



HHS Public Access

Author manuscript

Dev Biol. Author manuscript; available in PMC 2019 January 15.

Published in final edited form as:

Dev Biol. 2018 January 15; 433(2): 177–189. doi:10.1016/j.ydbio.2017.08.032.

Bioelectric Signaling in Regeneration: Mechanisms of Ionic Controls of Growth and Form

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Abstract

The ability to control pattern formation is critical for both the embryonic development of complex structures as well as for the regeneration/repair of damaged or missing tissues and organs. In addition to chemical gradients and gene regulatory networks, endogenous ion flows are key regulators of cell behavior. Not only do bioelectric cues provide information needed for the initial development of structures, they also enable the robust restoration of normal pattern after injury. In order to expand our basic understanding of morphogenetic processes responsible for the repair of complex anatomy, we need to identify the roles of endogenous voltage gradients, ion flows, and electric fields. In complement to the current focus on molecular genetics, decoding the information transduced by bioelectric cues enhances our knowledge of the dynamic control of growth and pattern formation. Recent advances in science and technology place us in an exciting time to elucidate the interplay between molecular-genetic inputs and important biophysical cues that direct the creation of tissues and organs. Moving forward, these new insights enable additional approaches to direct cell behavior and may result in profound advances in augmentation of regenerative capacity.

Keywords

bioelectricity; ion channel; resting potential; voltage; patterning

Introduction

A critical goal of regenerative biology and medicine is to understand and control the mechanisms underlying the processes directing growth and patterning. Alongside conventionally-studied transcriptional networks and chemical cues, additional inputs enable cells to cooperate and make decisions necessary for the repair and remodeling of complex anatomical structures. Endogenous ion flows serve as important regulators of cell behavior, coordinating cell activity during pattern homeostasis. Located within cell membranes, ion channels, pores, and pumps create a complex language of bioelectric signals that is tightly

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integrated with gene regulatory networks to direct cell behavior toward the creation and maintenance of functional tissues and organs. Here we discuss the known roles of ion-based physiological processes in directing cell behavior during pattern formation and regeneration. Specifically excluded in this review are the fast-acting action potentials associated with neurons and muscle cells, externally-applied electromagnetic fields and radiation, and ultra-weak photon emission.

What is developmental bioelectricity?

All cells drive and respond to changes in transmembrane voltage potential (V_{mem}). Unlike fast-spiking currents normally associated with nerve and muscle cell activity, ion pumps, channels, and pores distribute specific ion species across cellular plasma membranes to produce slowly-changing spatial patterns of resting potential (Figure 1). In addition, groups of cells can be electrically connected via the diffusion of small molecules between cells through electrical synapses known as gap junctions (Fitzharris and Baltz, 2006; Mathews and Levin, 2017). These transmembrane potentials, fluxes of individual ions, and iso-electric cell compartments established by gap junctions, convey information to target cells, their neighbors, and in some instances, to distant locations. This signaling modality is used to process and transmit information about regenerative parameters such as cell type, tissue size, positional information, axial polarity, and organ identity (Levin, 2014; Levin et al., 2017; Pitcairn and McLaughlin, 2016). Importantly, these signals (unlike the familiar mRNA and protein signals) can only be characterized in the living state. Furthermore, the ability of channels and gap junctions to open and close post-translationally means that bioelectric cell states are a complex function of a given cell's microenvironment history, impinging physiological signals, and expression levels of electrogenic machinery.

Over a century of observations in developmental bioelectricity - a historical perspective

The “electrical properties of living tissues” have been discussed by scientists for over a century (Mathews, 1903); prescient workers such as Burr (Burr and Northrop, 1935a) and Lund (Lund, 1947) characterized bioelectrical gradients in developing and regenerative systems, and used applied voltages to show that bioelectric signals were not merely epiphenomena of housekeeping physiology but were instructive for specific changes in growth and patterning in a range of fungal, plant, invertebrate, and vertebrate species. Marsh and Beams spearheaded some of the useful studies supporting an instructive role for bioelectric signaling during tissue patterning. By applying external electric fields to worm fragments, they demonstrated the ability to specifically alter the anterior-posterior polarity of regenerating fragments of planaria (Marsh and Beams, 1947, 1952). Subsequent instrumental work by several researchers including Lionel Jaffe, Richard Nuccitelli, Richard Borgens, Colin McCaig, and Ken Robinson, found that the electrical properties of single cells, neural tissues, epithelia, and entire appendages, were able to direct growth, morphology, and tissue polarity during regeneration of a wide range of model species (Borgens, 1982, 1983, 1986; Borgens, 1989; Jaffe, 1980, 1981, 1982; Jaffe et al., 1974; McCaig et al., 2005; Nuccitelli and Jaffe, 1974, 1976; Robinson, 1983).

With the advent of modern molecular, cellular, and genomic methodologies, more recently researchers have built upon these early studies to characterize proteins responsible for generating the bioelectric signals, transduction machinery that converts voltage change into second-messenger cascades, the gene regulatory networks downstream of bioelectric signaling, and ultimately the underlying mechanisms that direct cell behavior. The development of molecular-resolution genetic and pharmacological tools to investigate and manipulate ion flow has revealed that changes in resting potential can control individual cell behaviors including: proliferation, cell death, migration, and differentiation, in of a wide variety of cells types (Figure 1). In addition, recent data implicate endogenous spatiotemporal patterns of V_{mem} in regulating processes during embryonic development, regeneration, and patterning, that when altered, are responsible for a wide range of channelopathies and birth defects (Matusik, 2017; Persson and Bondke Persson, 2016).

Cell-level control of behavior by ion-mediated processes

During the creation of organized tissues, the ability to control cell behavior is critical for the formation of properly patterned structures. Large-scale morphogenesis necessitates the coordination of individual cells whose function is regulated via the integration of molecular cues and endogenous bioelectrical signals (as outlined in Figure 1). Both the creation of functional organs during development and regeneration of missing structures post-injury, require careful regulation of cell movement and positioning. Examples of cell migration events include the movement of progenitor cells towards the injury site observed in: planaria (Salo and Baguna, 1985), zebrafish (brains, hearts, fins) (Salo and Baguna, 1985; Tahara et al., 2016; Zupanc, 2006), and stem cell homing (Chute, 2006). Over half a century ago, several groups reported that electric fields could be used to direct cell behavior to orient cells either parallel or perpendicular to the field line, extend cell processes, or direct migration relative to the positioning of an anode or cathode (Anderson, 1951; Hyman and Bellamy, 1922). Although there is some debate over which cell types respond to physiologically relevant electric fields (Robinson and Cormie, 2008), subsequent work has shown numerous embryonic and somatic cell types exhibit galvanotaxis in electric fields *in vivo* (Pullar and Isseroff, 2005; Stump and Robinson, 1983; Yao et al., 2008; Zhao et al., 1997). It has been postulated that during embryogenesis these electric fields serve to both polarize the early vertebrate embryos as well as provide important positional cues that direct cell movements necessary for morphogenesis and pattern formation (Pitcairn et al., 2017; Shi and Borgens, 1995). This is especially relevant for guiding innervation and the movement of epithelial cells to close wounds – key components of regenerative response (Cao et al., 2013; Reid et al., 2005; Yamashita et al., 2013). Studies examining the migration of pigment cell derivatives originating from the neural crest, demonstrated altering bioelectrical events during early stages of embryogenesis caused melanocytes to inappropriately colonize tissues and organs in *Xenopus* tadpoles (Blackiston et al., 2011a; Morokuma et al., 2008). Similarly, a mutation in zebrafish that disrupts the pore function of an inwardly rectifying potassium channel (Kir7.1) altered the migration, but not the differentiation, of melanosomes (Iwashita et al., 2006).

