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Development of Motor Networks in Zebrafish Embryos

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ABSTRACT

General mechanisms of motor network development have often been examined in the spinal cord because of its relative simplicity when compared to higher parts of the brain. Indeed, most of our current understanding of motor pattern generation comes from work in the lower vertebrate spinal cord. Nevertheless, very little is known about the initial stages of motor network formation and the interplay between genes and electrical activity. Recent research has led to the establishment of the zebrafish as a key model system to study the genetics of neural development. The spinal cord of zebrafish is amenable to optical and electrophysiological analysis of neuronal activity even at the earliest embryonic stages when the network is immature. The combination of physiology and genetics in the same animal model should lead to insights into the basic mechanisms of motor circuit formation. This paper reviews recent work on the development of zebrafish motor activity and discusses them in the context of the current knowledge of embryonic and larval zebrafish spinal cord morphology and physiology.

INTRODUCTION

SPONTANEOUS ELECTRICAL ACTIVITY is a common feature of developing neuronal networks.[1–3] This activity is thought to refine synaptic connections by providing cues for appropriate neural wiring.[4–6] Spontaneous activity has also been proposed to affect cell fate determination,[7] and more recently, the control of neurotransmitter expression in embryonic neurons.[8,9] Developing vertebrate embryos, either in the egg or womb, undergo a period of spontaneous motor activity that is generated by the developing motor network. This spontaneous motor activity has been shown in many animal models to emanate from a central pattern generator that is independent of sensory inputs.[10–17] Perturbing this early pattern of spontaneous activity has recently been shown to affect motor axon guidance in the chick embryo[18] and disturb the assembly of spinal motor networks in the mouse.[19]

Most of what we know about the vertebrate central pattern generator (CPG) for locomotion has been learned from decades of work studying swimming in *Xenopus* larvae and lamprey.[20–23] The limited genetic and molecular tools for these animal models have restricted our understanding of motor circuit formation to the cellular level. The zebrafish has recently emerged as an important model to study the genetics of neural circuit development.[24,25] In the past decade, mutagenesis screens have yielded mutants affecting the central nervous system.[26,27] In addition, electrophysiological and imaging techniques have been developed to study the electrical activity of zebrafish embryos and larvae *in vivo*.[28–40] Previous reviews have covered the early steps in the development of the spinal cord in zebrafish such as neurogenesis,[41,42] axon guidance,[43–45] and the emergence of motor circuits.[42,46,47] This review will focus on what is currently known about the emergence and maturation of zebrafish motor behaviors and the progressive changes in the neuronal cir-

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cuits that are necessary to produce these activity patterns.

SPONTANEOUS MOTILITY AND EARLY SPINAL CORD MORPHOLOGY

Zebrafish embryos show their first motor activity at 17 h postfertilization (hpf).[48,49] This immature motor behavior consists of spontaneous repeating, alternating coils of the tail that persist over the course of several hours. The frequency of these coils in dechorionated embryos peaks at 1 Hz at 19 hpf and slowly decreases to 0.1 Hz by 26 hpf. The spontaneous coils were shown to be neural in origin by abolishing the coils with injections of nicotinic acetylcholine receptor blockers.[49,50] The substrate essential for the appearance of spontaneous coils is located completely within the spinal cord, as lesions that remove all brain structures above the spinal cord do not affect the frequency or strength of the coils.[49]

Zebrafish spontaneous motor activity appears at a developmental time when the spinal cord is immature. Indeed, the first postmitotic neurons in the zebrafish spinal cord extend their axons at 15 hpf, a mere 2 h before the appearance of motor behavior[51–53] and even by 21 hpf there are only six types of neurons bearing axons (Fig. 1A). The spinal cord of a zebrafish embryo is divided into repeating segments, or somites. At the dorsal most edge of each segment we find two–three Rohon-Beard sensory neurons, these cells are the first spinal cord neurons to extend axons in the dorsolateral fasciculus (DLF) of the spinal cord. Dorsolateral ascending (DoLA) interneurons have one–two somata per segment, are located slightly more ventrally than Rohon-Beard neurons and project their axons rostrally in the DLF. Commissural primary ascending (CoPA) interneurons have one–two somata per somite that are located dorsally and project axons first ventrally before crossing to the other side of the spinal cord and projecting rostrally in the DLF.

There are only two types of interneurons that project descending axons ipsilaterally along several somites as early as 17 hpf. First, the IC interneurons (ipsilateral caudal axon) are a population of early born neurons, with numbers ranging from one to two per segment, that span the hindbrain/spinal cord border but are not present after the sixth somite.[54,55] The other descending interneuron type is the ventral lateral descending (VeLD). VeLD cell bodies are located throughout the spinal cord with about two cells per segment.[51,52] Before 26 hpf there are only three motoneurons per side of each segment: the CaP (caudal primary), MiP (middle primary), and RoP (rostral primary) motoneurons.[56–58] The first contact between motoneurons and muscle is attained at 17 hpf. These initial contacts are coincident with the appearance of the spontaneous behavior, suggesting that the spinal circuitry may be active slightly before motoneuron–muscle contact.

These morphological observations suggest that a maximum of six cell types located wholly within the immature embryonic spinal cord are responsible for the appearance of spontaneous motor activity. These low numbers of neurons suggest that a simple neuronal circuit underlies the earliest form of behavior in zebrafish.

A GAP JUNCTION-MEDIATED IMMATURE SPINAL NETWORK

The strong rhythmic pattern of behavior observed in the embryo suggests that the electrical activity in the spinal cord should also be rhythmic in nature, although the type of electrical activity and method of propagation are unclear. In order to visualize the source of these motor patterns, *in vivo* whole-cell patch clamp recordings of spinal neuron activity were obtained from paralyzed embryos.[30,38] Cell attached recordings of spiking activity were first obtained from motoneurons.[59] The activity observed in the cell-attached configuration consists of repetitive patterns of one or more spikes (Fig. 1B). As the embryos mature, the bursts become less frequent but contain more spikes. As would be expected for a centrally generated and sensory independent behavior, the average frequency of bursting in motoneurons from paralyzed embryos matches the average frequency of the spontaneous coiling behavior observed at all ages.[59]

