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Gap Junctional Signaling in Pattern Regulation: Physiological Network Connectivity Instructs Growth and Form

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Abstract

Gap junctions (GJs) are aqueous channels that allow cells to communicate via physiological signals directly. The role of gap junctional connectivity in determining single-cell functions has long been recognized. However, GJs have another important role: the regulation of large-scale anatomical pattern. GJs are not only versatile computational elements that allow cells to control which small molecule signals they receive and emit, but also establish connectivity patterns within large groups of cells. By dynamically regulating the topology of bioelectric networks *in vivo*, GJs underlie the ability of many tissues to implement complex morphogenesis. Here, a review of recent data on patterning roles of GJs in growth of the zebrafish fin, the establishment of left-right patterning, the developmental dysregulation known as cancer, and the control of large-scale head-tail polarity, and head shape in planarian regeneration has been reported. A perspective in which GJs are not only molecular features functioning in single cells, but also enable global neural-like dynamics in non-neural somatic tissues has been proposed. This view suggests a rich program of future work which capitalizes on the rapid advances in the biophysics of GJs to exploit GJ-mediated global dynamics for applications in birth defects, regenerative medicine, and morphogenetic bioengineering.

Keywords

gap junctions; networks; bioelectric; patterning; morphogenesis

INTRODUCTION

Gap junctions (GJs), formed by connexin or innexins proteins, form aqueous channels directly connecting the internal cytoplasmic space of nearby cells for the transfer of ions and other small molecules (Phelan, 2005; Scemes et al., 2007). Along with ion channels, they are a key component by which most types of somatic cells organize into physiological networks (Fig. 1). The molecular biology and physiology of GJs, as well as their many roles in the nervous system, have recently been expertly reviewed (Sohl and Willecke, 2004; Steyn-Ross et al., 2007; Pereda et al., 2013; Baker and Macagno, 2014). Here, we focus not on the

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cellular-level signaling mechanics of GJs, but on their role in determining large-scale pattern formation. Tissue and organ patterning includes embryonic morphogenesis, regeneration of limbs and other organs in a range of model species, and continuous tumor suppression in adult metazoan organisms. Coordinating cell behaviors *in vivo* toward the creation and repair of complex structures depends on a tight coordination among cells. This coordination is implemented by a rich system of information-bearing signals that propagate throughout the body. Because GJs enable small molecule physiological signals (e.g., ion flow) to pass among cells, they serve as an important modality for cell–cell communication (Trosko, 2007). In this overview, we focus on intercellular channel functions of GJs, and do not discuss permeability-independent functions of connexin proteins (Dang et al., 2003, 2006; Vinken et al., 2012; Clasadonte and Haydon, 2014) or hemichannels.

GJ-mediated intercellular signaling functions in parallel with the better-understood secreted gradients of extracellular signaling molecules (Ben-Zvi et al., 2011; Rogers and Schier, 2011; Hironaka and Morishita, 2012). GJ channels are restricted to small molecules (unlike extracellular signaling, which is often mediated by sizeable proteins). However, GJ signaling networks are extremely versatile because they allow cells to control coupling at the transcriptional, translational, and physiological levels (Solan and Lampe, 2014). GJs can be gated by a range of regulatory inputs (phosphorylation, voltage, pH, etc.) (Peracchia, 2004) and depending on their specific connexin composition, can exercise considerable selectivity over permeant molecules (Goldberg et al.,2004). Because of this, GJ-coupled cell networks can set up very rich patterns of connectivity *in vivo*—potentially, with far greater complexity than is possible for diffusing chemicals. Thus, it is no surprise that now classical (Chuang-Tseng et al., 1982; Weir and Lo, 1982; Warner, 1985; Guthrie and Gilula, 1989; Lo, 1996) and recent data have implicated gap junctional communication (GJC) in a range of patterning events in both vertebrate and invertebrate model systems (Sutor and Hagerty, 2005; Elias and Kriegstein, 2008; Ahir and Pratten, 2014; Irion et al., 2014; Merrifield and Laird, 2015).

GJs are a powerful mechanism for coordinating long-range signaling during pattern regulation, which is only beginning to be understood. Cells often upregulate gap junctional communication when they need to share ions with their neighbors (Aslanidi et al., 1991; Larre et al., 2006). Thus, GJC is a perfect conduit for information flow during development, which depends on the ability of cells and tissues to communicate. The converse, however, is also paramount—embryos contain independent compartments (Rela and Szczupak, 2004; Sutor and Hagerty, 2005) that must remain isolated for proper morphology to result. Mutations in GJ genes are now known to be involved in a wide spectrum of patterning defects (Table 1); however, the true impact of GJC will be realized not only through ever finer-resolution analysis of the protein dynamics in single cells, but also through an appreciation of their contribution to network dynamics. Here, we review several non-neural contexts in which GJ-mediated signals instruct growth and form. We also present a novel perspective on this field, proposing that GJs may underlie information processing during somatic remodeling that is very similar to the role of these electrical synapses (Bennett, 1997) in implementing plasticity and memory in the brain. This hypothesis sheds new light on emerging data in this exciting field and suggests a research program focused on understanding the global dynamics of GJ-mediated physiological networks.

GJS IN CELLULAR REGULATION

The first patterning tasks required of any animal is embryogenesis—the remarkable self-assembly of a complex body from the descendants of a single egg cell. This process necessarily involves partitioning the embryo into diverse components with different fates and functions.

Endogenous Expression and GJC-Dependent Domains

Embryos of frog, fruit fly, fish, and nematodes have long been known to possess compartments—discrete regions of GJ-coupled cells with distinct selectivity of permeable signals (Lo and Gilula, 1979; Weir and Lo, 1982, 1984; Blennerhassett and Caveney, 1984; Pitts et al., 1988; Bozhkova, 1998). For example, the wing imaginal disk in *Drosophila* is subdivided into a number of communication compartments during differentiation (Weir and Lo, 1984). Such developmental domains are cell groups with distinct physiological properties, which thus could underlie the diversification and patterning of different embryonic regions. Early mollusk development includes a number of specific GJC patterns that functionally determine cell determination (de Laat et al., 1980). Chick limbs exhibit a gradient of GJC along the AP axis (Coelho and Kosher, 1991). The highest GJC was observed in cells adjacent to the zone of polarizing activity, while no GJC was present at the opposite end of the limb bud. Mesenchymal tissues in the middle of the limb had an intermediate level of dye coupling and it has been hypothesized that polarizing region cells communicate to anterior mesenchyme cells via GJs (Allen et al., 1990).

The potential complexity of GJ-based regionalization is augmented by the fact that GJs composed of different connexin subunits provide a degree of functional compensation (Vink et al., 2004), but also confer the ability to sense different signals upon cells that express them (Elfgang et al., 1995). For example, homomeric Cx32 channels are permeable to both cAMP and cGMP, whereas heteromeric Cx32/Cx26 channels retain permeability to cAMP but prevent transfer of cGMP (Bevans et al., 1998). Indeed, substitution studies in tissues such as lens showed that distinct connexins play different roles in development, forming signaling conduits that are not interchangeable (White, 2002). In addition to molecular selectivity, GJs also enable rectification and unidirectional transfer (Robinson et al., 1993; Bruzzone and Giaume, 1999; Zhang et al., 2003; Fan et al., 2005; Palacios-Prado et al., 2014), enabling embryogenesis to take advantage of a very rich and dynamic set of signaling paths. For example, unidirectional junctions are thought to form between Cx32 and Cx43 (Robinson et al., 1993; Xin and Bloomfield, 1997), potentially allowing an embryo to establish one-way signaling paths.

GJC and Stem Cells

Pattern formation is the result of a tight spatiotemporal regulation of cell proliferation, differentiation, migration, and programmed cell death. Recent data have shown that GJ-mediated signaling is a powerful regulator of these properties, especially in stem cells (Oviedo and Levin, 2007; Wong et al., 2008). GJs are involved in regulating multipotency (Dyce et al., 2014), differentiation (Bani-Yaghoub et al., 1999; Zhang et al., 2002; Araya et al., 2003; Gu et al., 2003b; Hirschi et al., 2003; Araya et al., 2005; Li et al., 2015),

self-renewal (Hitomi et al., 2015), proliferation (Paraguassu-Braga et al., 2003; Pearson et al., 2005; Starich et al., 2014), and motility (Huang et al., 1998a; Xu et al., 2001; Zahler et al., 2003; Marins et al., 2009; Kotini and Mayor, 2015). Neural stem cells exhibit a unique signature based on GJC and ion transporters, and GJC based on Cx43 and Cx45 is essential for their survival and proliferation (Cai et al., 2004). For example, the GJ protein Zero Population Growth (zpg) is required for germ cell differentiation in Drosophila ovary. In the absence of ZPG, the stem cell daughter destined to differentiate instead dies (Gilboa et al., 2003). Germ line stem cells differentiate upon losing contact with their niche, which likely involves GJC; as an example, it has been proposed that GJ-mediated cAMP signaling between blastomeres and somatic cells results in changes in somatic cell gene expression (Burnside and Collas, 2002). Many instances of GJC-dependent regulation is bidirectional, with several cell types exchanging instructive information, such as occurs in the communication between stem cells and their neighbors in spermatogenesis during germline differentiation (Smendziuk et al., 2015).

Pigmentation Control Networks

One of the exciting recent developments is the elegant merging of genetic evidence for GJC roles with a mathematical analysis of the resulting pattern formation that has taken place in the context of zebrafish pigment patterning. In 1953 Alan Turing wrote an article entitled “The chemical basis of morphogenesis.” In it, Turing describes a mathematical analysis of reaction-diffusion systems that could account for the formation of stable periodic patterns in organisms. His system consisted of two substances with differing diffusion rates. One of those unknown compounds was an activator that could enhance its own formation and that of the other unknown compound, the inhibitor. The inhibitor not only was capable of inhibiting the formation of the activator, but it also possessed the faster diffusion rate of the two (Turing, 1953). The model predicted self-organization of spatial patterns, which would change as the size of the organism changed, but the chemical identity of the morphogens was unknown (Schiffmann, 2005). Confirmation came in 1995 when Kondo and Asai found that pigmentation patterns on angelfish changed as the fish grew, and showed a traveling wave that behaved similar to the computer simulations of Turing’s reaction-diffusion system (Kondo and Asai, 1995). In order to elucidate the molecules that played the role of the activator and inhibitor, research began to focus on zebrafish pigmentation patterns due to the increasing amount of molecular tools available for experimentation in that model organism (Asai et al., 1999; Kondo, 2002; Iwashita et al., 2006; Watanabe et al., 2006; Yamaguchi et al., 2007; Watanabe and Kondo, 2014).

It was found that the zebrafish *leopard* mutation, which confers spots instead of the typical stripes, gives rise to distinct pigmentation changes that suggested a reaction-diffusion wave as the mechanism controlling pigment pattern (Asai et al., 1999). Interestingly, it was then discovered that the *leopard* mutation is in *connexin41.8* (Watanabe et al., 2006), suggesting that in this case of pigment system morphogenesis, the relevant signaling molecules may be moving through GJs. The zebrafish mutant, *jaguar*, also showed changes in pigmentation pattern. This mutant had irregular spacing of its stripes that was caused by a mutation in the gene for the inward rectifying potassium channel 7.1 (Kir7.1) (Iwashita et al., 2006). Since Kir7.1 is responsible for maintaining transmembrane voltage potential and is required in the

black pigment cells (melanophores), experiments were done to determine how membrane potential may be involved in pigment patterning changes. These experiments found that melanophore membrane potential was more depolarized in the *jaguar* mutants and that this interfered with the transient depolarization signal conferred by contact with xanthopores, the yellow pigment cells. Loss of this transient signal resulted in melanophores that stayed in close contact with xanthopores, rather than moving away from each other as was the case in the wildtype (Inaba et al., 2012).

