Cellular identity and Ca^{2+} signaling activity of the non-reproductive GnRH system in the *Ciona* larva

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ABSTRACT

Tunicate larvae have a non-reproductive GnRH system with multiple ligands and receptor heterodimerization enabling complex regulation. In the *Ciona* larva, one of the *gnrh* genes, *gnrh2*, is conspicuously expressed in the motor ganglion and nerve cord, which are homologous structures to the hindbrain and spinal cord, respectively, of vertebrates. The *gnrh2* gene is also expressed in the proto-placodal sensory neurons, which are the proposed homologue of vertebrate olfactory neurons. The tunicate larvae occupy a non-reproductive dispersal stage, yet the roles of their GnRH system remain elusive. In this study, we investigated neuronal types of *gnrh2*-expressing cells in the *Ciona* larva and visualized activity of these cells by fluorescence imaging using a calcium sensor protein. Some cholinergic neurons as well as dopaminergic cells express *gnrh2*, suggesting that a role of GnRH in the control of swimming behavior. By contrast, none of the *gnrh2*-expressing cells overlap with glycinergic or GABAergic neurons. A role in the motor control is also suggested by correlation between activity of some *gnrh2*-expressing cells and tail movements. Interestingly, *gnrh2*-positive ependymal cells in the nerve cord, known as a kind of glia cells, actively produced Ca^{2+} transients, suggesting that neuroendocrine signaling occurs in glia cells of the nerve cord.

Keywords: gonadotropin-releasing hormone (GnRH); ascidian; calcium imaging;

Introduction

Gonadotropin-releasing hormone (GnRH) is a key regulator of reproductive functions in vertebrates (Okubo and Nagahama 2008; Oka 2009). Non-reproductive roles of GnRH have been suggested in the nervous system and during development (Dolan et al. 2003; Albertson et al. 2008; Sherwood and Wu 2005; Wu et al. 2006; Abraham et al. 2008; Kanaho et al. 2009; Ramakrishnan et al. 2010). Compared to its reproductive roles, the non-reproductive roles of GnRH are less well understood.

Tunicates are the sister group of vertebrates (Delsuc et al. 2006; Putnam et al. 2008). A conspicuous non-reproductive GnRH system has been reported in the larva of the sessile tunicate *Ciona* (Kusakabe et al. 2012; Kamiya et al. 2014). Six GnRH peptides and four receptors are encoded by the *Ciona* genome (Adams et al. 2003; Kusakabe et al. 2003; Tello et al. 2005; Sakai et al. 2010, 2012). In the *Ciona* larva, the GnRH genes are strikingly expressed in the central nervous system (CNS) through the entire antero-posterior body axis (Kusakabe et al. 2012). Correspondingly, the GnRH receptor genes are specifically expressed in the tissues and organs located along the CNS, namely the notochord, the tail muscle, and epidermal sensory neurons (Kusakabe et al. 2012). One of the *Ciona gnrh* genes, *gnrh2*, is conspicuously expressed in the motor ganglion and nerve cord of the larva, which are homologous structures to the hindbrain and spinal cord, respectively, of vertebrates. The *gnrh2* gene is also expressed in the proto-placodal sensory neurons, which are the proposed homologue of vertebrate olfactory neurons (Abitua et al. 2015). *Ciona* GnRH has been implicated to play a pivotal role in the control of metamorphosis (Kamiya et al. 2014). Considering the complex and well-developed nature of the larval GnRH system in *Ciona*,

GnRH may play diverse and important roles in developmental and physiological processes in the *Ciona* larva. To date, however, the roles of the *Ciona* GnRH system remain elusive.

In this study, we investigated neuronal types of *gnrh2*-expressing cells in the *Ciona* larva and visualized activity of these cells by fluorescence imaging using a calcium sensor protein. Some cholinergic motor neurons as well as unique cholinergic cells along the nerve cord express *gnrh2*, suggesting that a role of GnRH in the control of swimming behavior. By contrast, none of the *gnrh2*-expressing cells overlap with glycinergic or GABAergic neurons. A role in the motor control is also suggested by simultaneous activation of some *gnrh2*-expressing cells with tail movements. Interestingly, *gnrh2*-positive ependymal cells in the nerve cord, known as a kind of glia cells, produced Ca^{2+} transients, suggesting that active signaling, presumably involving GnRH, occurs in the nerve cord.

Results

Gnrh2 is expressed in proto placode-derived sensory neurons and caudal glial ependymal cells

The 4.3-kb upstream region of *gnrh2* connected with a fluorescence reporter can recapitulate the expression patterns of *gnrh2* (Kusakabe et al. 2012) (Fig. 1). This upstream region was used to express mCherry and G-CaMP8 in cells expressing *gnrh2* (Figs. 1,2 and 3). Cell types were identified by double fluorescent staining of larvae with cell type-specific markers.

First, we examined whether *gnrh2*-expressing cells include glutamatergic neurons. In the *Ciona* larva, glutamate is a major neurotransmitter in the peripheral sensory neurons and photoreceptor cells (Horie et al. 2008). Some interneurons in the

posterior brain is also glutamatergic (Horie et al. 2008). As previously reported (Abitua et al. 2015), proto-placode derived *gnrh2*-expressing epidermal neurons (aATENs) are glutamatergic (Fig. 2A). aATENs are the only glutamatergic neurons that express *gnrh2*. None of the glutamatergic neurons express *gnrh2* in the CNS.

Then we examined whether any of GABAergic/glycinergic neurons express *gnrh2* using vesicular inhibitory amino acid transporter (VIAAT) as a marker. None of the VIAAT-positive cells were overlapped with the reporter expression under the control of the *gnrh2 cis*-regulatory region (Fig. 2C and 2D). In the anterior tail region, there are two pairs of VIAAT-positive neurons called ACINs, which align with glial ependymal cells in the lateral wall of the anterior nerve cord (Horie et al. 2010; Nishitsuji et al. 2012). The ACINs do not express *gnrh2* whereas the lateral ependymal cells express *gnrh2* (Fig. 2D). The *gnrh2* expression in the lateral wall ependymal cells of the nerve cord is consistent with the *in situ* hybridization data previously reported (Kusakabe et al. 2012).

Cellular retinaldehyde binding protein (CRALBP) is specifically localized in the glial ependymal cells in the brain vesicle and the motor ganglion (Tsuda et al. 2003; Kusakabe et al. 2009). CRALBP-positive cells were never overlapped with *gnrh2*-epxressing cells (Fig. 2B). Thus, in contrast to the conspicuous *gnrh2* expression in the ependymal cells of the nerve cord, *gnrh2* is not expressed in the ependymal cells in the brain vesicle and the motor ganglion.

Some cholinergic and dopaminergic neurons express *gnrh2*

Acetylcholine is a major neurotransmitter at the neuromuscular junctions of the *Ciona* larva (Yoshida et al. 2004; Horie et al. 2010). Cholinergic neurons were visualized by a

fluorescence protein expressed under the control of *cis*-regulatory region of the *vacht* gene (Yoshida et al. 2004). *Gnrh2*-expressing neurons were shown to be cholinergic both in the brain vesicle and the motor ganglion (Fig. 3A).

The caudal part of the CNS (nerve cord) mainly consists of non-neuronal ependymal cells (Katz 1983). The nerve cord also contains two types of neurons: ACINs and bilateral pairs of cholinergic caudal neurons (Horie et al. 2010). Some of these cholinergic caudal neurons seem to express *gnrh2* (Fig. 3B).

Another neurotransmitter that controls swimming of *Ciona* tadpoles is dopamine (Razy-Krajka et al. 2012). Dopaminergic neurons are present in the brain vesicle (Moret et al. 2005; Razy-Krajka et al. 2012; Horie et al. 2018). Dopaminergic neurons were labeled with mCherry expressed under the control of the *cis*-regulatory region of the dopaminergic cell-specific gene *fer2* (Razy-Krajka et al. 2012; Horie et al. 2018; previously described as *Ptf1a*, see Gyoja & Satoh 2013 for the orthologous families of the bHLH transcription factors). Double fluorescence imaging of dopaminergic neurons and *gnrh2*-expressing cells revealed that some of dopaminergic neurons express *gnrh2* (Fig. 3C).

