

RESEARCH ARTICLE

Development and circuitry of the tunicate larval Motor Ganglion, a putative hindbrain/spinal cord homolog

Katarzyna M. Piekarz | Alberto Stolfi

School of Biological Sciences, Georgia
Institute of Technology, Atlanta, Georgia, USA

Correspondence

Alberto Stolfi, School of Biological Sciences,
Georgia Institute of Technology, Atlanta,
Georgia, USA.
Email: alberto.stolfi@biosci.gatech.edu

Funding information

National Institutes of Health, National
Institute of General Medical Sciences,
Grant/Award Number: GM143326; National
Science Foundation, Division of Integrative
Organismal Systems, Grant/Award Number:
1940743

Abstract

The Motor Ganglion (MG) is a small collection of neurons that control the swimming movements of the tunicate tadpole larva. Situated at the base of the tail, molecular and functional comparisons suggest that may be a homolog of the spinal cord and/or hindbrain ("rhombospinal" region) of vertebrates. Here we review the most current knowledge of the development, connectivity, functions, and unique identities of the neurons that comprise the MG, drawn mostly from studies in *Ciona* spp. The simple cell lineages, minimal cellular composition, and comprehensively mapped "connectome" of the *Ciona* MG all make this an excellent model for studying the development and physiology of motor control in aquatic larvae.

KEYWORDS

Central Pattern Generator, chordates, *Ciona*, connectome, Motor Ganglion, tunicates

1 | INTRODUCTION

Tunicates are marine invertebrates and the sister group to the vertebrates (Delsuc et al., 2006). They have evolved diverse life histories and body plans that are quite divergent from those of other chordates (Lemaire, 2011). Most tunicates are traditionally classified in the paraphyletic "class" Ascidiacea (ascidians), defined by a "biphasic" life cycle that alternates between a swimming, tadpole-like larva and a sessile, filter-feeding adult. There are a few exceptions, like species with immotile larvae that have evolutionarily lost the ability to swim (Fodor et al., 2021), or species that undergo "maximal direct development" that bypasses any recognizable larval stage (e.g., *Polycarpa tinctor*) (Millar, 1962).

Unlike the larvae of many other marine organisms, ascidian tunicate larvae do not feed. They have only a short period of time (ranging from a few minutes to several hours) to find a place to settle and undergo metamorphosis. This process of species dispersal involves swimming through the water column, guided by various environmental cues (Athira et al., 2022; Bone, 1992; Bostwick et al., 2019; Mast, 1921; Salas et al., 2018; Zega et al., 2006). Underlying these simple behaviors is a simple nervous system. In the laboratory model *Ciona intestinalis*, this nervous system is made of 177 central nervous system (CNS) neurons

and ~50 peripheral sensory cells (Ryan et al., 2016, 2018). The larvae of other solitary ascidian tunicates have a CNS of similar size and organization as *Ciona*, though colonial species have much larger larvae with a mostly uncharacterized nervous system (Berrill, 1947, 1948a, 1948b).

In *Ciona*, a particular group of CNS neurons currently known as the Motor Ganglion (MG) represents the most well-studied structure of the larval nervous system. Previously known as the "Visceral Ganglion" or "Trunk Ganglion," the MG is formed by a core network of 7–8 bilateral pairs of neurons that includes the two pairs of motor neurons (MNs) that form the bulk of the synaptic input onto the tail muscles (Ryan et al., 2016). Evidence suggests that the MG is a Central Pattern Generator (CPG) that is sufficient to drive the tail muscle contractions necessary for swimming (Akahoshi et al., 2021; Hara et al., 2022), modulated by various sensory inputs that converge onto it (Borba et al., 2021; Bostwick et al., 2019; Kourakis et al., 2019). It has been proposed as a homolog of spinal cord, hindbrain, or both (as a "rhombospinal" structure) (Ikuta & Saiga, 2007; Meinertzhagen & Okamura, 2001; Meinertzhagen et al., 2004). Given the cellular simplicity of the tunicate larva and the unique phylogenetic position of tunicates as the sister group to vertebrates, the MG has emerged as an attractive model in which to study chordate-specific features of motor circuit development and function.

Here we describe the most recent advances in understanding the development and function of the MG of solitary tunicate larvae, focused mainly on the most heavily studied genus, *Ciona*. First we review the recent studies that have revealed MG neuron function and connectivity. Then we summarize what is known about MG development, including the cell lineages and gene networks that are important for the specification and differentiation of neurons in this structure. We will not revisit the evolutionary theories proposing homology to various vertebrate or invertebrate CNS compartments, which have been discussed elsewhere (Borba et al., 2021; Ikuta & Saiga, 2007; Meinertzhagen & Okamura, 2001; Meinertzhagen et al., 2004). Likewise, we focus exclusively on the MG, as excellent reviews of the larval nervous system have recently been published (Hudson, 2016; Nishino, 2018; Olivo et al., 2021).

1.1 | MG cell composition

The neurons in the MG can be broadly divided into two main types: MNs (or motoneurons) and interneurons. MNs are responsible for directly exciting the tail muscles through neuromuscular synapses and controlling their contraction. Interneurons, on the other hand, act as intermediaries between the MNs and other CNS compartments or sensory neurons, as well as allowing for connections between neurons within the MG. This way, the MG is able to integrate sensory inputs with motor outputs to ensure that tail movements are appropriately timed, coordinated, and modulated.

The cellular composition of the *Ciona* MG (Figure 1a) is relatively simple compared to similar compartments in more complex nervous systems. The most detailed description of the MG comes from the *C. intestinalis* larval connectome, only the second full connectome ever

documented (Ryan et al., 2016, 2018). According to the connectome, the MG consists of: MNs, interneurons (MGINs), and a pair of descending, decussating neurons (ddNs). These names have now supplanted earlier names based on the cell lineage nomenclature system of Conklin (Conklin, 1905c). Interspersed among these are ependymal-like cells that line the neural tube and might be the progenitors of the adult nervous system (Horie et al., 2011). Located a bit more posterior than the rest of the MG are ascending contralateral (sometimes “commissural” or “caudal”) inhibitory neurons (ACINs), while a group of ascending MG peripheral interneurons (AMGNs), previously called “contrapelo cells,” are situated just dorsal to the core MG. Because of the more peripheral locations, ACINs and AMGNs are sometimes not considered part of the “core” MG, though they provide extensive synaptic input onto the MNs and other MG neurons. Unlike in more complex nervous system, each left/right MG neuron pair (with the possible exception of the ACINs) appears to be unique. This is based on several morphological, molecular, and synaptic studies (Imai et al., 2009; Ryan et al., 2016, 2017; Stolfi & Levine, 2011); there is little evidence to suggest the existence of MG neuron “subtypes” represented by more than a single pair of cells. Although *Ciona* development is largely (but not completely) invariant or stereotyped, the degree of variation in the cellular composition of the MG from individual to individual is currently unknown.

