and precedes terminal bulb relaxation. The only unusual feature of the EPGs we recorded from wild-type L3 stage larvae was the absence of M3 IPSPs during the plateau

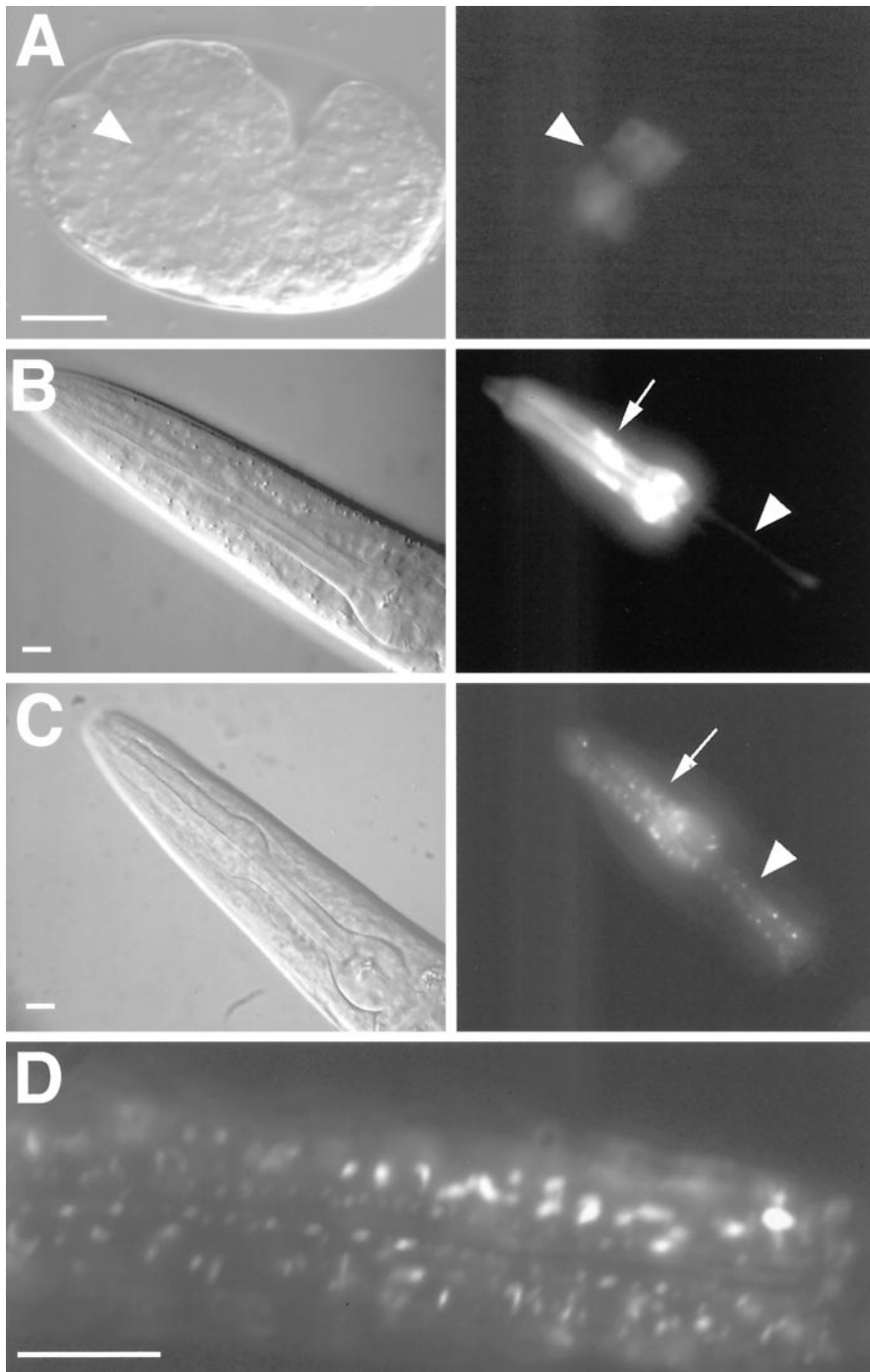


Figure 3. *inx-6* expression pattern during development. Differential interference contrast (left) and GFP fluorescence images (right) of transcriptional (*inx-6::GFP*) and translational (*inx-6::INX-6::GFP*) reporters. (A) Expression of the *inx-6::GFP* in embryo (anterior to the left) begins at the comma stage in anterior cells within the presumptive pharyngeal mesoderm (indicated by arrowhead). (B) *inx-6::GFP* is expressed in the corpus muscles (arrow) and isthmus marginal cells (arrowhead) throughout postembryonic development. (C) Expression of the *inx-6::INX-6::GFP* translational fusion reporter in a young adult hermaphrodite reveals a characteristic punctate expression pattern in corpus muscles (arrow) and isthmus marginal cells (arrowhead). The translational fusion construct fully rescued the mutant phenotype at 25°C, suggesting that the punctate GFP expression pattern may faithfully depict the expression of INX-6 in vivo. (D) A magnified image shows the punctate expression pattern in the corpus. Bars, 10 μm.

phase. This is an effect of levamisole, which we used to paralyze the worms for video recording.