Since it is a prerequisite for metastasis, much research has focused on targeting cell migration as way to hinder cancer progression. Ion transport proteins are easily accessible in

cell membranes and are often overexpressed or activated in cancer (Becchetti et al., 2017; Funk, 2015; Pollak et al., 2017); thus, inhibition of the mechanisms underlying metastasis offers great therapeutic potential. Numerous studies have implicated bioelectric signaling as one of the mediators of electric guidance in migrating metastatic cancer cells (Fraser et al., 2005; Litan and Langhans, 2015; Mycielska and Djamgoz, 2004; Schwab, 2001; Schwab et al., 2012; Schwab and Stock, 2014).

Integration of cell proliferation into the overall pattern of a tissue and its size during regeneration can be controlled by membrane potential. As a general rule, proliferating cells tend to be more depolarized than non-proliferating (often differentiated) cells. This observation was further explored in a comparative analysis examining the membrane voltage of numerous cell types that revealed a striking functional connection between resting V_{mem} and the ability to progress through the cell cycle (Binggeli and Weinstein, 1986; Levin, 2012; Rao et al., 2015). Although the control of cell division by bioelectric signals is not always cell-autonomous (Morokuma et al., 2008; Pai et al., 2015a), in several studies proliferation appears to be controlled predominantly by a cell's own membrane potential (Arcangeli et al., 1993; Cone, 1974).

While the underlying mechanisms have not been completely elucidated, there is growing evidence that ion flow plays a role in regulating cell division (MacFarlane and Sontheimer, 2000; Ouadid-Ahidouch and Ahidouch, 2008, 2013; Ouadid-Ahidouch et al., 2016; Putney and Barber, 2003; Valenzuela et al., 2000). More specifically, numerous studies have implicated K^+ currents as playing a key role in mediating cell division and cell cycle progression (Deng et al., 2007; MacFarlane and Sontheimer, 2000; Rouzair-Dubois et al., 1993; Urrego et al., 2014). In the zebrafish, a mutation that reduces intracellular K^+ concentration, and consequently hyperpolarizing cells, results in a dramatic increase in the fin and barbel size due to increased proliferation (Perathoner et al., 2014). Early observations that wide-spectrum potassium channel blockers inhibit proliferation in T-lymphocytes (DeCoursey et al., 1984) provided some of the first evidence that K^+ concentration could regulate cell cycle progression in mammalian cells. More recently, numerous studies examining a diverse array of cell types have demonstrated potassium channel activity is functionally linked to cell proliferation. For example, the voltage-gated potassium channel $K_V1.3$ (KCNA3) has been implicated in the control of cell cycle in many cell types including: proliferating oligodendrocyte progenitors during G_1/S transition (Chittajallu et al., 2002), microglia cells (Kotecha and Schlichter, 1999), and macrophages (Vicente et al., 2003).

The activity of chloride channels has also been shown to modulate cell proliferation. For instance, inhibition of volume-regulated Cl^- channels (VRCCs) results in p27 accumulation and G_1 cell cycle arrest in T-cell leukemia cells and human embryonic kidney (HEK) cells (Renaudo et al., 2007). Similarly, blocking the function of TMEM16A (a calcium-activated Cl^- channel), arrests colorectal cancer cells in G_1 (Sui et al., 2014). In addition, gliomas often express higher levels and activity of voltage dependent (CLC family) chloride channels, contributing to depolarized membranes (Olsen et al., 2003). A reduction of Cl^- channel activity via exposure to chemical inhibitors or siRNA-mediated knockdown both reduces Cl^- currents and inhibits proliferation of gliomas (Lui et al., 2010; Yang et al.,

2006). The induction of proliferative states via altering membrane potential (Sundelacruz et al., 2013b) offers an important addition to regenerative biologists seeking to induce growth after injury.

Biological processes such as regeneration require a careful balance between cell proliferation and the elimination of cells through programmed cell death (PCD). Thus, in addition to regulating cell division, induction of PCD must also be controlled. Although counterintuitive, research in diverse model systems revealed that dying cells can secrete mitogens that directly stimulate stem or progenitor cell proliferation (Chera et al., 2009; Fan and Bergmann, 2008a; Gauron et al., 2013; Huh et al., 2004; Li et al., 2010; Perez-Garijo et al., 2004; Sakurai et al., 2008). Coined the “phoenix rising pathway” by Li *et al.*, wound healing, cell proliferation, and formation of blastema cells, are regulated in part by dying cells (Li et al., 2010). Since the original observations over forty years ago, the process of apoptotic-induced compensatory proliferation has been found to be an evolutionarily conserved process (Fogarty and Bergmann, 2017). Importantly, not only can dying cells induce a mitotic response in neighboring cells, but studies in *Drosophila*, *Xenopus*, *Hydra*, mice, and planaria have demonstrated a role for apoptotic cells in repair and regeneration (Chera et al., 2009; Fan and Bergmann, 2008a, b; Hwang et al., 2004; Tseng et al., 2007).

Irrespective of the specific route cells employ to undergo programmed cell death, typically extrinsic and intrinsic pathways lead to similar biological events including: a reduction in cell volume, caspase activation, nuclear condensation, DNA fragmentation, and apoptotic body formation. Regardless of the apoptotic stimuli, a reduction in cell volume is a universal feature of programmed cell death that is evolutionarily conserved among species ranging from worms to mammals (Bortner and Cidlowski, 1998, 2007; Lang et al., 2007). This cell shrinkage (termed apoptotic volume decrease [AVD]) results from a significant loss of intracellular potassium, sodium, and chloride ions that are essential for the activation of caspases and nucleases (Bortner and Cidlowski, 2007). Numerous studies have demonstrated prevention of AVD inhibits the execution of programmed cell death in most cell types (Bortner and Cidlowski, 2002, 2004; Heimlich et al., 2004; Yu and Choi, 2000). Hence, in order to create a permissive environment for the apoptotic machinery to function, the underlying flux of ions in dying cells must be carefully regulated. One of the regulators of apoptosis appears to be resting potential, even of distant cells, as has been shown in the patterning of the *Xenopus* brain (Pai et al., 2015a) and the regeneration of the planarian head (Beane et al., 2013).