There are five pairs of MNs (MN1–MN5, Figure 1b). For the sake of simplicity, we will refer neurons from only one side of larva from here on, but it should be understood that these are all pairs of left/right cells. The MNs extend their axons caudally and form morphologically distinct neuromuscular synapses onto the tail muscle cells. MN1 and MN2 form the vast majority of synaptic connections onto the tail muscles, while the slightly more posterior MN3–5 pairs form far fewer and smaller neuromuscular synapses (Ryan et al., 2016).

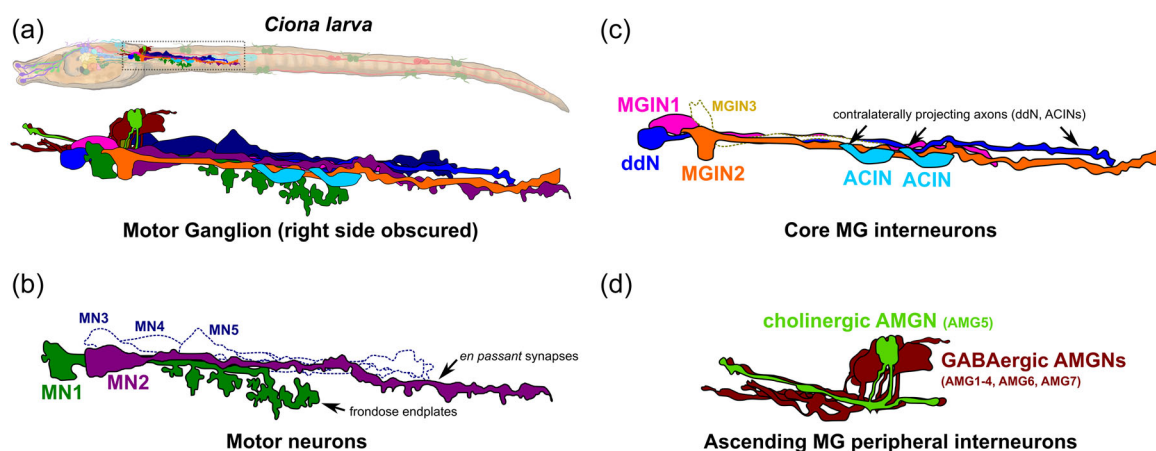


FIGURE 1 Neurons of the Motor Ganglion (MG) in *Ciona*. (a) Illustrated diagram of a *Ciona intestinalis* larva with the MG in the inset highlighted and magnified below. Neuron outlines based on the connectome reconstructions from Ryan et al., 2016, 2017, 2018 and Ryan & Meinertzhagen, 2019. Only neurons of the left side and the midline (AMG neurons) are shown. Larva artwork by Lindsey Leigh. (b) Motor neurons of the MG. Only neurons of the left side shown, even though all occur in left/right pairs. (c) Interneurons of the “core” MG including the ddN, MGIN1–3, and ACINs. Only neurons of the left side are shown, even though ddN and ACIN axons cross the midline. (d) AMG neurons including GABAergic AMG neurons (AMG1–4, AMG6, and AMG7) and the sole cholinergic AMG neuron (AMG5). MN3–5 and MGIN3 represented by dashed outlines due to lack of characterization outside the connectome studies. ddNs, descending decussating neurons.

However, as explained further below (see “Motor Ganglion cell lineages”), MN3-5 have not been observed in any studies apart from the connectome. Older reports of five or six pairs of MNs were based on the assumption that MGINs and ddNs were also MNs, a notion that was finally dispelled by the connectome studies.

The ddNs (formerly known as the A12.239 pair of neurons) are the most anterior pair of neurons still considered to be part of the MG (Figure 1c). Due to their characteristic contralateral projections and connectivity, it was suggested that the ddNs are homologous to giant reticulospinal Mauthner cells (M-cells) of fish and amphibians (Ryan et al., 2017; Takamura et al., 2010). In zebrafish, M-cells mediate the startle response in fish, though the function of ddNs in *Ciona* has not been ascertained. Other interneurons present in the core MG include three excitatory interneuron pairs in the “core” MG (MGIN1-3, Figure 1c). Of these, the most well-characterized is MGIN2, previously known as the A11.117 neuron (Stolfi & Levine, 2011). MGIN1 likely represents the neuron previously characterized as A13.474 (Imai et al., 2009; Stolfi & Levine, 2011), while nothing else is known about MGIN3. There are also two pairs of decussating, inhibitory ACINs (Figure 1c). Although the connectome only reported three total ACINs, this is likely to be a developmental anomaly or delay. Previous *in situ* messenger RNA (mRNA) hybridization, immunostaining, and reporter plasmid electroporations have consistently labeled two left/right pairs of ACINs (Horie et al., 2010; Kourakis et al., 2019; Nishino et al., 2010; Nishitsuji et al., 2012). Finally, there are seven AMGNs (AMG1-7). Unlike the other neurons, AMGNs do not occur in left/right pairs, but as single cells situated right on the dorsal midline (Figure 1d). Of the seven AMGNs, only AMG5 appears to be excitatory (cholinergic) and the rest inhibitory (GABAergic), based on mRNA *in situ* hybridization and fluorescent reporter plasmid data (Kourakis et al., 2019; Popsuj & Stolfi, 2021; Takamura et al., 2010). The AMGNs exhibit unusual axons, some bifurcated and projecting either anteriorly or posteriorly (Ryan & Meinertzhagen, 2019; Ryan et al., 2018). We propose the alternate name “AMG5^{ACh}” (or “AMG5-ACh”) for AMG5 as the sole cholinergic AMG neuron, and “AMGX^{GABA}” for the remaining GABAergic AMG neurons (AMG1-4^{GABA}, AMG6^{GABA}, and AMG7^{GABA}).

Additional neurons that have been reported even more peripheral to the core MG include two unpaired posterior MG interneurons (PMGNs, also known as PMGINs) (Ryan et al., 2016), ovoid cells (Imai & Meinertzhagen, 2007), and planate/mid-tail MNs (Imai & Meinertzhagen, 2007; Ryan et al., 2016). These are all poorly characterized or are not considered part of the MG. Thus, we do not discuss them further in this review.