Video recording of *inx-6(rr5)* L3 stage worms confirmed that the coordination of pharyngeal muscle contraction is abnormal. Contraction of the procorpus muscles is weak and their relaxation is premature. As shown in Figure 5C, often the muscles of the procorpus do not contract at all, whereas the muscles of the metacorpus and terminal bulb (not visi-

ble) contract almost normally and in synchrony. The EPG trace of the stunted *inx-6(rr5)* L3 stage worms was faint and difficult to interpret (our unpublished data).

To better understand the effects of *inx-6* on the electrical activity of the pharynx, we examined the somewhat larger L4 and adult *inx-6(rr5)* escapers. In spite of the presence of levamisole, we found negative spikes characteristic of M3 IPSPs in the EPG trace of the *inx-6(rr5)* mutants. If these

Table 2. *inx-6(rr5)* reduces pharyngeal contraction frequency but does not affect the capacity to respond to serotonin (5HT)

	Genotype	Pumping rate (contractions/min)	
		-5HT	+5HT
15°C	N2	134.4 ± 11.2	149.3 ± 16.6
	<i>inx-6(rr5)</i>	131.9 ± 12.1	141.2 ± 15.1
	<i>eat-2(ad465)</i>	17.9 ± 4.6	16.9 ± 4.6
25°C	N2	165.8 ± 15.3	182.6 ± 15.1
	<i>inx-6(rr5)</i>	76.8 ± 12.3	103.6 ± 16.2
	<i>eat-2(ad465)</i>	16.6 ± 5.1	17.4 ± 5.8

Wild-type N2 animals were used as positive control. All animals were examined at the L1 stage by mounting on 2% agarose pads with M9 buffer with (+5HT) or without (-5HT) 5 mM serotonin. The pumping rate was monitored using a compound microscope by counting the rhythmic motion of the grinder in the terminal bulb of individual animals over the course of 1 min beginning immediately after location of the specimens and adjustment of focus. Values are expressed as contractions/min ± SD observed from 20 different animals (n = 20).

were M3 IPSPs, they would be absent in an *avr-15(ad1051)* mutant background because *avr-15* encodes a glutamate-gated chloride channel subunit that is necessary to form the M3 postsynaptic receptor on the pharyngeal muscle (Dent *et al.*, 1997). However, even in the *inx-6(rr5); avr-15(ad1051)* double mutant background, these negative spikes persist (Figure 5, D and E).

The only other electrical activity known to produce negative spikes is the spontaneous repolarization of the pharyngeal muscles, mediated in part by the *exp-2* voltage-gated potassium channel (Davis *et al.*, 1999). Usually, there are at most two of these spikes: R1 and R2 (described above). Because a loss-of-function allele (*ad1426*) of the *exp-2* gene results in the reduction or absence of negative spikes resulting from muscle repolarization, we made the *inx-6; exp-2 avr-15* triple mutant. The negative spikes were absent in this triple mutant, indicating that the spikes in *inx-6(rr5)* are the result of EXP-2 mediated muscle repolarization (Figure 5F). The fact that there are a series of downward spikes in the *inx-6(rr5)* single mutant indicates that muscle repolarization is uncoordinated. It is also interesting to note that whereas in wild-type animals there are one or two distinct E spikes, initiation of the action potential in *inx-6(rr5)* is characterized by a broad upward deflection rather than a spike, which is consistent with a lack of coordination in muscle depolarization as well.

The effect of the *inx-6(rr5)* mutation on the motion of the pharyngeal muscle of L4 stage worms is also consistent with premature and uncoordinated muscle repolarization and relaxation. Instead of a rapid relaxation that occurs immediately after the first R spike, relaxation occurs slowly over a period of 30–60 ms beginning shortly after the second negative spike and well before the last spike (Figure 5E). The premature relaxation is evident first in the procorpus, indicating that these muscles are less electrically coupled than the metacorpus.

Cell-Cell Coupling of the Corpus Is Compromised in *inx-6(rr5)* Mutants

Because our electrophysiological data indicate that the procorpus of the *inx-6(rr5)* mutant has abnormalities in electrical coupling, we investigated whether *inx-6(rr5)* may cause gap junction defects. One effective way to test whether the procorpus of *inx-6(rr5)* mutants is appropriately coupled by gap junctions would be to monitor the diffusion of a fluorescent dye (carboxyfluorescein) among pharyngeal muscles. When animals are soaked in saturated carboxyfluorescein solution, the dye diffuses into all the tissues with the exception of the pharyngeal muscles. Instead it accumulates in the pharyngeal lumen, including the grinder in the terminal bulb. A single weak laser pulse directed at the posterior edge of the grinder is enough to perforate the pharyngeal lumen to allow the dye in the grinder to diffuse into terminal bulb muscles. The dye then diffuses via endogenous functional gap junctions to progressively more anterior muscles. Because the laser pulse can sometimes permanently damage the pharynx, we only considered animals that continued pumping and responded to serotonin appropriately after laser pulse.

In 100% of the wild-type animals (n >30) that were successfully operated, the dye diffused evenly throughout all pharyngeal muscles within 60 s after the laser pulse (Figure 6, A–D). In successfully operated *inx-6(rr5)* animals, dye spread into the terminal bulb, the isthmus, and the metacorpus at a similar rate as wild type, but failed to diffuse into the procorpus in all cases observed (n >50) (Figure 6, E–H). These data are consistent with the *inx-6::GFP* expression pattern and strongly indicate that the gap junctions required for coupling the procorpus with the metacorpus are functionally compromised in *inx-6(rr5)* mutants.