In addition to cell migration, proliferation, and apoptosis, distinctive cell types must be present for functional tissues and organs to regenerate. Early studies using tissue from *Rana pipiens* embryos, demonstrated that ventral ectoderm explants differentiate into different cell types by modulating the ion content of the extracellular medium (Barth and Barth, 1974a; Barth and Barth, 1974b). More recently, studies demonstrated that the combination of BMP signaling and Ca^{2+} -mediated electric activity regulates the appropriate differentiation of the spinal neurons during embryogenesis (Sundelacruz et al., 2009; Swapna and Borodinsky, 2012). Furthermore, bioelectric control of cell differentiation has recently been demonstrated in human cells, such as mesenchymal stem cells (Sundelacruz et al., 2008, 2013a). In addition to the role for bioelectric signaling during development, numerous studies have

shown altering V_{mem} can not only direct the differentiation of tissues during embryogenesis, but can also direct the fate of a wide range of regeneration-relevant stem cells, including neural, hepatic, mesenchymal, and cancer (Biagiotti et al., 2006; Wang et al., 2005; Wenisch et al., 2006). Studies in fish have identified a role for, and transcriptional targets of, gap junction-mediated signaling in control of skeletal and joint elements in zebrafish fin regeneration (Banerji et al., 2016; Ton and Iovine, 2012, 2013).

Due to their ability to self-renew via mitotic divisions, combined with the role ion channels play during cell cycle progression, it is not surprising that multiple functional channel currents have been observed in stem cell populations (Li and Deng, 2011; Liebau et al., 2013; Moore, 2005). Another important characteristic of stem cells is their ability to differentiate into a diverse range of specialized cell types. Consequently, numerous studies have revealed a role for bioelectric signaling during the differentiation of precursor cells. For instance, not only do adipose-derived mesenchymal stem cells (AD-MSCs) express several voltage-gated ion channels (VGICs) channels (Bai et al., 2007), blocking the function of these channels inhibited the osteogenic differentiation of these stem cells (Zhang et al., 2016). Moreover, both differentiation and proliferation are controlled by changes in V_{mem} in numerous cell types including: human mesenchymal stem cells (HMSCs) (Sundelacruz et al., 2009; Sundelacruz et al., 2008), cardiomyocytes (Genovese et al., 2008; Lan et al., 2014a; Lan et al., 2014b; van Vliet et al., 2010), hepatocytes (Bautista et al., 2017), embryonic stem cells (Ng et al., 2010; Yamada et al., 2007), and neural stem cells (Aprea and Calegari, 2012; Lange et al., 2011). Combined, mounting evidence implicates transmembrane potential as a broadly-conserved mechanism used to direct the migration, proliferation, apoptosis, and differentiation of cells. Because of the development of new tools to manipulate membrane resting potential, V_{mem} control is an attractive target for manipulating cells in regenerative applications both *in vitro* and *in vivo*.

Tissue-level pre-patterns mediated by bioelectric control mechanisms

Although controlling an individual cell's behavior is important, in order to create a functional organ during development or replace a missing structure via regeneration, the behavior of groups of cells must be carefully regulated to produce properly patterned structures. Over the years, gradients of growth factors and other transcriptional activators have been implicated in mediating the differential gene activation observed during the formation of complex structures. However, gene regulatory networks must work in concert with physical sources of information to establish anatomical order on multiple scales, from organs to the entire body plan. While physical forces such as pressures and tensions (Mammoto and Ingber, 2010; Navis and Bagnat, 2015) are well-known to be important, it is becoming increasingly appreciated that spatio-temporal patterns of V_{mem} are also an important instructive feature of large-scale pattern regulation.

In the early 20th century, Yale University biologist Harold S. Burr and colleagues postulated that some of underlying patterning information that directed groups of cells stemmed from what he called *electro-dynamic fields*. Burr's early studies in 1930s and 1940s focused on carefully measuring and correlating voltage gradients with future developmental patterns in numerous organisms (Burr, 1944; Burr and Northrop, 1935b). He speculated these voltage

gradients were not only quantitatively predictive of future morphology, but also contained important patterning information. Similar to the *electro-dynamic fields* described by Burr eighty years ago, researchers examining amphibian development discovered an increase in the resting potential in the developing neural plate, that is absent from adjacent ectodermal cells (Blackshaw and Warner, 1976). Inhibition of this increase in resting potential resulted in abnormal morphology, with craniofacial structures the most severely malformed (Messenger and Warner, 1979). These initial observations were further characterized by Shi and Borgens who measured the gradients of extracellular electric fields found in amphibian embryos and speculated that these gradients provided positional information to the developing animal (Shi and Borgens, 1995). Experiments by Vandenberg and colleagues (2011) in the frog embryo revealed the existence of a bioelectric pre-pattern in the developing face that predicted the prospective location of eyes and other craniofacial structures (Figure 2A). When these bioelectric pre-patterns were perturbed experimentally, the prospective boundaries of craniofacial gene expression, and the subsequent gross morphology of craniofacial structures, were altered (Vandenberg and Morrie, 2011). More recent work has revealed this “bioelectric face” pattern to underlie the misregulation of craniofacial patterning genes observed in human syndromes with facial dysmorphias (Adams et al., 2016). Similar bioelectric patterns, recently characterized in the *Xenopus* brain (Pai et al., 2015b), are responsible for the balance of proliferation and apoptosis needed to produce a brain that is size-matched to the surrounding tissues, an important aspect of regenerative response.

While there are numerous examples of the existence of endogenous bioelectric gradients across anatomical distances, in order to direct the creation of complex structures the ion-mediated information must be carefully regulated across populations of cells. In addition to receptor-mediated signal exchange, information can also be distributed via the direct cell-cell exchange of small molecules passing through gap junctions. This extremely flexible system for communication allows for the rapid synchronization amongst cells in a tissue that can be regulated at multiple levels. Gap junctions have been shown to both regulate a cell's ability to sense extracellular electric fields, but also organize cells into functional domains (Caveney, 1985; Cooper, 1984; Cooper et al., 1989; Levin, 2007a; Warner, 1985). Thus, gap junctional communication provides the ability to facilitate the rapid flow of information in a multicellular organism, or in some cases, to isolate groups of cells and thus create boundaries between compartments (Pitts et al., 1988; Relat and Szczupak, 2004; Sutor and Hagerty, 2005). Importantly, cells linked by gap junctions can react as a single unit to stimuli such as the weak electric fields commonly found in developing and regenerating tissues (Cooper, 1984). In addition, gap junctions not only shape electrical properties of populations of cells, but they themselves are sensitive to changes in pH and transmembrane potential. These characteristics provide a way for groups of cells to use both positive- and negative-feedback mechanisms to regulate level of activity and spatial organization of bioelectric properties within tissues *in vivo*.