Further research by Kondo et al. showed that interactions between the two main pigment cells, xanthophores and melanophores, also involved Delta/Notch signals. These signals were activated in the cells by long processes that connect the different chromatophores. The xanthophore processes is short and presents the Delta ligand that binds to the Notch receptor on the membrane of the melanophore and promotes its survival. If the melanophore is near a xanthophore then it can bind to the Delta ligand and survive. This relationship would satisfy the requirement for an activator with a short range. In addition, the melanophores have a long process that reach out to xanthophores, but after a certain distance they can no longer reach, resulting in long-range inactivation (Hamada et al., 2014; Watanabe and Kondo, 2014). However, it is possible that Delta/Notch works in conjunction with a molecule that still depends on diffusion through GJs (Hamada et al., 2014). Indeed, recent work on mutated Connexins 41.8 and 39.4 show that heterotypic GJs do play a part in zebrafish pigmentation patterning. These experiments also show that a third class of chromatophore called an iridophore is involved in patterning on the trunk of the animal but not on the tail, and that this relationship is possibly mediated by molecules that are diffused by GJs (Bullara and Decker, 2015; Irion et al., 2014; Kondo, 2002; Takagi and Kaneko, 2005). Together, these studies form an elegant body of work on the relationship of voltage and gap junctional signaling in pattern regulation.

The theoretical discussion as to the identity of these diffusible molecules has suggested the GJC-permeable molecules cAMP and ATP may be the relevant Turing couple. Whereby cAMP is the short range activator and ATP a long range inhibitor (Schiffmann, 1991). However, the small molecule serotonin may also play a role in the reaction-diffusion model of zebrafish coloring pattern formation, as it does in other examples of GJC-regulated patterning (Blackiston et al., 2015; Fukumoto et al., 2005b; Gairhe et al., 2012). Recently it was found that the zebrafish *leopard* mutation was correlated with behavioral abnormalities that pointed to lowered levels of serotonin transport and that treatments with serotonergic drugs resulted in altered pigmentation patterns (Maximino et al., 2013; Stewart et al., 2013).

GJC and Nerve Sprouting Control

In addition to development, GJs are also important for physiological maintenance (Maeda and Tsukihara, 2011), regeneration (Umino and Saito, 2002; Hoptak-Solga et al., 2008), and remodeling. A key cell type involved in these processes is the neuron. Axonal processes must interact with surrounding tissues and make decisions about direction and magnitude of sprouting, in order to correctly pattern and maintain innervation to target organs. The signaling underlying this, and the involvement of GJs, was recently revealed by using a transplantation assay in which eye primordial from a fluorescently labeled donor embryo

were transplanted onto the flank of a host (Vandenberg et al., 2014). Normally, the ectopic eye produced one major nerve bundle, which extended to the host's spinal cord. However, when the host cells were depolarized, the eye instead generated a huge overproliferation of nerve which spread throughout the tissue. Testing a number of transduction mechanisms for this effect on nerve growth, the authors showed that inhibiting GJC in the host abolished the ability of depolarization to cause hyperinnervation from the implanted organ. The authors showed that the effect only applied to ectopic innervation (not the host's native nerve pattern), and that serotonergic signaling was both required for the hyperinnervation and could rescue the blockade of GJC. A model was advanced in which ectopic nerve, searching for guidance cues, was making proliferation and extension decisions based on serotonin molecules arriving via GJs. As will be seen in the discussion of left-right patterning below, the movement of serotonin via GJs, under an electrophoretic force, is a conserved theme among a number of patterning systems.

GJs in Cancer

Another example in which GJs play an important role is the occasional defection of cells away from the normal anatomical plan toward carcinogenesis (Omori et al., 1998; Mesnil et al., 2005; Trosko, 2005) and subsequent metastasis (Defamie et al., 2014). In a sense, cancer is the derangement of developmental patterning—a disease of geometry (Rubin, 1985; Maffini et al., 2005; Soto and Sonnenschein, 2011; Tarin, 2012; Chernet and Levin, 2013a). Cancer cells abandon correct morphogenesis in favor of unrestrained growth reminiscent of unicellular organisms prior to the appearance of multicellularity and to the GJ-mediated coupling of cells to other cells within a larger somatic context (Loch-Caruso and Trosko, 1985). Reduction of GJ-dependent communication with other somatic tissues is an important early step in the process in which cells begin to treat the rest of the body as an "external environment" within which they must survive by any means necessary.

Studies have long noticed that lowered GJC was associated with induction of tumorigenesis (Potter, 1980; Yamasaki et al., 1984; Loch-Caruso and Trosko, 1985); normal tissue generally possesses a much higher degree of GJC than tumor tissue, and a loss of GJC accompanies early steps in neoplastic transformation (Pitts et al., 1988). A neoplastic phenotype can be induced in cell culture by ectopic closing of GJs using pharmacological agents or dominant-negative constructs (Omori and Yamasaki, 1998). Most interestingly, neoplastic characteristics can be suppressed by ectopic induction of GJC in tumor tissue (Mehta et al., 1991; Rose et al., 1993; Hellmann et al., 1999). Connexin32-deficient mice have a 25-fold increased incidence of spontaneous liver tumors (Temme et al., 1997), and Connexin32 is also an anti-invasive agent in renal cell carcinoma (Yano et al., 2006). Thus, gap junctional isolation is known to be a tumor-promoting agent (Loewenstein and Kanno, 1966; Loewenstein, 1969, 1979, 1980; Rose et al., 1993; Yamasaki et al., 1995; Leithe et al., 2006; Mesnil et al., 2005). This is consistent with a view of GJs as mediating morphogenetic cues that could be keeping cells under differentiation and growth limitation consistent with adult morphostasis. It should be noted, however, that a few studies have indicated the opposite (Stoletov et al., 2013), for example the finding that Connexins 26 and 43 mediate metastasis in melanoma and breast cancer (Stoletov et al., 2013), and a role for GJs in promoting metastasis by transfer of cGAMP (Chen et al., 2016).

The fact that some connexins are pro-, and others anti-oncogenic, or even can exert both types of effects in different contexts (El-Saghir et al., 2011; Sin et al., 2012), is less puzzling if GJs are considered not as typical proteins that induce or suppress a particular phenotype, but rather as conduits for cell–cell communication. The outcome is expected to be a function of what signals arrive via the GJs (Mendoza-Naranjo et al., 2007; Lim et al., 2011), and are thus hard to predict from knowledge of the connexin type, because it's partially dependent on the neighboring cells, and physiological parameters that dictate the open/closed state of the GJs and what permeant molecule might be traversing it. This has implications for therapies targeting GJs in cancer and other diseases (Kandouz and Batist, 2010; Grek et al., 2014; Cogliati et al., 2016), because it shifts the focus onto the signaling molecules and their transfer profiles, in addition to targeting connexin gene products themselves.

In *Xenopus*, cells expressing KRAS mutations make tumor structures; the incidence of tumors can be significantly reduced, despite the strong presence of the oncogenic mutation, by hyperpolarizing cells *in vivo* by misexpression of hyperpolarizing ion channels (Chernet and Levin, 2013b). Remarkably, however, this also works if cells at the opposite end of the animal are targeted (Chernet and Levin, 2014). How can such long-range communication of physiological state occur? Functional experiments implicated the movement of serotonin and butyrate in bioelectric control of tumorigenesis (Blackiston et al., 2011; Lobikin et al., 2012; Chernet and Levin, 2014), and a recent study looked at the role of GJs in this process. Chernet et al. showed that loss of GJC taking place within nascent tumor sites, within remote host tissues, or between the host and the prospective tumor region significantly lowered the incidence of tumorigenesis, with the most pronounced suppression taking place when GJC inhibition occurred far from the oncogene-expressing cells (Chernet et al., 2015). In contrast, overexpression of wild-type Connexin26 increased tumor incidence. The authors presented a mechanistic model, based on an oscillating signal that propagates through GJs and controls proliferation, that quantitatively explained these puzzling data (and made an unexpected, and subsequently validated, prediction about the role of the left–right axis in this process).

From Single Cell Properties to Multiscale Pattern

Active GJ communication allows cells to make sophisticated decisions comparing relative levels of specific compounds between themselves and their neighbors (Moreno, 2008), and to integrate information across anatomical distances. Thus, they underlie the transmission of physiological patterning signals (Levin and Mercola, 1998b; Levin and Mercola, 1999; Warner, 1999; Levin and Mercola, 2000; Lecanda et al., 2000; Pizard et al., 2005; Levin, 2007; Jin et al., 2008; Schiffmann, 2008; Dobrowolski et al., 2009; Oviedo et al., 2010). Cells utilize GJs to communicate directly with their neighbors, or with more distant cells via tunneling nanotubes—extended cell processes with GJs at their end (Wang et al., 2010; Wittig et al., 2012; Antanaviciute et al., 2014; Sherer, 2013; Rimkute et al., 2016).

Some of the earliest data implicating GJs in development came from studies with functional antibodies. Introduction of antibodies raised to specific portions of connexin proteins in mouse embryos resulted in developmental defects (Becker et al., 1995). In *Xenopus*, microinjection of antibodies has been reported to disrupt axial patterning (Warner et

al., 1984); the treated embryos contained differentiated mesodermal derivatives such as notochord and muscle tissue and Warner et al. argued that GJC has a role in pattern formation *per se*, rather than in the induction of specific cell types.

In invertebrate systems, *Caenorhabditis elegans* and *Drosophila* have been paramount in revealing GJC roles via genetic approaches. Numerous defects were observed in a *C. elegans* innexin-3 mutant, including rupture of hypodermis, failure of elongation, and failure of pharynx to attach to anterior (Starich et al., 2003). Likewise, epithelial organization and polarity of the embryonic epidermis is dependent on heteromerization of innexins 2 and 3 in *Drosophila* (Bauer et al., 2004; Lehmann et al., 2006). Establishment of the proventriculus requires Innexin-2, which is a target of Wingless signaling (Bauer et al., 2001, 2002). This work is particularly interesting because it links GJC to a canonical signaling pathway. Wnt signaling is likewise upstream of GJC patterns in vertebrates (Olson et al., 1991; Olson and Moon, 1992; van der Heyden et al., 1998; Ai et al., 2000), suggesting possible conservation of signaling modules involving GJC. Interestingly, overexpression of Cx43 provides a partial recovery of the formation of the cerebellum in Wnt-1 knock-out mice (Melloy et al., 2005), strengthening the link to the Wnt pathway and suggesting that establishing ectopic domains of cell:cell communication may be a useful modality to control certain patterning defects. Gene targeting studies of connexins in mice have provided evidence that GJs are important for cardiac septation (Kirchhoff et al., 2000) and morphogenesis of the outflow tract (Gu et al., 2003a) and endocardial cushions (Kumai et al., 2000). Many of these effects appear mediated by effects on the behavior of neural crest (Ewart et al., 1997; Lo et al., 1997; Huang et al., 1998a,b; Sullivan et al., 1998; Lo et al., 1999; Waldo et al., 1999; Waller et al., 2000; Xu et al., 2001; Li et al., 2002).

