Studying Electrical Activity in Development: Challenges and Solutions Using the Zebrafish Model

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ABSTRACT

Many developmental regulators play multiple and varied roles throughout development and during the adult life of the organism. Recent studies suggest that electrical activity may similarly orchestrate a wide variety of developmental processes in addition to its classic role in regulating function of the mature nervous system. Studies utilizing several model systems indicate electrical activity is an important regulator of neuronal differentiation, neurite outgrowth, axon pathfinding, cell adhesion molecule expression, myelination, synapse stability, and cell death or survival decisions. However, very little is known regarding the mechanisms by which precise patterns of electrical activity are transduced into signals which can produce long-lasting effects on development. The study of electrical activity as a developmental regulator is a new field and poses many technical challenges. These challenges include observing and manipulating endogenous patterns of electrical activity in living, developing embryos, and evaluating the effects of altered activity patterns on the embryonic nervous system. The rise of the zebrafish as a model organism for developmental studies provides many strategies to overcome these challenges and has led to valuable insights into electrical regulation of development.

CONTENTS

CHALLENGES AND APPROACHES TO STUDYING ELECTRICAL ACTIVITY

Study of the role of electrical activity during embryogenesis faces the challenge of interfacing molecular, cellular, and electrophysiological approaches to embryological studies. To effectively study the roles that electrical activity plays in directing development, we need tools that let us (1) observe endogenous patterns of electrical activity, (2) manipulate activity in developing embryos, and (3) evaluate the effects. The establishment of the zebrafish as a model system has provided new ways to study electrical activity in the embryonic nervous system. The following review will highlight strategies used to overcome these challenges. We also cri-

Invited Review

tically evaluate new tools designed to meet these challenges, many of which have been effectively used in the zebrafish system. Finally, we summarize recent studies that have demonstrated roles for electrical activity in development of zebrafish and other organisms.

Observing electrical activity in the developing nervous system

Challenges

To understand the functions of electrical activity in development, we must first be able to record endogenous patterns of activity in the developing embryo. For this to be possible, neurons and other electrically excitable cells must be accessible to observation during the developmental periods of interest. Further, we must have reliable indicators of electrical activity that allow characterization in single cells or neuronal networks. These indicators must be able to respond rapidly to the changes in electrical properties of cells that occur on a millisecond time scale during firing of action potentials. Additionally, it is important that the methods used to detect electrical activity in the developing nervous system do not themselves change the electrical properties of the cells being studied.

Approaches

As in other organisms, electrophysiology has been widely used to study electrical activity in the developing zebrafish (**Box 1**). Electrical currents and membrane potentials can be recorded from single cells within the developing nervous system, thus allowing biophysical characterization of embryonic neurons, many of which are readily identifiable due to their well-characterized anatomy and availability of transgenic lines expressing fluorescent reporters in specific cells. Taking advantage of the basic physical principles that

govern electrical activity we can alter various conditions during electrophysiological recordings to isolate specific ionic currents and determine how individual ion channels contribute to the overall properties of the cell. Electrophysiological methods record electrical signals with unsurpassed temporal resolution. Furthermore, by studying activity in specific neuronal populations at different points in development, we can identify how patterns of electrical activity change over time. However, given the invasive nature of electrophysiological approaches, we cannot easily record from single cells over extended periods of time during development. Moreover, while patch-clamp methods allow control of the chemical environment both inside and outside the cell, they also change them from the native state. In particular, intra-cellular factors that regulate currents may be washed out and thus one might record currents in the absence of important physiological regulators.

Recent studies have taken advantage of the transparent nature of zebrafish embryos to image neuronal activity in live embryos using fluorescent markers of intracellular calcium or membrane voltage (e.g., Friedrich and Korsching 1998; Higashijima *et al.* 2003, respectively). Cells within the developing nervous system can be directly visualized using standard microscopy in living, intact zebrafish embryos. Further, because electrical activity leads to changes in calcium concentrations within the cell, intracellular calcium can be used as a marker of electrical activity. In the zebrafish, calcium indicator dyes can be injected into embryos at the one-cell stage to label all cells or can be injected into a single blastomere at the 8-64 cell stage to mosaically label individual cells. Alternatively, the genetically-encoded *cameleon* construct can be used to measure calcium activity in transgenic fish (**Table 1**). Both the *cameleon* construct and calcium indicator dyes alter their fluorescence properties upon binding calcium. The fluorescence intensity of the indicator can be compared to the intensity of a signal (e.g., emission of the same or a different fluorophore at

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Box 1 Electrical Characterization of the Developing Nervous System

Recent studies have demonstrated that the zebrafish embryo allows multidisciplinary analysis of development of electrical activity. Electrophysiological studies allow characterization of membrane properties and electrical activity of single cells within the nervous system. Transparency of zebrafish embryos has also allowed observation of network activity within the developing nervous system. Voltage-sensitive and calcium-sensitive dyes have been used to image activity of neuronal networks in live, behaving embryos with single-cell resolution and to characterize the neurotransmitters that mediate these behaviors.