Patterning and morphogenesis of tissues and organs: bioelectric inputs

Beyond the control of cell fate decisions, several studies demonstrated bioelectric cues can also alter the morphogenesis and position of whole organs, which can occur even when

differentiation remains unperturbed. For example, recently research investigating the role of a cation channel during early embryogenesis demonstrated that altering channel function during embryogenesis affected the expression of important genes directing organ position and morphogenesis, resulting to mispositioned hearts with abnormal morphology, but genes directing the differentiation of the cardiac precursor cells such as, *Xbra*, *nkx2.5*, Cardiac troponin-T were unaltered (Pitcairn et al., 2017). A role for bioelectric signaling during large-scale patterning has also been observed in mammals, revealed by the numerous channelopathies that cause dysmorphic features. For example, human patients with Andersen-Tawil Syndrome that carry mutations in another inwardly rectifying potassium channel, Kir2.1, have limb and craniofacial abnormalities including, mispositioned ears, dental defects, fusion of digits (syndactyly), shortening of the digits (brachydactyly), and malformed or cleft palate (Plaster et al., 2001; Yoon et al., 2006). The Kir2.1 knockout mouse also has many of these malformed and mispositioned structures further supporting the channel function is needed for the normal development of these tissues and organs (Dahal et al., 2012; Zaritsky et al., 2000). Recently studies in *Drosophila melanogaster* examining the role of a potassium channel Kir2.1 homolog, Irk2, reported reduction of Irk2 ion channel resulted in the abnormal patterning of wings that was most likely caused by disrupting Dpp/BMP signaling (Dahal et al., 2012; Dahal et al., 2017).

The role of currents during appendage regeneration provides a more complex example of morphogenetic control of tissues and organs directed by bioelectric cues. Over half a century ago, Becker postulated that the bioelectric state of cells in regenerating organisms contained information that directed limb regeneration (Becker, 1961). Years later this theory was further probed by several investigators including Borgens and Lassalle who measured the bioelectric state in regenerating and non-regenerating organisms post limb amputation (Borgens, 1982, 1983; Lassalle, 1979, 1980). In both regenerating and non-regenerating animals examined immediately after injury, the wound surface was strongly negatively charged relative to surrounding tissue. Once the epithelium covered the wound, a switch to a positive charge was observed. However, only in regenerating organisms does the potential reverse a second time to return to a negative state. Since these currents persist over a prolonged period of time and can be detected weeks after the initial injury, it is unlikely that the resulting changes in electric fields are simply a byproduct of passive ion leaking from damaged cells. Importantly, functional experiments where these bioelectric gradients were altered via shunting, ion channel blockers, electrical isolation, or exogenous reversal of the gradient, inhibited regeneration and serve to provide further evidence that biophysical events are necessary components for regeneration (Borgens, 1982; Hotary and Robinson, 1992; Jenkins et al., 1996; Novak and Sironval, 1975). Combined, these experiments provided the groundwork that links bioelectric state during limb regeneration to critical information needed to direct normal anatomy of structures.

Going beyond the endogenous role for ion channel activity during regeneration, numerous studies have examined the sufficiency of bioelectric cues in inducing or augmenting regeneration (Sisken, 1992; Sisken et al., 1993). More recent experiments in the rodent limb have used marker analysis to confirm the augmentation of regenerative response induced via bioelectric stimulation (Leppik et al., 2015). To date, several labs have demonstrated the application of exogenous fields can induce the regeneration of appendages in less-

regenerative organisms, including anuran amphibians (Borgens et al., 1977; Sharma and Niazi, 1990; Smith, 1967), chick (Sisken and Fowler, 1981), and even mammals (Becker, 1972; Sisken et al., 1984; Smith, 1981). For example, the use of ionophores to induce depolarization has been shown to trigger leg regeneration in post-metamorphic (non-regenerative) frogs (Tseng and Levin, 2013) (Figure 3A-B).

The tadpole tail of the frog *Xenopus laevis* is able to regenerate a complete tail (including muscle, spinal cord, epidermis, vasculature, and notochord), making it an excellent model system for investigating tissue repair and regeneration and potential therapeutics at post-regenerative life stages (Tseng and Levin, 2008) (Figure 3C-F). Over the last decade researchers have identified multiple molecular mechanisms that regulate tail regeneration such as: Wnt-FGF, TGF- β , Notch, and BMP (Beck et al., 2003; Ho and Whitman, 2008; Lin and Slack, 2008; Slack et al., 2004). As with limb regeneration, in addition to the signaling molecules that mediate the regrowth of the amputated structure, tadpole tail regeneration is also regulated by biophysical factors. For example, the amount of current detected in amputated tadpole tails correlated with regenerative ability and the restoration of missing tissue was reduced manipulation when bioelectric cues were altered (Reid et al., 2009). Additionally, a combination of pharmacological and molecular-genetic approaches implicated the continuous pumping of H^+ at wound site mediated by the V-ATPase hydrogen pump as an instructive factor mediating proliferation and pattern formation during tail regeneration (Adams et al., 2007a). Not only is this current required during normal regeneration, but during stages when tadpoles cannot regenerate ablated tail tissue, regeneration can also be artificially induced via expressing a yeast H^+ -ATPase (PMA-1) to manipulate H^+ efflux (Adams et al., 2007a; Masuda and Montero-Lomeli, 2000). The expression and function of bioelectric machinery such as NaV1.2 channel revealed an important molecular marker that distinguishes true regeneration from wound healing. These initial observations were extended to demonstrate that a small molecule cocktail targeting sodium flux was sufficient to trigger complete tail regeneration even after a nonpermissive wound epithelium had formed (Tseng et al., 2010b) (Figure 3C-F). Furthermore, recent advances in optogenetics demonstrate this approach can be used to trigger regenerative response of a complex multi-tissue appendage (the *Xenopus* tail, including spinal cord) via light stimulation of appropriate bioelectric states (Adams et al., 2013).

Changes in membrane potential have also been implicated in eye development and patterning. For example, experiments conducted in *X. laevis* demonstrated that the cell fields that will eventually contribute to eye formation are demarcated by hyperpolarization long before any of the eye transcription factors are expressed. Altering the pattern of these hyperpolarized regions induced changes in eye-specific gene expression and defects in eye morphogenesis; more importantly, when other cells were artificially hyperpolarized in early *Xenopus* embryos, ectopic eye tissue was induced (Pai et al., 2012) (Figure 2B); this could even occur far from the anterior neural field (such as the gut), suggesting revision of standard views of competence restrictions in various tissues.

Axial patterning

Axis determination is a critical component of creating complex structures during development and regeneration. Seminal experiments conducted by Marsh and Beams showed that simply applying external electric fields to the regenerating planarian fragments caused specific reversals of normal anterior-posterior polarity (Marsh and Beams, 1947, 1952). In these experiments, when the anterior cut faced the negative cathode, regeneration proceeded normally. In sharp contrast, when the anterior cut was exposed to the positive anode, double-headed worms were created. This work drove the formulation of models of electrophoretic redistribution of morphogen signals (Lange and Steele, 1978). Further evidence that membrane voltage controls head-versus-tail identity during planarian regeneration was reported in more recent studies that took advantage of a chemical genetics approach to examine the effects of ion flux on axial patterning. In this study, researchers revealed H^+/K^+ -ATPase-mediated depolarization of the regenerating worm blastema drives head formation, even at posterior-facing wounds (Beane et al., 2011) (Figure 4A). Subsequent work showed that modulation of bioelectric network connectivity can switch genetically wild-type planaria to a permanent 2-headed state (regenerating as bi-axial forms after repeated, additional cuts in pure water) (Oviedo et al., 2010). Recent evidence reveals that the endogenous body-wide bioelectric gradient is the determinant of head-tail identity in animals that can regenerate fragments with 2 heads despite the original worm's normal anatomy, histology, molecular marker expression, and stem cell distribution (Durant et al., 2017) (Figure 4B). Remarkably, planaria can be converted between 1-head (normal phenotype) and 2-head (mispatterned) forms by transient, brief modulations of a bioelectric circuit that dictates radically different body plans in future rounds of regeneration in plain water; this model system provides one of the best examples of the instructive role of bioelectrics in setting, and executing, the pattern memory that guides large-scale regeneration.