One major contribution of GJs to large-scale patterning is by sculpting signaling via developmental bioelectricity—spatial gradients of distinct resting potential across tissues which instructs pattern regulation in embryogenesis and regeneration (Bates, 2015; Levin and Stevenson, 2012; Levin, 2012a,a,b). GJs both determine cellular voltage (by allowing current from nearby cells) and in turn are regulated by resting potential (Peracchia, 2004). They, thus, implement physiological feedback loops with complex behavior, akin and parallel to the bidirectional coupling between genes and the transcription factors they encode. These regulatory layers function in parallel (Fig. 2), each supporting unique dynamics that instruct pattern regulation and physiology. The brain and non-neural tissues both exploit GJ networks for processing information; indeed, there are remarkable parallels between the way bioelectric signaling, gene regulatory networks, and signals from the environment integrate within and outside the CNS, to generate instructive cues (Fig. 3). We next discuss a number of specific recent examples.

GAP JUNCTIONAL COMMUNICATION IN GROWTH AND PATTERNING OF THE ZEBRAFISH FIN

Zebrafish fin patterning is an excellent model of joint formation due to the ability of the fin to regenerate once amputated, and the continuation of fin growth in adult animals. The caudal fin is made up of 16–18 fin rays, which are made up of multiple bone segments

separated by multiple joints (Sims et al., 2009). GJs have been shown to regulate the morphology of bone segments and joints in the zebrafish fins. The fin length mutant *short fin* (*sof*) was found to be caused by mutations in the expression of Connexin43 (Cx43) protein (Iovine et al., 2005). Analysis showed that reduced GJC caused by lower expression of Cx43 or missense alleles reduces the total number of dividing cells that form the fin ray segments, resulting in shorter segments. However, Cx43 could also be allowing the diffusion of signaling molecules from proximal regions to distal regions acting as a biological ruler that could inform the formation of new segments that extend the length of the fin (Hoptak-Solga et al., 2007). The new bone segments in the fin are dependent on new joint formation that occurs in a proximal to distal manner. Cx43 was found to take part in joint formation by first determining the location of the future joint in the mesenchymal compartment. Later, the Cx43 proteins polarize themselves toward the medial surface of the newly differentiating cells that will form the new joint. Lowered levels of Cx43 resulted in premature joint formation and small fins, while increased expression resulted in joint failure and large fins (Hoptak-Solga et al., 2007).

Recently, microarray investigation into the mechanism by which Cx43 exerts its effect on fin size found that expression of the gene encoding the signaling molecule Semaphorin3d (Sema3d) was perturbed in Cx43 mutants. Semaphorins are involved in the patterning of a variety of tissues and organs. The semaphorin receptors Neruopilin2a (Nrp2a) and PlexinA3 (PlxnA3), were found to be involved in the changes in cell proliferation and joint forming phenotypes associated with Cx43 expression in fin formation, respectively (Ton and Iovine, 2012). Cx43 involvement in suppressing joint formation was also found to influence the timing of expression of the homeobox gene that encodes the transcription factor Even Skipped 1 (Evx1). Overexpression of Cx43 resulted in loss of evx-1 and decreased expression resulted in evx-1 expression that was more distal than wildtype and led to the premature expression of the genes involved in joint formation (Ton and Iovine, 2013).

The other gene identified in the microarray investigation into downstream effects of changes in Cx43 expression, was hyaluronan and “proteoglycan link protein 1a” (hapln1a). Hapln1a is a protein that links the carbohydrate polymer, hyaluronic acid (HA) with proteoglycans in the extracellular matrix (ECM). This link stabilizes HA creating the right ECM environment that works in parallel with the Sema3d pathway for correct fin formation (Govindan and Iovine, 2014). Further studies on Cx43 expression in zebrafish and how it affects fin formation have found that the gene called “establishment of cohesion1 homolog 2” (esco2) is responsible for regulating Cx43 levels. ESCO2 is an acetyltransferase that modifies cohesion proteins that are responsible for joining sister chromatids during mitosis. Mutations in esco2 cause disruptions in mitosis that increase cell apoptosis and decrease cell proliferation. Together, these data reveal elements both upstream and downstream of connexin function. However, the perturbation in mitosis caused by the mutant form of esco2 in humans could not completely explain the phenotype seen in the resulting syndrome. This syndrome, called Robert’s syndrome is characterized by craniofacial abnormalities and limb malformation that could be a more severe form of oculodentodigital dysplasia (OCDD) that arises from mutations in the human Cx43 (Banerji et al., 2016).

GJS AND LEFT-RIGHT PATTERNING

Establishing Vertebrate Laterality

All vertebrates, and many invertebrates, exhibit a fundamental asymmetry of the bilateral bodyplan, with some internal organs (the heart, viscera, and brain) consistently biased to one side of the midline (Speder et al., 2007; Vandenberg and Levin, 2009). Early discoveries of left- and right-specific transcriptional signaling cascades had suggested that each side develops independently (Levin, 1998; Ramsdell and Yost, 1998). However, surgical experiments in the chick showed that in the absence of the right side, the left-sided (and not directly manipulated) tissue became confused, sometimes failing to turn on left-sided markers such as *Sonic hedgehog* and *Nodal* (Levin and Mercola, 1999). Seeking a mechanistic understanding of why distant tissue is required for laterality decisions, gap junctional communication was tested as a mechanism to coordinate sidedness decisions across the whole early embryo in frog and chick models (Levin and Mercola, 1998b). The data showed that either universal blockade of GJs with dominant negative connexins, or universal expression of constitutively-open connexins, randomized both the expression of laterality marker genes and the sidedness of the internal organs. Targeted misexpression experiments in the frog showed that normal asymmetry requires open GJC across the embryos' dorsal side, while lack of GJC is required across the ventral midline. These endogenous differences in GJC had been shown to be set up by Wnt pathway signaling as part of dorso-ventral axis determination (Olson et al., 1991; Olson and Moon, 1992).

A model was proposed, in which signals traversed the blastoderm in a chiral (left to right) manner, propagating through GJs and accumulating on one side of a midline zone of junctional isolation (Levin and Nascone, 1997; Levin and Mercola, 1998a). This scheme was suggested by the molecular functional data, although LR asymmetric GJ transfer was actually present in earlier data examining connectivity among frog blastomeres (Turin and Warner, 1980; Guthrie, 1984; Guthrie et al., 1988; Nagajski et al., 1989). Subsequent work identified the neurotransmitter serotonin (Fukumoto et al., 2005a,b; Adams et al., 2006; Vandenberg et al., 2012) as one left-right morphogen that moves through GJs (long before the nervous system appears) to control downstream gene expression via HDAC1-dependent chromatin modification (Carneiro et al., 2011) and several proton pumps and potassium channels that provide the electrophoretic force for unidirectional accumulation of charged morphogens (Levin et al., 2002; Adams et al., 2006; Aw et al., 2008; Morokuma et al., 2008; Aw et al., 2010). These mechanisms and the role of GJs in left-right axial patterning appear to be implemented very similarly in chick and frog, despite quite different early embryonic architectures. In chick, the relevant connexin appears to be Cx43, which is also important for laterality in zebrafish, where it is required for morphogenesis of the Kupffer's Vesicle (Hatler et al., 2009). Together, these data have also led to quantitative models of the distribution of serotonin through long-range GJ paths, and a study of the main system-level behaviors of this electrophoretic system (Esser et al., 2006; Zhang and Levin, 2009). A number of open questions remain, including possible roles of unidirectional transfer (by distinct connexins expressed in neighboring cells).

Innexin-Based GJs also Pattern the Nematode LR Axis

Interestingly, despite a completely different bodyplan and long evolutionary distance, GJs are also involved in LR patterning in the nematode *C. elegans*. An elegant set of studies (Chuang et al., 2007) focused on neural lateralization showed that establishing left-right asymmetry in *C. elegans* olfactory neurons involves Ca^{++} signaling, tight junctions, and communication through GJs. The central nervous system of *C. elegans* contains two bilaterally symmetrical odor sensory neurons (AWC). During late embryogenesis only one of these neurons will express the *str-2* G-protein coupled olfactory receptor. The determination of which neuron is going to express *str-2* (AWC^{ON}) is stochastic, and the remaining neuron acquires the AWC^{OFF} phenotype. Chuang et al. identified *nsy-5*, a gene encoding a member of the innexin/pannexin GJ family of proteins as *nsy-5* mutants are unable to sense odorants normally processed by AWC^{ON} neurons. Reduction of *nsy-5* function resulted in both neurons having an AWC^{OFF} phenotype. NSY-5 can form functional hemichannels and provide electrical coupling between cells. Genetic approaches showed that *nsy-5* has specific site of actions within different neuronal cell bodies. Strikingly, *nsy-5* can act autonomously to induce the future AWC^{ON} neuron based on a feedback mechanism between both AWC neurons. Subsequent work showed that calcium levels in non-AWC sensory neurons, determined the AWC^{ON} bias, demonstrating that a network of neurons communicate with AWC via signaling dependent on *nsy-5* to determine asymmetric expression of the *str-2* gene. It was also found that the modulation and propagation of the calcium signals was not mediated by serotonin or inositol triphosphate (IP3) (Schumacher et al., 2012). It is important to note that NSY-5 mediated connectivity also works in parallel with the NSY-4 claudin, which is related to the gamma transmembrane protein subunit of voltage-activated calcium channels and genetically down-regulates calcium channels which inhibits the calcium signaling pathway in the AWC^{ON} cell (VanHoven et al., 2006; Chuang et al., 2007). Inhibition of the calcium signaling pathway, calcium-calmodulin-dependent kinase II (CaMKII)-MAP kinase, is also achieved by an unknown *nsy-5* mechanism and also by the *mir*–71 miRNA that is stabilized by *nsy-4* and *nsy-5* (Alqadah et al., 2013). Once AWC asymmetry is achieved by *nsy-5* and *nsy-4*, maintenance of that asymmetry depends on olfactory signaling, transcriptional regulation including the zinc finger transcription factor *die-1*, and TGF-β signaling (Cochella et al., 2014; Hsieh et al., 2014).

The data revealed some striking similarities between how GJs are used in invertebrates to their functions in vertebrate development (Levin, 2002). In chick and frog embryos, the left and right sides of the embryos communicate with each other to correctly assign left-right identity before the onset of asymmetric gene expression. In both *Xenopus* and *C. elegans*, both over- and under-expression of GJ proteins lead to defects in laterality. The native pattern of communication mediated by GJs in both vertebrates and nematodes is between the Left and Right sides and involves a zone of junctional isolation (in this case, provided by an extracellular matrix layer). Moreover, the experiments by Chuang et al. revealed an asymmetry in how the left and right AWCs respond to *nsy-5* expression, mirroring the differences in gap-junctional permeability that has been described on the left and right sides of the early frog embryo (Guthrie et al., 1988). Crucially, the discovery of this intrinsic bias in *C. elegans* shows that as in frog and chick, the communication via GJs does not initiate left–right information but functions as an intermediate step of the pathway.