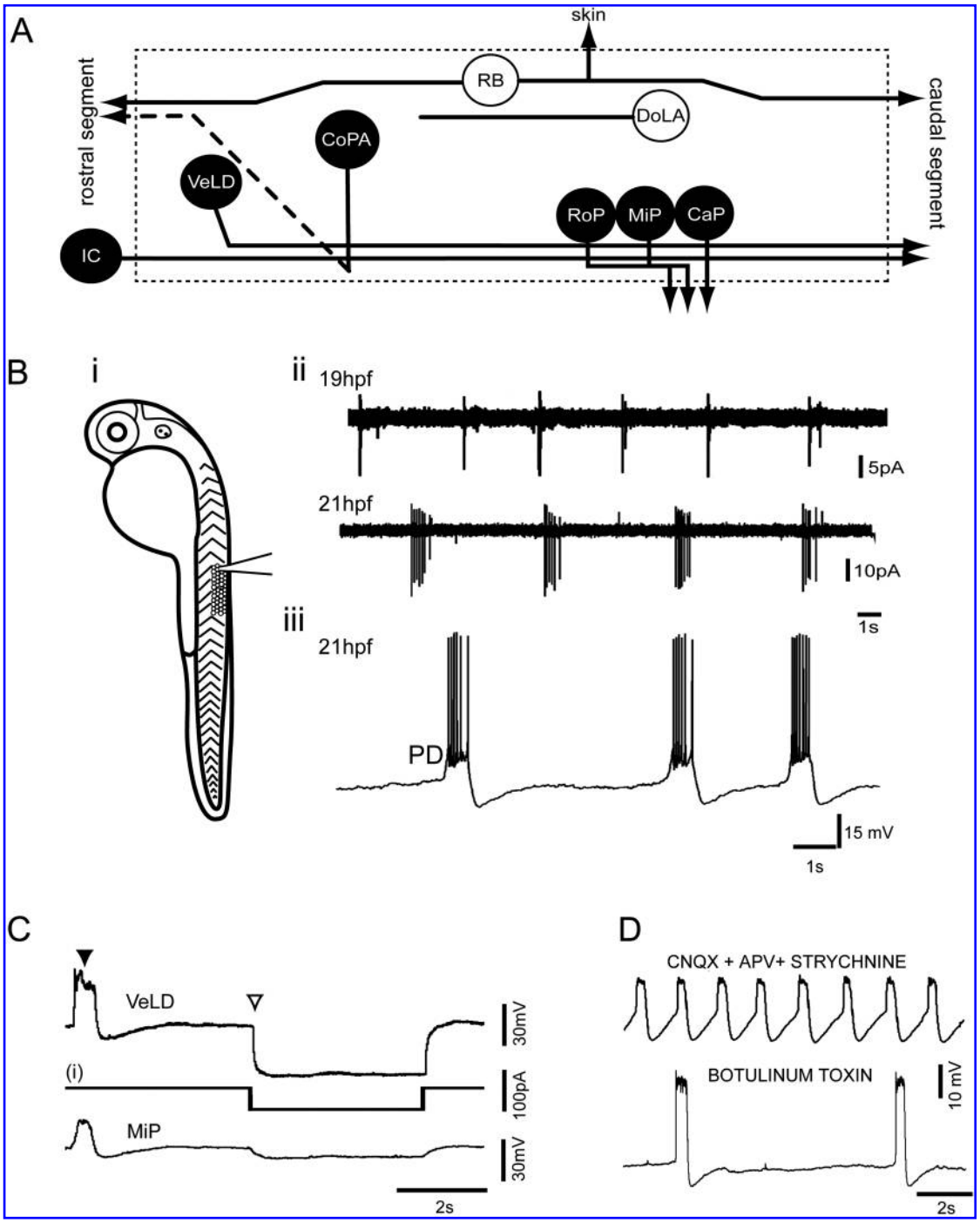

FIG. 1. Embryonic spinal cord. (**A**) Schematic diagram representing the spinal cord neurons that are present from 17 to 20 hpf. *Black neurons* are active during spontaneous activity, *white neurons* are silent. *Dashed lines* represent commissural projections. In this and other diagrams, dorsal is *up*, caudal is to the *right*. (**Bi**) Cartoon of a 1-day-old embryo showing the *in vivo* dissection and patch clamp approach; (**Bii**) cell attached traces from a primary motoneuron at 19 and 20 hpf showing spontaneous repetitive bursting activity; (**Biii**) whole cell recording from a 21 hpf primary motoneuron showing the periodic depolarizations (PD) that generate the spontaneous behavior. (**C**) Paired recording revealing synchronous activity between an interneuron and a motoneuron (*black arrowhead*) and the presence of strong electrical coupling (*white arrowhead*). (**D**) Pharmacology of the PDs, showing a lack of requirement for chemical neurotransmission. APV, CNQX, and strychnine are respectively NMDA, AMPA, and glycine receptor blockers. See Abbreviations for full chemical names. Figures modified from Refs. 38, 59, and 60.

When observed under whole-cell configuration, all primary motoneurons show repetitive spontaneous periodic depolarizations (PD) (Fig. 1C). These PDs consist of sustained voltage increases lasting 300 to 500 ms upon which action potentials are often superimposed. Recordings from other spinal neurons further revealed that several interneurons also showed PDs. Indeed, PDs are observed in all IC and VeLD interneurons and in most of the CoPA interneurons from 19 hpf to 24 hpf (Fig. 1A, black neurons). Interestingly, Rohon–Beard and DoLA neurons never show PDs or spontaneous activity of any kind during the period of spontaneous activity in embryos.[60] Paired simultaneous recordings from spinal neurons showed that PDs are synchronous in all active neurons and that these are electrically coupled to each other by gap junctions while inactive neurons are not coupled to any neurons[59,60] (Fig. 1D). The gap junctions between spinal neurons were found to be necessary and sufficient for propagating the PDs.

Blocking chemical neurotransmission with cocktails of glutamatergic, GABAergic, and glycinergic receptor antagonists or blocking synaptic release with botulinum toxin does not abolish PDs (Fig. 1E), whereas procedures that block gap junctions extinguish the PDs.[59] It is interesting to note that all neurons with PDs have axons that either course longitudinally in the VLF (IC, VeLD) or have axons that have ventral projections (CoPA), whereas inactive neurons (RB, DoLA) do not have any ventral projections. This observation suggests that either the active neurons contact each other at various points in the ventral spinal cord or that long descending axons link all the neurons together en passant. The finding that two CaP motoneurons in sequential somitic segments are electrically coupled to each other[60] even though they have no possible direct point of contact strongly suggests that longitudinal axons can link other neurons to the electrotonic network all along the spinal cord. The connections are presumably axo-axonal or axo-somatic as the cell bodies of spinal neurons are devoid of dendrites at these early time points in development.