1. **Electrophysiology.** Electrophysiological studies allow the electrical properties of single cells to be characterized with temporal resolution on the scale of milliseconds. Electrophysiology has demonstrated rhythmic bursts of firing in motor neurons that mimic tail beat frequencies in behaving embryos. As embryos develop they first acquire the spontaneous tail contractions, burst swimming, and finally acquire beat-and-glide type swimming. Frequency of electrical activity in motor neurons also changes during development in correspondence with these behavioral changes. Using pharmacological manipulation of specific channels and by varying the ionic composition of intracellular and extracellular solutions, studies have also identified the intercellular connections and neurotransmitters that mediate these motor neuron firing patterns (Ali *et al.* 2000; Buss and Drapeau 2001; Buss *et al.* 2003; Drapeau *et al.* 1999; Saint-Amant and Drapeau 2000). Additional studies have begun to show the usefulness of electrophysiology in analyzing changes in membrane properties and electrical activity of specific cell types during development and identifying the ion channel subtypes that mediate these changes (Pineda *et al.* 2005; Ribera and Nüsslein-Volhard 1998).
2. **Network imaging and behavior.** Calcium-sensitive indicator dyes and the recent development of transgenic lines expressing a genetically-encoded calcium-sensitive indicator have allowed the characterization of early neuronal networks. The use of calcium indicators is generally less invasive than electrophysiological experiments, and although indicators trade temporal resolution for high spatial resolution, some indicator dyes have been reported to detect even single action potentials. Imaging studies that monitor activity in multiple neurons simultaneously have identified subsets of interneurons that are part of embryonic motor circuits. Interestingly, different subsets of interneurons and motor neurons are active to generate an escape response than to mediate slow swimming behaviors (Fetcho and O'Malley 1995; Ritter *et al.* 2001; Higashijima *et al.* 2003). A curious pattern has emerged with more dorsally located interneurons and motor neurons active during high frequency swimming and more ventrally-located interneurons and motor neurons active during low frequency swimming (McLean *et al.* 2007). It has been suggested that this pattern correlates with early-born and later-born neuronal populations, respectively.
3. **Gap junctions and spontaneous electrical activity.** Electrical activity begins in the developing nervous system before synaptic connections are established. This early electrical activity is responsible for spontaneous trunk contractions in 17hpf embryos, which is the earliest behavior to develop in the zebrafish embryo (Saint-Amant and Drapeau 2000). A combination of electrophysiological and imaging studies identified the subsets of interneurons that are electrically coupled through gap junctions in this early pre-motor network. The pre-motor network includes interneurons that cross the midline to innervate contralateral neurons, suggesting this early network is already set up to coordinate activity on both sides of the spinal cord (Saint-Amant and Drapeau 2001). While electrical coupling in most spinal cord neurons and in fast muscle cells disappears as the embryo develops, slow muscle cells continue to be connected by gap junctions in the *shocked* mutant (Luna and Brehm 2006). Interestingly, electrical coupling persists throughout much of development in the *shocked* mutant. *Shocked* homozygous embryos prematurely terminate swimming and exhibit a delay in development of rapid touch-evoked responses, suggesting that while electrical coupling is important for early motor behaviors its presence at later times can be detrimental (Luna *et al.* 2004).

Table 1 Genetic markers to visualize cells.

Construct	Properties	Reference
GFP/RFP	Used to visualize specific cell types in live or fixed embryos when expressed under specific promoters	All in **Table 4**
Kaede	Fluoresces green until illuminated with UV light, at which point the fluorescence changes to red. Can be used to trace lineage, development, or migration of single cells. Can be expressed under cell-specific promoters.	Sato *et al.* 2006
Dronpa	Modified green fluorescent protein that can be reversibly turned off and on by irradiation at different wavelengths. Fluorescence is turned off by strong blue light and can be turned back on by violet or ultraviolet light.	Aramaki and Hatta 2006
cameleon	Genetically-encoded, ratiometric calcium indicator. The protein consists of CFP and YFP linked by calmodulin. Increased calcium concentrations inside the cell induces a conformational change that increases the YPF:CFP fluorescence ratio. Can be used to measure calcium activity non-invasively in living, behaving embryos.	Higashijima *et al.* 2003

Table 2 Pharmacological modulators of electrical activity.

Drug	Mechanism of action	Route of administration (or notes on use in zebrafish)
Tetrodotoxin (TTX)	Blocks conductance of most voltage-gated sodium channels	Inject into embryo. Can be applied to the entire embryo.
α-scorpion toxin (α-ScTX)	Slows sodium channel inactivation	Inject into embryo. Can be applied to the entire embryo.
α-bungarotoxon (BTX)	Competitive inhibitor of nicotinic ACh receptors	Inject into the yolk sac, preferably after 12 hpf, to get it into the extracellular space. Effects are long-lasting (embryos injected at 12 hpf are still paralyzed at 8 days)
Strychnine	Glycine inhibitor	Inject or put in bath and make holes in yolk sac. Can be applied to the entire embryo.
Bicuculline	GABA$_A$ receptor antagonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
Picrotoxin	GABA$_A$ receptor antagonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
Muscimol	GABA$_A$ receptor agonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
Atropine	Competitive antagonist of muscarinic cholinergic receptors	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
EGTA	Chelates calcium	Inject into the embryo. Use for dissected preparations because it is toxic if applied to the whole embryo.
Curare	Competitive antagonist of ACh at NMJs	Inject or put in bath and make holes in yolk sac. Can be applied to the whole embryo.
Tricaine	Blocks conductance of voltage-gated sodium channels	Apply to embryo media; effects are eventually reversible by transferring embryo to fresh media and allowing the tricaine that has been accumulated to leave the embryo.
Tetraethylammonium chloride (TEA-Cl)	Potassium channel inhibitor	Highly toxic if applied to the whole embryo. Only use for physiology experiments with dissected preparations!
Kynurenic Acid	NMDA receptor antagonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
CNQX	AMPA/Kainate receptor antagonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
APV	NMDA receptor antagonist	Inject or put in bath and make holes in yolk sac. Use for dissected preparations because it is toxic if applied to the whole embryo.
Cobalt	Calcium channel inhibitor	Highly toxic if applied to the whole embryo. Only use for physiology experiments with dissected preparations.
Cadmium	Calcium channel inhibitor	Highly toxic if applied to the whole embryo. Only use for physiology experiments with dissected preparations.
Heptanol	Gap junction inhibitor	Highly toxic if applied to the whole embryo. Only use for physiology experiments with dissected preparations. Heptanol can also non-specific effects.

another wavelength) that is not affected by calcium activity to measure changes in calcium concentrations.

Disadvantages of calcium indicators include cytotoxic effects due to calcium chelation or photodamage associated with excitation of fluorophores. Further, compared to the time resolution of electrophysiology, optical imaging techniques provide much lower temporal resolution. However, if used carefully, calcium imaging can be a relatively non-invasive method for monitoring changes in calcium activity in cells of a single developing embryo over an extended period of time. Additionally, activity can be monitored in many cells at once, allowing characterization of neuronal network activity during development.

Manipulate electrical activity in the embryonic nervous system

Challenges

While many studies have used electrophysiology and calcium imaging to study electrical activity during development, fewer show developmental effects of changing electrical activity. For results to be meaningful, it is important that the methods we employ change electrical activity without themselves affecting other developmental processes.

Additionally, the embryo must survive from the point of manipulation to later points in development when the effects of manipulation can be assessed.

Approaches

Zebrafish embryos are particularly amenable to both pharmacological and genetic manipulation of electrical activity. Pharmacological modulators of electrical activity can be delivered to zebrafish embryos by several different routes (**Table 2**). Injection into either the yolk or the cell of the one-cell embryo will distribute the drug into intracellular compartments. Alternatively, for drugs to reach the extracellular space, they can be injected into the yolk sac of older embryos or simply added to the embryo media and be taken up into the circulation. A wide variety of drugs are available that modulate electrical activity by altering the function of specific types of ion channels, neurotransmitter systems, pre-synaptic activity, or postsynaptic activity, allowing specific aspects of electrical activity to be modified. Additionally, the consequences of altering electrical activity at specific points in development can be studied by applying modulators only at the time of interest. Overall, this allows a high degree of precision to be achieved in studying electrical activity in development.