GAP JUNCTION-MEDIATED SIGNALING IN HEAD PATTERNING

Long-Range Control of Brain Formation

The developing brain must coordinate its growth and morphogenesis. In *Xenopus* embryos, it was recently shown that brain size and patterning is in part regulated by endogenous gradients of transmembrane resting potential (Pai et al., 2015a). Experimentally modulating resting potential (by expressing hyperpolarizing or depolarizing channels) in neural cells, or even in distant (ventral) cells, could control cell proliferation and overall patterning in the nascent brain. This effect was mediated by gap junctional communication: blocking GJC either genetically or pharmacologically rescued hyperpolarization-triggered brain malformations, and prevented ventral hyperpolarization from inducing proliferative effects in the brain. The authors suggest that GJC is involved in coordinating growth across long distances in the embryo, allowing the developing brain to match its proliferative profile to the rest of the body.

Moreover, induction of specific voltage gradients via ion channel misexpression was shown to induce ectopic brain tissue in posterior regions (Pai et al., 2015a). Interestingly, brain markers are also induced in *neighbors* of cells that misexpress the brain-inducing channels. However, with time, these neighboring cells turn off the ectopic brain markers, leaving only the cells whose V_{mem} is continuously set to a brain-specific pattern to be able maintain brain fate. The authors speculate that the brain-inducing bioelectric state is spread non-cell-autonomously via GJs, but with progressive restriction of GJ communication as development proceeds and individual tissue compartments refine, cells that do not drive abnormal resting potentials with their own channels go back to their normal fates. This normalization also works in the opposite direction, often enabling neighbors to suppress aberrant functions in cells with unique resting potentials that can otherwise induce foci of ectopic eyes (Pai et al., 2012) or tumors (Chernet and Levin, 2013b).

Species-Specific Head Morphology During Planarian Regeneration

Planarian flatworms are complex creatures, with a true brain, bilateral symmetry, a complex behavioral repertoire, and many body organs (Sarnat and Netsky, 1985; Gentile et al., 2011). They also have the remarkable ability to regenerate completely from partial body fragments (Reddien and Sanchez Alvarado, 2004; Salo et al., 2009; Lobo et al., 2012). Their capacity for self-repair serves as a paradigm case of dynamic morphostasis and continuous remodeling toward a specific target morphology. While many details of molecular pathways required for correct stem cell differentiation and regenerative capacity are becoming clear (Handberg-Thorsager et al., 2008; Aboobaker, 2011), the mechanisms that determine the correct shape of the head, and cease growth and remodeling when that shape is achieved, are almost completely unknown (Lobo et al., 2012, 2014). Examining physiological inputs into this process, we recently asked two questions: (1) could modification of overall bioelectric network connectivity give rise to coherent patterning changes during regeneration, and (2) is it possible to obtain evolutionarily relevant patterns. The results suggested that shifting among different regions of planarian morphospace (Stone, 1997) is possible by physiological perturbation alone (Emmons-Bell et al., 2015).

When GJC was systemically disrupted in fragments of *Girardia dorotocephala (GD)*, it induced the expected finer-grain V_{mem} regionalization among the endogenous bioelectric network (since GJC can normally act to establish isopotential cell fields). Remarkably, the resulting worms regenerated heads with an altered shape morphology that quantitatively resembles that of multiple other flatworm species (Emmons-Bell et al., 2015). This resemblance was more than skin deep: not only was the external shape of the head converted to the shapes of distant planarian relatives, but they also manifested different species-specific brain morphologies and stem cell distributions. The exact same treatment of a cohort of GD worms produced four types of worm heads in characteristic frequencies (proportional to their evolutionary distance from GD). It is not yet known whether this stochastic property is a consequence of the still relatively crude method of network topology perturbation (soaking in GJ blocker such as octanol), or whether it is an intrinsic aspect of the dynamics of this system. The ability to induce a different species' head shape in a genetically wild-type worm suggests that the bioelectric network is a profound regulator of species-specific morphology. It remains to be seen whether changes in the dynamics of bioelectrical circuits have been widely exploited by evolution to explore variations of anatomical structure.

How to infer the large-scale outcomes (which kind of head, how many heads, etc.) from cell-level properties and signals? This question has been addressed for gene regulatory networks using dynamical state spaces built to describe transcriptional circuits have been used to map complex system behavior (Huang, 2011; Huang et al., 2009; Halley et al., 2012). More directly relevant to physiological networks, similar approaches have been used to understand global behaviors of electrical activity in neural networks during decision-making. Importantly, however, in planarian regeneration, as in the brain, circuit dynamics are not directly revealed from ion channel expression data but are complex and nonlinear. Such dynamics must be modeled quantitatively to understand their emergent properties (Cervera et al., 2015; Law and Levin, 2015). One possibility is that different anatomical outcomes correspond to specific attractors in the dynamical state space of the bioelectric network formed by the planarian body; in this paradigm, bioelectric perturbations can shift the system from a default (genome-specified) attractor to another nearby one (Fig. 4). We are currently working to computationally model this process, to quantitatively map stable attractors to underlying physiological details, and thus gain more control over the resulting shapes.

GAP JUNCTIONAL SIGNALING UNDERLIES ANATOMICAL PATTERN MEMORY

In order for a bisected worm to regenerate as two normal worms, the cells at the posterior-facing edge of the wound must make a tail, while those at the anterior-facing edge must make a head. This simple fact reveals that the adult stem cells which implement regeneration in each blastema are not locally controlled: those cells were direct neighbors until the scalpel separated them, and thus have identical positional information, and yet they form completely different structures. Local position is not sufficient for blastema cells to know what shape to build; rather, they must communicate with the remaining tissue to decide what anatomical structures must be formed. Searching for the mechanism of this long-range coordination,

(Nogi and Levin, 2005) found that inhibition of GJC in the species *Dugesia japonica* for just 2 days resulted in subsequent regeneration of worm fragments forming heads at both ends (Nogi and Levin, 2005; Oviedo et al., 2010). These bipolar forms were viable, and could also be phenocopied by RNAi targeting 3 Innexins. By itself, this supported a role for GJ communication in anterior-posterior patterning in this species of flatworm. However, the real surprise came when the 2-headed worms were analyzed long after the GJ blocker (octanol) was gone (as shown by HPLC).

Weeks later, when these 2-headed animals had their heads and tails amputated again (in plain water, with no further perturbation), the same 2-headed phenotype resulted (Fig. 5), and this was repeated upon subsequent rounds of amputations (Oviedo et al., 2010). These data showed that a transient perturbation of physiological cell:cell communication via GJs could stably change the pattern to which the animal regenerates upon damage—its target morphology. This represents a clear example of physiological change permanently rewriting major anatomical features, despite a normal genome—a novel aspect of epigenetics in the original sense of the word (Noble, 2015). While chromatin modification processes may be involved, they are not a sufficient explanation for the effect, since the ectopic heads (tissue which might be suggested to have been epigenetically reprogrammed into a head state from its original tail identity) are *discarded* at each generation of cutting. What remains is a normal gut fragment, which has been reprogrammed to form 2 heads, not 1, upon future regeneration; the information about basic anatomical polarity and body organization must be stored in a distributed form throughout the animal.

GJS AS ELECTRICAL SYNAPSES IN BIOELECTRIC NETWORKS: FUNCTIONAL IMPLICATIONS

Synapses among cells are a key component of multi-cellularity, enabling cell–cell communication and information processing necessary for complex patterning and adaptive (regulative) physiology (Baluska and Mancuso, 2014). GJs are an especially important type of synapse because of the ability to regulate current based on voltage—this makes them similar to transistors or memristors (Jo et al., 2010; Pershin and Di Ventra, 2010), which are a basic unit of computation or logic gates. The central nervous system utilizes both chemical and electrical synaptic transmission. Chemical synapses use extracellular bursts of neurotransmitter release to relay digital information between adjacent cells. Electrical synapses use GJs to directly connect the interiors of the cells to one another, which results in a local and long-range relay of analog information through small molecules or changes in resting membrane potential (Pereda, 2014). These electrical synapses have been found to be particularly important in the imprinting of emotional memories, such as fear conditioning (Bissiere et al., 2011).

The main connexin in the mammalian brain is Connexin36, but there appear to be other connexins involved as well (Baker and Macagno, 2014). The regulation of connexin expression and the post-translational gating of the channels affects the strength of electrical synapses as does the nature of the connection formed by distinct connexin hemichannels. GJs that are formed by heterotypic channels comprised of different connexins have different

conductivities than homotypic channels comprised of identical connexins (Palacios-Prado et al., 2014). Electrical synapses are bidirectional, but not necessarily equally conductive in both directions (Marder, 2009; Palacios-Prado, 2009). They are also not dependent on action potentials; therefore they are capable of sensing the simultaneous sub-threshold depolarizations of a population of connected cells. In neurons, this detection can result in synchronous firing or asynchronous firing due to changes in neuronal thresholds and frequency of firing (Saraga et al., 2006; Gutierrez and Marder, 2013). The synaptic strength of a neuron can be determined by these thresholds and is modulated by the "coupling potential" that is imparted on the neurons by the GJ conductance but also by the capacitance and resistance of the connected neurons (Pereda et al., 2013). One of the most important functional features implemented by these versatile electrical gating elements is that of plasticity: history-dependent conductive processes.

Plasticity of electrical synapses occurs when the strength of the electrical synapse is changed by any one of a number factors related to prior electrical activity (Postma et al., 2011). Neighboring inhibitory chemical synapses can change the coupling potential between cells by shunting away excitatory currents (O'Brien, 2014). Ion channel expression and activation in neurons can change the resistance of connected cells (Curti and Pereda, 2004). Neurotransmitters can also change the conductivity of the GJs themselves (Piccolino and Neyton, 1984). Dopamine is able to increase cAMP, activating cAMP-dependent protein kinase A (PKA) that phosphorylates sites on Cx36 and decreases its conductance (Urschel et al., 2006). Other neurotransmitters including nitric oxide, histamine, and noradrenaline also have a regulatory effect on GJ conductance (Hatton and Yang, 1996; Rörig and Sutor, 1996; Zsiros and Maccaferri, 2008). Glutamate from local glutamatergic synapses has a potentiating role on electrical synapses by increasing the availability of calcium that activates CaMKII, which then phosphorylates sites on Cx36 that are both unique to and the same as PKA (Alev et al., 2008). This binding further affects the plasticity of the GJ conductivity, usually resulting in an increase of coupling (Pereda et al., 2013). In this way, memory and computational circuits can be formed as physiological state changes alter GJ-dependent connectivity shaping subsequent intercellular signaling dynamics, resulting in feedback loops that can stabilize transient bioelectric states into stable cascades of downstream signaling pathways.

An important source of neuronal activity modulation in the brain is the astrocyte. These are specialized cells that support neuronal metabolism and housekeeping, provide a structural framework, and are capable of releasing a variety of signaling molecules called gliotransmitters including glutamate, D-serine, and ATP to affect neuronal spiking thresholds (Hamilton and Attwell, 2010). Astrocytes have many processes and each one can connect to a large number of neurons. Although astrocytes do not form GJs with neurons, they do form GJs between themselves. These GJs allow the passage of calcium waves that are induced in response to patterns of neuronal activity, which are conveyed to astrocytes via glutamate (Wade et al., 2011). The propagation of these calcium waves through GJs is important for the coordination of neuronal groups that are not directly linked to one another, as these waves are capable of influencing any neuron that is coupled to the astrocytic network through which it is propagating (Pereira and Furlan, 2010). The coordination of information processing in astrocytes via GJ network plasticity is thought

to underlie important aspects of cognitive information-processing in the brain (Verwey and Edwards, 2010; Stehberg et al., 2012). Since many of the same mechanisms (bioelectric signaling, calcium waves, cAMP, etc.) occur in numerous other somatic cell types, it is possible that some of the patterning plasticity observed during regulative development and regeneration could be implemented by brain-like circuits of GJs that stabilize altered downstream signaling events as a function of prior physiological state.