1.2 | The MG connectome

The connectivity of the MG has been completely described in a series of historic connectome papers (Ryan et al., 2016, 2017, 2018). The connectome was documented by serial-section transmission electron microscopy of a single larva of the species *C. intestinalis* (Type “B”), and at the time represented only the second complete connectome

reported, after that of the nematode *Caenorhabditis elegans* (White et al., 1986). Despite the overall simplicity of the MG, it displays surprisingly complex connectivity patterns (Figure 2). However, because the connectome is currently based on a single individual, it is not yet known how much this connectivity varies in different individual larvae.

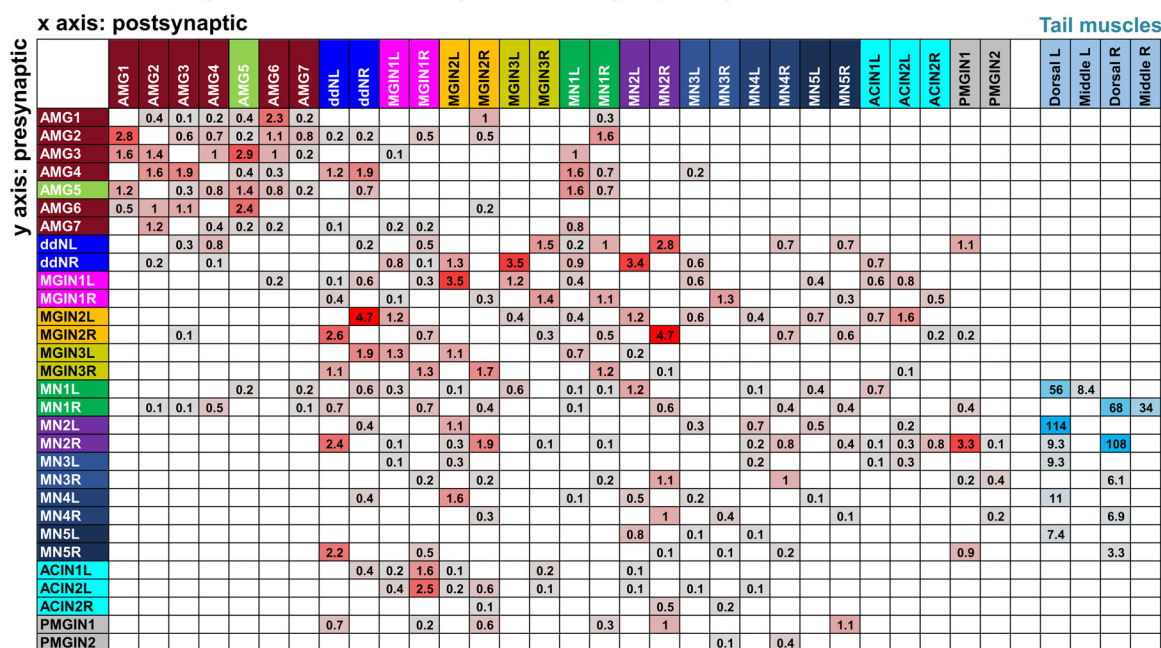
In general, MNs innervate their ipsilateral muscle cells, which in tunicate larvae are mononucleated but electrically coupled (Bone, 1992). While MNs innervate only the most dorsal or anterior muscle cells, more ventral or posterior cells are believed to be stimulated through gap junctions (Horie et al., 2010). According to the connectome (Figure 2a), MN1 (previously known as the A11.118 neuron) and MN2 (previously known as A10.57, later corrected to A10.64) form 200–360 large synapses each, comprising the vast majority of synaptic contact between the CNS and the muscles. MN2 forms the largest numbers of synapses, forming en passant along the dorsal edge of the tail muscles, while MN1 forms prominent “frondose” (i.e., “leaf-like”) endplates that contact both dorsal and middle muscles in the very anterior portion of the tail. The three remaining MNs (MN3–MN5) form 15–50 smaller synapses each.

The MG connectome is left/right asymmetric, as for example the right MN1 forms more neuromuscular synapses (onto the muscle cells of the right side) than the left MN1, while MN3–5 inputs are more heavily right-sided. Muscle innervation by the MN2 pair is more symmetrical, however. MNs and MGINs also synapse extensively onto each other (Figure 2a), and can be electrically coupled to each other by gap junctions as well (Figure 2b). Interestingly, MGIN1 is heavily coupled to MN1, while MGIN2 is coupled to MN2, suggesting that inputs onto these two different MG interneurons might stimulate muscle contractions in distinct ways. These interconnections also exhibit left/right asymmetries. Connectivity between the left and right halves is mediated mainly by the ddNs and ACINs, as predicted by their contralaterally projecting axons. Surprisingly, the inhibitory ACINs do not synapse primarily onto the contralateral MNs but rather the MGINs. Communication across the left and right halves of the MG is presumably carried out by the MGIN1 pair, which forms a large number of gap junctions between the left and right neurons. Finally, most synapses in the MG are axo-axonal, as very few *Ciona* larval neurons have dendrites or dendritic arbors. However, the precise subcellular locations of each synapse has not to, to our knowledge, been summarized and published. Such information will be crucial for precise modeling of MG neuron and circuit functions.

1.3 | Neuronal function in the MG

The *Ciona* MG must allow for a variety of unidirectional tail “flicks” and spontaneous or photosensitive swimming patterns in straight, circular, or spiral trajectories. The swimming behavior of *Ciona* was recently dissected in a detailed manner using machine vision to extract postural features, identifying six basic shapes (or “eigencio-nas”) generated during swimming (Athira et al., 2022). This advanced movement tracking of the body midline, and not just centroids,

(a) Motor Ganglion connectome (chemical synapses)



(b) (electrical synapses)