These results are consistent with gap junctions having a critical role in the propagation of PDs, but the mechanism by which PDs are initiated is still unknown. Tetrodotoxin (sodium channel blocker) blockade reveals that voltage dependent sodium channels are necessary for the generation of PDs, suggesting an important role for sodium influx.[60] The increase of PD duration during apamin (Kca channel blocker) application further suggests that that there is significant calcium entry during PDs, and that calcium-activated potassium channels may play a role in PD termination.[60] It will be interesting to determine whether the zebrafish rhythm originates from a pacemaker core as is the case for the lobster stomatogastric ganglion, where a few neurons have inherent pacemaker properties and drive the whole network[61] or whether the zebrafish spinal cord pacemaker is more distributed and requires the active contribution of many different cell types and multiple electrical synaptic connections.

The presence of a pure electrotonic network in a developing vertebrate may seem unusual but there is a growing body of evidence that gap junctions play an important part in synchronizing early rhythms in the developing nervous system. During early development of the eye in mammals, waves of depolarizations spread across the retina.[3] Dye-coupling experiments showed that gap junctions are present within retinal ganglion cells and amacrine cell populations, and the use of gap junction blockers demonstrated that retinal waves require gap junctions to propagate at very early stages.[62] Disruption of retinal waves adversely affects axonal projections to the superior colliculus and eye-specific segregation of retinogeniculate projections.[63] Interestingly, when chemical neurotransmission is perturbed at later stages when gap junctions are not normally required, gap junctions seem to upregulate and are able propagate the retinal waves.[64] The leech has been shown to have extensive gap junctional coupling between neurons implicated in the production of motor activity.[65–69]

Gap junction-mediated networks also seem to play a role in the formation of the motor networks. In the developing chick spinal cord, gap junction blockers such as octanol and carbenoxolone were shown to reversibly abolish

spontaneous bursting activity.[70] Drug applications that alter the pattern of this bursting activity in the chick embryo have recently been shown to impair motor axon outgrowth to appropriate muscle targets.[18] In addition, extensive dye junctional coupling in the spinal cord was recently observed at a very early stage in the embryonic mouse,[71] and it is thought that the synchronization provided by this extensive coupling is permissive for the innervation of muscle by multiple motor axons during embryogenesis. After one week of postnatal life, as the electrical coupling diminishes, the synchronicity is lost and the normal course of synapse elimination is enabled.[72] These results in many vertebrate animal models all point to a central importance of gap junction mediated synchronicity for the correct development of neuronal networks.

The appearance of the gap junction network in embryonic zebrafish may be a prerequisite for the formation of excitatory connections. The widespread electrical activity provided by the gap junction network may shape the formation of spinal circuitry. One of the roles of this network may be to synchronize the neurons and provide an electrical environment required for chemical synapse formation. It should be noted that experiments in which electrical activity is reduced during embryonic development[49] support a significant role for activity-independent mechanisms in spinal circuit development, making the contribution of spontaneous activity unclear. Alternatively, the role of the gap junctions may not necessarily be to spread electrical activity but rather to connect prospective synaptic partners metabolically and enable the rapid spread of cellular messengers. Future experiments in which spontaneous activity is modified globally or in subsets of neurons while simultaneously monitoring efficacy at identified synapses are needed to test for the role of spontaneous activity in circuit formation.

TACTILE RESPONSES AND CHEMICAL NEUROTRANSMISSION

The next step in the progression of motor behaviors in the zebrafish embryo is the appearance of the touch response at 21 hpf. This new behavior suggests a change in the early gap junction-mediated motor network and implies at the very least, new functional connections with the sensory system. At this early time point, analysis of the touch response is complicated by the high frequency of spontaneous coiling.[49] But by 23 hpf, the frequency of spontaneous coiling is sufficiently low to enable a detailed observation of the touch response. At this stage, head and tail stimulation produces the same response, which typically consists of a strong flexion of the trunk on the contralateral side, followed by one or two weaker alternating contractions. The initial contractions in response to touch are always stronger than the spontaneously occurring coils, suggesting a stronger recruitment of the musculature during touch responses. Although the isolated trunk responded to touch, the full flexure and strength are only seen when the hindbrain is intact. Brain structure above the hindbrain are not necessary for touch responses at 24 hpf.[49] Touch responses are completely abolished when blockers of glutamatergic transmission are injected into the embryos (Fig. 2Bi), whereas spontaneous coiling continues at a normal frequency in the presence of the blockers (Fig. 2Bii). These behavioral results clearly show that hindbrain projections and chemical neurotransmission are already present by 21 hpf in the zebrafish embryo and are required for normal touch responses.

The morphology of the spinal cord is changing rapidly at the stage when touch responses first appear. After 21 hpf, at least four more types of interneurons are added the spinal cord[51–53] (see gray neurons in Fig. 2A). Commissural secondary ascending (CoSA) interneurons project their axons like the CoPAs, first ventrally, then contralaterally and rostrally, but have smaller somata and extend their growth cones slightly later than CoPAs. Circumferential descending (CiD) interneurons send their axons first ventrally then caudally. Circumferential ascending (CiA) interneurons project ventrally to the floor plate, then rostrally on the same side of the embryo. Commissural bifurcating (CoB) project ventrally, cross the midline, and then project axons both rostrally and caudally. Of these four later ar-