CONCLUSION

What are the GJ-Permeable Signals?

What sorts of signals endogenously traverse GJs during patterning? In general, a variety of small molecules and metabolites are thought to permeate GJs, including cAMP (Burnside and Collas, 2002; Webb et al., 2002; Bedner et al., 2003), ATP (Bao et al., 2004; Pearson et al., 2005), cGAMP (Chen et al., 2016), Ca^{++} (Toyofuku et al., 1998; Blomstrand et al., 1999; Paemeleire et al., 2000), serotonin (Wolszon et al., 1994; Fukumoto et al., 2005b; Gairhe et al., 2012; Hou et al., 2013), and histamine (Chaturvedi et al., 2014). These are in general impossible to watch traversing GJ in situ, as these molecules are too small to be labeled (fluorescently) without radically altering their properties. However, in addition to small molecule metabolites, several recent reports have demonstrated that molecules much larger than the normal ≈1 kDa size cutoff can penetrate through GJs under some circumstances (Brooks and Woodruff, 2004; Valiunas et al., 2005). These include siRNA's (Wolvetang et al., 2007; Katakowski et al., 2010; Lim et al., 2011; Rimkute et al., 2016) and even the protein calmodulin (Brooks and Woodruff, 2004; Woodruff, 2005); one possibility is that the crucial parameter is shape, not overall size, and that long, thin molecules may be able to traverse GJ channels. The alignment of such molecules to facilitate GJC-mediated transfer could be provided by an endogenous electric field (Woodruff, 2005). Understanding the full range of endogenous permeant signals is an important area for future investigation.

Junctional selectivity can result in radically different permeabilities of GJs to different types of molecules (Bevans et al., 1998; Goldberg et al., 1999; Nicholson et al., 2000; Goldberg et al., 2002). This may have important consequences for the morphogenetic system, and means that studies of this process *in vivo* may be strongly dependent upon the experimental probe used. For example, at 7.5 days, the mouse embryo was found to be subdivided into at least nine GJC compartments with respect to Lucifer Yellow (LY) transfer, but only two domains with respect to ionic coupling (Kalimi and Lo, 1989). In the loach (*Misgurnus fossilis*), LY, fluorescein, and DAPI showed consistent differences in their ability to transfer between tissues during mesoderm induction and patterning (Bozhkova and Rozanova, 2000). Moreover, the chemical selectivity of the GJs connecting early embryonic cells changes appreciably during loach development (Bozhkova, 1998), suggesting that embryonic patterning can utilize regulation of not only the amount of GJC but also of the various types of molecules being passed through the GJs. Significant advances can be expected to result from future development of versatile fluorescent methods to reveal patterns of distinct GJ paths *in vivo*.

GJC and Bioelectricity: Two Key Components of Computational Networks

In addition to chemical metabolites, one of the most important signals propagated through GJ paths is current. This allows GJs to demarcate isopotential compartments of cells with similar V_{mem} (Sherman and Rinzel, 1991). This is thought to underlie normalization of aberrant founder cells in the production of ectopic brain tissue (Pai et al., 2015a) or tumors (Chernet et al., 2015). GJs and resting potential are bidirectionally linked, since V_{mem} can regulate GJ opening, while GJs regulate the sharing of V_{mem} among neighboring cells, but are not interchangeable. Genetic crosses have revealed that in the zebrafish tail, connexins and ion channels have distinct roles (Hoptak-Solga et al., 2008; Perathoner et al., 2014). And in the case of voltage control of nerve sprouting (Blackiston et al., 2015), depolarization cannot induce the hyperinnervation phenotype if GJ is shut down. Indeed, GJs allow multiple cells to act as one in response to electric fields (Cooper, 1984; Tsutsui et al., 2014) and alter their bioelectric dynamics (Baigent et al., 1997; Donnell et al., 2009). One of the best recent linkages between physiology and transcriptional regulation was characterized in the highly GJ-coupled heart tissue, in which Wnt1 controls the bioelectrical gradient via regulation of the L-type calcium channel (Panakova et al., 2010).

Thus, one mechanism by which GJ activity regulates pattern formation is by shaping endogenous bioelectric gradients. The importance of these gradients have been confirmed by numerous functional studies implicating spatiotemporal V_{mem} differences as an instructive parameter for cell behavior and large-scale patterning (Jaffe, 1981; Nuccitelli, 2003; McCaig et al., 2005; Funk, 2013; Levin, 2014b). Ion channel-mediated changes in V_{mem} not only affects individual cell behaviors such as proliferation, differentiation, apoptosis, and migration (Sundelacruz et al., 2009), but also determines large-scale parameters such as organ size, shape, and axial patterning of the entire body (Beane et al., 2011; Perathoner et al., 2014). In a range of vertebrate and invertebrate model systems, V_{mem} regulates the formation of the brain, eye, wing, and face, and controls patterning along the anterior–posterior and left–right axes during embryonic development (Levin et al., 2002; Dahal et al., 2012; Pai et al., 2015a). Moreover, experimental control of bioelectric gradients has enabled induction of regenerative ability in non-regenerative contexts (Tseng et al., 2010; Leppik et al., 2015), induced reprogramming of gut tissue into complete eyes (Pai et al., 2012), and normalized tumors (Chernet and Levin, 2013b). Electrical synapses (GJs) and neurotransmitters like serotonin are a key component of several patterning systems, having been implicated in embryonic left–right asymmetry, bone patterning, tumor suppression, and brain size control (Levin and Mercola, 1998b; Iovine et al., 2005; Levin, 2007; Chernet et al., 2015; Pai et al., 2015a). As in the brain, these elements often work together, such as the bioelectrically controlled movement of serotonin through GJs during left–right patterning and control of nerve growth (Levin et al., 2006; Blackiston et al., 2015).

In addition to the known downstream targets (Pai et al., 2015b) of the bioelectric signals propagated and limited by GJ paths, specific molecular endpoints for GJ include NFATc1 downstream of Cx37 signaling (Kanady et al., 2015), semaphorin downstream of Cx43 function (Ton and Iovine, 2012), and numerous targets of SP1 and SP3 transcription factors (Stains et al., 2003; Stains and Civitelli, 2005). Because so many downstream targets are impacted by GJC, it is a kind of master regulator node, like Ca^{++} (Slusarski and Pelegri,

2007) signaling or the RAS gene (Jindal et al., 2015). Thus, one of the exciting aspects of GJC function, as discussed above in the context of selecting alternate head morphologies in planaria, is the ability to regulate large-scale system-wide properties by targeting a single element GJC (or even individual connexins). An interesting recent example is the demonstration that decreasing Connexin36 GJ coupling compensates for overactive KATP channels to restore insulin secretion and prevent hyperglycemia in a mouse model of neonatal diabetes (Nguyen et al., 2014). GJs will surely play an increasing role in the understanding and management of circuit disorders, in the fields of birth defects and cancer (Huang et al., 2009).

Next Steps: Finer-Scale Reductive Studies

Confocal microscopy uncaging and FRET methodologies will greatly facilitate the study of endogenous GJC paths in embryos (Bedner et al., 2003; Braet et al., 2003; Cannell et al., 2004; Dakin et al., 2005; Di et al., 2005). Key future research areas include the characterization of factors which set up patterns of differential GJC in various embryonic tissues (at the transcriptional level, as well as at the level of controlling GJC states, such as by endogenous patterns of pH and voltage gradients (Ek-Vitorin et al., 1996; Morley et al., 1997; Calero et al., 1998; Francis et al., 1999; Gu et al., 2000), and the mapping of paths which exist in and between different tissues to molecules of various charges and sizes.

Experiments in mouse (and other systems) must, of necessity, be performed in the context of multiple connexin knock-outs, since it is becoming increasingly clear that due to compensation and redundancy, single loss of GJ genes can obscure important phenotypes (Simon et al., 2004). Indeed, knock-in of dominant negative mutants with different specificities for endogenous connexin families is likely to reveal many important and novel roles (Paul et al., 1995; Fiorini et al., 2004; Beahm et al., 2006). Likewise, more sophisticated technologies allowing expression of dominant negative mutants restricted in space and time during development will allow the circumvention of embryonic lethal phenotypes and likely to lead to the discovery of novel patterning mechanisms (Becker et al., 1992; Bakirtzis et al., 2003). While we now have some understanding of downstream transcriptional changes that occur when gap junctional signaling is perturbed (Kim et al., 2005), next generation sequencing analysis of GJC-inhibited tissues may lead to the discovery of proximal early response genes to GJC-permeable morphogens. Perhaps the most impactful tool would be the ability to selectively close or open GJ channels, in the way that optogenetics currently allows for ion channel function (Bernstein et al., 2012).

Next Steps: Systems-Level Integration

Perhaps even more important than increasing the molecular resolution with which we understand GJ function at the cellular level, is the converse task of synthesizing reductive data into a systems view of cellular networks coupled by dynamic GJC paths. Long-range GJ-mediated signaling, and feedback loops such as the one implemented by calmodulin, which regulates GJC (Peracchia et al., 1983; Burr et al., 2005; Dodd et al., 2008) but is itself a GJ-permeable signal (Brooks and Woodruff, 2004; Woodruff, 2005), ensure regulatory modes that cannot be captured via simple pathway models. Especially important is the investigation of GJs in reducing the impact of physiological variability and noise in

individual cells to facilitate robustness (Hallett, 1989), and their converse roles in sharpening cell responses to weak stimuli (Cooper, 1984). Because of the inherent complexity and nonlinearity of the behavior of such networks, the first task is systems-level modeling. Theoretical analyses of GJC signaling have begun via mathematical models (Cooper, 1984; Cooper et al., 1989; Vogel and Weingart, 1998; Nygren and Giles, 2000; Hofer et al., 2002; Vogel and Weingart, 2002; Fortier and Bagna, 2006; Cervera et al., 2015), and in future work it will be very important to apply these to the understanding of specific patterning processes in embryonic and regenerative morphogenesis (Esser et al., 2006; Zhang and Levin, 2009). Such models, especially as they become integrated with bioelectric and transcriptional networks, will become an essential tool for understanding endogenous pattern formation and developing interventions to control complex remodeling events for regenerative medicine and bioengineering applications.