Active Ca^{2+} transients in aATENs and *gnrh2*-expressing cells of the posterior CNS

G-CaMP8 was used to monitor temporal changes in intracellular Ca^{2+} in *gnrh2*-expressing cells. Active Ca^{2+} transients were observed in aATENs, the motor ganglion, and the caudal nerve cord (Figs. 4-6; Movies S1-3). The larva contains two aATENs, each has a sensory cilium (Abitua et al 2015, Fig. 4A,B). Both aATENs showed Ca^{2+} transients but their activity was not synchronized (Fig. 4C; Movie S1).

Cholinergic *gnrh2*-expressing neurons at the posterior part

of the motor ganglion exhibited active Ca^{2+} transients (Figs. 5 and 6). Periodic Ca^{2+} transients of a *gnrh2*-expressing neuron in the motor ganglion were observed at 19 hours after fertilization (*arrowheads* in Fig. 6B). In the tail region, Ca^{2+} transients were observed though the entire length of the nerve cord (Figs. 5 & 6). Both cholinergic neurons and ependymal cells showed Ca^{2+} transients in the tail nerve cord. For example, a narrow cell indicated by an *arrow* in Fig. 5A is presumably cholinergic neurons. Many of cells showing Ca^{2+} transients were block-shaped cells, characteristics of caudal ependymal cells (Fig. 6C).

Ca^{2+} transients were not synchronized between cells, including neurons and ependymal cells, located at different sites of the larva (Figs. 5 and 6). However, simultaneous activation of cells at different sites were occasionally observed as indicated by an arrowhead in Fig. 5B and Fig. 6B, suggesting the presence of a neural circuit connecting *gnrh2*-expressing cells at different sites.

Correlation between tail movements and Ca^{2+} transients in the motor ganglion and the anterior nerve cord

A neural circuit in the motor ganglion and the anterior nerve cord is thought to control muscle contraction of the tail (Nishino et al. 2010; Horie et al. 2010; Kusakabe 2017). Because active Ca^{2+} transients in *gnrh2*-expressing cells were observed in these regions, we examined temporal correlation between tail movement and the activity of *gnrh2*-expressing cells. Ca^{2+} transients were frequently observed when the tail stopped movement, whereas the Ca^{2+} transients were seemingly suppressed during the term when the tail was moving (Fig. 7). This finding suggests possible involvement of *gnrh2*-expressing cells in the control of swimming behavior.

Discussion

In this study, we identified cell types of *gnrh2*-expressing cells and visualized their activity in the *Ciona* larva. Previously, the cells expressing GnRH-encoding genes had been only partially identified in *Ciona*. The caudal ependymal cells and the aATEN epidermal neurons were reported to express *gnrh2* (Kusakabe et al. 2012; Abitua et al. 2015). We confirmed these findings and further identified CNS neurons expressing *gnrh2*.

In the brain vesicle, dopaminergic neurons and a limited number of cholinergic neurons express *gnrh2*. Pharmacological and behavioral analyses have suggested that dopaminergic cells modulate the light-off-induced swimming behavior of *Ciona* larvae (Razy-Krajka et al. 2012). The role of cholinergic neurons in the *Ciona* brain vesicle has not been elucidated. Current observation reveals previously unknown heterogeneity of cholinergic neurons in the brain vesicle and would provide a clue to investigate roles of these neurons.

Cholinergic neurons in the motor ganglion have been implicated in the regulation of tail muscle contraction (Horie et al. 2010; Nishino et al. 2010, 2011; Kusakabe 2017). Here we show that one subtype of cholinergic neurons in the motor ganglion expresses *gnrh2* (Fig. 3A). These neurons posteriorly send axons but it is unclear whether they are motor neurons directly innervating muscle cells or interneurons connecting to other CNS neurons in the caudal nerve cord. In the nerve cord, another class of cholinergic cells also expresses *gnrh2* (Fig. 3B). A potential role of cholinergic/GnRH neurons in the motor ganglion and the nerve cord may be the control of swimming behavior. These neurons may also play a role in metamorphosis because GnRH has been known to be involved in the regulation of metamorphosis (Kamiya et al.

2014).

Calcium imaging has been applied to studies of *Ciona* development (Hackley et al. 2013; Abdul-Wajid et al. 2015; Akahoshi et al. 2017). These previous studies focused on Ca^{2+} transients in embryos but not in mature larvae. The present study for the first time reported spatio-temporal patterns of Ca^{2+} transients in mature larvae of *Ciona*. Our observation includes four novel findings: i) active Ca^{2+} transients in the proto-placode-derived aATENs, ii) periodic spikes in the motor ganglion of young larvae, iii) correlation between Ca^{2+} transients and the tail movements, and iv) active Ca^{2+} transients in ependymal cells of the nerve cord.

The proto-placode-derived aATENs share morphological and molecular properties with vertebrate olfactory neurons and thought to be chemosensory cells (Abitua et al. 2015). However, olfactory receptors have not been identified in *Ciona* and chemical cues that stimulate aATENs are not known. Calcium imaging with *gnrh2*>G-CaMP8 could help us search for chemical cues that triggers activation of aATENs in future studies.

Periodic Ca^{2+} transients observed in the motor ganglion of young larvae are reminiscent of spontaneous rhythmic activities observed in developing nervous systems of vertebrates (Gu et al. 1994; Wong et al. 1995; Feller et al. 1996; Zhou 1999; Kerr et al. 2005; Chang and Spitzer 2009). These periodic neuronal activities are thought to be important for the development of neural circuits in the central nervous system and the retina (Zhou 1999; Spitzer et al. 2000; Feller 2006). Similar rhythmic oscillation of Ca^{2+} transients were reported in the developing motor ganglion of the *Ciona* embryo (Akahoshi et al. 2017). By contrast, we observed rhythmic Ca^{2+} transients only in young larvae. Swimming behavior of *Ciona* larvae reveals ontogenic changes and their photo-

responsiveness appears a few hours after hatching (Nakagawa et al. 1999; Tsuda et al. 2003; Zega et al. 2006). Thus, the spontaneous rhythmic Ca^{2+} transients may play an important role in the neural circuit development of *Ciona* larvae.

We observed correlation between the tail movements and Ca^{2+} transients in the motor ganglion and the nerve cord. This suggests that *gnrh2*-expressing cells are involved in the control of swimming locomotion. Ca^{2+} transients appeared when the tail stops movement and Ca^{2+} signal was low when the tail was moving (Fig. 7B). In other words, the tail movement precedes the Ca^{2+} spike. This pattern suggests that these *gnrh2*-expressing cells are not motor neurons. In fact, majority of the cells expressing *gnrh2* in the nerve cord are ependymal cells and we observed Ca^{2+} transients correlating with tail movements in ependymal cells. An intriguing possibility is that Ca^{2+} spikes are induced in ependymal cells by muscle contraction or motor axon excitation. If so, the ependymal cells may monitor activity of muscle or motor neurons. It has been reported that various types of glia cells exhibit Ca^{2+} transients in response to neuronal activities and regulate neuronal functions in vertebrates (Reist and Smith 1992; Newman and Zahs 1997; Robitaille 1998; Haydon 2001). The ependymal cells of *Ciona* larva may have similar regulatory roles, suggesting a deep evolutionary conservation of glia function between tunicates and vertebrates. Given the simplicity of its nervous system, the *Ciona* larva could serve as a unique model for the study of glia-neuron interaction.

In conclusion, the present study reveals dynamic Ca^{2+} transients of *gnrh2*-expressing cells at various sites in the *Ciona* larva. Our findings suggest a connection between activity of *gnrh2*-expressing cells and tail movements of the larva. An important yet unsolved question is whether GnRH2 is involved in these processes.

Future studies should address developmental and physiological roles of *gnrh2*-expressing cells and GnRH peptides based on the findings of this study.