Table 3 Mutant lines with altered electrical activity.

Mutant	Protein affected	Phenotype	Reference
macho	Unknown	Reduced sodium current in Rohon-Beard sensory neurons and retinal ganglion cells, enhanced survival of Rohon-Beard cells, reduced touch sensitivity with normal spontaneous swimming, first identifiable at 27 hpf, defects in retinotectal mapping	Granato *et al.* 1996; Trowe *et al.* 1996; Ribera and Nüsslein-Volhard 1998; Gnuegge *et al.* 2001
relaxed	Dihydropyridine receptor beta-1 calcium channel subunit	Immotile due to defective excitation-contraction coupling, muscle degeneration at later stages, cardiac edema	Granato *et al.* 1996; Zhou *et al.* 2006
sofa potato	Muscle-specific acetylcholine receptor delta subunit	Paralysis due to lack of synaptic transmission	Granato *et al.* 1996; Ono *et al.* 2001, 2004
nic1 *chrna1*	Muscle-specific acetylcholine receptor alpha subunit	Immotile due to lack of synaptic transmission, death by 7 dpf	Westerfield *et al.* 1990; Granato *et al.* 1996; Sepich *et al.* 1998
twister *chrna1$^{dtbn12/dtbn12}$*	Muscle-specific acetylcholine receptor alpha subunit (allelic to *nic1*)	Increased neuromuscular activity, uncoordinated movement and hypercontraction of trunk muscles, aberrant pathfinding and branching of motor axons, disorganized muscle fiber structure	Lefebvre *et al.* 2004
ache or *zieharmonika*	Acetylcholine esterase (AChE)	Progressive paralysis, muscle fiber defects, death of PMNs and SMNs, early death of Rohon-Beard cells	Behra *et al.* 2002; Downes and Granato 2004
unplugged	Muscle-specific kinase	Acetylcholine receptor clustering defects, aberrant pathfinding of motor axons	Zhang *et al.* 2004
twitch once	Muscle rapsyn	Abnormal touch response with use-dependent fatigue, lack of spontaneous swimming	Granato *et al.* 1996; Ono *et al.* 2002
shocked	Glycine transporter 1	Reduced spontaneous contractions, abnormal touch response, lack of swimming, persistent electrical coupling	Granato *et al.* 1996; Cui *et al.* 2004; Luna *et al.* 2004; Cui *et al.* 2005
bandoneon	Glycine receptor beta subunit	Abnormal touch response characterized by bilateral trunk muscle contraction rather than alternating trunk movements	Hirata *et al.* 2005

While pharmacological agents target specific classes of ion channels or neurotransmitter systems, they rarely target a single molecule or cell type and consequently several ion channels or receptors expressed in numerous cells are affected. Forward and reverse genetics approaches allow examination of the consequences of altering expression and/or function of a single gene. Large-scale mutagenesis screens looking for mutants with abnormal behavior, altered development of neurons, or aberrant axons have identified a wide variety of genes involved in development of the nervous system (**Table 3**). Some of these mutants display altered patterns of electrical activity during development, suggesting roles for specific aspects of electrical activity in regulating nervous system development (Luna *et al.* 2004; Ribera and Nüsslein-Volhard 1998). These mutants serve as valuable models that will greatly enhance our understanding of the genes that regulate electrical activity in the developing nervous system.

Although efficient methods for targeted gene knockouts in zebrafish have yet to be developed, gene expression can be easily altered through the use of antisense morpholino technology. Morpholinos are modified oligonucleotides that can be designed to target a specific RNA and either prevent correct splicing of the transcript or inhibit protein translation (Nasevicius and Ekker 2000). Morpholinos can be injected into the yolk or the cell of one-cell embryos allowing distribution throughout the embryo. This is an efficient way to study the effects of loss of a specific ion channel or loss of another component in the electrical signaling system. While morpholinos are highly stable, they eventually turn over so that the effects typically last only 3-5 days (Nasevicius and Ekker 2000). However, the vast majority of nervous system development in the zebrafish embryo is completed within the first 5 days of development, so the duration of gene knock-down achieved by antisense morpholinos is sufficient for studying most aspects of nervous system development.

Recent studies also demonstrate that it is possible to deliver morpholinos to a subset of cells within the embryo by injecting the oligonucleotide into a single cell of a 128-cell embryo (e.g., Pineda *et al.* 2006). Consequently, embryos are generated that have targeted protein knock-down in individual cells or groups of cells randomly distributed throughout the embryo (mosaic knock-down). This allows study of the roles of electrical activity in specific subsets of cells. Mosaic knock-down could also be used to study the effects of knock-down of some proteins that are lethal when knocked out in the entire embryo.

Evaluate the effects of altered electrical activity

Challenges

For studies manipulating electrical activity to be meaningful, we need reliable tools that allow us to assess the effects on development. We must be able to reliably evaluate alterations in development at the molecular, cellular, and behavioral levels.

Approaches

While a wide variety of developmental processes may be affected by electrical activity, the zebrafish system allows examination of these effects at the level of changes in gene expression, cellular and axonal morphology, and behavior. Patterns of mRNA expression can be studied in the zebrafish embryo using reverse transcription-polymerase chain reaction to evaluate expression of genes in entire or portions of embryos, or using *in situ* hybridization to localize expression signals to specific tissues or even to individual cells within a tissue. *In situ* signals can be detected using a color reaction (nitro blue tetrazolium chloride/5-bromo-4-chloro-3'-indolyphosphate p-toluidine salt, diaminobenzidine, FastRed) or can be co-localized with another signal using fluorescent detection (FastRed, tyramide signal amplification systems [PerkinElmer], biotinylated antibody systems). Further, recent studies have generated methods for double fluorescent *in situ* hybridization, allowing comparison and co-localization of two *in situ* signals. Similarly, protein expression can be examined by immunocytochemistry (ICC) with either colorimetric or fluorescent detection. Of particular interest, *in situ* and ICC signals can be easily visualized in the transparent zebrafish embryo using traditional microscopy or confocal microscopy without the need to section the tissue.