A Speculative Hypothesis: Somatic Memories

Beyond modeling the biophysics of GJ-coupled cells, the field will truly mature when it also develops conceptual tools to explain information and computation propagating through GJ-coupled cell networks (Friston et al., 2015; Pezzulo and Levin, 2015). GJs are known to be important for memory and computation in the brain (Wang and Belousov, 2011; Wu et al., 2011; Dere and Zlomuzica, 2012), and the ability of most general anesthetics to serve as GJ uncoupling agents have motivated models of cognition and consciousness based on GJ-mediated integration across the brain (Mantz, 1992; Juszczak and Swiergiel, 2009). Interestingly, recent work has applied the same types of Hebbian plasticity concepts to understand the function of the heart (Chakravarthy and Ghosh, 1997; Zoghi, 2004) and pancreas (Goel and Mehta, 2013). We have recently extended the parallelism between brain and non-neural tissues' use of GJ-dependent synapses by proposing that target morphologies for regenerating systems are encoded via a memory-like mechanism within somatic tissues (Friston et al., 2015; Pezzulo and Levin, 2015). Because of the molecular and functional similarity in the use of GJs in the brain and in non-neural patterning tissues (Fig. 3), it is possible that circuits that guide self-limiting, flexible remodeling and regeneration programs are implementing true memories. The central involvement of electrical synapses and the holographic-like nature of patterning information that can be stably re-written (e.g., permanently 2-headed planaria) suggest models in which the target morphology is actually stored (encoded) within the real-time current dynamics, perhaps akin to storage of spatial memory in neural networks or similar proposed processes of memory in non-neural tissues (McConnell et al., 1959; Turner et al., 2002; Zoghi, 2004; Levin, 2011; Baluska and Mancuso, 2012; Levin, 2012b; Shomrat and Levin, 2013). This view makes two major predictions, currently being addressed in our lab. First, it suggests that models taken from cognitive neuroscience for understanding the system-wide dynamics of neural networks (Balduzzi and Tononi, 2009; Friston, 2010; Ebner and Hameroff, 2011; Friston, 2013; Hoel et al., 2013; Pezzulo et al., 2015) may be appropriate formalisms for efficient prediction and control of morphogenesis.

Second, it suggests that patterning could be controlled top-down, in a complementary approach to today's focus on bottom-up (molecular-level) interventions. If GJ-coupled tissues are indeed information processing agents as they are in the brain, then it may be

possible to efficiently exploit the plasticity and Hebbian-like learning capabilities facilitated by connexins in non-neural tissues by literally training them to desired outcomes: providing positive and negative reinforcement for collections of cells undergoing morphogenesis to encourage specific types of growth patterns. As with training in cognitive animal models, rewarding for final outcome (as opposed to directly regulating molecular signaling at each point in the tissue) may allow bioengineers to capitalize on the inherent modularity and plasticity of pattern formation (Sullivan et al., 2016). This may enable the induction of desired anatomical patterning changes without facing head-on the complexity explosion that stymies attempts to construct complex organs such as limbs directly.

Training non-neural tissues and treating them like “neural” networks with plasticity and memory could be feasible as shown by recent studies that heart and pancreas cells are capable of memory. The repolarization of the ventricles in the heart, referred to as the T-wave, can be inverted using atrial pacing. Depending on the duration and frequency of that pacing, the inversion strength and duration of inversions after pacing are increased, displaying a Hebbian-like memory (Rosenbaum et al., 1982; Chakravarthy and Ghosh, 1997; Zoghi, 2004). Networks of pancreatic islet beta-cells release insulin due to calcium waves that travel through GJs, and display a loss in GJC in response to heightened levels of glucose that can be modeled using learning theories also based on Hebbian-like memory (Benninger et al., 2008; Goel and Mehta, 2013). These data suggest that GJ-based plasticity outside the brain could be exploited to offload the computational complexity of micromanaging pattern of physiology onto the cells themselves (as occurs during reward circuit shaping in behavioral training). A whole new paradigm for medicine that incorporates tissue training could arise if these two important tissues can be physiologically entrained to correct certain heart arrhythmias and insulin secretion disorders.

Looking to neuroscience for examples of how neural networks are trained can inform new strategies to train non-neural cellular networks for desired GJC. A number of neuronal plasticity studies grow neurons on microelectrode arrays (MEAs) that can then be used to monitor the patterns of firing neurons and to also deliver electrical impulses that can stimulate the firing of certain neurons in the network (DeMarse and Dockendorf, 2005; le Feber et al., 2008; Franke et al., 2012; Pimashkin et al., 2013). Cardiomyocytes, pancreatic islet cells, and fibroblasts can all be monitored for electrical activity using MEAs (Parak et al., 1999; Bornat et al., 2010; Pfeiffer et al., 2011; Spira and Hai, 2013; Schönecker et al., 2014). However, the appropriate method for training GJ connectivity in non-neural networks might differ from neuronal networks.

Adaptive learning in neural networks *in vitro* requires an appropriate stimulation and feedback mechanism. In two studies, which utilized extracellular microelectrode arrays, low frequency stimulation was delivered to a culture of dissociated rat cortical neurons or murine hippocampal neurons via a stimulation electrode, and the spiking frequency was read on the other recording electrodes. Whenever the output at a specific recording electrode was over a certain threshold that considers previous output (response-to-stimulus ratio), then the next recurrence of the low frequency stimulation at the stimulation electrode was delayed (Shahaf and Marom, 2001; Pimashkin et al., 2013). This strategy for training neuronal networks has shown more promise than open-looped strategies, since it results in more space exploration

and greater changes in connectivity (le Feber et al., 2010). Indeed, closing the loop by using embodied networks that are capable of interpreting spiking outputs as instructions for robotic movement and in turn take sensory information from those robots and translate them into changes in stimulation patterns is showing great promise as way to demonstrate neuronal plasticity (Bakkum et al., 2004, 2008; Chao et al., 2008). Linking spiking outputs to flight simulator software controls with one stimulating electrode for pitch and the other stimulating electrode for roll can be used to train neuronal networks to fly a plane in the simulator and be able to correct for changes in headwind (DeMarse and Dockendorf, 2005). New optogenetic stimulation of action potentials allows for even better time resolution of output spiking due to the lack of an artifact that occurs when using electrode stimulation (Dranias et al., 2013).

Adapting these neuronal training techniques to non-neural GJs can be achieved using electrodes and optogenetics to change membrane potentials and thus affect the GJC between those and surrounding cells. While the details could be connexin- and cell-specific, one class of training strategies could deliver dopamine or glutamate as ways to provide GJ-modulatory feedback to the network. Microfluidics can be used to implement stimulation and also to directly measure the GJC between cells (Bathany et al., 2012). Real-time monitoring of calcium wave patterns using transgenic cell lines could also provide better measurement of output (Shigetomi et al., 2010).

Thus, one way to control the patterning outcome of GJ-coupled cell networks may be to modify their information content, whether by rewriting shape memories directly (via optogenetic-like approaches), or providing stimuli that exploit learning. Whether the neural computation analogy turns out to be the best way of understanding GJ dynamics, it is clear that GJs are a central hub for cellular information. Cells rely on GJ-permeable signals to regulate their individual activities toward large-scale construction, remodeling, and repair of complex anatomical structures. Thus, gaining predictive control over GJ dynamics is likely to be at the forefront of future efforts to exploit the ability of biological systems to regulate not just gene expression and physiology, but three-dimensional structure. The implications will be not only in fields like birth defects, regenerative medicine, and cancer, but are likely to impact evolutionary biology, unconventional computation, and the design of robust hybrid technologies. The technological revolution precipitated by the introduction of transistors in electronics was driven not only by their physical properties, but also by the appreciation of their unique computational capabilities. GJs support similar functions at the cellular level, with similar implications for computational capabilities of GJ-coupled networks (Scarle, 2008; Simon, 2009). Thus, it may not be unreasonable to predict that fully understanding and making use of GJ signaling dynamics in biological tissues may lead to the same scale of revolutionary advances in basic biology and applied biomedicine.

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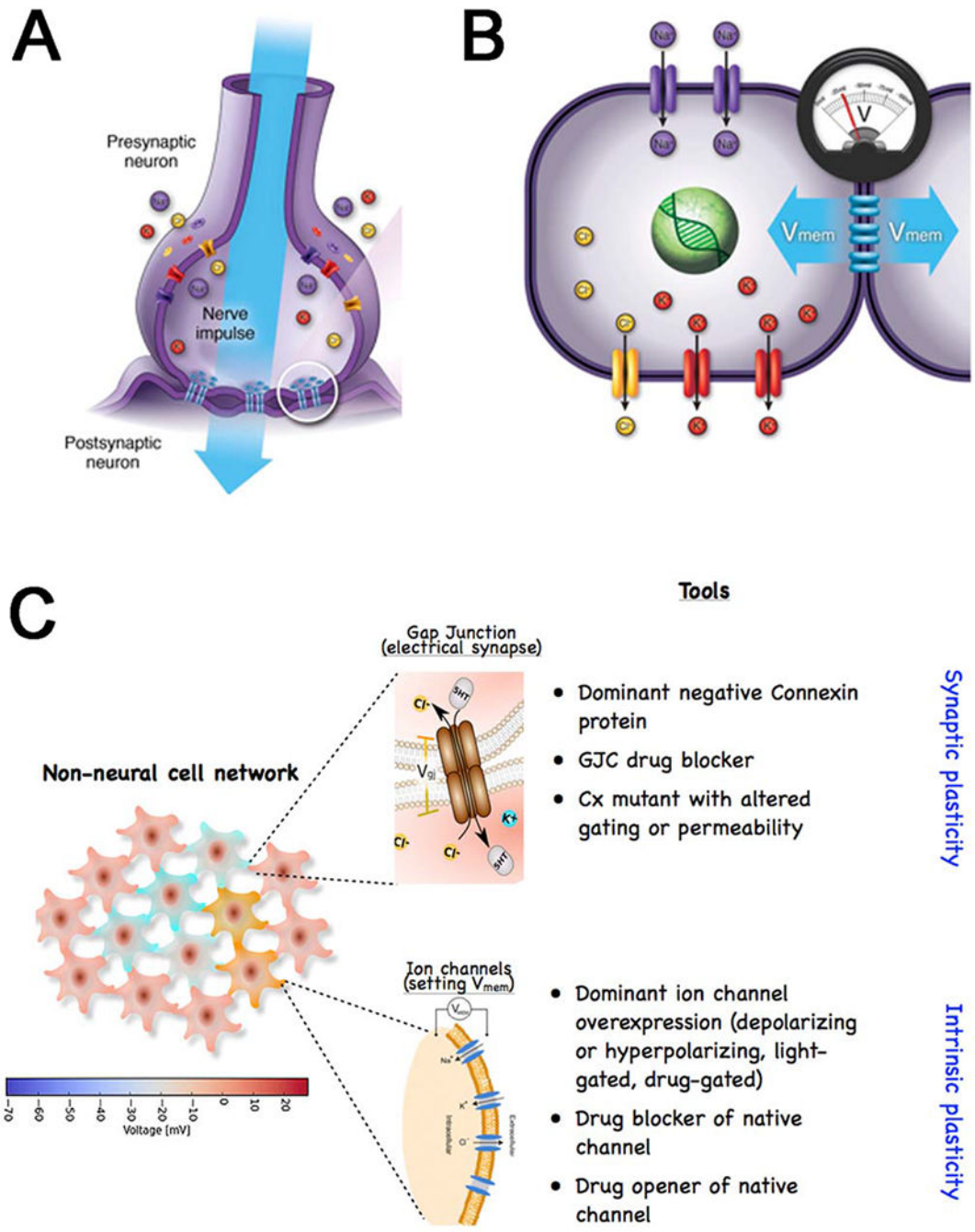

Figure 1.