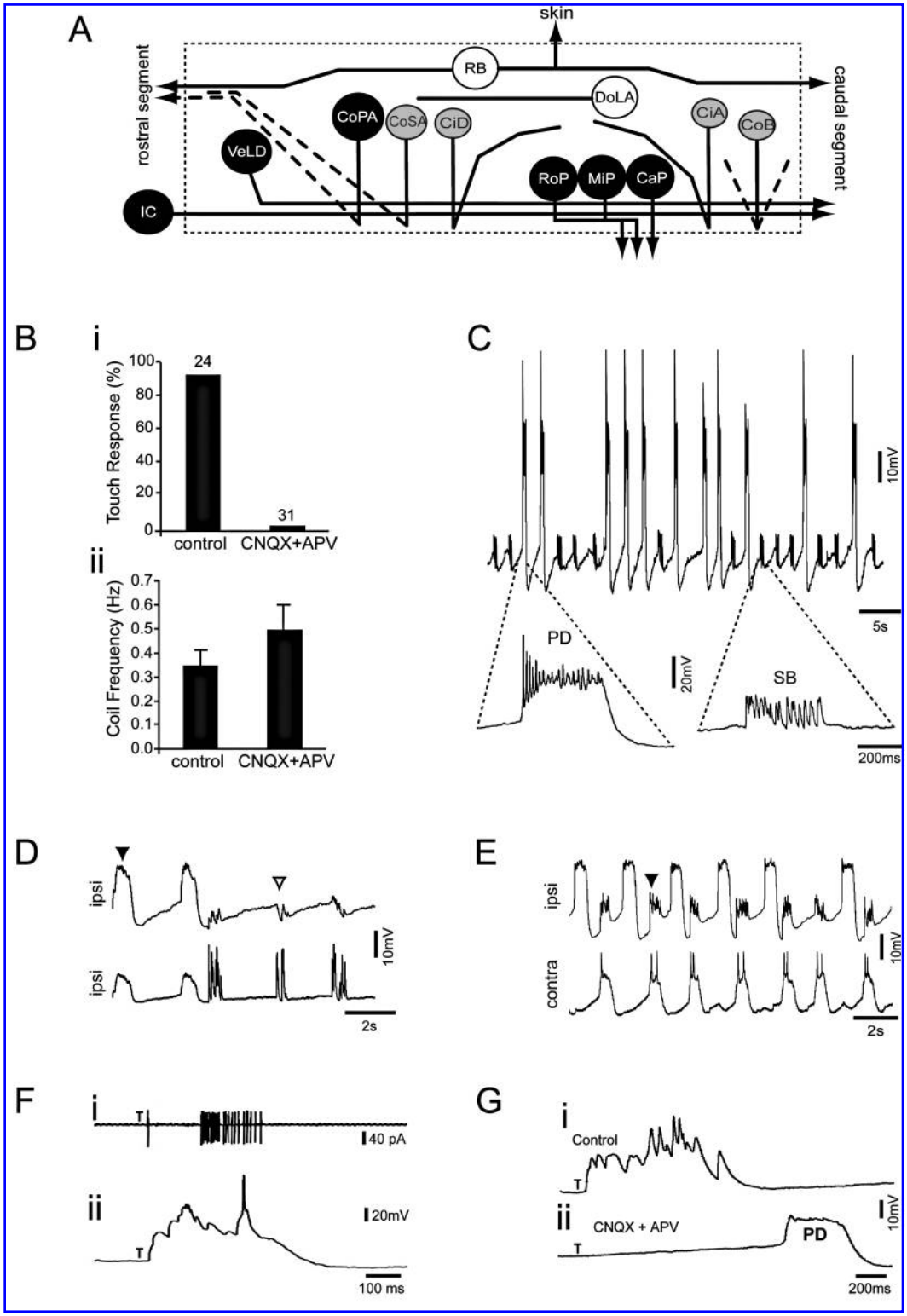

FIG. 2. Development of chemical synapses. (**A**) Schematic diagram representing the spinal cord neurons that are present from 21 to 26 hpf. (**Bi**) Block of the touch response by glutamatergic receptor blockers; (**Bii**) spontaneous contractions are unaffected by blocking glutamatergic transmission. (**C**) Whole cell patch clamp recording showing the appearance of bursts of glycinergic activity at 20 hpf (SB). (**D**) Just as PDs (*black arrowheads*), SBs are synchronous in ipsilateral neurons (*white arrowhead*). (**E**) SBs are coincident with contralateral PDs. (**F**) Touch response recorded from a motoneuron in the cell-attached (**i**) and whole cell configuration (**ii**). (**G**) Touch responses are blocked by glutamatergic blockers (**i**) but PDs are unaffected (**ii**). See Abbreviations for full names. Figures modified from Refs. 38 and 60.

riving neurons, CoSAs are the only ones that reliably cross more than one somitic boundary before 24 hpf. A significant minority (45%) of these later arriving neurons showed PDs (23/51), suggesting that these neurons quickly wire into the existing gap junction network, presumably through their ventral projections.

Whole cell patch clamp from embryos after the onset of touch responses show that over 80% of primary motor neurons (PMN) display a new type of spontaneous rhythmic event in addition to the PDs that are already present. These events were named synaptic bursts (SB) because they are composed of rapid depolarizations that quickly return to baseline as opposed to the sustained drive during PDs (Fig. 2C). Synaptic bursts are present in most spinal neurons at the same frequency as the PDs but are sensitive to strychnine, a blocker of glycinergic transmission.[59] Paired recordings revealed that SBs are simultaneous in pairs of ipsilateral spinal neurons (Fig. 2D), but are coincident with PDs on the contralateral side of the spinal cord (Fig. 2E). Due to high intracellular chloride, the glycine mediated events have been shown to be depolarizing at these early stages,[59,73] but they are nevertheless thought to have an inhibitory shunting effect in motor networks as application of strychnine causes an abnormal synchronous activation of both sides of the animal.[74]

These results suggest that SBs represent commissural inhibition during contralateral excitation, akin to the midcycle inhibition seen during swimming in *Xenopus* larvae, which is required to prevent coactivation of both sides of the axial musculature during swimming.[75] In *Xenopus* larvae, the glycinergic neurons responsible for the midcycle inhibition are the commissural interneurons, which project a commissural axon which bifurcates and extends both rostrally and caudally upon crossing the spinal cord.[76] In the zebrafish, there are only three different types of commissural neurons at this stage: the CoPAs, CoSAs, and CoBs. The CoPA interneurons and a subset of CoSA interneurons were found to be positive for the presence of vesicular glutamate transporter mRNA at 32 hpf, indicating that these neurons are presumably glutamatergic interneurons[77] and therefore not causing the SBs. The CoB interneurons and a second subset of CoSA interneurons on the other hand were found positive for mRNA encoding the neuronal glycine transporter,[78] suggesting that they are good candidates for the commissural inhibitory interneurons responsible for the SBs.