Methods

Animals and embryos

Mature adults of *Ciona intestinalis* Type A (also called *Ciona robusta*) were provided by the Maizuru Fisheries Research Station of Kyoto University and by the Misaki Marine Biological Station of the University of Tokyo through the National Bio-Resource Project (NBRP) of MEXT, Japan, and maintained in indoor tanks of artificial seawater (ASW) (Marine Art BR, Tomita Pharmaceutical Co., Ltd., Tokushima, Japan) at 18°C. The adults were also collected from the pond on the Fukae campus of Kobe University, Kobe, Japan and from the fishing harbor in Murotsu, Hyogo, Japan. Eggs and sperm were obtained surgically from the gonoducts, and eggs were fertilized *in vitro*. After insemination, embryos were raised in ASW containing 50 μg/ml streptomycin sulfate (S6501, Sigma-Aldrich, St. Louis, MO, USA) at 18°C.

Preparation of reporter constructs and electroporation

Construction of the *vglut>kaede* was described previously (Horie et al., 2011, Razy-Krajka et al. 2012). The *vacht>cfp* plasmid was made by inserting the 3.8-kb upstream region of *Ciona vacht* (Yoshida et al. 2004) into the *Sal*I/*Bam*HI site of pSP-CFP (Horie et al. 2011). The 2.4-kb upstream region of *Ciona fer2* (Gene ID KH.L116.39) was previously cloned in the pSP-CFP vector (Razy-Krajka et al. 2012). The reporter sequence was replaced with a DNA fragment coding for mCherry to generate *fer2>mcherry*

using *Not*I/*Eco*RI sites. The *gnrh2>kaede* and *gnrh2>mcherry* plasmids were made by inserting the 4.3-kb upstream region of *Ciona gnrh2* (Kusakabe et al. 2012) into the *Xho*I/*Not*I sites of the pSP-Kaede vector and pSP-mCherry vector, respectively (Hozumi et al. 2010). The *gnrh2* upstream region was also used to generate the *gnrh2>g-camp8* construct. The Kaede coding sequence of pSP-Kaede was replaced with a DNA fragment coding for G-CaMP8 (Ohkura et al. 2012) using *Not*I/*Eco*RI sites. The *gnrh2* upstream region was amplified from the *gnrh2>kaede* plasmid using a pair of nucleotide primers (5'-GAATCGGCCAACGCGGGATCCAGGAGCAGACGTCATAAGTA-3' and 5'-TGACGCGGCCGCTGTTACGTTATCTCTCTAGAAG-3'), digested with *Bam*HI and *Not*I, and then inserted into the *Bam*HI/*Not*I sites upstream of the G-CaMP8 in the pSP vector. Plasmid DNA constructs were electroporated into *Ciona* fertilized eggs as described by Corbo et al. (1997).

Immunofluorescent staining

Immunofluorescent staining was carried out according to the method described by Nishitsuji et al. (2012). Fluorescent images were obtained by using a laser scanning confocal microscope (FV1200 IX83; Olympus, Tokyo, Japan).

To visualize localization of cell-type specific proteins, a mouse antiserum against *Ciona* VIAAT (Horie et al. 2010) or a rabbit antiserum against *Ciona* CRALBP (Tsuda et al. 2003) was diluted 1:1000 in 10% goat serum in T-PBS (0.1% Triton X-100 in PBS) and used as the primary antibody. The secondary antibody was an Alexa Fluor 594−conjugated anti-mouse IgG (A11005; Thermo Fisher Scientific) or an Alexa Fluor 594−conjugated anti-rabbit IgG (A11012; Thermo Fisher Scientific).

Primary antibodies used to visualize localization of

fluorescent reporter proteins are rabbit anti-Kaede polyclonal (PM012; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan; for Kaede), rabbit anti-GFP polyclonal (A11122; Thermo Fisher Scientific; for G-CaMP8 and CFP), rat anti-RFP monoclonal (5F8; ChromoTek GmbH, Martinsried, Germany; for mCherry), and rat anti-GFP monoclonal (GF090R; Nacalai Tesque, Kyoto, Japan; for G-CaMP8 double stained with anti-CRALBP) antibodies. All the primary antibodies are diluted 1000-fold as described above. Secondary antibodies were an Alexa Fluor 488−conjugated anti-rabbit IgG (A11008; Thermo Fisher Scientific) for G-CaMP8 and CFP, an Alexa Fluor 488−conjugated anti-rat IgG (A11006; Thermo Fisher Scientific) for G-CaMP8, and an Alexa Fluor 594−conjugated anti-rat IgG (A11007; Thermo Fisher Scientific) for mCherry.

In vivo Ca^{2+} imaging

Electroporated *Ciona* larvae expressing the G-CaMP8 transgene was placed in ASW on a 35-mm glass based dish (coverslip diameter 12 mm, #3931-035; Iwaki, Japan). For imaging, a microscope (IX81, Olympus, Tokyo, Japan) equipped with an EMCCD camera (EVOLVE512, Photometrics, USA) was used. Images were taken with a 50-ms exposure time per 1 second and 1×1 binning. For each animal, 300 images were taken in 6 minutes. Changes in intracellular calcium concentrations were measured as the changes of green fluorescence of G-CaMP8. MetaMorph image analysis software system (Molecular Devices) was used for the analysis of imaging. Image processing was also performed with ImageJ (U. S. National Institutes of Health, Bethesda, Maryland, USA, https://imagej.nih.gov/ij/).

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References

Abdul-Wajid, S., Morales-Diaz, H., Khairallah, S. M. & Smith, W. C. T-type calcium channel regulation of neural tube closure and EphrinA/EPHA expression. *Cell Rep.* **13**, 829-839 (2015).

Abitua, P. B., Gainous, T. B., Kaczmarczyk, A. N., Winchell, C. J., Hudson, C., Kamata, K., Nakagawa, M., Tsuda, M., Kusakabe, T. G. & Levine, M. The pre-vertebrate origins of neurogenic placodes. *Nature* **524**, 462–465 (2015).

Abraham, E., Palevitch, O., Ijiri, S., Du, S. J., Gothilf, Y. *et al.* Early development of forebrain gonadotrophin-releasing hormone (GnRH) neurons and the role of GnRH as an autocrine migration factor. *J. Neuroendocrinol.* **20**, 394–405 (2008).

Adams, B. A., Tello, J. A., Erchegyi, J., Warby, C., Hong, D. J. *et al.* Six novel gonadotropin-releasing hormones are encoded as triplets on each of two genes in the protochordate, *Ciona intestinalis*. *Endocrinology* **144**, 1907–1919 (2003).

Akahoshi, T., Hotta, K. & Oka, K. Characterization of calcium transients during early embryogenesis in ascidians *Ciona robusta* (*Ciona intestinalis* type A) and *Ciona savignyi*. *Dev. Biol.* **431**, 205-214 (2017).

Albertson, A. J., Talbott, H., Wang, Q., Jensen, D. & Skinner, D. C. The gonadotropin-releasing hormone type I receptor is expressed in the mouse cerebellum. *Cerebellum* **7**, 379–384 (2008).

Chang, L. W. & Spitzer, N. C. Spontaneous calcium spike activity in embryonic spinal neurons is regulated by developmental expression of the Na^+, K^+-ATPase β3 subunit. *J. Neurosci.* **29**, 7877-7885 (2009).

Corbo, J. C., Levine, M. & Zeller, R. W. Characterization of a notochord-specific enhancer from the *Brachyury* promoter region of the ascidian, *Ciona intestinalis*. *Development* **124**, 589-602 (1997).

Delsuc, F., Brinkmann, H., Chourrout, D. & Philippe, H. Tunicates and not cephalochordates are the closest living relative of vertebrates. *Nature* **439**, 965–968 (2006).

Dolan, S., Evans, N. P., Richter, T. A. & Nolan, A. M. Expression of gonadotropin-releasing hormone and gonadotropin-releasing hormone receptor in sheep spinal cord. *Neurosci. Lett.* **346**, 120–122 (2003).

Dravid, S. M. & Murray, T. F. Spontaneous synchronized calcium oscillations in neocortical neurons in the presence of physiological [$Mg(^{2+})$]: involvement of AMPA/kainate and metabotropic glutamate receptors. *Brain Res.* **1006**, 8–17 (2004).