The development of green fluorescent protein (GFP)-expressing transgenic zebrafish lines has further facilitated assessment of many developmental processes. Transient transgenic zebrafish are generated by injecting the transgene construct into the one-cell embryo. When the transgene incorporates into the genome in germ line cells, founders for stable lines are also generated by this method. As

Table 4 Transgenic lines with neural expression patterns.

Neuronal promotor	Expression pattern	Reference
HuC	Neuronal cell bodies and axons in the CNS, and ventral motor nerves	Park *et al.* 2000
Hb9	PMN and SMN cell bodies and axons	Flanagan-Steet *et al.* 2005
ngn1 (-8.4 promoter)	Epiphysis, dorsal midbrain, hindbrain, Rohon-Beard sensory neurons, dorsal root ganglia, telencephalon, cells throughout the D-V extent of the spinal cord, somatic and axonal labeling	Blader *et al.* 2003
ngn1 (-3.1 promoter)	Epiphysis, dorsal midbrain, hindbrain, Rohon-Beard sensory neurons, dorsal root ganglia, restricted subset of intermediate spinal cord neurons, somatic and axonal labeling	Blader *et al.* 2003
gata2	SMNs and their axons, brain, and eyes	Meng *et al.* 1997
islet1	Cranial motor neurons, Rohon-Beard sensory neurons, telencephalon, mesencephalon, medial macula, posterior otolith, dorsally-projecting motor neurons, somatic and neuritic labeling	Higashijima *et al.* 2000
alpha-1 tubulin	Pan-neuronal expression from neuronal commitment through synaptogenesis	Gulati-Leekha and Goldman 2006
flh	Developing midline, pineal organ, parapineal organ, ventrally-projecting PMNs	Gamse *et al.* 2003; Pineda *et al.* 2006
pax2.1	Forebrain, midbrain-hindbrain boundary, hindbrain, rhombomeres 3 and 5, ear, pronephros, restricted populations of spinal cord neurons	Picker *et al.* 2002
olig2	Ventral diencephalon, cerebellar purkinje cells, spinal motor neurons, oligodendrocytes and their precursors	Shin *et al.* 2003
GAP-43	Brain, cranial ganglia, retina, olfactory epithelium, Rohon-Beard sensory neurons, spinal interneurons, SMNs (not PMNs). Expressed during neuronal differentiation and axogenesis.	Udvadia *et al.* 2001

the efficiency of this process increases with new technology, more and more transgenic lines are being developed with GFP, red fluorescent protein (RFP), or other markers expressed under a wide variety of promoters in specific, well-characterized subsets of cells (**Tables 1, 4**). Additionally, several recent studies have successfully utilized transposable elements to perform enhancer trapping in zebrafish. These enhancer trap projects have resulted in large-scale production of novel transgenic lines, many of which display neural expression patterns (Parinov *et al*. 2004; Choo *et al*. 2006; Scott *et al*. 2007). Transgenic embryos generated by either method with expression in subsets of neurons and their axons represent a useful tool to assess changes in cellular morphology, axon pathfinding, or neuronal differentiation in response to altered electrical activity.

Alternately, cellular and axonal morphology can be examined in mosaically labeled embryos or through the use of single-cell labeling techniques. Mosaically-labeled embryos, generated by injection of a fluorescent tracer dye into a single cell of a blastula-stage embryo, contain isolated fluorescent cells and small clusters of fluorescent cells randomly distributed throughout the embryo. This technique results in labeling of a wide variety of cell types in many different tissues. Individual cells can also be labeled by iontophoretic injection of a fluorescent tracer. Single-cell and mosaic labeling both allow examination of cellular morphology and axonal trajectories in individual cells and can be used to detect changes that result from altered electrical activity.

While the tools discussed above allow us to investigate the molecular and cellular changes in processes that are regulated by electrical activity, behavioral studies in zebrafish

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Box 2 Zebrafish Neural Development

Development of the zebrafish nervous system has been well characterized at the cellular, molecular, and electrophysiological levels. The following is a guide to some of the important developmental processes that occur during zebrafish nervous system development.

1. **Induction and specification**. Induction and specification of nervous tissue begins during gastrulation of the zebrafish embryo. Numerous signals from the mesoderm, the notochord, and the floor plate are involved in neural induction and specification. Many classic developmental signaling molecules, such as BMP, FGF, hedgehog, and chordin also play critical roles in zebrafish neural induction and specification (Appel and Eisen 1998; Park *et al*. 2004; Londin *et al*. 2005).

2. **Primary neurogenesis.** Primary neurogenesis occurs during late gastrulation from about 9 to 12 hpf in zebrafish embryos. In the spinal cord, primary neurogenesis results in the generation of 3 primary motor neurons (Myers *et al*. 1986; Beattie *et al*. 1997; Gray *et al*. 2001), 2-3 Rohon-Beard sensory neurons, and several classes of interneurons in each hemisegment (Hale *et al*. 2001).

3. **Secondary neurogenesis.** Secondary neurogenesis begins after primary neurogenesis. For motor neurons secondary neurogenesis starts at about 16 hpf and continues past 25 hpf. The end result is 30-40 SMNs per hemisegment. (Myers *et al*. 1986; Beattie *et al*. 1997). SMNs are molecularly distinct from primary motor neurons and mediate fast swimming, whereas primary motor neurons regulate slow swimming (McLean *et al*. 2007). Although most classes of interneurons cannot be definitively classified as either primary or secondary neurons, interneurons continue to be added throughout the period of secondary neurogenesis.

4. **Axon outgrowth and pathfinding.** Axon outgrowth and pathfinding occurs over an extended period of time with timing and regulatory factors that are highly specific to different neuronal populations. For example, in each hemisegment primary motor axons leave the spinal cord from 18-19 hpf in the ventral nerve root and travel together in the ventral nerve root until they reach the horizontal myoseptum, where they encounter the choice point. At the choice point CaP axons continue ventrally as part of the ventral nerve; RoP axons extend rostrally along the horizontal myoseptum; and MiP axons turn 180 degrees dorsally and travel in the dorsal nerve. Secondary motor axons begin to leave the spinal cord at 26 hpf in the ventral nerve root and axons continue to be added until about 34 hpf (Myers *et al*. 1986). Although secondary motor axons have similar trajectories to primary motor axons, they are guided by different factors (Beattie 2000; Zeller *et al*. 2002; Pineda *et al*. 2006).