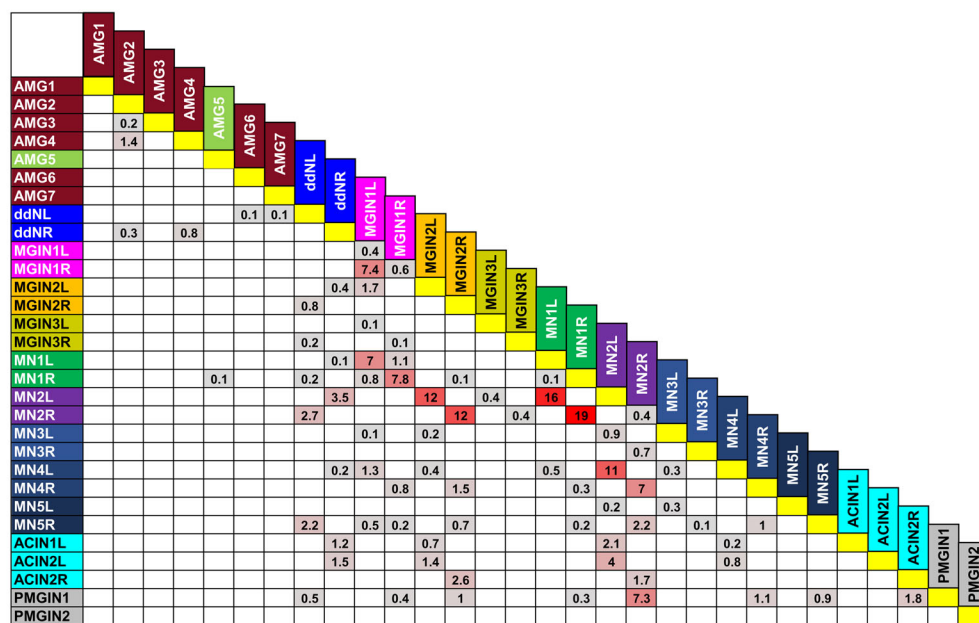


FIGURE 2 *Ciona* MG connectome tables. (a) Table of cumulative depth of chemical synaptic contact (um) measured between specific presynaptic (y axis) and postsynaptic (x axis) neurons of the MG. (b) Table of cumulative depth of gap junction contact between electrically coupled neurons in the MG. Note that MGINs and MN2R were noted as forming gap junctions with themselves. Data from Ryan et al., 2016. For bilateral cell pairs, L, left side; R, right side. Color coding: values increase from gray to red (for neuron-neuron pairs) or blue (for neuromuscular synapses). See text for neuron abbreviations. PMGIN = PMGN.

allowed the determination of those six shapes accounting for 97% of larval postures during natural swimming, and the identification of a novel startle-like maneuver. The authors even asked: might this startle-like behavior be mediated by the ddNs, which are proposed to be homologous to the M-cells that mediate startle behaviors in fish? One clue comes from their finding that the *Ciona* startle-like behavior

is suppressed by administration of serotonin, which also regulates startle behavior in fish (Pantoja et al., 2016).

CPGs underlie numerous locomotor behaviors, and the MG is no exception. A preparation of the tail, nerve cord, and MG generates alternating swimming movements (Hara et al., 2022; Nishino et al., 2010). Unlike vertebrate locomotion, the MG is not serially

repeated, and the entire tail is essentially a single motor unit. The location of the CPG was further pinpointed by careful investigation of the locomotor ability of over 200 anterior and posterior larval fragments cut at different positions. Mid-body fragments including the trunk-tail junction showed periodic bursts of tail beating of ~2.5 s, at ~20 s intervals. This is exactly where the core MG is located, including the ACINs (Ryan et al., 2016). While acetylcholine is the major excitatory neurotransmitter in the MG (Horie et al., 2010; Kourakis et al., 2019; Ohmori & Sasaki, 1977; Takamura et al., 2010), glycine appears to be the key inhibitory neurotransmitter in this CPG (Nishino et al., 2010). Loss of left-right alternation was observed upon administration of strychnine, a glycine receptor antagonist (Nishino et al., 2010), while GABA had no such effect on left-right coupling (Brown et al., 2005). The ACINs project contralaterally and are labeled by glycine immunostaining (Nishino et al., 2010), suggesting they likely mediate this left-right alternation through inhibition of the contralateral side. Although it has been proposed that ACINs might be GABAergic based on their expression of the *VGAT/Slc32a1* gene encoding a vesicular GABA/glycine transporter protein, they do not express GAD, which encodes the glutamic acid decarboxylase enzyme required for GABA biosynthesis (Zega et al., 2008).

Another interesting feature of the *Ciona* motor system is the ability to generate graded muscle contractions (Nishino et al., 2011). The nicotinic acetylcholine receptors (nAChRs) at the neuromuscular junctions in the tail are inwardly rectifying, and the muscle cells exhibit high Ca²⁺ permeability. These two traits allow the muscles to contract in a graded manner in response to graded release of acetylcholine. This Ca²⁺ permeability was shown to be due to a single amino acid residue (glutamate) in the channel pore of one of the non- α nAChR subunits, "BDGE3" (KyotoHoya gene model ID KH.C7.476). It was proposed that Ca²⁺ flux through these nAChRs, together with Ca²⁺ influx through L-type voltage-gated calcium channels, stimulates ryanodine receptors to direct calcium-induced Ca²⁺ release and allowing graded excitation-contraction coupling (Nishino et al., 2011). The presence of nAChR clusters along the dorsal muscle band, innervated primarily by MN2 axons (Nishino et al., 2011), suggests that this graded control of muscle contractions is carried out primarily by MN2, not MN1.

MN2 also plays a crucial role in generating the early tail flicks during the development of the larva (Akaoshi et al., 2021; Utsumi et al., 2023). Intracellular Ca²⁺ oscillations in MN2 begin in early- to mid-tailbud stages (st. 20–22), though no muscle contraction is detected then. At late tailbud (st. 23), MN2 activity begins to coincide with Ca²⁺ levels in the ipsilateral tail muscle, which is when tail flicks are first observed. These tail flicks were abolished by photoablation of MN2, while optogenetic stimulation of MN2 was sufficient to trigger them (Akaoshi et al., 2021). A gradual development of synchronicity between the left and right MN2, encompassing seven distinct developmental phases, was revealed by longer-term imaging (Utsumi et al., 2023). By later larval stages (~stage 26), MN1 and MGIN2 were also active, suggesting additional MG neurons are recruited to the final swimming larva CPG (Akaoshi et al., 2021; Utsumi et al., 2023). The voltage-gated sodium channel NaV1

(encoded by the *Scna.a* gene) is expressed in the ddN and MN1 (Gibboney et al., 2020). Furthermore, tunicate NaV1 proteins have the chordate-specific "anchor" motif that allows for enrichment of these channels at the axon initial segment, via association with Ankyrin and Spectrin (Hill et al., 2008; Nishino & Okamura, 2018). This suggests that ddNs and MN1 are capable of firing action potentials that may be indispensable for the fully mature swimming CPG.