Feller, M. B. Spontaneous correlated activity in developing neural circuits. *Neuron* **22**, 653-656 (1999).

Feller, M. B., Wellis, D. P., Stellwagen, D., Werblin, F. S. & Shatz, C.

J. Requirement for cholinergic synaptic transmission in the propagation of spontaneous retinal waves. Science 272, 1182–1187 (1996).

Gu, X., Olson, E. C. & Spitzer, N. C. Spontaneous neuronal calcium spikes and waves during early differentiation. *J. Neurosci.* **14**, 6325-6335 (1994).

Gyoja, F. & Satoh, N. Evolutionary aspects of variability in bHLH orthologous families: Insights from the pearl oyster, *Pinctada fucata*. *Zool. Sci.* **30**, 868–876 (2013).

Hackley, C., Mulholland, E., Kim, G. J., Newman-Smith, E. & Smith, W. C. A transiently expressed connexin is essential for anterior neural plate development in *Ciona intestinalis*. *Development* **140**, 147-155 (2013).

Haydon, P. G. Glia: listening and talking to the synapse. *Nat. Rev. Neurosci.* **2**, 185–193 (2001).

Horie, T., Kusakabe, T. & Tsuda, M. Glutamatergic networks in the *Ciona intestinalis* larva. *J. Comp. Neurol.* **508**, 249–263 (2008).

Horie, T., Nakagawa, M., Sasakura, Y., Kusakabe, T. G. & Tsuda, M. Simple motor system of the ascidian larva: neuronal complex comprising putative cholinergic and GABAergic/glycinergic neurons. *Zool. Sci.* **27**, 181–190 (2010).

Horie, T., Shinki, R., Ogura, Y., Kusakabe, T. G., Satoh, N. & Sasakura, Y. Ependymal cells of chordate larvae are stem-like cells that form the adult nervous system. *Nature* **469**, 525–528 (2011).

Horie, T., Horie, R., Chen, K., Cao, C., Nakagawa, M., Kusakabe, T. G., Satoh, N., Sasakura, Y. & Levine, M. Regulatory cocktail for dopaminergic neurons in a protovertebrate identified by whole-embryo single-cell transcriptomics. *Genes Dev.* **32**, 1297-1302 (2018).

Hozumi, A., Kawai, N., Yoshida, R., Ogura, Y., Ohta, N., Satake, H.,

Satoh N. & Sasakura, Y. Efficient transposition of a single *Minos* transposon copy in the genome of the ascidian *Ciona intestinalis* with a transgenic line expressing transposase in eggs. *Dev. Dynam.* **239**, 1076-1088 (2010).

Kamiya, C., Ohta, N, Ogura, Y., Yoshida, K., Horie, T., Kusakabe, T. G., Satake, H. & Sasakura, Y. Nonreproductive role of gonadotropin-releasing hormone in the control of ascidian metamorphosis. *Dev. Dynam.* **243**, 1524–1535 (2014).

Kanaho, Y. I., Enomoto, M., Endo, D., Maehiro, S., Park, M. K., *et al.* Neurotrophic effect of gonadotropin-releasing hormone on neurite extension and neuronal migration of embryonic gonadotropin-releasing hormone neurons in chick olfactory nerve bundle culture. *J. Neurosci. Res.* **87**, 2237–2244 (2009).

Katz, M. J. Comparative anatomy of the tunicate tadpole, *Ciona intestinalis*. *Biol. Bull.* **164**, 1–27 (1983).

Kerr, J. N., Greenberg, D. & Helmchen, F. (2005) Imaging input and output of neocortical networks in vivo. *Proc. Natl. Acad. Sci. USA* **102**, 14063–14068.

Kusakabe, T. G. Identifying vertebrate brain prototypes in deuterostomes. *Brain Evolution by Design: From Neural Origin to Cognitive Architecture* (Springer), pp. 153-186 (2017).

Kusakabe, T., Mishima, S., Shimada, I., Kitajima, Y. & Tsuda, M. Structure, expression, and cluster organization of genes encoding gonadotropin-releasing hormone receptors found in the neural complex of the ascidian *Ciona intestinalis*. *Gene* **322**, 77–84 (2003).

Kusakabe, T. G., Takimoto, N., Jin, M. & Tsuda, M. Evolution and the origin of the visual retinoid cycle in vertebrates. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **364**, 2897–2910 (2009).

Kusakabe, T. G., Sakai, T., Aoyama, M., Kitajima, Y., Miyamoto, Y. *et al.* A conserved non-reproductive GnRH system in chordates.

PLoS One **7**, e41955 (2012).

Moret, F., Christiaen, L., Deyts, C., Blin, M., Joly, J.-S. & Vernier, P. The dopamine-synthesizing cells in the swimming larva of the tunicate *Ciona intestinalis* are located only in the hypothalamus-related domain of the sensory vesicle. *Eur. J. Neurosci.* **21**, 3043–3055 (2005).

Nakagawa, M., Miyamoto, T., Ohkuma, M. & Tsuda, M. (1999) Action spectrum for the photophobic response of *Ciona intestinalis* (Ascidiacea, Urochordata) larvae implicates retinal protein. *Photochem. Photobiol.* **70**, 359–362.

Newman, E. A. & Zahs, K. R. (1997) Calcium waves in retinal glial cells. *Science* **275**, 844–847.

Nishino, A., Okamura, Y., Piscopo, S. & Brown, E. R. A glycine receptor is involved in the organization of swimming movements in an invertebrate chordate. *BMC Neurosci.* **11**, 6 (2010).

Nishino, A., Baba, S. A. & Okamura, Y. A mechanism for graded motor control encoded in the channel properties of the muscle ACh receptor. *Proc. Natl. Acad. Sci. USA* **108**, 2599-2604 (2011).

Nishitsuji, K., Horie, T., Ichinose, A., Sasakura, Y., Yasuo, H. & Kusakabe, T. G. Cell lineage and *cis*-regulation for a unique GABAergic/glycinergic neuron type in the larval nerve cord of the ascidian *Ciona intestinalis*. *Dev. Growth. Differ.* **54**, 177–186 (2012).

Ohkura, M., Sasaki, T., Sadakari, J., Gengyo-Ando, K., Kagawa-Nagamura, Y., Kobayashi, C. et al. Genetically encoded green fluorescent Ca^{2+} indicators with improved detectability for neuronal Ca^{2+} signals. *PLoS ONE* **7**, e51286 (2012).

Oka, Y. Three types of gonadotrophin-releasing hormone neurones and steroid-sensitive sexually dimorphic kisspeptin neurones in

teleosts. *J. Neuroendocrinol.* **21**, 334–338 (2009).

Okubo, K. & Nagahama, Y. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. *Acta Physiol.* **193**, 3–15 (2008).

Putnam, N. H., Butts, T., Ferrier, D. E., Furlong, R. F., Hellsten, U., *et al.* The amphioxus genome and the evolution of the chordate karyotype. *Nature* **453**, 1064-1071 (2008).

Ramakrishnan, S., Lee, W., Navarre, S., Kozlowski, D. J. & Wayne, N. L. Acquisition of spontaneous electrical activity during embryonic development of gonadotropin-releasing hormone-3 neurons located in the terminal nerve of transgenic zebrafish (*Danio rerio*). *Gen. Comp. Endocrinol.* **168**, 401–407 (2010).

Razy-Krajka, F., Brown, E. R., Horie, T., Callebert, J., Sasakura, Y., Joly, J.-S., Kusakabe, T. G. & Vernier, P. Monoaminergic modulation of photoreception in ascidian: evidence for a proto-hypothalamo- retinal territory. *BMC Biol.* **10**, 45 (2012).

Reist, N. E. & Smith, S. J. Neurally evoked calcium transients in terminal Schwann cells at the neuromuscular junction. *Proc. Natl. Acad. Sci. USA* **89**, 7625–7629 (1992).

Robitaille, R. Modulation of synaptic efficacy and synaptic depression by glial cells at the frog neuromuscular junction. *Neuron* **21**, 847–855 (1998).