5. **Synaptogenesis.** When axons reach their targets, they form synapses that allow electrical signals to be translated into chemical signals. This process is tightly regulated and while some synapses are strengthened and survive, others are eliminated (Liu and Westerfield 1990). Synapse strengthening and elimination are mediated by neurotrophic signals. Some of these signals are activity-dependent, and they can be regulated by competition with other axons (Downes and Granato 2004; Hua *et al*. 2005). In zebrafish embryos, synaptic clusters can be seen as early as 16-24 hpf with identifiable synapses forming by 20 hpf as developing axons enter the periphery. Synaptogenesis continues throughout embryonic and larval development (Panzer *et al*. 2005).

6. **Development of motor behaviors.** Zebrafish embryos develop stereotyped motor behaviors as they develop. The earliest movements, consisting of spontaneous contractions of the trunk and tail, begin at about 17 hpf. As the embryo develops further, it will respond to touch by alternating tail contractions, starting at about 22 hpf. At approximately 27 hpf the embryo becomes capable of swimming in response to a head tap. Swimming frequency and the fidelity of the escape response increase until about 48 hpf (Saint-Amant and Drapeau 1998, 2000). These behaviors are commonly used as indicators of sensorimotor function in the embryo.
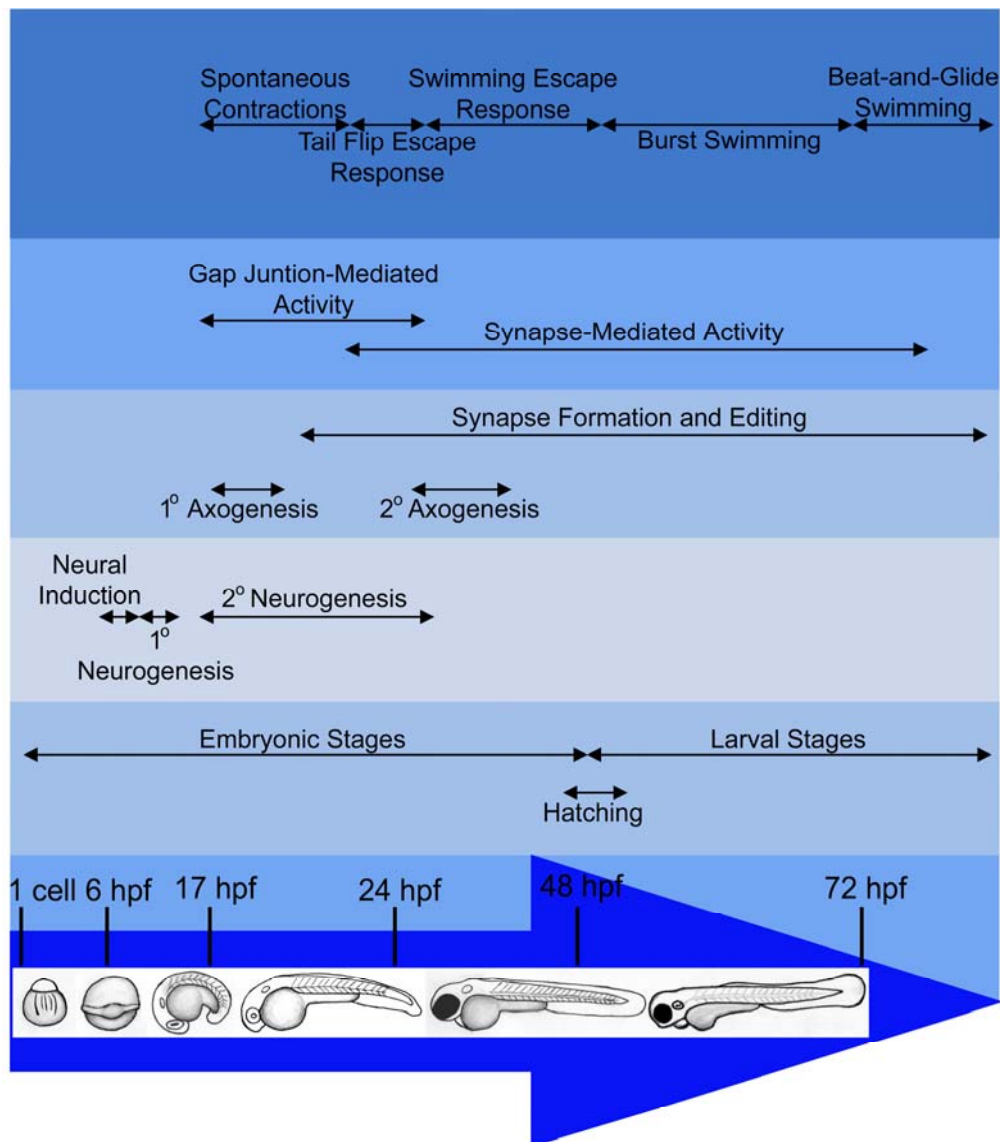
Fig. 1 Timeline of zebrafish nervous system development. Critical events in development of the zebrafish nervous system occur within the first five days of development.

embryos provide a way to link cellular changes to function. Zebrafish embryos display several predictable behaviors that can be easily tested during development (**Box 2; Fig. 1**). For example, motor system function is first apparent at 17 hours post fertilization (hpf) when embryos begin to twitch spontaneously. As the nervous system matures, embryos gain the ability to generate alternating trunk contractions and eventually develop burst type swimming and finally become capable of beat-and-glide swimming. These behaviors occur either spontaneously or in response to sensory stimuli, providing a good test for sensorimotor function. Taking advantage of these and other well-characterized behaviors in developing zebrafish we can assess the functional and behavioral consequences of many of the developmental changes that result from altered electrical activity.

ROLES OF ELECTRICAL ACTIVITY IN ZEBRAFISH EMBRYOS

Several recent studies, reviewed below, have taken advantage of the zebrafish system to investigate the roles of electrical activity in development. These studies demonstrate that in addition to regulating the physiological functions of the mature nervous system, electrical activity also has much longer-lasting effects on development. Altered electrical activity in zebrafish embryos has been shown to regulate such developmental processes as synapse stabilization, refinement of the retinotectal map, programmed cell death of sensory neurons, and axon branching and fasciculation *in vivo* (**Fig. 2**).

Synapse stabilization

Electrical activity plays a role in synapse formation and plasticity. In zebrafish, the existence of mutants with abnormal levels of the transmitter acetylcholine (ACh), or the postsynaptic ACh receptor (AChR), provides the opportunity to study the role of electrical activity in development of cholinergic synapses (**Table 3**). Downes and Granato (2004) utilized these mutants to show that the neuromuscular junction can form in the presence of abnormally high levels of the transmitter. The synapse, however, fails to be maintained and abnormal neuromuscular junctions are present at later stages. These data suggest that at least some synapses can form independently of activity but are dependent on activity for stabilization (Downes and Granato 2004).