1.4 | Modulation of MG function by sensory inputs

Although the MG likely constitutes a CPG for tail flicks and rhythmic swimming, it is heavily modulated by sensory inputs that limit the activity of spontaneous swimming or initiate different swimming movements (Zega et al., 2006). This modulation is likely a result of the extensive synaptic inputs onto MG neurons from other CNS compartments or peripheral nervous system (PNS) networks (Ryan et al., 2016, 2018). While acetylcholine and glutamate provide excitatory inputs from the brain (Horie et al., 2008; Kourakis et al., 2019; Yoshida et al., 2004), GABA was identified as an important inhibitory modulator of swimming, as the administration of GABA decreased swimming periods (Brown et al., 2005). In contrast, picrotoxin (a GABA receptor antagonist) increased the frequency and duration of swimming and electrical activity in the tail (Brown et al., 2005; Kourakis et al., 2019). GABA immunoreactivity is observed prominently in the brain, in the bipolar tail neurons (BTNs) in the middle of the tail, and in the GABAergic AMG neurons (Brown et al., 2005; Kourakis et al., 2019), while distinct GABA receptor genes are expressed in different brain and MG neurons (Gibboney et al., 2020; Zega et al., 2008).

In mutant ("*frimousse*") larvae missing the anterior sensory vesicle brain region, spontaneous swimming is increased (Kourakis et al., 2019). A single oscillatory, GABAergic neuron in the anterior sensory vesicle region was recently identified as modulating spontaneous "casting" swims that are an important component of the negative phototaxis behavior of *Ciona* larvae (Chung et al., 2023). Ablation of this neuron (identified in the connectome as the Coronet-associated Brain Vesicle Intrinsic Neuron 78, or cor-assBVIN78) phenocopied the long swimming periods observed in some *frimousse* mutant larvae in the absence of light cues. According to the connectome, this neuron synapses onto 5 of the 6 Photoreceptor Relay Neurons (prRNs) of the posterior sensory vesicle brain region, which in turn connect to the MG (Ryan et al., 2016).

Which specific connections mediate the different sensory inputs onto the MG? It was shown that relay interneurons in the brain likely mediate different light-sensitive behaviors (Figure 3a) (Kourakis et al., 2019). However, there are no clearly delineated interneuron subtypes using specific neurotransmitters dedicated to these different tasks. For instance, different classes of brain interneurons such as the prRNs or Photoreceptor-AMG Relay Neurons (pr-AMGRNs) are a mix of cholinergic and GABAergic subtypes, connecting inputs from different groups of photoreceptors to mostly MGIN1 and MGIN2

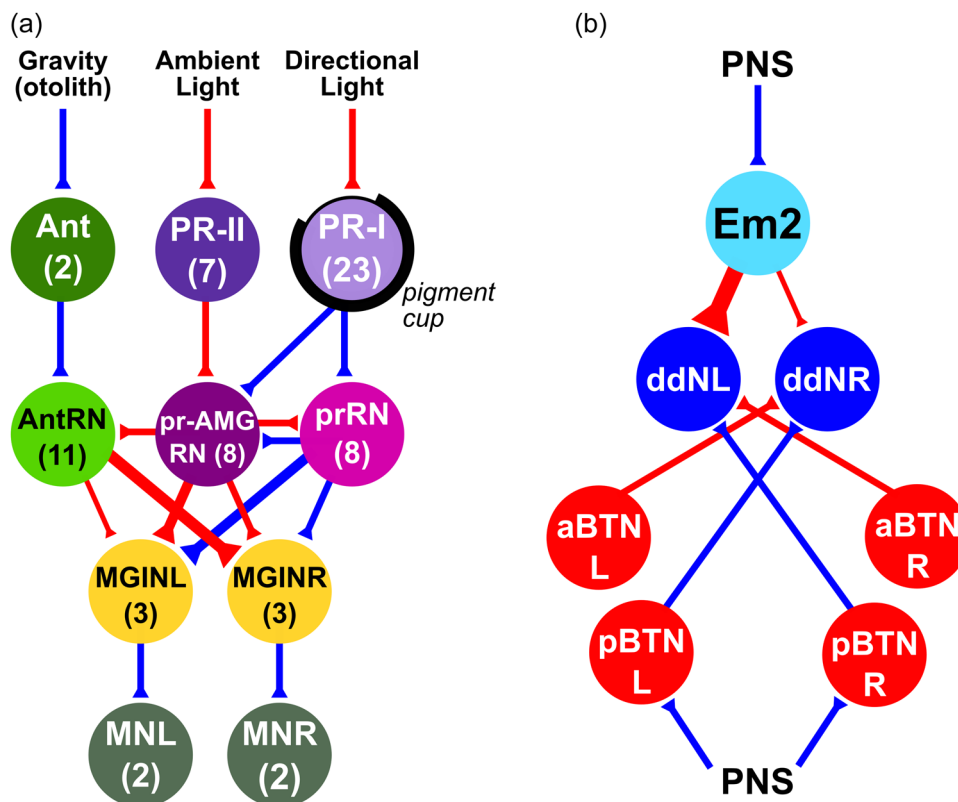


FIGURE 3 Major sensory inputs into the MG. (a) Simplified connectivity diagram depicting the light- and gravity-dependent synaptic inputs into the MG, based on work from Bostwick et al., 2019; Kourakis et al., 2019, and Borba et al., 2021. Number of total cells in each category shown in parentheses. *Ciona* photoreceptors are likely hyperpolarized by light, as in vertebrates (see Kourakis et al., 2019). (b) Simplified diagram of peripheral nervous system (PNS, e.g., RTENS, ATENS, PTENS, and DCENS) relay inputs onto the ddNs, based on Ryan et al., 2017 and Ryan et al., 2018. PNS neurons may mediate mechanosensory and/or chemosensory inputs. For simplicity, AMGNs, which also relay PNS inputs to the ddNs and other MG neurons, are not shown. For both subpanels, blue lines depict excitatory inputs, red lines depict inhibitory inputs, and left(L)/right(R) asymmetric inputs are shown through differential thickness of lines. aBTN, Anterior Bipolar Tail Neurons (putatively GABAergic); Ant, Antenna Cells; AntRN, Antenna Relay Neurons; ddN, Descending Decussating Neurons; Em2, Eminens Cell 2; PR-I, Type I photoreceptors; PR-II, Type II photoreceptors; pr-AMGRN, Photoreceptor-AMGN Relay Neurons; prRN, Photoreceptor Relay Neurons; MGIN, MG interneurons (MGIN1-3); MN, Motor neurons (MN1-5); pBTN, Posterior Bipolar Tail Neurons (putatively cholinergic). Putative aBTN/pBTN neurotransmitter type distinction based on GAD/VACHT reporter plasmid labeling in Kim et al. 2020. See text for additional details. VACHT, vesicular acetylcholine transporter.