EAT-5 Can Partially Substitute for INX-6 Function In Vivo

Previous studies showed that the *eat-5* mutants also demonstrated a pharyngeal pumping defect similar to that observed in *inx-6(rr5)* mutants. EAT-5 is another *C. elegans* innexin family member and is a close homolog to INX-6, sharing >30% sequence identity over the length of the entire protein.

The *inx-6* and *eat-5* expression patterns overlap in the pharyngeal muscles of the metacorpus, and they are expressed in adjacent cells (marginal cells and muscles, respectively) of the isthmus. Based on the above-mentioned information, it is possible that *inx-6* and *eat-5* may function together or have analogous roles in regulating pharyngeal pumping during larval development.

To test whether EAT-5 and INX-6 are functionally interchangeable, we performed a gene substitution experiment in which the *inx-6* promoter was used to drive the *eat-5* coding sequence to induce EAT-5 expression in the region where *inx-6* would normally be expressed. The construct was injected into *inx-6(rr5)* mutants, and stable transgenic animals were monitored at restrictive temperature to determine whether the *inx-6::EAT-5* transgene could rescue the *inx-6(rr5)* mutant phenotype. Strikingly, we found that the newly hatched transgenic animals could grow to adulthood at the restrictive temperature (Table 3). These transgenic animals are healthy and fertile, although they grow some-