In addition to a role in anterior-posterior axis determination, bioelectric cues are also essential for patterning the left-right (L-R) axis in a wide range of organisms (Levin, 2005; Levin, 2012; Vandenberg and Levin, 2010, 2013). For example, in *Xenopus* the asymmetric localization of 4 electrogenic proteins, including the H^+/K^+ -ATPase exchanger and V-ATPase H^+ pump, results in differential L-R voltage and pH gradients that help establish normal L-R patterning during embryogenesis (Adams et al., 2006a; Aw et al., 2010; Levin et al., 2002). Although the timing of activity varies, the role of bioelectric cues in L-R axis establishment has also been observed in invertebrates such as the sea squirt and sea urchin as well as in other vertebrates including zebrafish, chick, mouse, and even human patients (Fukumoto et al., 2005; Hibino et al., 2006; Kawakami et al., 2005; Levin et al., 2002; Miyachi, 2017; Shimeld and Levin, 2006; Vandenberg and Levin, 2010).

While the details of how voltage gradients couple to early events are somewhat divergent among phyla with different body plans, work in amphibians has provided the most detailed picture of one evolutionary strategy. In *Xenopus*, the bioelectric gradients formed in early embryogenesis provide the motive force for a charged molecule, serotonin, to pass through gap junctions and become localized on the right side of the embryo where it functions as an electrophoretic morphogen (Fukumoto et al., 2005; Vandenberg et al., 2014; Zhang and

Levin, 2009). Altering serotonin signaling or channel function results in the randomization of canonical L-R positioning proteins such as *Nodal*, *Lefty*, *Pitx2* and *situs ambiguus* (heterotaxy). The L-R pathway provides a good illustration of the molecular mechanisms by which bioelectrics regulates global pattern, as all of the steps are known – from cytoskeletal machinery that regulates channel positioning (Lobikin et al., 2012), to the transduction of voltage gradient information from single cell chirality into body-wide asymmetric transcriptional cascades of canonical genes (McDowell et al., 2016a; McDowell et al., 2016b). The latter process is executed by lateralized epigenetic silencing of left-sided genes such as *Nodal* by electrophoretic movement of morphogens (Carneiro et al., 2011), revealing a tight integration of bioelectrics and biochemical signaling to link the dynamics of single cells to the creation of complex structures. Given the importance of epigenetic transcriptional control during regeneration in amphibia, fish, and planaria (Anderson et al., 2009a; Hamada et al., 2015; Robb and Sanchez Alvarado, 2014; Tseng et al., 2011a), it is likely that the interplay of bioelectrics and chromatin modification machinery will be increasingly characterized in regeneration, as it has been in development (Carneiro et al., 2011) and tumorigenesis (Chernet and Levin, 2014).

Ion flux and control of the size of structures

During the regeneration of missing structures, in addition to proper patterning, the ability to regulate the final size of newly created structures is equally important. This is accomplished via the coordination of the number of each cell type required to make a tissue with the final dimensions of a regenerating structure. In the late 1940s Moment conducted a systematic study of earthworm regeneration to understand the mechanisms utilized to control growth and cell division (Moment, 1947; Moment, 1949a, b). He presented evidence that electrical events were correlated with posterior regeneration and postulated regenerating worms generated a critical inhibitory voltage which functioned as a limiting factory to regulate growth (Moment, 1949a). A few years later Kurtz and Schrank conducted additional experiments that served to further confirm the initial findings demonstrating changes in voltage accompany cessation of regenerative growth in earthworms (Kurtz and Schrank, 1955). In zebrafish, a gain-of-function mutation in a potassium channel, *K2P* (encoded by the *kcnk5b* gene) resulted in allometric overgrowth of the fins (Perathoner et al., 2014), identifying and characterizing a new electrogenic control of appendage size.

Bioelectric cues in plants

The involvement of bioelectric signaling in regenerative processes is not just restricted to animals; it is also a mechanism used to direct cell behavior in plants. Some of the earliest studies in this field involved plants, addressing both the endogenous bioelectric correlates of plant morphogenesis (Burr and Sinnott, 1944; Rehm, 1938) and the effects of applied fields on plant growth (Berry et al., 1947; Rehm, 1939). Some of the pioneering studies by Lionel Jaffe and colleagues over forty years ago recognized that pollen tubules behave as electrical dipoles, with inward currents leaking the apical parts of the tube, and outward (positive) currents arising from the grain (Weisenseel and Jaffe, 1976; Weisenseel et al., 1975); this work gave rise to one of the first models of bioelectrical self-organization and polarity (Bentrup and Jaffe, 1968; Jaffe, 1968). Similar to pollen tubules, the outgrowth of root hairs

occurs in a polar fashion and is also guided by an external electrical field (Michard et al., 2009b). The role of ion flux is not restricted to the normal growth of pollen tubules and root hairs but is also important for regenerative processes. For example, studies have shown that both alternating (Cogalniceanu et al., 1998) and continuous (Rathore and Goldsworthy, 1985) weak electric current can increase the proficiency of *in vitro* regeneration in tobacco tissue cultures. Molecular studies have revealed the details of physiological gradients driving pollen tube extension and patterning (Cortal et al., 2008; Michard et al., 2009a; Michard et al., 2008; Robinson and Messerli, 2002), including bi-directional feedback between the activity of ion transporters and the distribution of small signaling molecules such as auxin and GABA (Goldsworthy and Rathore, 1985; Ramesh et al., 2015; Schrank, 1951). More recently, studies using examining *Arabidopsis* root regeneration *in vivo*, demonstrated that the regeneration process of the root tip apical meristem could be perturbed by a brief exposure of the stump to an external electric field (Kral et al., 2016). Thus, electric fields are capable of directing cellular processes in a wide spectrum of tissue types as well as across diverse taxa.