Retinotectal mapping

Acuity of the visual system relies on a precisely organized retinotectal map that translates the visual world into electrical signals in the tectum. During development, a rough map is formed, with retinal ganglion cells forming large arbors that are subsequently refined. Sharpening of this map is thought to be dependent on electrical activity, and indeed studies in zebrafish have begun to identify roles for electrical activity in this developing system. While there have been conflicting reports as to the necessity of activity in retinotectal mapping, Gneugge *et al.* (2001) suggest these inconsistencies may arise from differences in timing of electrical blockade. The *macho* mutant, first identified for
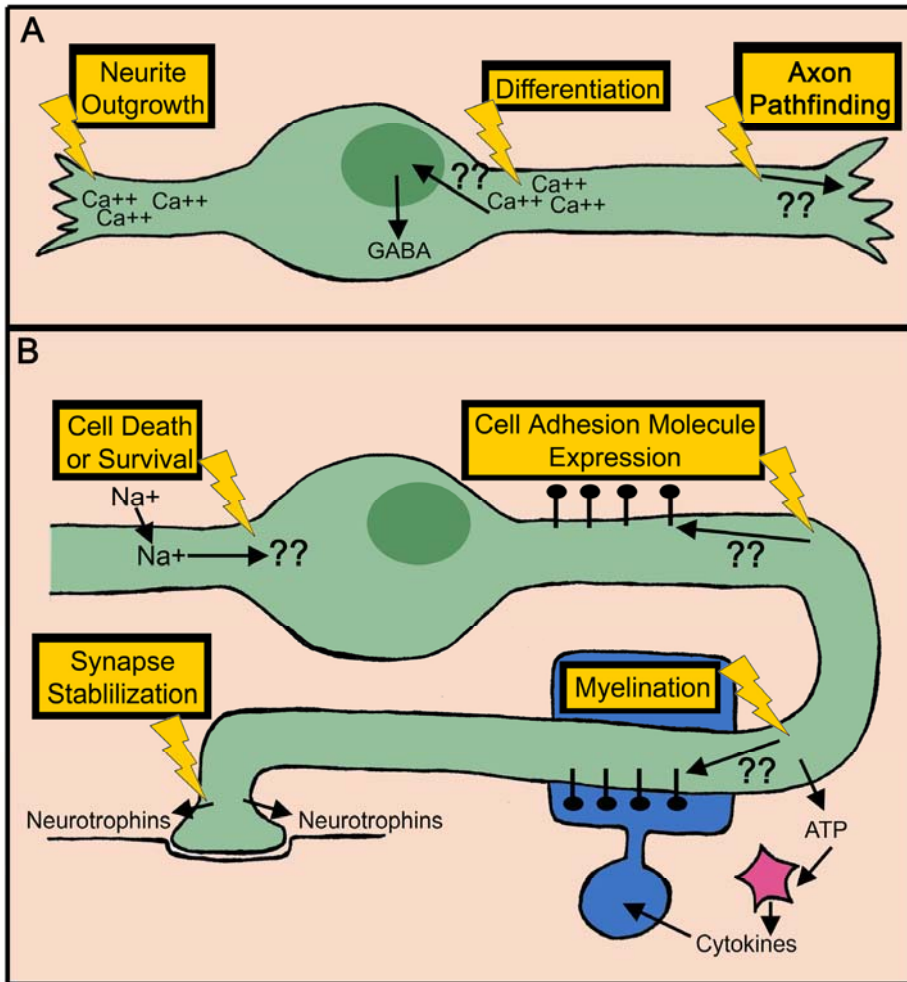
Fig. 2 Electrical activity plays many roles in neural development. (**A**) Roles of electrical activity in early developmental processes as neurons initiate morphological differentiation. (**B**) Roles of electrical activity in later developmental processes as neurons form and maintain synapses. Lightning bolts represent electrical activity within the cell membrane.

defects in locomotion and retinotectal mapping (**Table 3**), was shown to lack action potentials in retinal ganglion cells, implicating loss of activity as a possible regulator of this defect (Granato *et al*. 1996; Ribera and Nüsslein-Volhard 1998; Trowe *et al*. 1996). Further, although blocking electrical activity by tetrodotoxin (TTX) application during early stages of mapping (before 4 days of development) does not affect the retinotectal map, TTX application from 4-6 days results in unusually large retinotectal arbors and abnormalities in visual behaviors, similar to those present in the *macho* mutant (Gnuegge *et al*. 2001). The finding that effects of electrical activity are restricted to specific developmental periods, known as sensitive periods, is a common theme in electrical regulation of development. Sensitive periods are also critical periods if the affected phenotypes do not recover at later times even after the perturbation is removed.

Further studies have provided insights into the molecular mediators of activity-dependent refining of the retinotectal map. Schmidt *et al*. (2000) used pharmacological blockade of NMDA receptors to show that a rough map is made in the absence of NMDA receptor activity, but as with electrical blockade the map is not refined and sharpened (Schmidt *et al*. 2000). Additionally, these effects can be phenocopied by inhibition of PKC and arachidonic acid, suggesting that electrical activity regulates refinement of the retinotectal map by modulating these messenger systems, possibly through activation of NMDA receptors (Schmidt *et al*. 2004). Collectively, these studies suggest that, in contrast to the extra-synaptic electrical signaling that regulates myelination, retinotectal mapping is mediated by synaptic activity.

Hua *et al*. (2005) revealed an additional role for electrical activity in regulating growth and branching of retinal ganglion cell axons. Interestingly, these effects were only apparent upon mosaic knock-down of electrical activity. Axon growth and branching appears to be unaffected by loss of electrical activity in all retinal ganglion cells. However, axon growth is inhibited in cells lacking electrical activity when activity is unaffected in neighboring cells, suggesting that activity serves as a competitive regulator of RGC axon growth and branching (Hua *et al*. 2005).

Programmed cell death

Programmed cell death is an important aspect of nervous system development. For example, in many species a population of mechanosensory early-born neurons, known as Rohon-Beard cells, are completely eliminated by programmed cell death as later developing dorsal root ganglia take over their function. In zebrafish, electrical activity has been suggested as a regulator of Rohon-Beard cell death. Svoboda *et al*. (2001) demonstrated that Rohon-Beard cells persist much longer in *macho* mutants, which have reduced sodium current in Rohon-Beard cells, than in wild-type fish. Similar reductions in Rohon-Beard cell death were seen in embryos treated with TTX, providing further evidence that Rohon-Beard cell death is regulated by electrical activity (Svoboda *et al*. 2001).