Sakai, T., Aoyama, M., Kusakabe, T., Tsuda, M. & Satake, H. Functional diversity of signaling pathways through G protein-coupled receptor heterodimerization with a species-specific orphan receptor subtype. *Mol. Biol. Evol.* **27**, 1097-1106 (2010).

Sakai, T., Aoyama, M., Kawada, T., Kusakabe, T., Tsuda, M. & Satake, H. Evidence for differential regulation of GnRH signaling via heterodimerization among GnRH receptor paralogs in the protochordate, *Ciona intestinalis*. *Endocrinology* **153**, 1841-

1849 (2012).

Sherwood, N. M. & Wu, S. Developmental role of GnRH and PACAP in a zebrafish model. *Gen. Comp. Endocr.* **42**, 74–80 (2005).

Spitzer, N. C., Lautermilch, N. J., Smith, R. D. & Gomez, T. M. Coding of neuronal differentiation by calcium transients. *Bioessays* **22**, 811–817 (2000).

Tello, J. A., Rivier, J. E. & Sherwood, N. M. Tunicate gonadotropin-releasing hormone (GnRH) peptides selectively activate *Ciona intestinalis* GnRH receptors and the green monkey type II GnRH receptor. *Endocrinology* **146**, 4061–4073 (2005).

Tsuda, M., Kawakami, I. & Shiraishi, S. Sensitization and habituation of the swimming behavior in ascidian larvae to light. *Zool. Sci.* **20**, 13–22 (2003).

Tsuda, M., Kusakabe, T., Iwamoto, H., Horie, T., Nakashima, Y., Nakagawa, M. & Okunou, K. Origin of the vertebrate visual cycle. II. Visual cycle proteins are localized in whole brain including photoreceptor cells of a primitive chordate. *Vision Res.* **43**, 3045-3053 (2003).

Wong, R. O., Chernjavsky, A., Smith, S. J. & Shatz, C. J. Early functional neural networks in the developing retina. *Nature* **374**, 716–718 (1995).

Wu, S., Page, L. & Sherwood, N. M. A role for GnRH in early brain regionalization and eye development in zebrafish. *Mol. Cell. Endocrinol.* **257–258**, 47–64 (2006).

Yoshida, R., Sakurai, D., Horie, T., Kawakami, I., Tsuda, M. & Kusakabe, T. Identification of neuron-specific promoters in *Ciona intestinalis*. *Genesis* **39**, 130–140 (2004).

Zega, G., Thorndyke, M. C. & Brown, E. R. Development of swimming behavior in the larva of the ascidian Ciona intestinalis. *J. Exp. Biol.* **209**, 3405–3412 (2006).

Zhou, Z. J. Direct participation of starburst amacrine cells in

spontaneous rhythmic activities in the developing mammalian retina. *J. Neurosci.* **18**, 4155-4165 (1998).

Figure Legends

Figure 1. Expression patterns of *gnrh2* in the *Ciona* larva. (A) Schematic diagram showing the central nervous system (CNS) of the larva. (B) Localization of *gnrh2* mRNA visualized by *in situ* hybridization. (C) Immunofluorescent localization of G-CaMP8 expressed under the control of the *cis*-regulatory region of *gnrh2*. *Scale bar*, 200 μm.

Figure 2. Immunohistochemical identification of types of cells expressing *gnrh2* in the *Ciona* larva. (A) Glutamatergic neurons and *gnrh2*-expressing cells were labeled with Kaede (*green*) and mCherry (*magenta*), respectively. The proto placode-derived sensory neurons (*arrows*) were shown to express *gnrh2*. (B) Cellular retinal aldehyde binding protein (CRALBP)-positive cells (*magenta*) were not overlapped with *gnrh2*-expressing cells (*green*). Arrows indicate *gnrh2*-expressing cells in the brain vesicle. (C,D) GABAergic/glycinergic neurons were visualized by immunostaining with anti-VIAAT antibody (*magenta*). *Arrows* in (C) indicate GABAergic/glycinergic neurons in the motor ganglion. *Arrows* in (D) indicate VIAAT-positive ACINs. Scale bars, 30 μm.

Figure 3. Some cholinergic and dopaminergic neurons express *gnrh2*. (A,B) Cholinergic neurons and *gnrh2*-exressing cells were labeled with CFP (*green*) and mCherry (*magenta*), respectively. *Arrows* indicate cells co-expressed both markers. (C) Dopaminergic cells and *gnrh2*-expressing cells were labeled with mCherry (*magenta*) and G-CaMP8 (*green*), respectively. Some dopaminergic cells were also labeled with G-CaMP8 (*arrows*). Scale bars, 30 μm.

Figure 4. Calcium imaging of *gnrh2*-expressing chemosensory

neurons. Fluorescence images of *Ciona* larvae expressing G-CaMP8 in the chemosensory apical trunk epidermal neurons (aATENs). (A) The putative sensory cilia of each aATEN was labeled with G-CaMP8 fluorescence (dotted circle). (B,C) An example of a larva showing dynamic Ca^{2+} transients in the pair of aATENs (*arrowheads*) at 19 hpf (hours post fertilization). (C) Representative images of the larva recorded at the time (s) indicated. The serial images of the larva shown in (B,C) is shown in **Movie S1**.

Figure 5. Interconnection between *gnrh2*-expressing cells in the larval central nervous system. (A) Fluorescence image of a larva at 19.5 hpf, showing G-CaMP8 fluorescence in the motor ganglion and the anterior nerve cord. (B) The graph shows temporal patterns of fluorescence intensity at the three sites indicated by circles in (A). Colors of lines correspond to the sites indicated by circles in respective colors. The Ca^{2+} transients occurred independently of each other, but sometimes occurred at the same time (*arrowheads*). (C) Representative images of the larva recorded at the time (s) indicated. The serial images of the larva shown in (A) is shown in **Movie S2**.

Figure 6. Periodic oscillation of Ca^{2+} transients in a *gnrh2*-expressing cell in the motor ganglion. (A) Fluorescence image of a larva at 20 hpf, showing G-CaMP8 fluorescence in the motor ganglion and the anterior nerve cord. (B) The graph shows temporal patterns of fluorescence intensity at the four sites indicated by circles in (A). Colors of lines correspond to the sites indicated by circles in respective colors. Ca^{2+} spikes were periodically observed at regular intervals in the cell indicated by the magenta circle in (A) (*arrowheads*). (C) Representative images of the larva recorded at the time (s) indicated. The serial images of

the larva shown in (A) is shown in **Movie S3**.

Figure 7. Correlation between Ca^{2+} transients in *gnrh2*-expressing cells and the tail movement. (A) Fluorescence image of a larva at 19.5 hpf, showing G-CaMP8 fluorescence in the anterior nerve cord. (B) The graph shows temporal patterns of fluorescence intensity at the four sites encircled by colored lines. Colors of lines in the graph correspond to the sites encircled by lines in respective colors. Gray vertical lines indicate the period when the tail was moving. Ca^{2+} transients generally occurred when the tail movement stopped. (C) Representative images of the larva recorded at the time (s) indicated. The serial images of the larva shown in (A) is shown in **Movie S4**.

Movie S1. Serial fluorescence images showing Ca^{2+} transients in the larva shown in Figure 4.

Movie S2. Serial fluorescence images showing Ca^{2+} transients in the larva shown in Figure 5.

Movie S3. Serial fluorescence images showing Ca^{2+} transients in the larva shown in Figure 6.

Movie S4. Serial fluorescence images showing Ca^{2+} transients in the larva shown in Figure 7.