Molecular mechanisms

Although initial observations that bioelectric cues play a role in the formation and patterning of complex structures span decades of research, it is only more recently that molecular mechanisms responsible for sensing, interpreting, and translating weak bioelectric signaling into changes and gene expression have been elucidated. Not surprisingly, bioelectric cues are found both upstream and downstream of traditionally studied genetic and biochemical elements (Figure 1). Though still only examined in a small handful of contexts, the study of bioelectrics is revealing a robust conservation of mechanisms. For example, galvanotactic guidance of cell migration occurs in fungi (Gow and Morris, 1995) as it does in mammalian cells during wound healing (Nakajima et al., 2015) and retinal patterning (Yamashita, 2013). Bioelectric gradients establish polarity in single yeast cells (Haupt et al., 2014; Minc and Chang, 2010) as in whole metazoan axes (Aw and Levin, 2009). The same voltage regulators, like the plasma membrane V-ATPase proton pump, are involved broadly from stem cell regulation in eye patterning (Nuckels et al., 2009) to the determination of axial polarity in L-R asymmetry (Adams et al., 2006b) and the induction of tail regeneration (Adams et al., 2007b). Even the downstream targets of voltage change appear to be well-conserved from amphibia to man, as revealed by recent microarray studies comparing transcriptional responses to V_{mem} change in frog, axolotl regeneration, and human mesenchymal stem cells (Pai et al., 2016).

How do changes in the resting potential at the plasma membrane regulate downstream gene expression? To date many studies have described mechanisms for transduction of ion flows and voltage gradients into second-messenger cascades that mediate changes in gene expression including: activation of calcium influx via voltage gated calcium channels (Deisseroth et al., 2004; Sasaki et al., 2000), conformational changes in integrin signaling (Arcangeli and Becchetti, 2006; Olivotto et al., 1996), voltage regulated phosphatase activity (Murata et al., 2005; Okamura and Dixon, 2011), clustering of RAS proteins (Zhou et al., 2015), and regulation of small morphogens' movement in and out of cells via voltage-regulated transporters (Blackiston et al., 2011a; Fukumoto et al., 2005).

Since calcium is one of the most widely used second messengers in cell biology and participates in numerous cell functions, it is not surprising that Ca^{2+} influx (via voltage-sensitive Ca^{2+} channels) has been linked to the regulation of downstream events modulating pattern formation (Barbado et al., 2009; Calabrese, 2014). Of particular interest is calcium's ability to control gene function, providing a link between membrane potential and gene expression. Unlike many other second messengers, it cannot be metabolized; while present in relatively high concentrations in the extracellular space, its presence inside cells is tightly regulated. Thus, the ability to control intracellular Ca^{2+} concentration and location is critical for regulating a wide variety of cell functions. Not only can Ca^{2+} control rapid-signaling processes like electrical excitability and neurotransmitter release, but it can also control cellular events that occur more gradually. Ca^{2+} often recruits intracellular signaling pathways (e.g., calmodulin, calcineurin, calpains) that persist longer in cells than the Ca^{2+} , to control cellular events that develop over a large time scale (Jaffe, 1995, 1999; Slusarski and Pelegri, 2007). Many studies have examined the role of voltage-dependent calcium channels in mediating gene expression in response to membrane depolarization. Some of the initial evidence that activity of voltage-gated calcium channels can direct gene regulation comes from studies demonstrating chronic depolarization of PC12 cells induced an increase in *c-fos* expression levels (Morgan and Curran, 1986). More recently, in experiments where Ca^{2+} signaling is inhibited via the addition of ryanodine, a reduction in the number of muscle progenitor cells and activated satellite cells was observed in the regenerating tadpole muscle tissue (Tu and Borodinsky, 2014). Another study examining the specification of commissural interneurons in the tadpole spinal cord, provided evidence for an interaction Ca^{2+} -dependent electrical activity and of Smad-mediated BMP signaling (Swapna and Borodinsky, 2012).

Other V_{mem} -transducing processes include adhesive receptor signaling mechanisms (Arcangeli and Becchetti, 2006; Liu et al., 2005; Meyers et al., 2004; Nesti et al., 2002). In fact, research dating back to the early nineties provided evidence that integrin-mediated cell adhesion to the extracellular matrix is often coupled with ion activation via a physical association between integrin receptors and channel proteins. This interaction can subsequently regulate cell behavior such as: cell migration, proliferation, apoptosis and differentiation (Becchetti et al., 1992; Schwartz, 1993). A good example of this interaction is observed in PC12 cells whose shift to a neuronal phenotype is induced by the physical interaction of voltage-gated Ca^{2+} channels and neural cell adhesion molecules/N-cadherins (Doherty et al., 1991). Over the last several decades, numerous studies have demonstrated adhesive receptors (especially integrins) physically and functionally associated with several classes of ion channels (Arcangeli and Becchetti, 2006).

The recent discovery that voltage-sensitive phosphatases (VSPs) can hydrolyze phosphoinositides upon depolarization of membrane potential provides a novel mechanism of how electrical activity can directly alter biochemical signaling. VSPs contain two functional modules: a voltage sensitive domain (VSD) and a phosphatase enzyme region (Murata et al., 2005; Murata and Okamura, 2007). The voltage sensitive domain regulates the enzymatic activity of VSP to dephosphorylate target molecules (either phosphatidylinositol 3,4,5-trisphosphate or phosphatidylinositol 4,5-bisphosphate). Interestingly, the cytoplasmic region of VSP has considerable sequence homology to a

tumor suppressor enzyme, PTEN (phosphatase and tensin homolog), suggesting VSP has enzyme activity similar to PTEN, a protein that regulates the activity of many genes via canonical mechanisms (Iwasaki et al., 2008). Thus, although additional research is needed better understand this class of proteins, VSPs constitute a novel mechanism for coupling intracellular gene regulatory networks to electrical activity at the plasma membrane. A comprehensive and elegant study implicated bioelectric control, mediated by PTEN machinery, of wound healing in the mammalian eye (Zhao et al., 2006).

Over the years, numerous studies have demonstrated the function of intracellular transporters of signaling molecules (e.g., serotonin) can be altered by changes in bioelectrical activity. This voltage-controlled movement of second messengers, such as neurotransmitters, facilitates the binding of intracellular receptors and transcription of target genes. For example, in early frog embryos bioelectrical gradients drive serotonin movement through gap junction-connected cell paths (by simple electrophoresis) and by modulating the SERT voltage-gated transporter (Fukumoto et al., 2005; Fukumoto and Levin, 2003; Levin et al., 2006). A very similar scheme involving electrogenic regulation of serotonin movement is observed in determining the amount of innervation from tissue transplants (Blackiston et al., 2011b; Blackiston et al., 2017), a finding that could be leveraged for advances in regenerative medicine.

There are several lines of evidence that suggest the epigenetic regulation of chromatin state is critical for pattern formation and regeneration. For example, in contrast to young tadpole limbs, the froglet limb no longer expresses important patterning genes like *Sonic Hedgehog* (*shh*) and reduced ability regeneration missing structures (Yakushiji et al., 2007). Sequence analysis of the limb enhancer region in young and old animals revealed that tadpole limbs were hypomethylated whereas froglet limbs were hypermethylated (Yakushiji et al., 2007). In addition, in zebrafish mutants carrying a loss-of-function allele of Dnmt-1 (DNA methyltransferase 1) are less able to regenerate ablated pancreatic cells (Anderson et al., 2009b). Because butyrate is an inhibitor of HDAC1, the movement of butyrate through an ion-dependent transporter SLC5A8 can also trigger epigenetic responses leading to tumorigenesis and altered regenerative ability (Chernet and Levin, 2013; Chernet et al., 2014; Davie, 2003; Gupta et al., 2006; Tong et al., 2004; Tseng et al., 2011b; Tseng and Levin, 2012). Hence, changes in membrane voltage can result in the acetylation of chromatin and modified rates of transcription.