Interneuron differentiation

Studies in zebrafish suggest that glycine receptors may regulate differentiation of interneurons. McDearmid *et al*. (2006) took advantage of the ease of antisense morpholinos to knock down a specific glycine receptor subunit, α-2, in embryonic zebrafish. Using molecular markers of different populations of neurons, they found that morphant embryos had decreased numbers of interneurons but not sensory or motor neurons. Furthermore, cell death was not increased

but an increase in mitotic divisions of neuronal precursor pools occurred in the morphant spinal cord. These data suggest that precursors of interneurons failed to exit the mitotic cycle and differentiate. Consequently, early network activity is altered in these embryos (McDearmid *et al.* 2006). It was not examined whether at later stages of neurogenesis more interneurons were formed as a result of the increase in the precursor population. Taken together with work done in *Xenopus* showing altered neurotransmitter phenotypes in neurons with altered electrical activity, these data reveal electrical activity as a possible regulator of neuronal differentiation.

Motor axon development

A recent paper by Pineda *et al.* (2006) provides evidence that voltage-gated sodium channels regulate development of specific populations of motor axons in zebrafish embryos. Morpholino knock-down of nav1.6a delays outgrowth of dorsally-projecting secondary motor neuron (SMN) axons. This effect recovers upon turnover of the morpholino, suggesting nav1.6a is required for outgrowth of dorsally-projecting motor axons.

Additionally, ventrally-projecting SMNs develop abnormally when the voltage-gated sodium channel nav1.6a is specifically knocked down by antisense morpholinos. These effects are detectable by 72 hpf, at which time morpholinos were still functional. However, unlike the effects of the morpholino on dorsal axons, the ventral axon phenotype continues to worsen at later stages when morpholino turnover is expected (Pineda *et al.* 2006). Interestingly, this suggests that nav1.6a acts during a critical period to regulate ventral motor axon development and the effects do not recover at later times even though channel synthesis resumes. Moreover, rather than recover, effects worsen with time. These data are particularly intriguing as they may shed some light on the developmental processes that lead to development of motor endplate disease in mice lacking functional Nav1.6, which has been suggested to model human dystonias.

Furthermore, the effects of loss of nav1.6a on ventral motor axon development are non-cell autonomous, suggesting that nav1.6a may be important in the functioning of a network that regulates axon development or possibly changes the cell adhesion properties of the environment. The secondary motor axon effects of loss of nav1.6a have not been directly linked to electrical activity, but certainly demonstrate nontraditional roles for voltage-gated ion channels and highlight the need for more research in this area (Pineda *et al.* 2006).

ELECTRICAL ACTIVITY IN THE EMBRYONIC VERTEBRATE NERVOUS SYSTEM

Studies in frog, chick and mouse suggest that electrical activity plays a variety of roles in patterning development of the nervous system (for review, Buonanno and Fields 1999). These studies show that in addition to molecular signals, the developing nervous system also responds to precisely tuned patterns of electrical activity. Particularly intriguing is the discovery that it is the pattern of electrical activity, rather than the presence of electrical activity itself, that seems to encode various aspects of development (Borodinsky *et al.* 2004; Itoh *et al.* 1997; Stevens *et al.* 1998). While few studies in these organisms examine the roles of electrical activity *in vivo*, they have provided valuable insight into the molecular mechanisms through which activity may regulate developmental processes. These results reveal a new level of developmental regulation and demonstrate the need to look beyond the traditional signaling molecules when investigating the regulation of developmental processes.

Myelination

Studies in mouse, primarily taking advantage of neuron-glia co-culture systems, show that electrical activity is an important regulator of myelination in both the central and peripheral nervous system (Demerens *et al.* 1996; Stevens and Fields 2000). One study shows that certain patterns of activity (low frequency spikes) inhibit myelination of peripheral axons by down-regulating expression of the cell adhesion molecule L1, whereas lack of electrical activity and high frequency spiking patterns permit axons to be myelinated. These patterns of electrical activity change during different stages of development, with low-frequency activity predominant during development of pre-myelinating Schwann cells and high-frequency activity predominant during the myelinating phases of development (Stevens *et al.* 1998). Furthermore, axonal activity-dependent signals have been shown to regulate proliferation of Schwann cells (Stevens and Fields 2000; Stevens *et al.* 2004).

Of particular interest, these studies have begun to elucidate the molecular mechanisms by which electrical activity can be transduced into developmental signals that regulate myelinating cells. In cell culture systems, axons of both peripheral and central neurons have been shown to secrete adenosine triphosphate (ATP) in response to electrical stimulation. This release of ATP regulates the myelinating activity of both Schwann cells and oligodendrocytes (Stevens and Fields 2000; Ishibashi *et al.* 2006). Curiously, the activity-dependent interactions between axons and oligodendrocytes are mediated indirectly via astrocytes. The ATP released by axons in response to action potentials induces neighboring astrocytes to synthesize and secrete the cytokine leukemia inhibitory factor, which in turn promotes myelination by oligodendrocytes (Ishibashi *et al.* 2006).

Cell adhesion molecules

Many diverse processes in nervous system development, from cell adhesion and migration to neurite outgrowth, pathfinding, and fasciculation, are regulated by cell adhesion molecules (for review, Takeichi 1995). Subsequently, expression patterns of cell adhesion molecules are tightly regulated. Studies of cultured mouse dorsal root ganglion neurons demonstrate that cell adhesion molecules can be differentially regulated by electrical activity (Stevens *et al.* 1998). Furthermore, some adhesion molecules (L1) are regulated only by specific frequencies of activity, while some (e.g., N-cadherin) are regulated by electrical activity at all frequencies tested, and others (e.g. NCAM) are unaffected by electrical activity (Itoh *et al.* 1997). This suggests a mechanism through which neurons can specifically regulate cell adhesion molecule expression as they functionally mature.