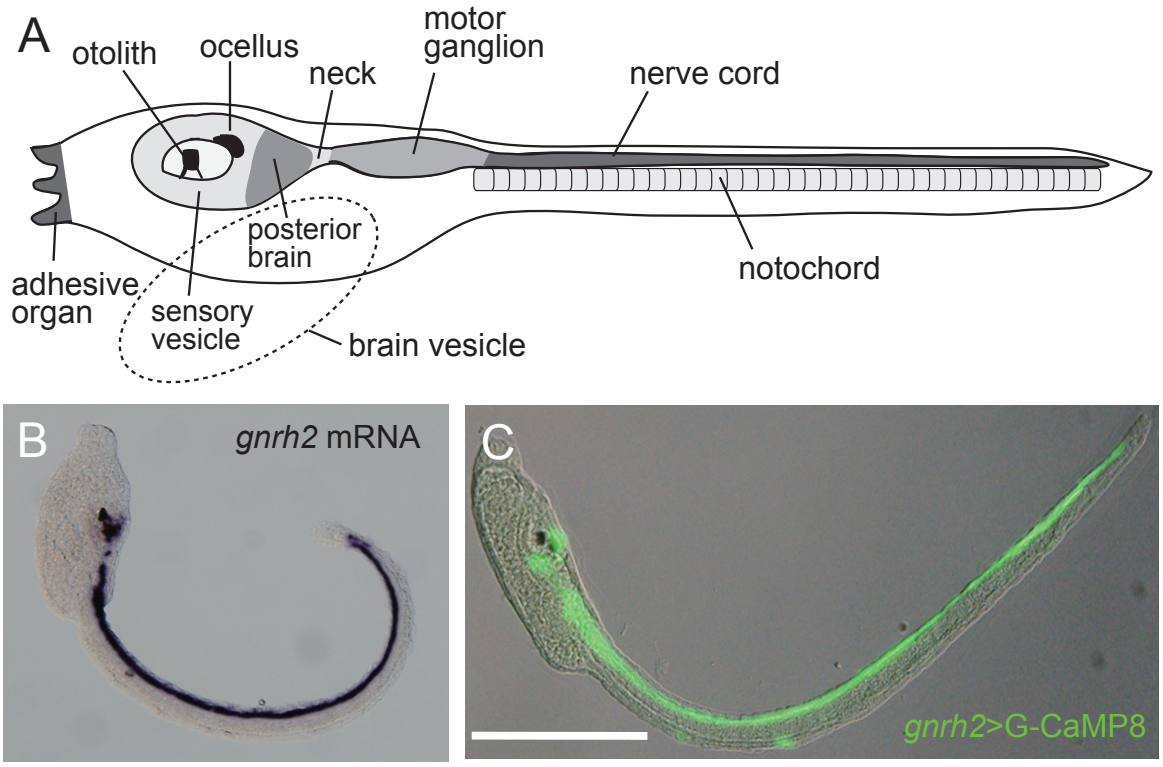
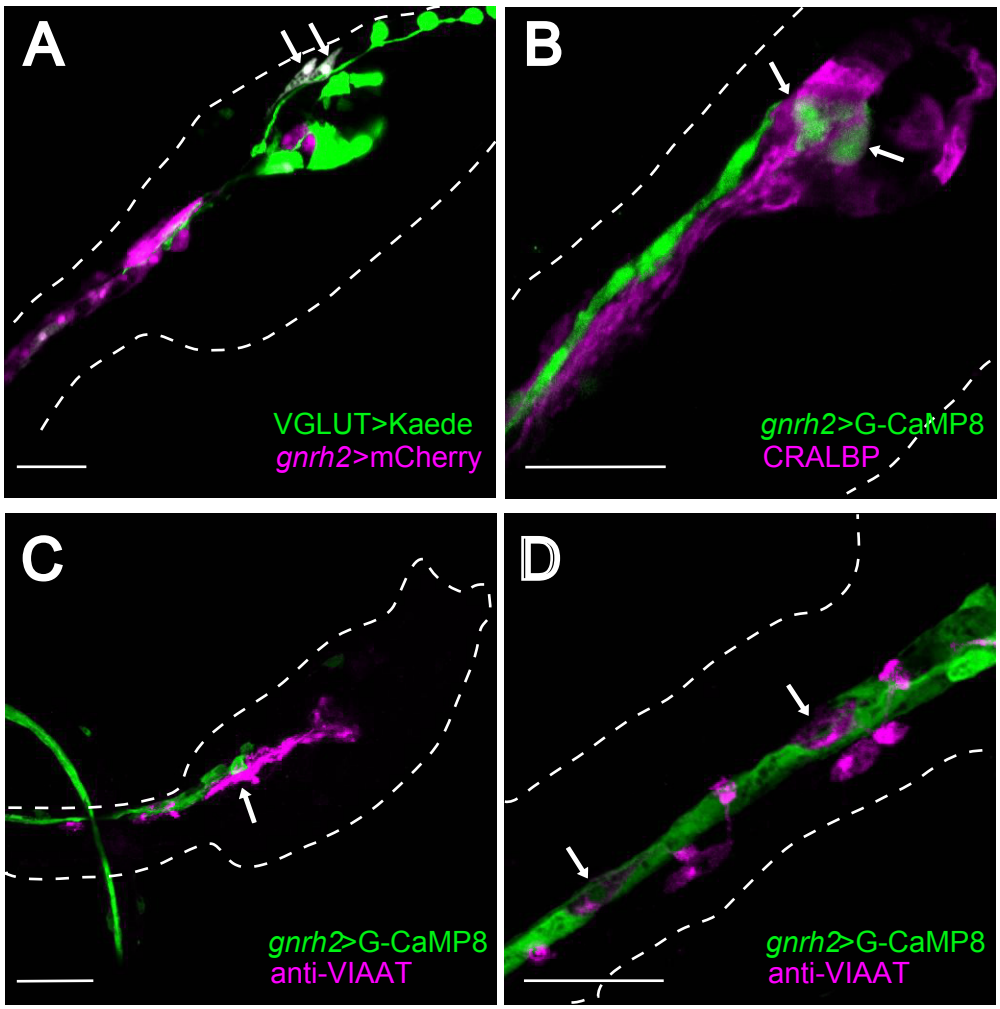

A
otolith
ocellus
neck
motor
ganglion
nerve cord
posterior
brain
notochord
adhesive
organ
sensory
vesicle
brain vesicle
B
gnrh2 mRNA
C
gnrh2>G-CaMP8

Figure 1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gnrh2>mCherry
B
gnrh2>G-CaMP8
CRALBP
C
gnrh2>G-CaMP8
anti-VIAAT
D
gnrh2>G-CaMP8
anti-VIAAT

Figure 2

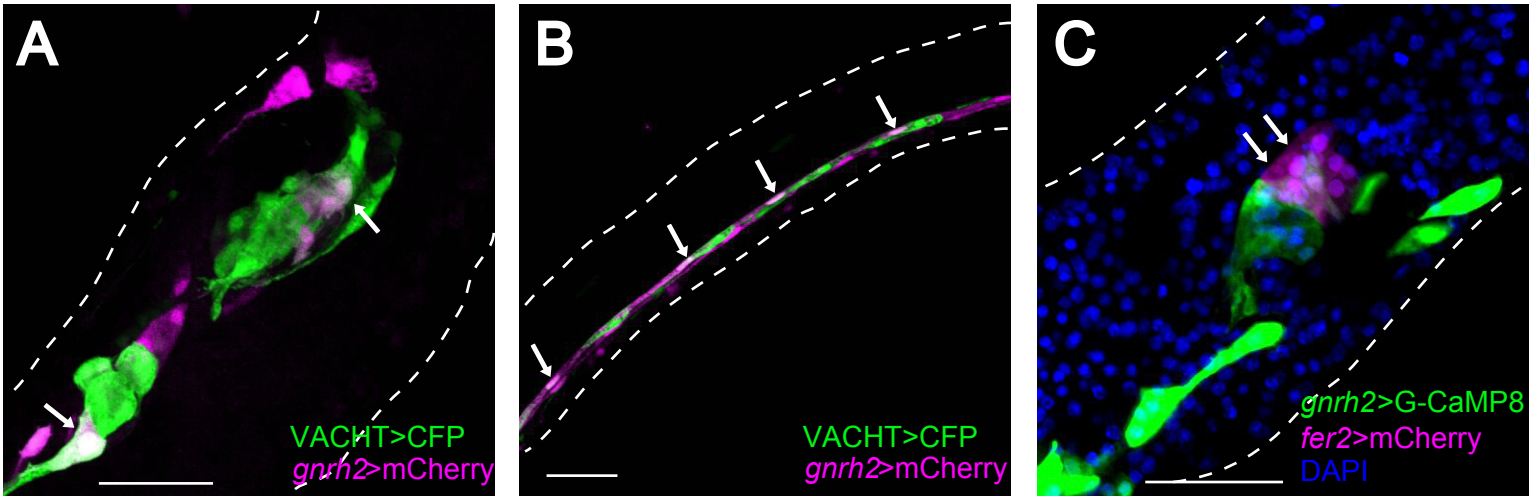

A
VACHT>CFP
gnrh2>mCherry
B
VACHT>CFP
gnrh2>mCherry
C
gnrh2>G-CaMP8
fer2>mCherry
DAPI