Recent transcriptomic and proteomic analyses of regeneration have revealed prominent bioelectric components within the regenerative response (Chang et al., 2017; Rabinowitz et al., 2017), and linked them functionally to other physiological signals such as reactive oxygen species (ROS) (Ferreira et al., 2016). Likewise, modern optical imaging of *in vivo* physiology is beginning to provide a comprehensive multi-dimensional picture of the biophysics of amphibian tail regeneration (Ozkucur et al., 2010).

Conclusions and next steps

Endogenous bioelectrical states serve as instructive signals in patterning at multiple levels of organization, from single cells to the whole body plan. V_{mem} gradients specify information

such as: initiating modules for complex self-limiting organogenesis (Adams et al., 2007b; Pai et al., 2015b), setting axial polarity (Beane et al., 2011; Durant et al., 2017; Levin, 2006; Oviedo et al., 2010; Stern and MacKenzie, 1983), serving as prepatterning for the layout of large regions (Adams et al., 2016; Pai et al., 2015a; Vandenberg et al., 2011), and even determining the shape and size of structures (Emmons-Bell et al., 2015; Perathoner et al., 2014). Despite the progress that has been made thus far, the field still faces a number of major questions. These include a more in depth understanding of the mechanisms by which cells compare bioelectric state across distances, elucidation of how bioelectric cues interface with chemical gradients and physical forces, and development of quantitative models of bioelectric circuits that are able to store patterning information needed to create complex structures. The ability of bioelectric signaling to direct cell behavior has been described in the literature for over a century, yet only recently are we gaining sufficient insight about mechanisms and global dynamics to enable biomedicine to unlock this valuable information. It is crucial to point out that continued advances in the control of regenerative patterning will require not only increase reductive detail on subcellular molecular pathways, but also integrative work to understand how large-scale pattern is established (and how growth is limited once appropriate anatomy has been restored) by large-scale bioelectrical circuits. Moving forward, researchers need to extend our knowledge about gene regulatory networks and signaling cascades to include information generated at the level of bioelectricity.

Acknowledgments

We appreciate the numerous helpful discussions with members of the McLaughlin, Levin, and Adams labs, as well as many others in the developmental bioelectricity field. We apologize to the many workers in this field whose work could not be discussed in detail due to length constraints. This paper is dedicated to R. O. Becker, a tireless investigator of bioelectricity in regeneration. We gratefully acknowledge support by an Allen Discovery Center award from the Paul G. Allen Frontiers Group (No. 12171), the G. Harold and Leila Y. Mathers Charitable Foundation (No. TFU141), the Templeton World Charity Foundation (No. TWCF0089/AB55), and the W. M. Keck Foundation.

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Highlights

- Endogenous ion flows are important regulators of cell behavior.
- Creating complex structures requires the integration of molecular inputs and biophysical cues.
- Bioelectrical states play a key role in the robust restoration of normal pattern after injury.
- Bioelectric cues serve as instructive signals in patterning at multiple levels of organization.

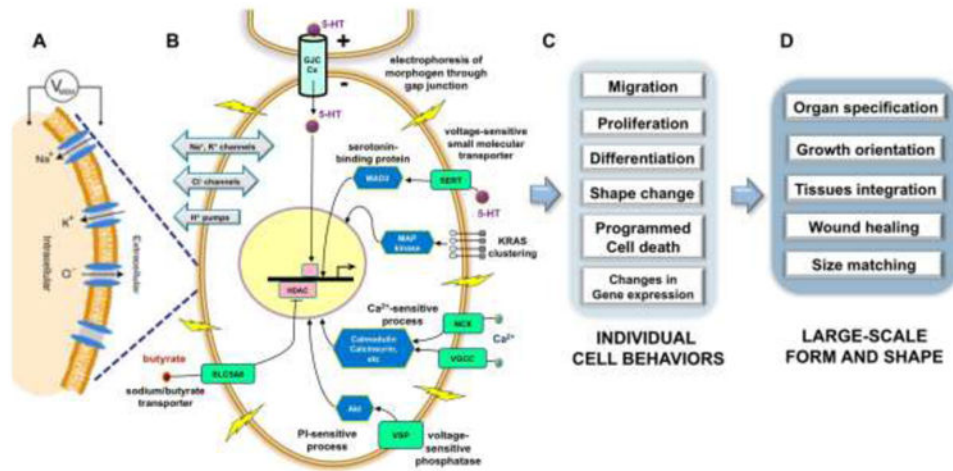


Figure 1. Bioelectrical signaling drives pattern formation at the level of the cell, tissue, and organism

(A) Changes in transmembrane voltage are transduced (B) by a set of membrane mechanisms (voltage-powered transporters of serotonin and butyrate, voltage-gated calcium channels, voltage-regulated phosphatases, and others) into second-messenger cascades that regulate gene expression, thus directing cell behavior (C) such as, migration, proliferation, cell death, differentiation, gene expression, and shape changes. (D) In turn, these changes in cell behavior enable the creation of complex structures. Abbreviations: 5-HT, 5-hydroxytryptamine, also known as serotonin; HDAC, histone deacetylase; MAD3, Max-interacting transcriptional repressor; Akt, serine/threonine-specific protein kinase; GJC, gap junction communication; NCX, Na^+/Ca^{2+} exchanger; VGCC, voltage-gated calcium channel; Cx, connexin; MAP kinase, mitogen-activated protein kinase. Lightning bolts represent changes in resting membrane potential. Panels A and B modified with permission (Levin, 2007b).

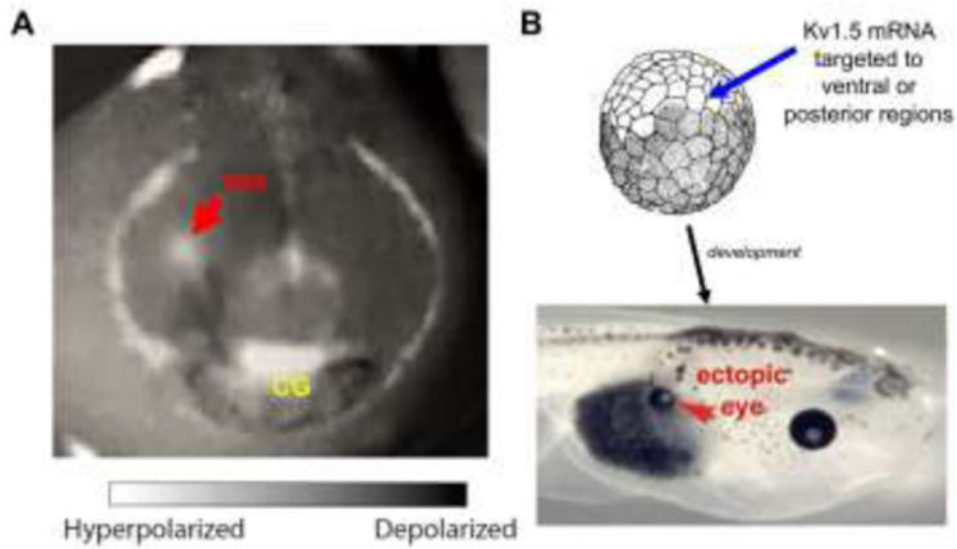


Figure 2. Bioelectric cues can specify pre-pattern information needed to create complex structures and direct the reprogramming of complete organs via non- cell-autonomous patterning signals