When a day-old embryo is touched while performing a cell attached recording from an ipsilateral motoneuron, a single spike is observed, followed by a long pause of 150–200 ms and a spiking burst of 200–300 ms (Fig. 2Fi). This pattern is consistent with the behavior where the first contraction is always on the side opposite to the touch (Fig. 2Fi). When recording in the whole-cell configuration, touching the animal gives rise to a long summated burst of synaptic potentials that is longer in duration and larger in amplitude than the PDs (Fig. 2Fii). This touch response presumably propagates through the gap junctions in addition to using a mix of chemical synapses as it is hard to imagine gap junctions being selective for PDs but not to other electrical signals occurring at the same time.

The response to touch when recording from a VeLD interneuron is also composed of a similar burst of synaptic potentials, suggesting that the whole gap junction coupled ventral spinal cord is undergoing the same activity pattern upon touch (Fig. 2Gi). The burst of synaptic potentials in response to touch is completely absent when glutamate receptor blockers are added to the bath, but the PDs are still present (Fig. 2Gii). Because of limited knowledge of the wiring of the spinal cord in the zebrafish, it is still unclear where this stringent requirement for glutamatergic transmission comes from. The continued propagation of PDs through the gap junction network during the glutamatergic blockade of touch responses suggests that the sensory input never reaches the interconnected premotor network. The strict requirement for glutamate transmission in touch responses may come from the sensory to premotor neuron connections. More work is needed to address this possibility.

The appearance of responses to tail touch after 21 hpf reveals that new connections are being made between sensory neurons and neurons in the motor network (Fig. 2F). The identification of the commissural CoPA in-

terneurons as a glutamatergic interneuron puts this neuron at the head of a very short list to carry sensory excitation to the contralateral side of the spinal cord.[77] Indeed, a very similar type of interneuron in the *Xenopus* larvae, the dorsolateral commissural sensory interneurons, is a glutamatergic interneuron responsible for relaying and amplifying sensory information from the Rohon-Beard sensory neurons to motoneurons and premotor interneurons on the opposite side of the spinal cord.[79,80] It remains to be demonstrated by electrophysiological techniques whether this neuron is activated during embryonic touch responses and functions downstream of Rohon-Beard neurons.

Weaker contractions after lesions of the hindbrain suggest that reticulospinal projections are required to obtain the full strength touch response at 24 hpf, but it is still unclear which hindbrain neurons participate in the touch responses at this stage. The Mauthner neuron is presumably involved in the first manifestations of touch responses as its axon is already at the third spinal segment by 21 hpf.[54,55] We can nevertheless hypothesize that the hindbrain neurons that get added to this early spinal circuit for the emergence of early touch responses continue to be involved in touch response with further development and may mediate the fast larval predator escape responses that can be seen after hatching.[81]

LARVAL SPINAL CORD, ESCAPE, AND SWIMMING

Embryonic zebrafish can be induced to swim as early as 28 hpf. The cycle (two alternating tail beats) frequency of the first observed swimming bouts is 8 Hz and the duration of the episodes is short lived. Slightly later in development, at 36 hpf, the embryos can swim at a 30 Hz cycle frequency.[49] After normally hatching at 2 days, the larvae do not show much spontaneous motor activity, although swimming can normally be seen after a tactile stimulation.[32,82] Two-day larvae swim in long uninterrupted bursts with tail beats that can reach a frequency of over 100 Hz and last tens of seconds.[32,82] After 3 days of development, swimming switches to an intermittent mode of swimming. These motor events are composed of a string of a few cycles of tail beats punctuated by periods of inactivity.[32,33,82] The level of spontaneous activity can be modulated; as recent research has shown that serotonin can modulate the level of motor activity in 3- to 4-day-old larvae by changing chloride homeostasis and therefore cell excitability in spinal neurons.[73,83]

After 4 days, larvae have been shown to exhibit four main types of motor behaviors. Two of these motor gaits were classified as turning behaviors, namely, routine turns and escape turns. Routine turns are slower and weaker than escape turns and occur mostly spontaneously whereas escape turns are usually triggered by tactile stimulation and are an integral part of the startle response.[84] The startle response is a predator escape motor behavior that has been very well studied in fishes, including the larval zebrafish.[85,86] The startle response in larval zebrafish usually consists of a pronounced contralateral bend of the tail, the C-start, which is usually followed by a weaker counter bend and an episode of swimming.[81,84,87] The other two motor gaits seen at 4 days were classified as swimming behaviors and have been designated slow swims and burst swims. Slow swims typically follow routine turns whereas burst swims are associated to tactile evoked escape responses. Slow swim episodes involve weaker bends of the tail, lower frequencies of tail beats and slower swimming speeds than burst swimming.[84]

This simultaneous presence of weaker and stronger motor behaviors in zebrafish larvae suggests some flexibility in the recruitment of muscle fibers during behaviors. Indeed, muscle recordings in 3-day-old zebrafish larvae have shown that while embryonic red and white muscle cells usually received the same input, embryonic red muscle could be recruited independently of embryonic white muscle during slow swim episodes. Additionally, input to embryonic red muscle was shown to be attenuated during burst swimming episodes.[33] This difference between slow and burst swimming is further highlighted by the observation that pectoral fins are used during slow swims but are tucked away during burst swimming.[88]

This differential recruitment of musculature suggests that that the motor circuitry may be composed of distinct subcircuits, or central pattern generators (CPG), that can act independently or in concert to produce different behaviors.[84] By 6 days of development, zebrafish larvae show a larger repertoire of motor behaviors that includes prey tracking[89] and prey capture,[90] suggesting involvement of higher brain structures. The variability in larval motor behaviors presumably comes from differences in the descending control provided by functional subsets of reticulospinal neurons.[36] Motor behaviors such as slow swims or routine turns may thus represent simple behavioral "building blocks" with which more complex behaviors are built.