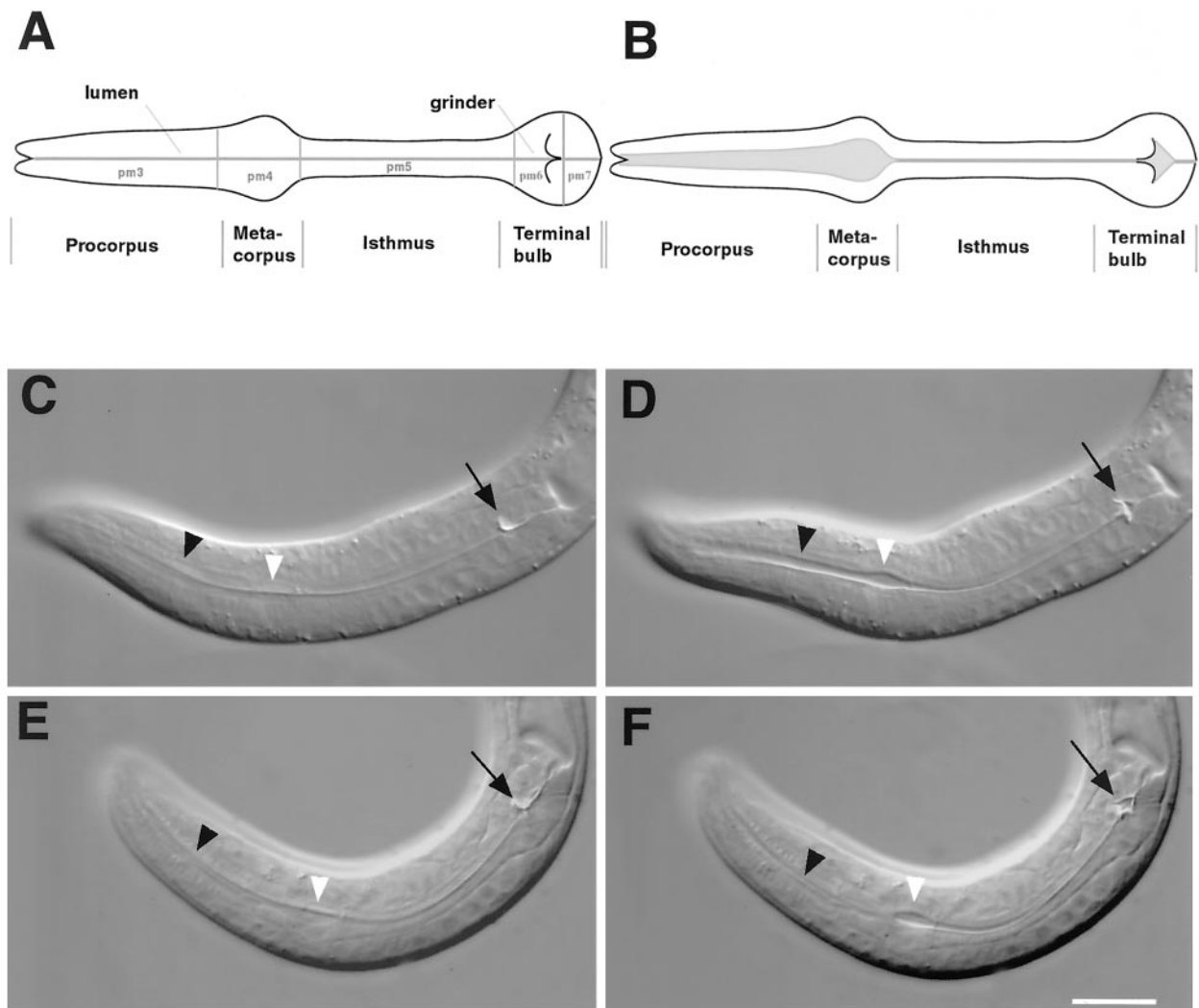


Figure 4. *inx-6(rr5)* disrupts appropriate luminal opening of the procorpus during pumping at restrictive temperature. (A) Lateral view of *C. elegans* pharyngeal anatomy with anterior to the left. The pharynx is divided into three functional components, which consist of five types of large muscles: the corpus, which can be further subdivided into the procorpus (pm3) and the metacarpus (pm4), the isthmus (pm5), and the terminal bulb (pm6 and pm7). (B) During pumping, the corpus contracts simultaneously with the terminal bulb, thereby opening the lumen of the pharynx to allow the entry of bacteria. This is then followed by simultaneous muscle relaxation (Avery and Thomas, 1997). (C and E) Pharyngeal muscles in a relaxed state between pumps in N2 and *inx-6(rr5)* L1 larvae. The black arrows indicate the grinder in the terminal bulb. The black arrowheads indicate the procorpus lumen, and the white arrowheads indicate the metacarpus lumen. In wild-type animals (D), the terminal bulb muscle contraction inverts the grinder and the procorpus and metacarpus contract simultaneously, thereby opening the lumen of the whole corpus and allowing the influx of bacteria. In *inx-6(rr5)* animals (F), the terminal bulb is inverted during pumping (black arrow). However, the procorpus lumen (black arrowhead) does not open. Bar, 10 μ m.

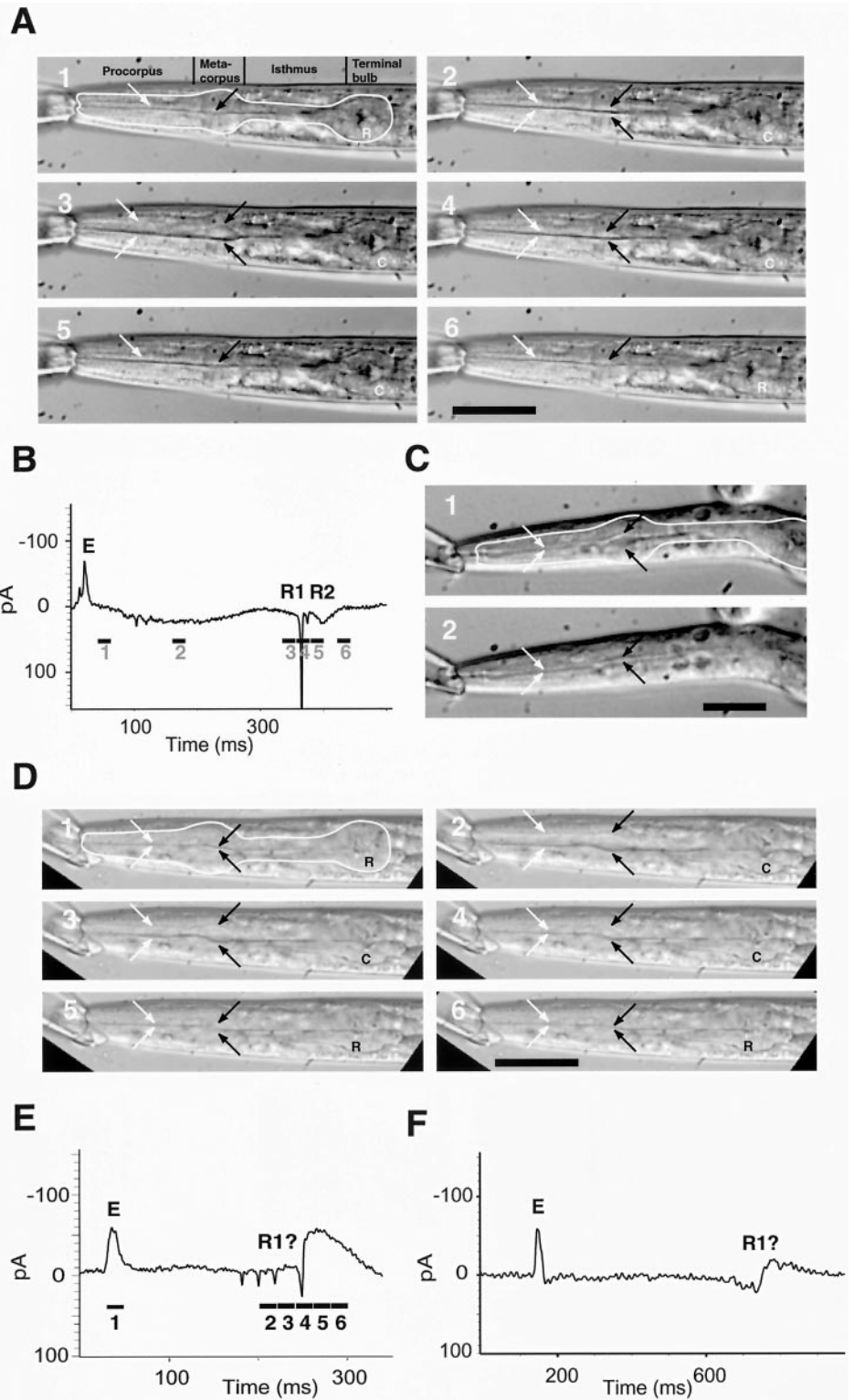
what slower and need \sim 88 h to reach the adult stage compared with \sim 40 h for wild-type animals at 25°C. The fact that EAT-5 can rescue a mutation in *inx-6(rr5)* is strong evidence that *inx-6* and *eat-5* serve similar but not identical functions in vivo.