(Borba et al., 2021; Kourakis et al., 2019). These connections mediate negative directional phototaxis or photosensitive tortuous escape behaviors ("looming shadow" response). The former occurs downstream of Type I photoreceptors that detect the directionality of light thanks to a "shield" of melanin from the ocellus pigment cup (Figure 3a). The latter is downstream of Type II photoreceptors that are not shielded by pigment and thus can detect changes in ambient light (Kawakami et al., 2002; Mast, 1921; Salas et al., 2018; Young & Chia, 1985). Interestingly, pr-AMGRNs synapse mostly onto MGIN1, and much less onto MGIN2 (Ryan et al., 2016). Additionally, there is left/right asymmetry in the inputs to the MGINs, which could drive the asymmetric, graded tail contractions needed for tortuous swimming or turning away from a source of light. Although similar neuron types and neurotransmitters are used in both circuits, their different connectivity architectures are predicted to ultimately result in distinct patterns of MN activity, resulting in the different swimming behaviors (Borba et al., 2021; Kourakis et al., 2019).

Finally, monoamines from the brain have also been shown to modulate the shadow response, suggesting additional neuromodulation of these circuits by a broader set of neurotransmitter systems (Borba et al., 2021; Razy-Krajka et al., 2012).

Overlaid onto the looming shadow circuit is a specialized gravitaxis circuit (Figure 3a). Up-facing larvae swim upwards in response to a decrease in ambient light, while down-facing larvae reorient themselves until they face upwards (Bostwick et al., 2019). It was proposed that asymmetric inputs from gravity-sensing circuits onto the MG results in left/right asymmetric tail movements that help turn larvae upright. However, this circuit is only disinhibited by the pr-AMGRNs of the looming shadow circuit upon sensing a decrease in ambient light, thus triggering reorientation and/or upwards swimming when the larva senses a shadow. It is likely that this behavior is essential for *Ciona* larvae to settle specifically on shaded undersides (like outcroppings or ship hulls), which they tend to prefer (Jiang et al., 2005). Presumably this circuit may be absent/altered in

larvae of species that do not prefer to settle upside down in shaded areas. One example is *Molgula occidentalis*, which produce swimming larvae that prefer to settle in sediment (Young, 1989).

While these light and gravity circuits seem to connect to the MG mostly via MGIN1 and MGIN2, the ddNs conspicuously lack synaptic inputs from these circuits (Ryan et al., 2016, 2017, 2018). Instead, ddNs receive inputs mostly from the PNS (Figure 3b). These inputs are downstream from putative mechano/chemosensory neurons embedded in the epidermis of head and tail, which contact either the external environment or the extracellular tunic that encases most of the larva (Terakubo et al., 2010; Yokoyama et al., 2014). While these putative mechano/chemosensory PNS inputs do not go to the MG exclusively through the ddNs (a substantial number of PNS inputs also converge onto the cholinergic AMG5 neuron, for instance), this suggests some degree of compartmentalization of inputs from distinct sensory modalities. Interestingly, although it has been proposed that the ddNs are homologous to M-cells and might be similarly involved in startle reflexes, the lack of obvious directionality in their PNS inputs suggests that their escape behavior may not be directional, but rather stochastic (Ryan et al., 2017).

1.5 | MG cell lineages

The embryos of *Ciona* and other solitary species develop according to extremely conserved (almost identical), invariant cell lineages (Conklin, 1905c; Hotta et al., 2020; Lemaire, 2009). Many fundamental discoveries about developmental biology have been made possible

thanks to these invariant lineages (Conklin, 1905a; 1905b; Satoh, 2013). More recently, transcriptomic and gene regulatory network (GRN) data in *Ciona* (mostly in *C. robusta*) have been overlaid onto these lineages, allowing us to understand larval neurodevelopment at single-cell resolution (Cao et al., 2019; Imai et al., 2006). The “core” MG (excluding the AMGNs and the still unidentified MGIN3 and MN3-5 cells) is derived from a single pair of vegetal pole-derived blastomeres at the 64-cell stage, named the A7.8 pair of cells according to the named according to the cell lineage nomenclature of Conklin (Figure 4). The A7.8 pair gives rise ultimately to the neural plate blastomeres that give rise to much of the CNS, including the core MG.

The invariant positions and unique morphologies of each MG neuron subtype has made it rather straightforward to reconcile cell lineages with the final connectome. Originally, the neurons of the core MG were thought to come from just two blastomeres of the neural plate: A9.30 and A9.29, the latter giving rise to MN2 and ACINs (Nishitsui et al., 2012). More recently, it was shown that MN2 is actually specified from the A9.32 blastomere, which also gives to ependymal cells in the tail (Navarrete & Levine, 2016). Therefore, in older papers the identify of MN2 is given as A10.57, but in fact it should be A10.64, as per the Conklin lineage nomenclature. The reason for the previous assumption is that MN2 is adjacent to the descendents of the A9.30 lineage at the larval stage, while the rest of the A9.32 lineage is further back in the tail. Additionally, the sister cell of A9.32 is A9.31, which gives rise to muscle cells at the very posterior tip of the tail (Hudson & Yasuo, 2008). It turns out that MN2 cells migrate anteriorly, “leapfrogging” the A9.29 lineage in the