It should prove interesting to study the changes in the underlying neural circuits that are required to go from the limited and stereotyped embryonic motor behaviors to the more complex and variable larval behaviors. One of the main substrates for these changes is the spinal cord. By 5 days of development, the spinal cord of zebrafish has changed dramatically from day 1 (Fig. 3A). Neurons that had short local projections such as the CiD in the embryo, now project axons several somites away from their cell bodies and spinal neurons now have extensive dendritic arborizations.[91] It also seems that some neurons may change or modify their axonal projections, CiDs for example now have an additional rostral going projection and VeLDs (MCoD) now project contralaterally. New neurons are also added to the spinal cord. The first type of neuron, the CoLA neuron, has long ipsilateral rostral and caudal dendrites and they project a long commissural ascending axon. The second type of neuron, the UCoD neuron, also has an extensive dendritic arbor but has a descending commissural axon.[91]

What is remarkable is that even though the spinal cord is more complex at this later stage, it is still possible to identify individual neurons. This ability enables researchers to map the role of identified spinal neurons in the production of different motor behavior. Indeed, *in vivo* imaging of calcium transients while monitoring behavior has shown that CiDs are active only during escape responses and not swimming, while conversely MCoDs are only active during swimming and not escape responses (Fig. 3B).[92] This intriguing result clearly shows that spinal circuitry is not simply shared for all behaviors and suggests that there are functional subdivisions in the zebrafish spinal cord. These findings support the concept that the spinal cord is composed of distinct, but presumably overlapping, CPGs. The inclusion or exclusion of a type of spinal neuron in the execution of a behavior likely results from differences in synaptic connectivity between reticulospinal neurons and their spinal targets.

Fictive motor activity can be recorded in the spinalized larvae under the right conditions, namely tonic NMDA application, but the spinal network is normally driven by reticulospinal inputs originating from the hindbrain.[34,81,87,93] An identified reticulospinal neuron, the Mauthner cell and its homologues, has been implicated in escape responses in a variety of teleosts as well as in zebrafish.[86,94,95] Experiments in zebrafish are beginning to assign behavioral functions to individual hindbrain neurons. Calcium imaging of the Mauthner and serial homologues has shown that a tail touch elicits activity in the Mauthner only, whereas a head touch recruits the Mauthner cell and homologues MiD2cm and MiD3cm.[81] This result reinforced the concept that the differential recruitment of hindbrain neurons may be responsible for the difference in behavior observed when touching either the head or the tail.[85] This concept of differential recruitment was tested by ablating these neurons from zebrafish larvae and then testing their escape responses. Ablating all three Mauthner homologs abolished the short latency, high amplitude escape response, whereas ablating only the Mauthner neuron affected solely the performance of the tail evoked response. These results support the idea that these three neurons are differentially recruited to produce the behaviors appropriate to sensory context.[93]

These results highlight the power of imaging neuronal activity to probe the role of neurons in the production of behavior. The information gained from optical experiments can in turn be used to design more targeted electrophysiological experiments aimed at answering questions of connectivity, direction, and timing of

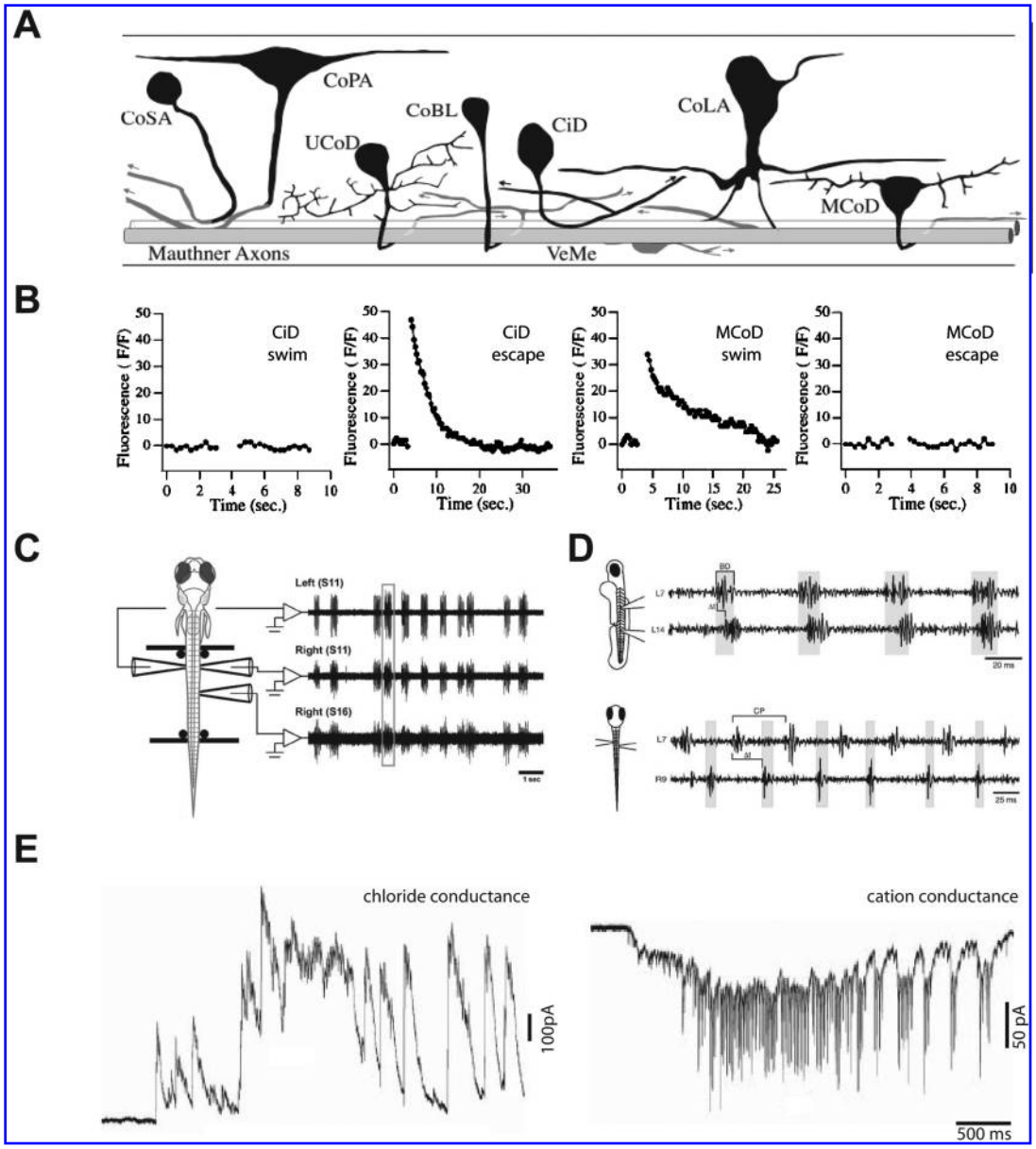

FIG. 3. The larval spinal cord. (**A**) Schematic diagram representing the spinal cord neurons that are present at 5 days. (**B**) Calcium imaging experiment showing that CiDs are only active during escape and MCoD are only active during swimming. (**C**) Schematic diagram of a ventral root recording technique and representative traces. (**D**) Ventral root recordings from spinalized larvae showing alternating activity. (**E**) Whole cell recordings from motoneuron during 2-day-old burst swimming showing the isolation of the chloride conductance (**i**) and the cation conductance (**ii**). See Abbreviations for full names. Figures modified from Refs. 91 (**A**), 92 (**B**), 40 (**C**), 39 (**D**), and 32 (**E**) with permission.