Ectopic Expression of inx-6 Caused Abnormalities

Because EAT-5 could substitute for INX-6, we were curious whether ectopically expressed INX-6 would contribute in a benign way to endogenous gap junctions or whether it might

interfere with the proper regulation of cell-cell coupling. The *inx-6* expression pattern showed that *inx-6* is only expressed in the corpus and isthmus tissues, suggesting that there must be other kinds of gap junction channels in the terminal bulb muscles to ensure the intact pharyngeal muscle contraction. If the expression of *inx-6* were expanded into the terminal bulb, it could potentially interact with endogenous innexins and interfere with their functions, or it could form inappropriate gap junctions between normally isolated cells. To test this, we used the *myo-2* promoter to drive *inx-6* coding sequence in the corpus, isthmus, and the terminal bulb muscles.

Figure 5. Repolarization and relaxation of corpus muscles is desynchronized in the *inx-6(rr5)* mutant. (A and B) Video and EPG, respectively, of wild-type L3 larva. In all frames, the white arrows indicate the position of the luminal walls in the procorpus and the black arrows indicate the position of the luminal walls in the metacarpus. In the first frame, the outline of the pharynx is traced in white. In the terminal bulb (TB) a C indicates that the TB muscles are contracted and an R indicates they are relaxed. The period of time over which each video image was captured is indicated by a black bar under the EPG trace with the video frame number corresponding to that time point indicated below the bar. E marks the excitation spike that signals the beginning of the action potential and R1 and R2 mark the repolarization of the corpus and terminal bulb muscle, respectively. In frame 1, depolarization has occurred but there is almost no contraction of the corpus or terminal bulb. In frame 2, the terminal bulb is contracted but the corpus contraction is modest. In frame 3, the corpus reaches maximal contraction just before repolarization. In frame 4, the corpus muscle repolarizes and relaxes almost simultaneously although relaxation is not quite complete. In frame 5, the terminal bulb has repolarized but is still contracted. In frame 6, all muscles are relaxed. Bar, 50 μm . (C) L3 *inx-6(rr5)* that escaped the restrictive temperature. In frame 1, the metacarpus has reached the point of maximum contraction during the pump but the procorpus is still relaxed. In comparison, frame 2 shows complete relaxation of the pharyngeal muscle during an interpump interval. Bar, 25 μm . (D and E) Video and EPG of a 3-d-old L4 *inx-6(rr5); avr-15(ad1051)* mutant worm that escaped the restrictive temperature. The E spike(s) is broadened and there are four downward spikes in spite of the absence of M3 neurotransmission as a result of the *avr-25(ad1051)* mutation. Frame 1, the pharynx just before contraction begins. All muscles are fully relaxed. Frame 2, all muscles are at their point of maximum contraction indicated by the wide-open lumen. Frame 3, the procorpus muscle, and to a lesser extent, the metacarpus muscles, have begun to relax and the lumen is more constricted. Frame 4, the procorpus muscles have relaxed but the metacarpus is still mostly contracted. Frame 5, the terminal bulb has now relaxed but the metacarpus is still slightly open. Frame 6, all of the muscles are completely relaxed. Bar, 50 μm . (F) EPG trace of *inx-6(rr5); exp-2(ad1426) avr-15(ad1051)* that escaped restrictive temperature. The *exp-2(ad1426)* mutation eliminates the potassium channel that mediates fast muscle repolarization and hence the R spikes. With *exp-2(ad1426)* in the background, there are no R spikes, only a slow wave indicating the repolarization.