Gap junctions form bioelectric circuits in the brain and beyond. (**A**) Neurons are often coupled by gap junctions, which allows electrical activity to propagate and integrate across cells. (**B**) The same scheme, involving ion channels to set V_{mem} levels and gap junctions to communicate bioelectric state to neighboring cells, is present in most somatic tissues. (**C**) Non-neural cells assemble into GJ-coupled networks that have many of the properties of neural networks. Manipulating the function of somatic tissues during pattern formation, by modulating GJ activity, makes use of two basic approaches, using pharmacological or

genetic techniques to target connectivity (GJ gating, akin to synaptic plasticity) or resting potential (ion channels, akin to intrinsic plasticity). Graphics courtesy of Alexis Pietak and Jeremy Guay.

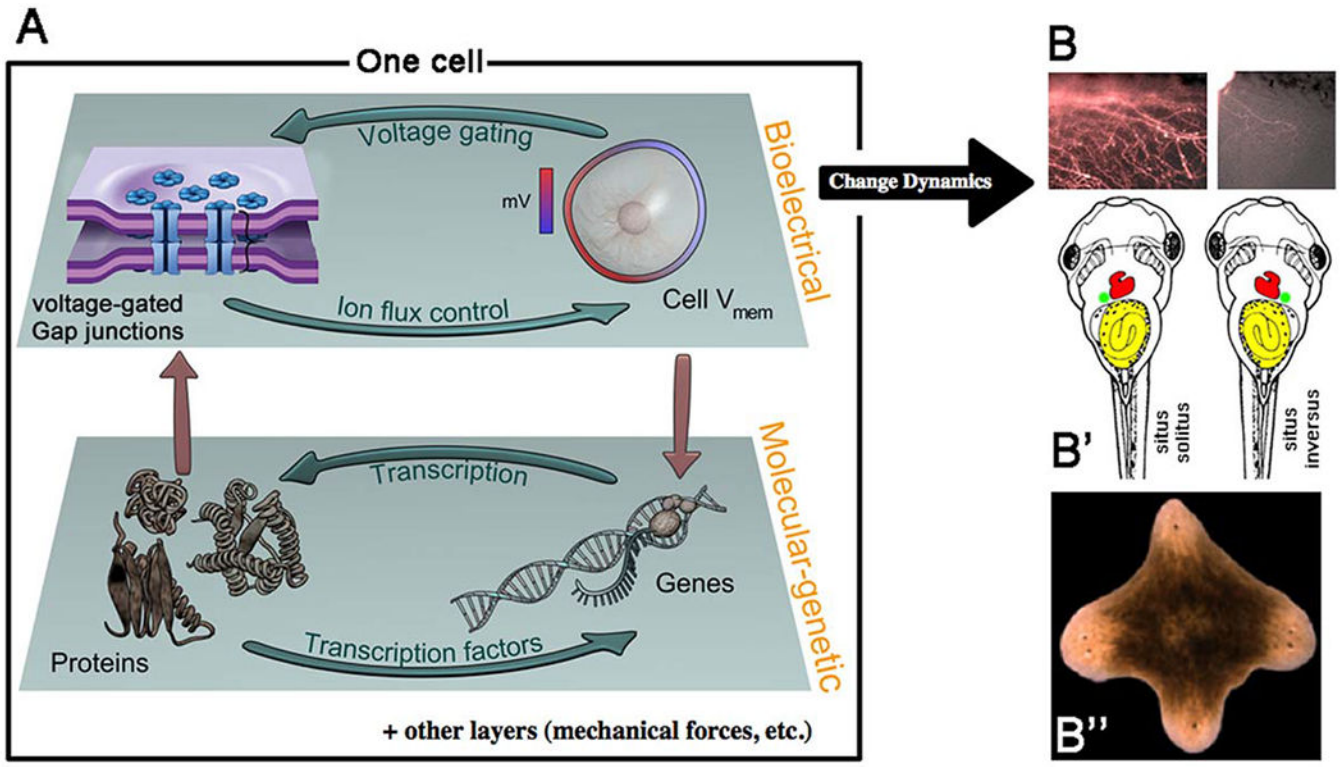

Figure 2.

Information in physiological circuits instructs pattern formation. Gap junctions are key regulators of bioelectric cell state. (**A**) Like gene regulatory networks, which contain numerous feedback loops among gene loci, gap junctions, and ion channels both regulate and are regulated by resting potential. This establishes an autonomous layer of physiological dynamics that is coupled to transcriptional cascades, but has its own unique information and functions. Modulating bioelectric dynamics by induced changes of GJC results in large-scale alterations of pattern formation, including hyperinnervation (**B**), left-right organ inversions (**B′**), and multiple head formation in regenerating planaria (**B″**). Graphics courtesy of Alexis Pietak and Jeremy Guay.

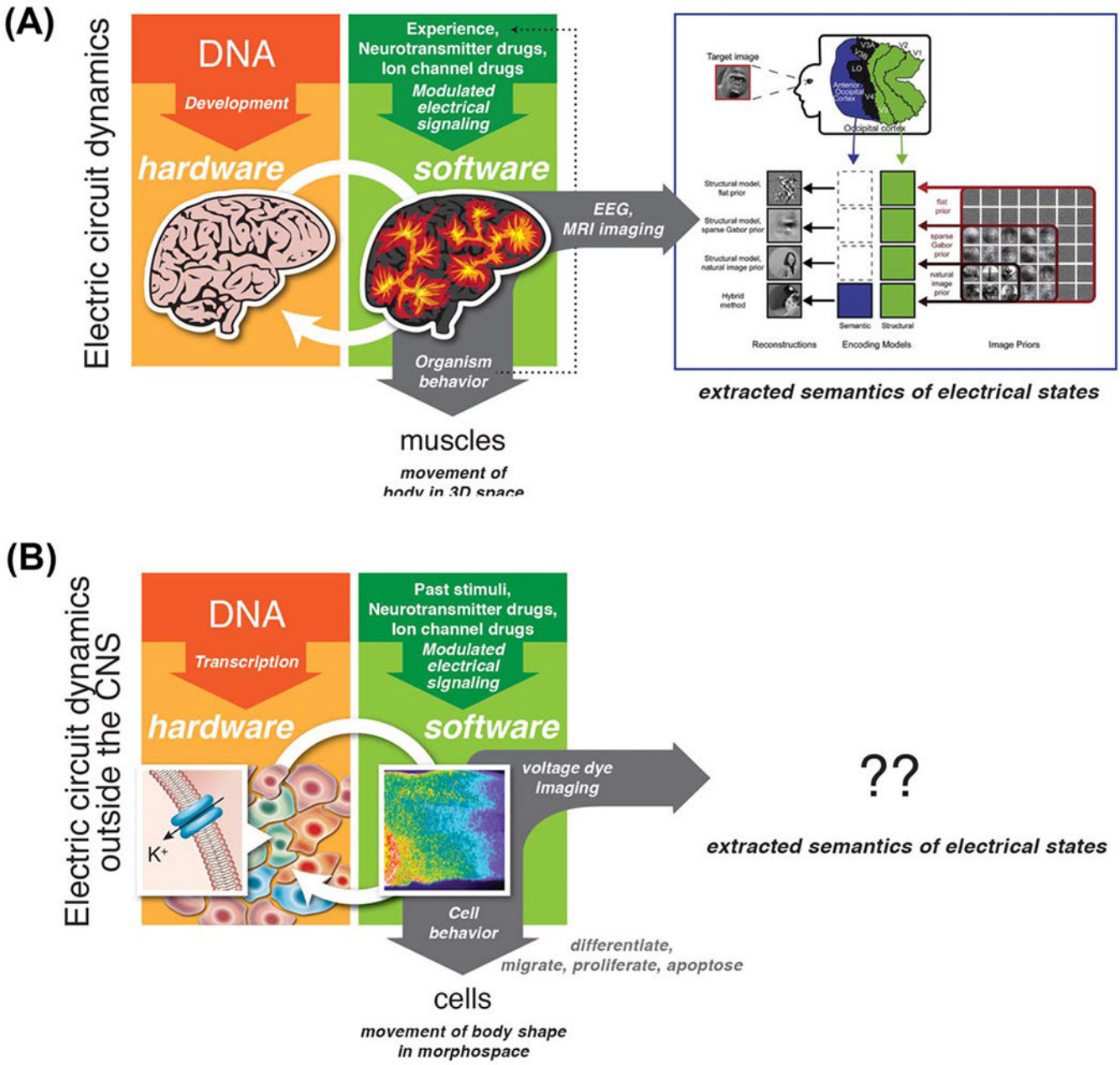

Figure 3.

Parallelism between brain and body. (**A**) In the brain, DNA sets the structure of the central nervous system—the hardware. Electrical circuit dynamics process information and store memories, resulting in experience-dependent (and self-organizing) patterns that control muscles resulting in the movement of the animal in three-dimensional space. Experiences (external sensory stimuli) and reagents targeting GJs, ion channels, and neurotransmitters alter the electric dynamics. Computational pipelines are beginning to be developed and applied to dynamics observed via EEG and MRI imaging, to extract the semantics—the memories represented by these bioelectric states. (**B**) In somatic tissues of developing or regenerating organisms, DNA sets the complement of connexins, ion channels, and

neurotransmitter machinery in all cells—the hardware. Bioelectric dynamics (slow patterns of resting potential within tissues) process information and establish prepatterns for gene expression and morphogenesis, resulting in chemical signal-dependent (and self-organizing) patterns that control cell functions like migration and differentiation, resulting in anatomical changes that move the body through morphospace. Stimuli (chemical and other signals) and reagents targeting GJs, ion channels, and neurotransmitters alter the electric dynamics. Computational pipelines need to be developed and applied to these dynamics as imaged with voltage-sensitive dyes and GJ tracers, to extract the semantics—the instructive anatomical patterns represented by these bioelectric states. Graphics by Jeremy Guay of Peregrine Creative and Alexis Pietak. Neural decoding panel in (A) is reproduced with permission from Naselaris et al., 2009. Voltage pattern panel in (B right) is courtesy of Douglas J. Blackiston.