information flow. Indeed, the combination of ventral root recordings and intracellular recordings from identified neurons has been used for several decades in the *Xenopus* and lamprey in order to map the circuitry responsible for swimming. These animal models have enabled some understanding of the cellular mechanisms underlying swimming in vertebrates but the precise cellular and ionic mechanisms of rhythm generation are still elusive.[20,21,23,80,96,97]

Recently, techniques have been developed to record from the peripheral nerves of larval zebrafish during fictive swimming in intact larvae[40] and in spinalized larvae[39] (Figs. 3C and 3D). These techniques will provide information about the phase relationships of the two sides of the spinal cord, information which is useful

when trying to relate the activity from individual neurons to the rest of the network. In addition, ventral root recordings from spinalized zebrafish during NMDA-induced fictive swimming have provided evidence that very few spinal segments are necessary for oscillating activity. Indeed, lesions have shown that only two spinal segments are necessary for generating NMDA-induced rhythmic activity,[39] which is consistent with results in other swimming vertebrates.[98] Additional techniques have been developed to record from muscle cells[33,99] and motoneurons.[32,100] These techniques have begun to reveal the synaptic drive to motoneurons during fictive swimming as well as a complex pattern of motoneuron recruitment at early larval stages. Indeed, whole cell recordings from intact and spinalized larvae has shown that glycinergic drive has a tonic component, while the glutamatergic drive seems to have a stronger phasic component[32,39] (Fig. 3E). The combination of these whole cell recordings from spinal motoneurons and interneurons with simultaneous ventral root recordings should enable researchers to identify the neurons that form the CPGs for simple motor behaviors in zebrafish. As we learn more about the networks that mediate the simpler sensory evoked motors behaviors we will be in a better position to understand how these hindbrain and spinal cord circuits are recruited by higher brain centers during more complex volitional behaviors.

MOTILITY MUTANTS

One of the great strengths of the zebrafish as an animal model is the ability to perform forward mutagenesis screens. One such mutagenesis screen performed in the Nüsslein-Volhard laboratory has generated over 30 distinct motility mutants with no visible muscle abnormality.[26] Studying the genetic roots of these motor mutants should yield insights into motor circuit function in normal animals.[24,101] Several of the motility mutants that have been studied to date affect motor activity by perturbing motor axon guidance,[102–105] the neuromuscular junction,[104,106–112] or excitation contraction coupling.[111,113–116] A few motor mutants have been studied in more detail and have been shown to directly affect the central nervous system and will be described here.

Six mutants isolated by the Nüsslein-Volhard laboratory showed a decreased sensitivity to touch, yet were able to swim spontaneously.[26] Three of these: *macho*, *steifftier*, and *alligator*, were selected for electrophysiological analysis because of the appearance of phenotypes early in development. All of these mutants were shown to have reduced voltage-dependent sodium currents in Rohon-Beard sensory neurons, which lead to the absence of overshooting action potentials.[29] This inability of sensory cells to spike in the mutants is presumably the cause of their lack of mechanosensation. None of the genes for these mutations has yet been identified, yet the characterization of the mutant *macho* has already provided insights into an activity dependent developmental mechanism. Rohon-Beard neurons undergo programmed cell death in zebrafish and are completely eliminated from the spinal cord by 5 days postfertilization (dpf).[117,118] The decreased activity in Rohon-Beard neurons of *macho* mutants seems to protect them from programmed cell death as they were shown to have less TUNEL positive staining and survive longer in *macho* mutants than in wild-type siblings.[119]

The *deadly seven* mutant was identified because of a somitogenesis defect, in which somite formation is disrupted after the sixth somite.[120] Although this mutant was not identified as a motility mutant *per se*, it does cause the appearance of supernumerary neurons including multiple copies of the Mauthner cells.[121] Kinematic analysis of the escape response in *deadly seven* mutants revealed little difference with wild-type siblings, suggesting that the extra Mauthner cells are either silent or integrate smoothly into the circuitry.[122] Further results showed that all supernumerary Mauthners are active during touch, but each of them makes fewer synaptic contacts with neurons in the spinal cord, enabling the mutants to have normal escape responses despite the extra copies of Mauthner neurons.[122] These results are interesting because they suggest a very high degree of plasticity in the development of the circuitry responsible for the escape response.

The *space cadet* mutant is part of the twitch twice category of motor defects because they respond to tactile stimulation at 96 hpf with multiple C-bends toward the same side instead of the normal touch response of a C-bend followed by a counter bend and swimming.[26] The large magnitude of the successive C-bends in the mutants suggested that the problem arose in the selection of the proper motor response and not the execution of the escape response itself, which pointed to a problem in the network of the Mauthner cell.[123] Indeed, analysis of hindbrain morphology revealed a very specific defect of axonal projection in *space cadet* mutants, including the complete absence of the spiral fiber projections through the third hindbrain commissure. Moreover, lesioning of the same commissures in wild-type zebrafish resulted in the same aberrant phenotype.[123] These missing projections normally give rise to the axon cap, which is a structure that has previously been shown to have a role in regulating the excitability of Mauthner cells in the goldfish.[124–126] Although the search for the *space cadet* gene is still underway, this mutant has already provided novel insights into the structure and function of hindbrain motor network by showing a clear role for the spiral fibers in the production of a specific motor behavior. When identified, this gene will undoubtedly provide additional insights into axonal pathfinding mechanisms and the wiring of motor networks.