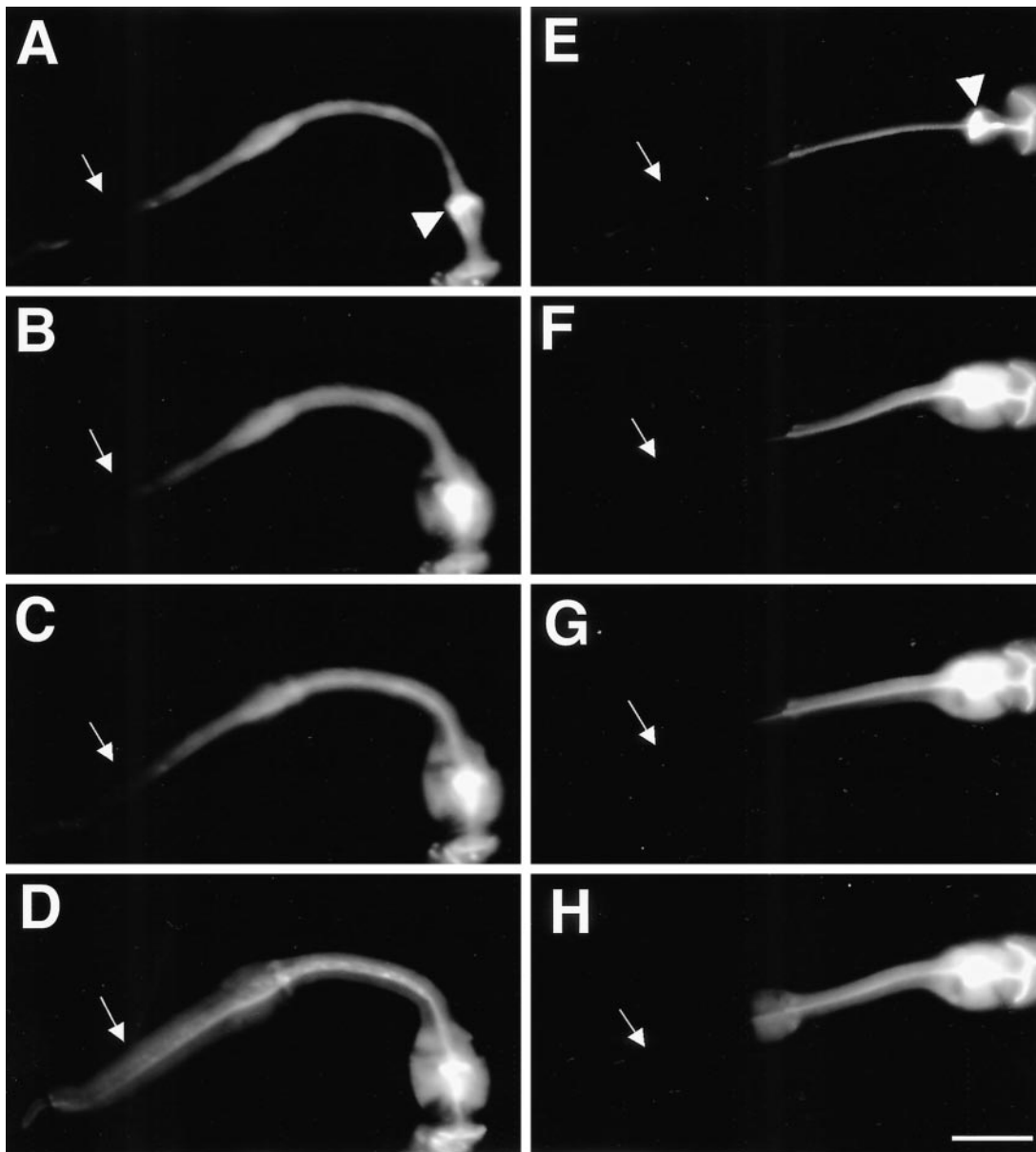


Figure 6. Cell-cell coupling is abnormal in the pharyngeal muscle of *inx-6(rr5)* mutants. Wild-type N2 (A) and *inx-6(rr5)* mutant animals (E) were allowed to ingest a saturated carboxyfluorescein solution. Dye was accumulated in the pharyngeal lumen and was selectively excluded from the pharyngeal muscle in wild-type and *inx-6(rr5)* animals but could enter the muscle and diffuse anteriorly after a single laser pulse focused at the posterior of the grinder. (A–D) Dye-coupling process in the wild-type L1 stage animal, showing diffusion of the fluorescent dye toward the procorpus after the laser pulse. (E–H) Same process in the *inx-6(rr5)* L1 mutant preincubated at restrictive temperature. Arrowheads indicate the grinder in the terminal bulb where the laser pulse was applied. White arrows indicate the procorpus. In wild-type animals, dye spreads anteriorly through the isthmus and throughout the whole corpus evenly within 60 s after laser treatment, whereas in *inx-6(rr5)* animals, dye quickly spreads into the metacarpus but could not cross into the procorpus. Images were captured within 60 s after laser treatment by using the same exposure time for each acquisition. The final dye-coupling pattern reached equilibrium and did not change as verified 10 min after the initial laser application. Bar, 10 μ m.

The *myo-2::INX-6* construct was injected into *inx-6(rr5)* mutants and wild-type animals. In the F1 generation, most transgenic animals developed relatively normally, including *inx-6(rr5)* mutants at restrictive temperature, which grew slightly more slowly than wild-type animals. However, in

the F2 generation, all the animals expressing the *myo-2::INX-6* transgene died as L1 larvae. We found that the pharyngeal lumen of most transgenic animals was wide open due to the hypercontracted pharyngeal muscles (our unpublished data), which presumably rendered the trans-

Table 3. *eat-5* can partially rescue the *inx-6(rr5)* mutation

Genotype	Distribution of worms at each postembryonic stage after 40 h at 25°C (%)				
	L1	L2	L3	L4	Adult
<i>inx-6; Ex[inx-6::GFP]^a</i>	99	1	0	0	0
<i>inx-6; Ex[inx-6::INX-6; inx-6::GFP]^b</i>	0	0	0	0	100
<i>inx-6; Ex[inx-6::EAT-5; inx-6::GFP]</i>	46	31	16	5	2

Expression of *eat-5* under the control of the *inx-6* promoter can rescue the *inx-6(rr5)* mutation although the rescued animals are compromised in growth rate. To identify transgenic animals at the L1 stage, the nonrescuing *inx-6* transcriptional fusion with GFP was used as the cotransformation marker: *inx-6(rr5)* mutants that possess a wild-type *inx-6* transgene (*inx-6::INX-6*) were used as a positive control. *inx-6(rr5)* mutants that carried the nonrescuing *inx-6::GFP* transcriptional fusion reporter were used as a negative control. L1 stage animals (100) from each strain were picked and maintained on food at 25°C for analysis. The distribution of animals at each stage was scored after 40 h, when all the animals in the positive control had reached the adult stage. More than 59% of the mutant strain *inx-6; Ex[inx-6::EAT-5; inx-6::GFP]* reached the adult stage only after 88 h (our unpublished data).