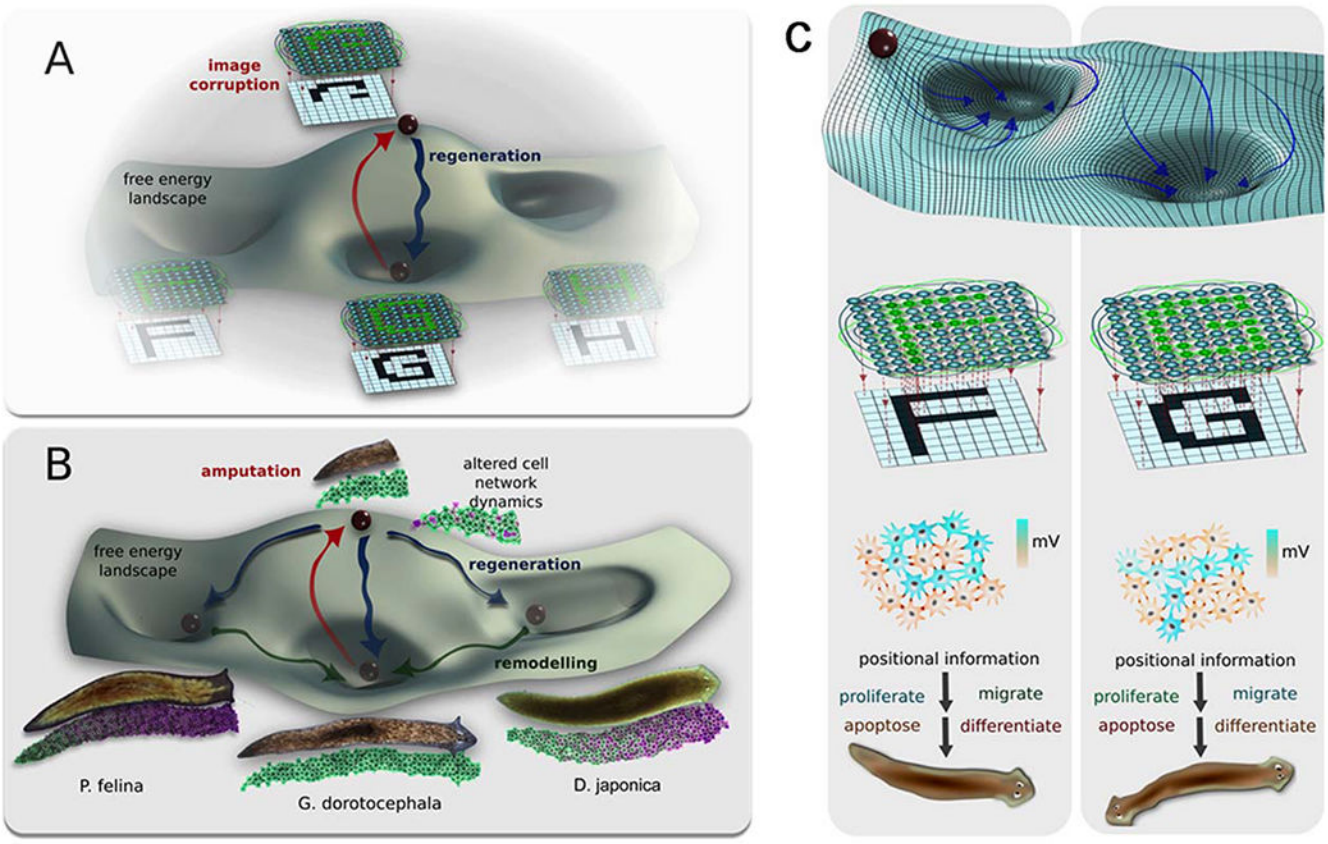

Figure 4.

Morphogenetic memories visualized as attractors in GJ network state space. GJ dynamics may offer an opportunity to understand information processing, not only molecular biophysics, but also of pattern formation regulated by physiological cell–cell signaling. One way to visualize planarian regeneration is (**A**) as the function of a large network of electrically coupled cells. Some such networks have been shown (in computational neuroscience and artificial intelligence research) to have a planaria-like property of holographic memory storage: a trained network can recreate the entire pattern despite deletions of the pattern or of the network components. A well-accepted mathematical paradigm for understanding the global properties of such networks is as an energy landscape, with attractors corresponding to specific stable modes of the network. In our analogy, amputation raises the energy of the system, temporarily pulling it out of the attractor to which the system tends to return. One hypothesis is that these networks are responsible for storing the pattern of a normal planarian, and when damaged, issuing cell-level commands (differentiate, proliferate, and other instructions) that restore the anatomy (in parallel to how recall of complex geometric memories can be triggered by stimuli and induce goal-directed behavior in cognitive science studies of animal behavior). This hypothesis makes a prediction: that coherent changes in patterning will result from experimentally induced changes of the bioelectric network's topology or dynamics. (**B**) It has been shown (Emmons-Bell et al., 2015) that altering the bioelectric connectivity in *G. dorotocephala* results in regeneration of one of four discrete head types. On this view,

amputation of head and tail causes the system to move to an unable state from its basin of attraction. Partial interruption of gap-junction communication between cells (reduced connectivity and thus altered dynamics of the network) induces head wounds to regenerate (yellow arrows) new heads that resemble closely-related flatworm species that are regions of stability in the regeneration morphospace landscape (left to right: *Schmidtea mediterranea, Dugesia japonica*, and *Philbertia felina*) as well as heads of the original species in the center basin. The probability of regenerating a certain head shape is proportional to the evolutionary distance from *Girardia dorotocephala*. These states are non-permanent (shallow basins of attraction) and over time will remodel into their final morphological state (white dashed arrows) to the deepest and most stable basin of attraction of the original head shape. (**C**) The same process can be modeled as a neural-like network, with stable modes visualized as stable attractors (a.k.a. memories in neural nets), which lead to specific instructive signals regulating proliferation, migration, and differentiation that induces different but coherent patterning outcomes. Graphics by Alexis Pietak.

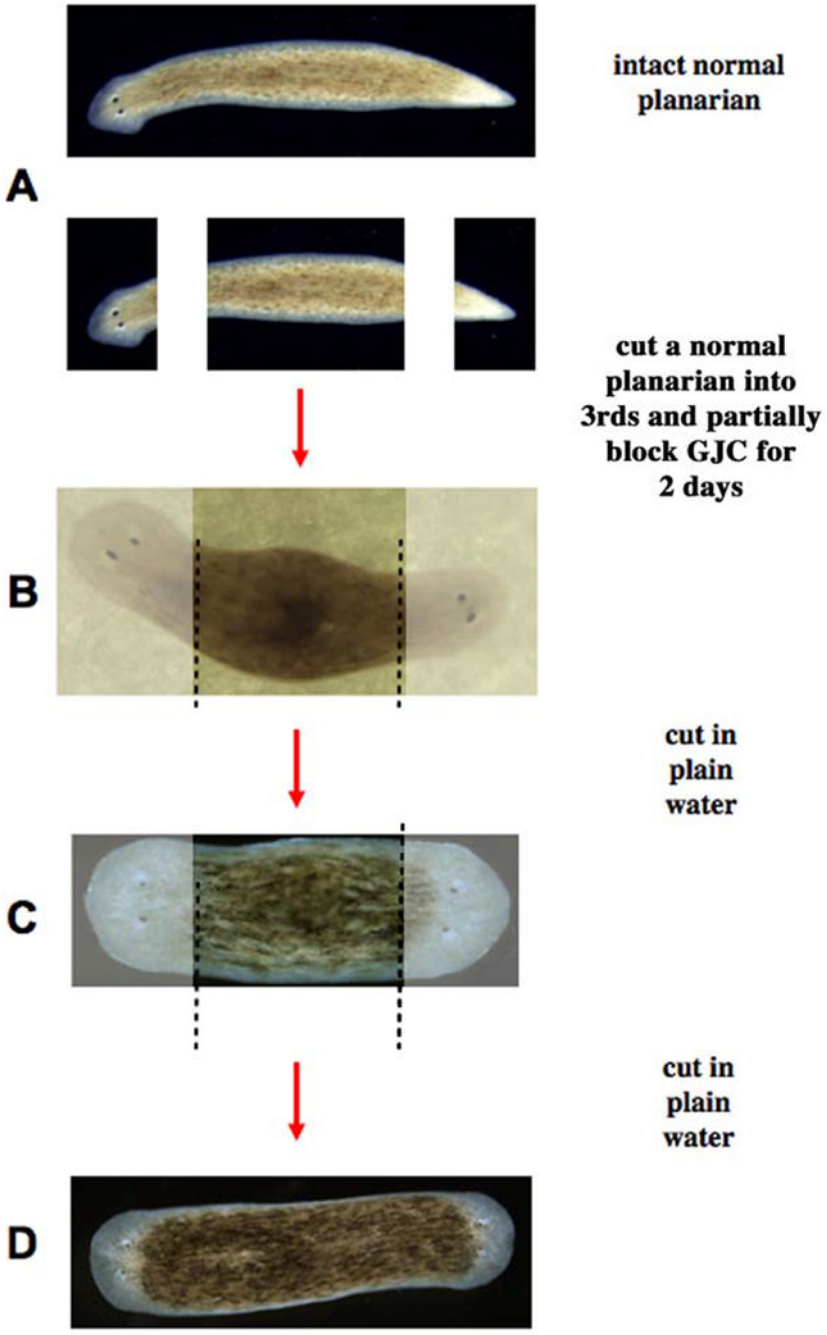

Figure 5.

Stable inheritance of target morphology change after GJ network perturbation. A normal planarian has a head and tail, and regenerates each at the appropriate end of an amputated fragment (**A**). When cut into thirds, and the middle fragment is briefly exposed to octanol, which temporarily blocks long-range bioelectrical signaling between the wound and mature tissues, a 2-headed worm results (**B**). GJC, gap junctional communication. Remarkably, upon further rounds of cutting in plain water (long after the octanol has left the tissues, as confirmed by HPLC), the 2-headed form is recapitulated (**C,D**; images of 2-headed worms

provided by Fallon Durant.). This change in the animal's target morphology (the shape to which it regenerates upon damage) appears to be permanent, and persists across the animal's normal reproductive mode (fissioning), despite the fact that the genomic sequence has not been altered. Chromatin modifications alone do not explain this, because the posterior wound cells, which could have been epigenetically reprogrammed to a head fate, are thrown away at each cut: the information encoding a bipolar 2-head animal is present even in the normal gut fragment—it is distributed throughout the body. We propose that this information is a kind of memory, encoded in electrical networks of somatic cells coupled by gap junctions, and is stored at the level of bioelectrical dynamics, not genetics.

Table 1

Channelopathies of GJ Genes and the Resulting Patterning Defects

Gap Junction Protein	Morphogenetic Role or LOF Phenotype	Species	Reference
Innexins	Gonad and germline morphogenesis	*C. elegans*	(Starich et al., 2014)
Innexin1,2	Epithelial patterning, foregut development	*Drosophila*	(Bauer et al., 2002)
Innexin 2	Eye size	*Drosophila*	(Richard and Hoch, 2015)
Innexins	Foregut, cuticle (epithelial) patterning	*Drosophila*	(Bauer et al., 2004)
Connexin 43	Oculodentodigital dysplasia (ODDD), heart defects (outflow tract and conotruncal), left-right asymmetry randomization, Osteoblast differentiation problems, craniofacial defects, myogenesis	Human, mouse, chick	(Britz-Cunningham et al., 1995; Reaume et al., 1995; Ewart et al., 1997; Becker et al., 1999; Levin and Mercola, 1999; Lecanda et al., 2000; Araya et al., 2003; Pizzuti et al., 2004; Debeer et al., 2005; Civitelli, 2008)
Connexin 37	Lymphatic system patterning	Mouse	(Kanady et al., 2011., 2015)
Connexin 45	Cardiac defects (cushion patterning)	Mouse	(Kumai et al., 2000; Nishii et al., 2001)
Connexin 50, Connexin 46	eye patterning (differentiation and proliferation, especially lens),	Mouse	(White, 2002)
Connexin 26	Cochlear development	Mouse	(Chang et al., 2014)
Connexin 41.8	Pigmentation pattern	Zebrafish	(Watanabe et al., 2006)
Connexin 43	Fin size and pattern regulation Craniofrontonasal syndrome	Zebrafish Mouse	(Iovine et al., 2005; Davy et al., 2006; Hoptak-Solga et al., 2008; Sims et al., 2009)
Innexin 4, Innexin 2	Germline differentiation and spermatogenesis	*Drosophila*	(Smendziuk et al., 2015)
Pannexin 3	Skeletal development	Mouse	(Oh et al., 2015)