Figure 3

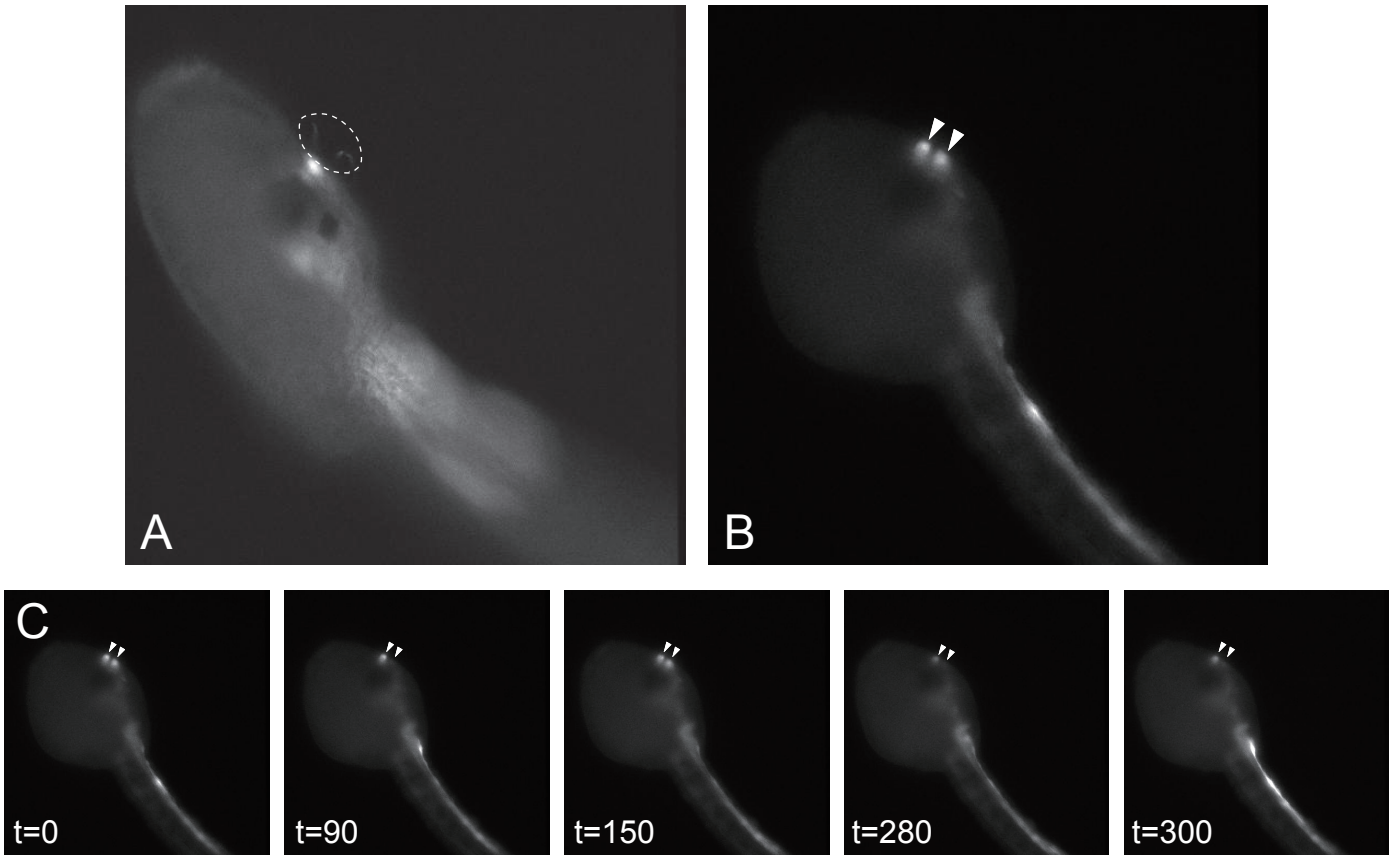


Figure 4

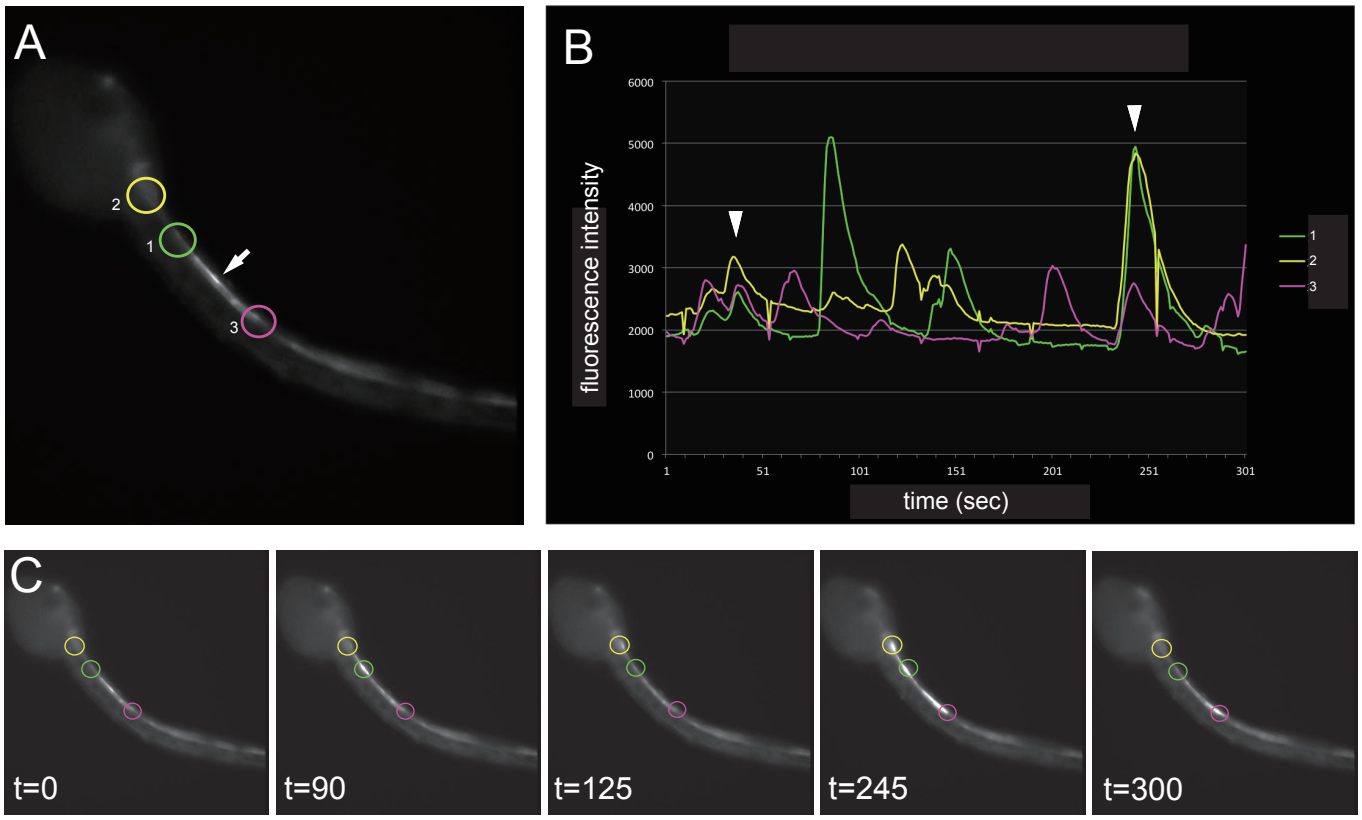
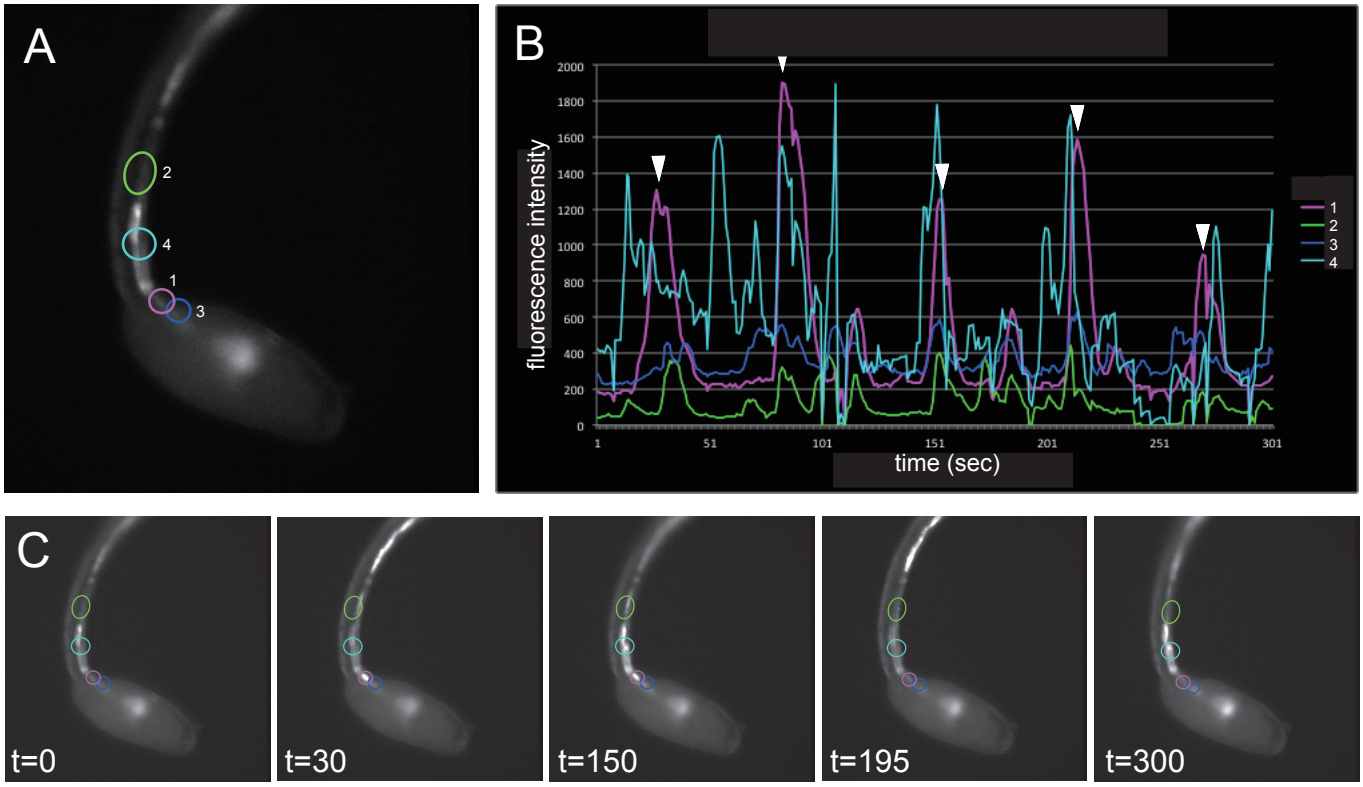

A
2
1
3
B
fluorescence intensity
6000
5000
4000
3000
2000
1000
0
1
51
101
151
201
251
301
time (sec)
1
2
3
C
t=0
t=90
t=125
t=245
t=300

Figure 5


A
2
4
1
3
B
fluorescence intensity