Axon guidance and fasciculation

Studies suggest that electrical activity regulates neurite outgrowth (Fields *et al.* 1990). *In vivo* studies in chick by Hanson and Landmesser (2004) show that electrical activity plays a role in motor axon guidance and fasciculation. While the role of electrical activity in synaptic pruning is well established, recent studies have identified specific patterns of spontaneous electrical activity in motor neurons during initial stages of axon outgrowth. When electrical activity is pharmacologically decreased during the developmental period when motor axons enter the limb bud, many axons fail to make the correct dorsal-ventral pathfinding decisions. Axons in control embryos defasciculate as they enter the limb bud and choose specific migration paths. However, when activity is decreased, axons fasciculate into clusters and collectively make aberrant pathfinding decisions. Further, guidance molecules involved in dorsal-ventral pathfinding decisions show altered expression patterns in embryos with decreased electrical activity (Hanson and Landmesser 2004). These findings are consistent with stu-

dies discussed above showing regulation of cell adhesion molecules in mouse cultured neurons and point to a physiological consequence of this regulation.

In addition to its effects on motor axon fasciculation, electrical activity has also been implicated in regulating fasciculation of axons in the mouse olfactory system (Serizawa *et al*. 2006). Specific odors are detected by specialized receptors on olfactory sensory neurons. Axons of olfactory sensory neurons that express the same odorant detector converge and innervate the same glomerulus, which then sends axons to the olfactory bulb and contributes to the development of an odorant map. Axon sorting based upon odorant receptor has been shown to be dependent on differential expression of cell adhesion and recognition molecules. Further, this odorant receptor-specific differential expression of cell adhesion molecules was shown to be disrupted in mice when electrical activity is reduced (Serizawa *et al*. 2006).

Neuronal differentiation

A series of studies in *Xenopus* demonstrated a role for calcium activity in regulating neurotransmitter phenotype and neurite extension (for review, Spitzer *et al*. 2004). Calcium imaging of developing neurons in culture revealed the presence of calcium spikes in neuronal cell bodies and calcium waves in growth cones. Decreasing calcium activity resulted in lower expression levels of the inhibitory neurotransmitter gamma aminobutyric acid (GABA), delays in channel maturation, and increased neurite length in cultured neurons. Interestingly, these defects could be corrected by stimulating calcium spikes and waves at frequencies normally seen in developing neurons, but not at other frequencies. This suggests that developing neurons are tuned to respond to specific frequencies of calcium transients. Further, normal development could not be rescued by depolarization at the correct frequency when calcium transients were blocked, nor could they be rescued in the presence of calcium when depolarization was inhibited. These data suggest that electrical activity can regulate neurotransmitter expression and neurite outgrowth through the generation of specific frequencies of calcium spikes and waves (Gu and Spitzer 1995).

Borodinsky *et al*. (2004) extended these studies by examining the effects of altered electrical activity *in vivo* before the development of synaptically-driven neuronal networks. By overexpressing either the human inward rectifier potassium channel to suppress calcium spikes or rat brain voltage-gated sodium channels to increase spikes, they demonstrated that electrical activity acts as a homeostatic regulator of neurotransmitter expression. In embryos with suppressed electrical activity, more neurons expressed excitatory neurotransmitters, while in embryos with increased electrical activity more neurons expressed inhibitory neurotransmitters. Interestingly, expression of molecular markers of specific neuronal populations was not changed, but identifiable neurons expressed inappropriate neurotransmitters. Finally, cultured neurons were shown to release the inappropriately-expressed transmitters upon stimulation, suggesting these changes are functionally significant (Borodinsky *et al*. 2004). Collectively, these studies suggest that electrical activity regulates neuronal differentiation and neurotransmitter phenotype to normalize overall network activity within the developing nervous system.

FUTURE DIRECTIONS

Certain themes have emerged from these studies that guide our future investigations into the role of electrical activity. First, a myriad of studies reveal the importance of the frequency of patterned activity in regulating development. These studies show that many developmental processes are tuned to respond only to precise frequencies, thus complicating interpretation of results that manipulate activity in an all-or-nothing manner. Second, electrical activity can have non-synaptic effects. While synaptic effects of electrical

activity have long been recognized, far more surprising is the discovery that electrical activity is often present before synaptogenesis occurs and disrupting this activity can have has developmental consequences. By activating intracellular signaling molecules and release of extracellular signaling molecules, activity can alter gene expression and thus regulate basic developmental processes such as differentiation and cell death or survival decisions. Third, studies have begun to reveal that electrical activity can be transduced into developmental signals in a variety of ways. In some instances, activity acts as a regulator of intracellular calcium, which subsequently modulates intracellular signaling cascades. In other instances, electrical activity leads to synaptic as well as non-synaptic release of factors that influence development of surrounding cells. Future studies need to consider that electrical activity is capable of acting through many molecular mechanisms.

Recent advances in technology have led to the establishment of the zebrafish as an important model system for the study of the role of electrical activity in development. Many pieces of the puzzle have been discovered as studies identify more and more patterns of electrical activity that are active during specific developmental processes. Studies identifying molecular regulators of this patterned activity and the importance of early networks in pattern generation have provided still more pieces. Nonetheless, relatively few studies to date have investigated the developmental consequences of altered electrical activity in the embryonic zebrafish. Consequently, the various pieces of the puzzle still need to be put together in an *in vivo* context to obtain a complete picture of electrical regulation of development. To that end, tools now exist in zebrafish that allow us to (1) observe endogenous patterns of electrical activity, (2) manipulate electrical activity in the developing embryo, and (3) evaluate the effects of altered electrical activity on development. With these tools we are poised to build upon the frame provided by pioneering studies in zebrafish and other organisms.

One tool, however, is notably absent from the zebrafish toolbox. There are currently no standard methods for generation of targeted gene knock-outs as is the case for mouse. However, the recent use of TILLING (targeting induced local lesions in genomes) in zebrafish has demonstrated the feasibility of screening fish generated by chemical mutagenesis for mutations in a gene of interest (Wienholds *et al*. 2003). This new method, along with classical linkage analysis and sequencing of mutant lines, has resulted in the production of many zebrafish lines containing null mutations in identified genes. Further, morpholino technology allows targeted knock-down of a protein, albeit for a limited period during the first days of development. While these alternative approaches can often substitute for a genetic knock-out, they do not allow the gene knock-down to be limited to a specific subset of cells, as is possible in mouse using the cre-lox system (Kuhn *et al*. 1995). One method that overcomes this difficulty is the generation of embryos that are either mosaic or chimeric with respect to the presence of a morpholino or a genetic mutation (Shinya *et al*. 1999; Pineda *et al*. 2006). Currently, these methods are inefficient and require extensive analysis in order to obtain embryos that have the appropriate complement of morphant/mutant and wild type cells. One possible approach to overcome this limitation is to develop transgenic lines using cell-specific promoters driving expression of RNAi targeting a gene of interest. We thus look forward with great anticipation to the production of these tools in zebrafish.

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