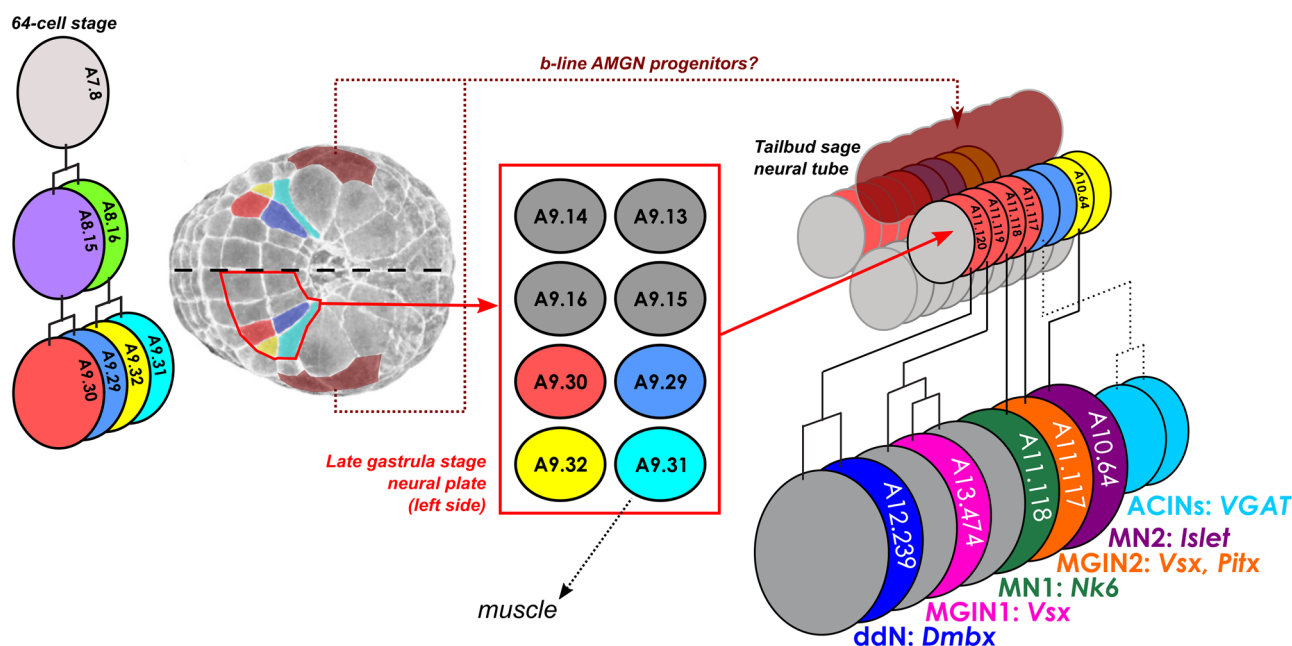


FIGURE 4 The invariant cell lineages of the Motor Ganglion in *Ciona*. Known or suspected cell lineages of the MG in *Ciona*. Cell identities on only one side of the embryo indicated, even though the embryo is bilaterally symmetric. Dashed lines indicate lineages that are not completely characterized. See text for details and references. Late gastrula image adapted from FABA/Tunicanato (Hotta et al., 2020). MG neuron subtype-specific reporters indicated next to each cell, based on Stolfi & Levine, 2011 and Yoshida et al., 2004.

process (Navarrete & Levine, 2016). Thus, ACINs come from A9.29 (Nishitsuji et al., 2012), MN2 also comes from A9.32 (Navarrete & Levine, 2016), and the remaining core MG neurons come from A9.30 (Stolfi & Levine, 2011).

In contrast to the vegetally-derived core MG, the AMGNs likely derive from animal pole lineages. This would be consistent with their more dorsal position above the core MG, as the dorsal cells of the neural tube (i.e., roofplate) are derived from the animal pole (Cole & Meinertzhagen, 2004; Nicol & Meinertzhagen, 1991). The sole cholinergic cell here, AMG5, was shown to come from *Msx*⁺ cells, likely corresponding to the animal pole-derived b8.19 lineage (Popsuj & Stolfi, 2021). However, because there is only one AMG5 cell per larva, it is unclear whether it is invariantly born from the left or right side of the embryo.

A current understanding of lineages of the MG is shown in Figure 4. However, some discrepancies or gaps remain:

- (1) The precise lineage and mitotic history of the AMGNs has not been studied. Only AMG5 has been shown to come from animal pole lineages, and presumably the other AMGNs likely have a common or similar developmental origin, but no definitive lineage has been documented for these cells. In fact, because all AMGNs occur as single cells (not pairs), they are likely specified after intercalating to form a single row of cells in the dorsal neural tube. This may be similar to how the ocellus/otolith cell fate choice is determined after intercalation of an otherwise equivalent left/right pair of pigment cell precursors in the dorsal row of the neural tube; the more posterior cell being specified as the ocellus due to contact with a posterior Wnt7 signal (Abitua et al., 2012).
- (2) The identity of the MGIN3 pair of interneurons has not been ascertained, neither by cell lineage studies nor by fluorescence microscopy. It is only known through its description in the connectome studies. Given its more dorsal position, it is possible that MGIN3 comes from an animal pole lineage, like the AMGNs.
- (3) The connectome reported the existence of “minor” MNs MN3-5 on either side of the tail that form relatively few synapses with the muscles. These minor MNs have never been observed by fluorescence microscopy nor by *in situ* hybridization of common MN markers. There may be a few explanations for this: (a) these neurons are molecularly distinct from MN1/MN2 and therefore we have simply not found markers/reporters to label them; (b) these neurons exist in *Ciona intestinalis* Type B, which was used for the connectome, but not in *Ciona robusta* (*intestinalis* Type A), which is the species primarily used for developmental studies; (c) the connectome was assembled from one individual that may have had an aberrant number of MNs specified. Further studies will be required to resolve these questions.

1.6 | Cell type specification in the MG

The invariant cell lineages of *Ciona* have allowed for a detailed understanding of MG neuron specification. The expression patterns

of regulatory genes (mostly transcription factors and signaling molecules) have been described at single-cell resolution over the course of MG development (Ikuta & Saiga, 2007; Imai et al., 2009). Furthermore, morpholino-knockdown of these regulatory genes have generated provisional GRN diagrams for each MG precursor cell and neuron subtype (Imai et al., 2009), connecting to the earlier GRN “blueprint” of the whole embryo (Imai et al., 2006).

Additional work has revealed the cell signaling events that result in proper specification of different MG precursors and differentiated neuron. At the late gastrula stage, Nodal, Delta/Notch, and FGF/MEK/ERK signaling pathways pattern the grid-like neural plate to precisely and invariantly specify the identity of each blastomere, including those that give rise to the core MG (A9.29, A9.30, and A9.32) (Hudson et al., 2007). During neural tube closure, the descendants of these blastomeres form the lateral rows of the neural tube posterior to the sensory vesicle of the head. In *Ciona*, the diminutive neural tube is formed by exact four anterior-posterior, single-file rows of cells. Thus, all core MG neurons are specified from the lateral rows of the neural tube, while the AMGNs, located dorsally relative to the core MG, are likely derived from the dorsal row, or roofplate. However, this simple tube likely evolved from a larger, more complex one like that of cephalochordates.

In spite of a reduced neurogenic domain, the developing MG features complex patterning along the anterior–posterior (A–P) axis, as evidenced by overlapping expression domains of conserved transcription factors (Imai et al., 2009; Stolfi, Wagner, Taliaferro, et al., 2011). However, the BMP and Shh morphogen gradients that pattern the vertebrate neural tube do not appear to function this way in *Ciona*, probably due to this highly diminutive neural tube layout (Hudson et al., 2011). So how is the MG patterned? One recurring “theme” of solitary ascidian development is that cell–cell contacts determine most cell fate inductive events, with little evidence for any morphogens that act at distances greater than a single cell diameter (Guignard et al., 2020). The MG is no exception, as it was shown that cell–cell Ephrin/Eph, FGF/MEK/ERK, and Delta/Notch signaling events set up the molecular diversity of the neurons in the A9.30 lineage (Stolfi, Wagner, Taliaferro, et al., 2011). The same is likely true for the A9.29 and A9.32 lineages, though this remains to be definitively shown.