The mutant *bandoneon* was placed in the *accordion* group of mutations, because tactile stimulation causes both sides of the mutant to briefly contract simultaneously which reduces the length of the trunk much like an accordion. The spontaneous contractions occur normally in *bandoneon* mutants, but the responses to touch in the same animals at 24 h are clearly of the accordion type.[74] When wild-type 24 hpf embryos are placed in the glycine receptor blocker strychnine, they exhibit the same phenotype of normal spontaneous contractions with abnormal bilateral touch responses. This result suggests that *bandoneon* affects glycinergic synaptic transmission. The *bandoneon* gene was found to encode for the â2 subunit of the glycine receptor (*glrb2*). The defect in *bandoneon* was shown to result from the complete absence of clustering of the á subunit of the glycine receptor at glycinergic synapses, leading to the absence of synaptic glycinergic currents.[74] The hypercontractility phenotype observed in bandoneon animals is reminiscent of what is observed in herperrekplexia, a disease involving impaired glycinergic transmission, causing patients to overreact with rigidity to unexpected stimuli. This mutant clearly highlights the fact that zebrafish mutants have potential to become important models to study genes implicated in human neurological diseases.

The *shocked* mutants were first isolated because of a lack of swimming.[26] Further analysis of the mutants revealed that they cease spontaneous contractions abruptly at 21 hpf and fail to respond to tactile stimulation at 24 hpf: by 48 hpf, the larvae respond to touch with a vigorous contralateral contraction but fail to initiate swimming.[127,128] By 4 days, the mutants seem to recover some motor activity and show what may be a compensatory increase in electrical coupling between muscle cells,[129] yet they start to die prematurely after 6 days. The mutation that causes shocked was found to inactivate the CNS glycine transporter (glyt1).[128] Glyt1 is expressed extensively by non-neuronal cells in the hindbrain and spinal cord of embryos and larvae. It is thought that the absence of the glycine transporter in *shocked* mutants leads to an aberrant accumulation of glycine levels in the CNS and the shunting of sustained activity.[128] Indeed, exposing the animals to strychnine recovers normal spontaneous coils in the embryos and partial swimming at 2 days, while removing glycine from the hindbrain during electrophysiological recordings uncovered a normal fictive swimming rhythm.

The investment of time and energy in the mutagenesis studies undertaken over a decade ago is beginning to bear fruit. The search for the locus of each mutation will identify proteins that are essential for the production of motor activity. It is surprising that a gene expressed in a glial cell can have a profound effect on the production of motricity, yet the mutant *shocked* provided evidence that absence of a glial cell glycine transporter prevents proper swimming rhythms. It is interesting to note that *bandoneon* and *shocked* both cause abrupt termination of motor rhythms, yet they are dif-

ferent sides of the same glycinergic coin. These mutations reveal that proper rhythm generation can only occur with the right dose of inhibition. The glycinergic drive to motoneurons was found to be more tonic than the glutamatergic drive.[32] These mutations reveal not only that this tonic drive is important but also that the amplitude of this tonic drive may be very critical. More work will be needed to uncover at which synapses and at which time glycinergic input to the motor pattern generator is critical for the production of rhythmic motor activity. Mutated genes are becoming easier to find as genomic sequence information is constantly being added to the databases. In the next decades we should be able to build a library of zebrafish mutants that affects every step required for motor behavior in order to characterize its development: from sensory perception to muscle contraction, and everything in between.

FUTURE RESEARCH

We still know very little about the circuitry required for complex motor behaviors in vertebrates. As shown above, optical techniques that take advantage of the transparent zebrafish larvae can be used to identify populations of spinal cord and hindbrain neurons that are active during swimming. Identification of these active neurons should be followed by lesion experiments ascribing a behavioral function to each type of neuron and ultimately to a detailed electrophysiological analysis of the synaptic drive mediated by these neurons during motor behaviors. This type of research will lead to a greater understanding of how vertebrate circuits generate rhythmic activity and behavior.

How these circuits initially develop is also a critical issue in biological research. The molecular mechanisms that lead to the precise synaptic connectivity during development in the embryonic spinal cord are still largely unknown. A variety of genetic tools and techniques are available in the zebrafish such as transgenic lines,[130,131] enhancer lines,[132–135] targeted protein knockdown with morpholinos[136] and motility mutants.[26] The combination of these various tools will permit researchers to modify the spinal cord expression patterns of any protein of interest *in vivo* while assaying the effects on neuron morphology, synaptic junction formation, network activity, and behavior. An area of promising interest to study the mechanisms involved in synaptic specificity is to look at proteins involved in cell–cell interactions such as the cadherin family of proteins.[137,138]

CONCLUSIONS

We are still at the beginning of this journey to the understanding of the genetics behind motor network assembly and function. One of the important determinants of the success of this journey is the development of powerful animal models that can reveal new information and promote new questions about circuit formation and function. The zebrafish is one such model, aligning the power of forward genetics, antisense knockdown, *in vivo* imaging, *in vivo* electrophysiology, and behavioral analysis. Research in the zebrafish has already shown that the motor network starts with a very simple circuit and builds complexity by adding layers of organization. Each new layer increases the range of possible motor acts from purely reflexive to volitional behaviors. Studying the formation of these basic motor networks and their maturation in zebrafish should lead to a better understanding of how animals go, as Grillner put it, "from egg to action."[139]

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ABBREVIATIONS

APV, DL-2-Amino-5-phosphonovaleric acid; CaP, caudal primary motoneuron; CiA, circumferential ascending; CiD, circumferential descending; CoB, commissural bifurcating; CNS, central nervous system; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; CoLA, commis-

sural lateral ascending; CoPA, commissural primary ascending; CoSA, commissural secondary ascending; CPG, central pattern generator; DLF, dorsal lateral fasciculus; DoLA, dorsal lateral ascending interneuron; glyR, glycine receptor; glyt1, glycine transporter 1; HPF, hours postfertilization; IC, interneuron with ipsilateral caudally directed axon; MCoD, multipolar commissural descending; MiP, middle primary motoneuron; NMDA, N-methyl-D-aspartate; PD, periodic depolarization; PMN, primary motoneuron; RB, Rohon-Beard sensory neuron; RoP, rostral primary motoneuron; SB, synaptic burst; UCoD, unipolar commissural descending; VeLD, ventral lateral descending interneuron; VLF, ventral lateral fasciculus.

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