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
51
101
151
201
251
301
time (sec)
1
2
3
4
C
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t=300

Figure 6

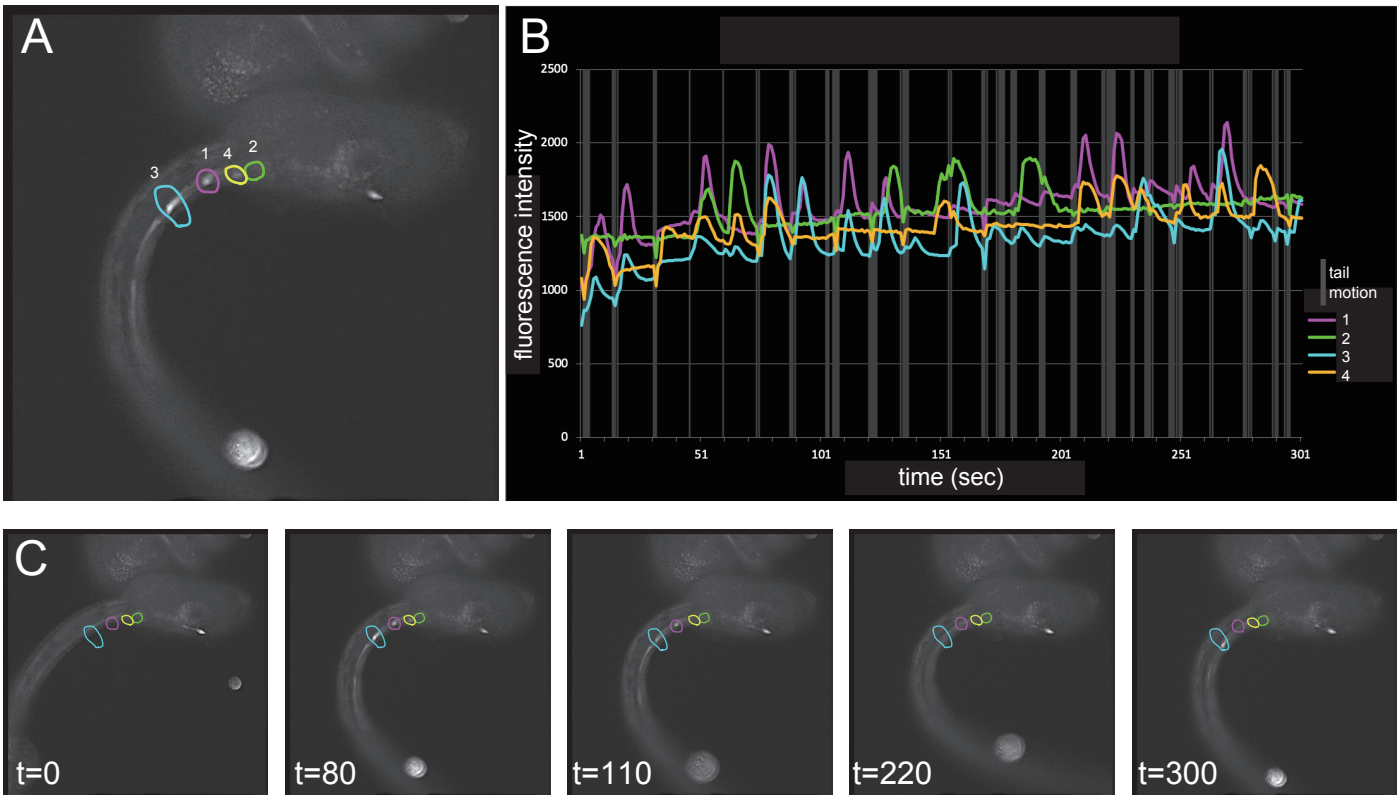
A
1
4
2
3
B
fluorescence intensity
2500
2000
1500
1000
500
0
1
51
101
151
201
251
301
time (sec)
tail motion
1
2
3
4
C
t=0
t=80
t=110
t=220
t=300

Figure 7