1.7 | MG neuron differentiation

After cell–cell contacts pattern the lateral rows of the neural tube into transcriptionally distinct MG neuronal precursors, there is still the matter of mitotic exit and differentiation. Most core MG neurons (with the exception of the ACINs) are cholinergic, based on their expression of the cholinergic locus encoding both Choline acetyltransferase (ChAT) and Vesicular acetylcholine transporter (VACHT) (Horie et al., 2010; Kourakis et al., 2019). It has been shown that the conserved transcription factor Ebf (also known as Collier/Olf/EBF, or COE) is required for cholinergic MG neuron differentiation (Kratsios et al., 2012). Furthermore, Ebf is required to directly activate the

ChAT/VACHT locus in AMG5, which is the only cholinergic AMG neuron (Popsuj & Stolfi, 2021). *Ebf* expression in the MG in turn depends on Neurogenin (Imai et al., 2009), a proneural bHLH which is activated in A9.30 and A9.30 by Delta/Notch signaling (Hudson et al., 2007; Imai et al., 2009), thus directly linking neurogenic potential to the patterning of the neural plate blastomeres. Neurogenin appears to be the main proneural regulator in the MG and rest of the CNS, in contrast to Achaete-Scute and/or Atonal in the PNS (Tang et al., 2013).

As each MG neuron pair is morphologically distinct, there are certainly neuronal subtype-specific differentiation programs deployed in parallel to more pan-MG ones. Less obvious than morphology, but just as important, are subtype-specific differences in electrophysiological properties, neurotransmitter receptor expression, neuropeptide repertoire, and other differences of developmental and functional importance. Transcriptome profile comparisons revealed substantial differences in gene expression between the ddN and MGIN2 that might underlie such properties (Gibboney et al., 2020). It is not yet known exactly how these different transcriptional profiles are established, but they undoubtedly involve the myriad transcription factors expressed in each neuron in different combinations (Imai et al., 2009). With the advent of single-cell RNA sequencing, it is now possible to analyze and compare transcriptome-wide differences between each individual cell in the MG at each stage of development (Cao et al., 2019).

1.8 | Evolution of the MG in tunicates

Although much of recent work on the MG has been in *Ciona*, it was not among the first species in which the MG was characterized. Some of the earliest characterizations of ascidian tunicate larvae were done without the benefit of fluorescence microscopy or even photographic equipment (Grave, 1926). In these earliest studies, the MG was often called the “visceral ganglion,” as its ventral position appeared to be associated with the viscera of the larva. Now we know that this structure does not innervate any visceral organs, and as such this is an obsolete misnomer.

In the molecular era, the earliest studies of gene expression in the MG was done in the larva of the solitary stolidobranch species *Halocynthia roretzi* (Katsuyama et al., 2005; Nagahora et al., 2000; Okada et al., 2002; Wada et al., 1995). Although the *Halocynthia* larva is almost twice as large as that of *Ciona*, its cell lineages are almost identical (Hirano & Nishida, 1997; Nishida, 1987; Taniguchi & Nishida, 2004), and those of the MG appear to be highly conserved, if not exactly the same. Even the presence of two distinct MN types (“Moto-b” likely corresponding to MN1, and “Moto-c” to MN2) and their different synaptic endplate morphologies appears to be conserved (Katsuyama et al., 2005; Nagahora et al., 2000). Work in another stolidobranch species, *Molgula occidentalis*, revealed that specification of MG neuron subtypes appears to be perfectly conserved relative to *Ciona* (Lowe & Stolfi, 2018). Even the ddNs of *M. occidentalis* project contralaterally, indicating perhaps perfect

conservation of MG circuitry over the hundreds of millions of years that separate *Ciona* and *Molgula/Halocynthia* (Delsuc et al., 2018).

And yet, exceptions exist. First, little is known about the MG in the much larger larvae of colonial species. Second, multiple species in the molgulid family have lost the motile larva and instead develop as tailless, immotile larvae instead (Fodor et al., 2021). While in some species the MG is still specified and patterned to some degree, it almost assuredly does not differentiate or function as it does in swimming, tailed species (Lowe et al., 2021). Finally, distantly related, pelagic tunicates in the class Appendicularia (also known as larvaceans) have evolutionarily lost the sessile adult phase, and retain a neotenic larval body plan through sexual maturity (Ferrández-Roldán et al., 2019; Nishino & Satoh, 2001). Appendicularians do not propel themselves forward with the same whip-like tail movements of ascidian tunicate larvae. Their movements through the water column are more precise yet quite complex (Kreneisz & Glover, 2015), and notably use the tail to capture food particles in an intricate external filter made of cellulose and glycoproteins (Fenaux, 1986; Hosp et al., 2012). While oikopleurid appendicularians inhabit their feeding filter and beat their tail to draw food-laden water through it (Kreneisz & Glover, 2015), fritillariids use their tail to inflate and deploy an external filter (Bone et al., 1979). In *Oikopleura*, these tail movements are likely controlled by a “caudal ganglion,” which is almost certainly homologous to part of the MG of ascidian tunicates, and additional MNs spread along the entire A-P axis of the tail (Cañestro et al., 2005; Søviknes et al., 2007). This more segmental-like distribution, more similar to the vertebrate spinal cord than the *Ciona* MG, likely underlies the more complex movements of the appendicularian tail (Kreneisz & Glover, 2015). In sum, there is still much work to be done to understand the full diversity of MG development and connectivity among tunicates.

2 | CONCLUSION

We have presented a case for why the MG of tunicate larvae is an intriguing model for understanding how CPG generate rhythmic movements, and how those circuits are set up within a chordate-specific developmental program. The cellular simplicity of the tunicate larva, the close genetic relationship between tunicates and vertebrates, and the first complete chordate connectome all contribute to positioning this as a potentially powerful system in which to answer these questions.

ACKNOWLEDGMENTS

We thank members of the lab and the wider tunicate neurobiology community for insightful discussions and Aakarsha Pandey for assistance in compiling references. This work is funded by NSF (IOS) grant 1940743 and NIH grant GM143326 to AS.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

There are no original data associated with this review article.

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How to cite this article: Piekarz, K. M. & Stolfi, A. (2024).

Development and circuitry of the tunicate larval Motor Ganglion, a putative hindbrain/spinal cord homolog. *Journal of Experimental Zoology Part B: Molecular and Developmental Evolution*, 342, 200–211. <https://doi.org/10.1002/jez.b.23221>