^a *inx-6; Ex[inx-6::GFP]* animals behave identically to *inx-6(rr5)* mutants. Most animals (>98%) did not progress beyond the L1 stage even after 88 h when maintained at 25°C.

^b *inx-6; Ex[inx-6::INX-6; inx-6::GFP]* is a fully rescued *inx-6(rr5)* mutant strain and it grows and develops as wild-type animals.

genic animals unable to feed. This phenotype is consistent with electrical coupling of cells that are not normally coupled, abnormally increased coupling between cells that are normally coupled, or the formation of open hemichannels on the surface of muscle cells.

DISCUSSION

Structure of *inx-6* Gap Junctions

Temperature-sensitive missense mutations such as *inx-6(rr5)* are generally thought to interfere with protein stability. Structure predictions based on sequence indicate that both innexins and connexins are membrane proteins with four transmembrane domains with both the amino and carboxyl termini on the cytoplasmic side. The homology between INX-6 and the other innexins is high in the putative transmembrane domains, so it, too, should have this topology. Based on this prediction, the *rr5* mutation is in the cytoplasmic C terminus, which has important roles in gap junction gating sensitivity in connexins (Morley *et al.*, 1996; Wang and Peracchia, 1998). However, the P353 residue affected by *rr5* is not well conserved among innexins and is in a non-conserved domain. Thus, an alternative hypothesis is that P353 could lie in a protein-protein interaction domain, and association with an INX-6-specific cytoplasmic protein is compromised at high temperatures by the P353L mutation.

Like connexins, innexins are probably multimeric, although whether *inx-6* forms a homomeric or heteromeric channel remains to be determined. The precise subunit composition is unknown for any gap junction formed by innexins, but there are indications that innexins do form heteromeric channels. UNC-7 and UNC-9 share considerable sequence similarity and also have similar mutant phenotypes (Starich *et al.*, 1993; Barnes and Hekimi, 1997). These innexins may associate to form a channel expressed in the nervous system. Similarly, direct electrophysiological and genetic evidence indicate that the *Drosophila* innexins *Dm-INX-2* and *Dm-INX-3* form obligate heteromeric channels (Stebbing *et al.*, 2000).

There are several *C. elegans* innexins expressed in the pharynx with which *inx-6* might associate. For example, *inx-6* expression overlaps that of *eat-5* (Starich *et al.*, 1996). It is interesting, therefore, that *eat-5* can substitute for *inx-6*, especially because *eat-5* does not form homomeric junctions in *Xenopus* oocytes (Landesman *et al.*, 1999). It may be that *eat-5* can rescue *inx-6(rr5)* because it can associate with and stabilize the mutant INX-6 encoded by *rr5*. On the other hand, ectopic expression of INX-6 under the *myo-2* promoter indicates that it may also form a homomeric channel. Work in *Drosophila* showed that expressing one subunit of an obligate homomer (*Dm-inx-2* or *Dm-inx-3*) has little effect, but when the two subunits are ectopically coexpressed, they cause severe developmental defects (Stebbing *et al.*, 2000). By analogy, the fact that ectopically expressed INX-6 has severe effects on the pharynx suggests that either INX-6 is capable of forming a homomeric channel, or it can associate promiscuously with endogenous innexin subunits.

Innexin Redundancy in the Pharynx

The temperature-sensitive period (TSP) of *inx-6(rr5)* suggests that other innexins may be functionally redundant. Although *inx-6* is expressed from mid-to-late embryogenesis until the adult stage, our TSP experiments indicated that the *inx-6* gene product is only required for the L1 and L2 larval stages. In spite of its embryonic expression, there seem to be no abnormalities in the *inx-6(rr5)* embryos raised at the restrictive temperature. Therefore, other innexins expressed in the pharyngeal muscles may act redundantly with *inx-6* to ensure pharyngeal differentiation and morphogenesis. The fact that cell-cell coupling was normal within the metacarpus in *inx-6(rr5)* animals, where the *inx-6::GFP* is strongly expressed in wild-type may reflect such redundancy. Other innexin members such as *eat-5* in the metacarpus may form functional gap junctions independent of *inx-6*, and thereby permit diffusion of dye and synchronization of action potentials among metacarpus muscles. That *inx-6(rr5)* animals can survive at the restrictive temperature as an adult may also reflect redundancy. However, at present we cannot exclude

that high temperature prevents assembly or transport of the mutant INX-6 protein, and conversely wild-type protein normally remains stable and persists.