(A) Spatial distributions of resting potential gradients reveal the existence of complex prepatterns *in vivo*. Imaging with a voltage-sensitive fluorescent dye in the *Xenopus* nascent face reveals the borders of patterning compartments and organ locations prior to the induction of face-specific patterning transcripts. Anterior/face, red arrow denotes position of future right eye field. Modified with permission from (Vandenberg and Morrie, 2011). (B) Manipulation of these endogenous patterns by misexpression of ion channels can result in organ-level reprogramming. For example, targeted V_{mem} change, via misexpression of ion channels in the frog embryo, induces the formation of ectopic structures such as complete eyes, even in regions normally not competent to form eyes such as the gut (red arrow). Modified with permission from (Pai et al., 2012).

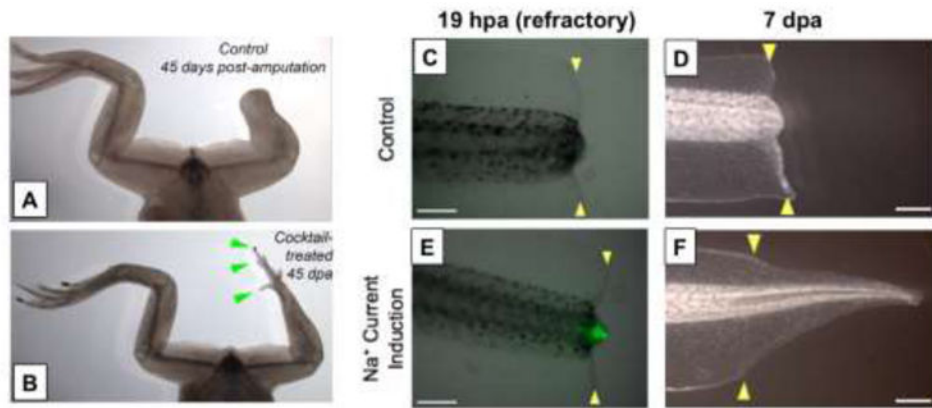


Figure 3. Large-scale bioelectric patterns are instructive for shape

(A-B) Limb regeneration does not normally occur in post-metamorphic froglets. After only 24 hours of exposure, an ionophore cocktail designed to specifically alter the bioelectric state of the blastema triggers growth of an entire limb (green arrowheads indicate the appearance of distal elements such as toes and toenails). Used with permission from (Tseng and Levin, 2013). (C-D) During the refractory period in *Xenopus*, tail regeneration does not occur. (E-F) A one-hour exposure of the animal to an ionophore cocktail induces sodium influx into the bud, which triggers the regeneration of an entire new tail. This example illustrates how a simple signal can trigger a complex, self-limiting downstream morphogenetic cascade appropriate in orientation, scaling, and location within the host organism. Exploiting such endogenous “master-regulator” triggers may be a powerful strategy for regenerative medicine, to restore complex organs long before we have the knowledge to micromanage its creation from specific cell types. Yellow arrows indicate location of amputation. Abbreviations: hpa, hours post amputation; dpa, days post amputation. Modified with permission from (Tseng et al., 2010a).

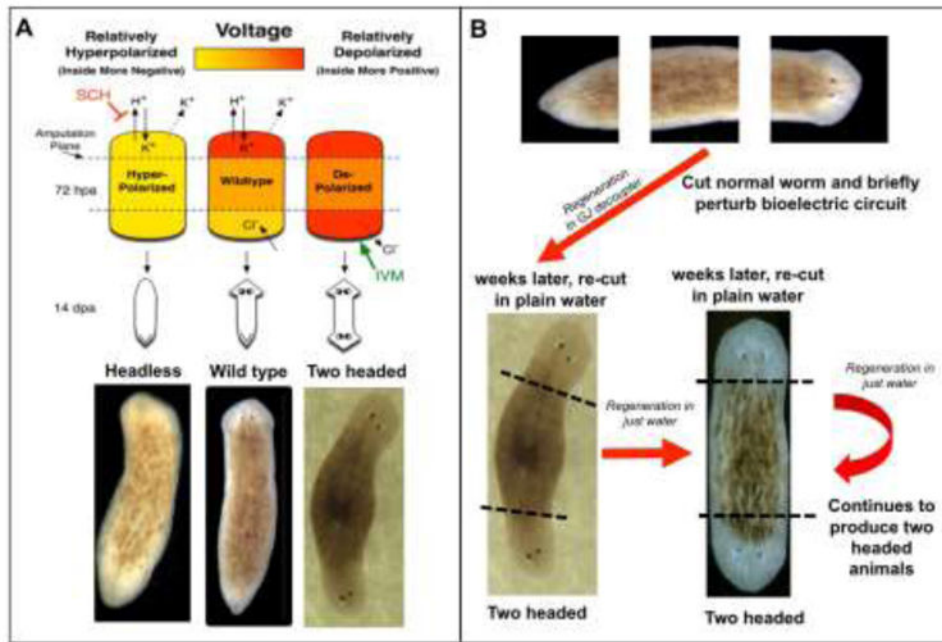


Figure 4. Large-scale bioelectric patterns direct the morphology of regenerated structures (A) Understanding of the bioelectric circuit that controls anterior–posterior specification in a fragment of regenerating planaria can be used to design drug cocktails that alter the regenerating anatomical structures produced by adult stem cells. Using this information, the desired target morphology can be created including inducing the posterior-facing blastema to build a secondary head in planaria. Modified with permission from (Beane et al., 2011). (B) Pattern memory encoded in bioelectric circuits can be altered by manipulating bioelectric cues. Planarian head-tail polarity is regulated in part by an endogenous voltage gradient. When cut fragments are briefly exposed to reagents to alter the topology of bioelectric cues (e.g., gap junction targeting drugs or RNAi targeting innexins), their regeneration results in the creation of two headed animals. Remarkably, weeks later, when these same animals are re-cut in plain water over multiple rounds of regeneration, the two-headed worm phenotype persists. Recent work (Durant et al., 2017) shows that these two-headed forms can be re-set back to a permanent one-head state by a manipulation of the H^+/K^+ -ATPase component of the circuit, and reveals how epigenetic long-term pattern memory that can be stored in bioelectric circuits.