If innexins are largely redundant, then ectopic expression of an innexin in a wild-type background should have no ill effects. In contrast, we found that ectopic expression of *inx-6* in the terminal bulb, not replacing but rather supplementing the innexins that are normally expressed there, causes the pharynx to hypercontract. Although we cannot rule out the possibility that overexpression from the multicopy transgene causes this phenotype, it is unlikely because *inx-6::INX-6*-containing transgenic arrays show no such effect. It is also unlikely that *inx-6* is associating with endogenous channels of the terminal bulb and acting as a dominant negative allele because reducing gap junctions should reduce the excitability of muscles, not cause hypercontraction. *inx-6*, acting alone or in association with endogenous innexins, might increase the degree of coupling. Variations on this model are that ectopically expressed *inx-6* is coupling cells that are normally isolated or that it forms permeable hemichannels in the cell membrane. Either way, the severe phenotype of *inx-6* expressed in the terminal bulb argues against a simple model of innexin redundancy wherein ectopic expression of an innexin in cells that already have innexins has no effect.

Innexin Specialization and the Role of Gap Junctions in Pharyngeal Muscle Contraction

The 20 muscle cells of the pharynx must contract with precise timing to ensure appropriate function of this organ. Thus, electrical coupling of pharyngeal muscles is of the utmost importance. The need for so many different innexins in a simple organ such as the pharynx is less clear. Our video, electrophysiological, GFP reporter expression, and dye-coupling data all suggest that *inx-6* is involved specifically in coupling the metacarpus to the procorpus. Whence the need for an innexin dedicated to this task?

One explanation is that the innexin subunits are functionally equivalent and the specialization is in their pattern of expression. By driving *eat-5* with the *inx-6* promoter, we showed that this transgene could rescue the larval arrest of the *inx-6(rr5)* mutants but, unlike an *inx-6::INX-6* transgene, could not entirely restore wild-type growth rate. Thus, the functional properties of *eat-5* and *inx-6* must be similar but not identical. Although *eat-5* and *inx-6* may be redundant in the metacarpus, *inx-6* seems to be better suited to couple the pro- and metacarpus.

If the innexin proteins are not perfectly interchangeable, what might be the properties that make them uniquely suited to couple specific cells? Here, we can only speculate. Subunit specific properties might include ability to form heterotypic interactions, voltage sensitivity, or regulation by second messengers. However, an understanding of the unique requirements of the pro- to metacarpus gap junctions may inform our speculation. Neuronal control is necessary for efficient pharyngeal function and the motor neurons seem to interact primarily with the metacarpus muscles. The motor neurons that regulate pharyngeal contraction and relaxation, MC and M3, are both corpus neurons and M3 synapses onto the metacarpus (Albertson and Thomson, 1976; Raizen and Avery, 1994; Raizen *et al.*, 1995). Presumably, the effects of these neu-

rons on metacarpus muscle membrane potential must be transmitted to the rest of the pharyngeal muscles to maintain synchrony. Thus, it seems that *eat-5* ensures the posterior propagation, and *inx-6* the anterior propagation, of changes in membrane potential originating in the metacarpus.

Our results indicate that although the metacarpus drives depolarization of both the procorpus and terminal bulb, there are different requirements for the control of current flow in each case. The multiple spikes in the *inx-6(rr5)* EPG indicate that when coupling to the metacarpus is compromised, the procorpus muscles repolarize prematurely. Although we were not able to correlate these spikes with relaxation of specific muscles, it is unlikely that these are R2 spikes, because, even in wild-type worms, the R2 spike that corresponds to terminal bulb relaxation is usually smaller than the R1 spikes seen in the *inx-6(rr5)* mutant (compare R2 in Figure 5B to the R spikes in Figure 5E). Rather, it seems that in the L4 stage *inx-6(rr5)* worms the corpus muscles contract normally (Figure 5D, frame 2) but that individual procorpus muscles begin relaxing prematurely. Thus, these spikes likely represent premature procorpus repolarizations. The implication is that the procorpus needs the metacarpus not only to initiate depolarization but also to maintain it. In contrast, the repolarization and relaxation of the terminal bulb muscles lags those of corpus. Moreover, in the *eat-5* mutant, uncoupling the corpus from the terminal bulb reveals that the terminal bulb seems to have its own pacemaker (Starich *et al.*, 1996),

It would make sense then that gap junctions formed by INX-6 would differ from those formed by EAT-5. Assuming that the metacarpus drives both depolarization and repolarization of the procorpus, the INX-6 gap junctions would be relatively passive. However, because (in this model) the metacarpus must drive terminal bulb depolarization but then be immune to the continued depolarization of the terminal bulb after the metacarpus repolarizes and relaxes, one might predict that the gap junction formed by EAT-5 would rectify.

CONCLUSIONS

The identification of a temperature-sensitive allele of the innexin *inx-6* offers an important opportunity to study the various cellular roles of gap junctions. Future work will focus on how these important proteins function together with other innexin family members to coordinate the precise electrical coupling essential for pharyngeal function and larval development. Because of the ease of generating mutants and the availability of promoters for ectopic expression, the pharynx could be very useful for elucidating the role of gap junction diversity in controlling current flow through coupled muscles.

ACKNOWLEDGMENTS

We thank Todd Starich and Jocelyn Shaw for sharing reagents, information, and discussion; Andy Fire for the GFP vector kit; the *C. elegans* Genetics Centre for strains; the Sanger Centre for cosmids; and Ron Chase for the spike counter. J.A.D. was supported by the Natural Sciences and Engineering Research Council of Canada. This work was supported by the National Cancer Institute of Canada with funds from the Terry Fox Run.

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