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ARTICLE ADDENDUM

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Physiological inputs regulate species-specific anatomy during embryogenesis and regeneration

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ABSTRACT

A key problem in evolutionary developmental biology is identifying the sources of instructive information that determine species-specific anatomical pattern. Understanding the inputs to large-scale morphology is also crucial for efforts to manipulate pattern formation in regenerative medicine and synthetic bioengineering. Recent studies have revealed a physiological system of communication among cells that regulates pattern during embryogenesis and regeneration in vertebrate and invertebrate models. Somatic tissues form networks using the same ion channels, electrical synapses, and neurotransmitter mechanisms exploited by the brain for information-processing. Experimental manipulation of these circuits was recently shown to override genome default patterning outcomes, resulting in head shapes resembling those of other species in planaria and *Xenopus*. The ability to drastically alter macroscopic anatomy to that of other extant species, despite a wild-type genomic sequence, suggests exciting new approaches to the understanding and control of patterning. Here, we review these results and discuss hypotheses regarding non-genomic systems of instructive information that determine biological growth and form.

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bioelectricity; epigenetics; regeneration; morphology; morphospace; patterning; planaria; Xenopus

> "Treasure your exceptions! Keep them always uncovered and in sight. Exceptions are like the rough brickwork of a growing building which tells that there is more to come and shows where the next construction is to be."
>
> —W. Bateson, The Methods and Scope of Genetics

Introduction: What determines anatomical pattern?

Biological patterning is at the nexus of most of the important problems facing basic biology and biomedicine. Understanding the instructive signals that ensure self-assembly and maintenance of complex 3-dimensional morphology is crucial for basic evolutionary and developmental biology. How do cells, all derived from the same fertilized egg (the original stem cell) and bearing the same DNA, become not only differentiated into distinct cell types, but arranged into stereotypical spatial patterns with no external guidance? This question is at the center of understanding evolutionary change because development is what links genetics (upon which mutation acts) with form and function (upon which selection operates). Moreover, the identification of and functional control over these processes will lead to transformative strategies for injury repair, reversal of degeneration and aging, and tumor reprogramming.[1] If we understood how the body created its structures in the first place, we could coax it to repeat the process throughout its lifespan as needed. Complex pattern control is thus at the heart of regenerative medicine as well as synthetic bioengineering.[2,3]

Importantly, pattern control does not solely occur during the feed-forward process by which a fertilized egg's genome progressively unrolls toward an invariant outcome. A view of embryogenesis as terminal shape simply emerging over time from the parallel interactions of molecular components is too limited. Patterning information also has to be stored, processed, and acted upon during *shape homeostasis*: regeneration, remodeling, and maintenance of existing adult structures against

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damage, aging, and cancer.[4-6] Salamanders readily replace amputated body-parts, growing exactly what's missing and stopping when the correct pattern has been achieved. Grafting the tail of one salamander to the flank of another reveals an even more complex pattern-homeostasis capability: over time, the grafted tail remodels to a limb – a structure more appropriate to its new location.[7] The most important aspect of regeneration and remodeling is that it requires feedback: it cannot simply be an emergent process from hardwired rules because the details of injury are unpredictable for the organism. Somatic plasticity requires an organism to be able to recognize damage and enact repair from a vast variety of starting states, toward the same (correct) target morphology.

How do organs, tissues, and entire bodyplans know what shape they are supposed to build and maintain (Fig. 1A)? It is known that genomes do not specify target morphology directly, but nevertheless it is thought that the origin of a species' specific anatomy, at multiple levels of organization, is ultimately in its genome. The genome is often held to be the entirety of the information medium that determines pattern, and the key factor that differentiates one species' body from another. Thus, the emphasis of modern synthetic biology is almost exclusively on rewriting the genome.[8] This view has to be expanded in several ways toward epigenetic controls of patterning (Fig. 1B). First is chromatin modification, and the genomic regulatory code that drives cell type-specific gene expression and thus cell specification and differentiation.[9,10] Second are biotic signals produced by commensal organisms such as microbes,[11,12] and abiotic aspects of the environment, such as light which determines brain structure and function in some species[13] and temperature which can determine sex.[14]

Feedback between genetic and physical forces is a key determinant of patern formation, enabling gene regulatory circuits to generate specific anatomies. By expressing molecules that couple to specific physical forces (e.g., adhesion proteins), genetic circuits can harness self-organizing physical dynamics such as tensegrity,[15] reaction-diffusion,[16,17] and many others.[18-22] Consistent left-right patterning[23-25] is a perfect example of how important physics is to pattern. While a cascade of regulatory molecules (a gene-regulatory network, GRN) is required for maintaining identity of the left and right sides of the body,[26-28] it is immediately clear that no genetic circuit can be sufficient to distinguish left from right in space *ab initio*. To establish proper laterality, the cascade of asymmetrically-expressed genes is functionally positioned

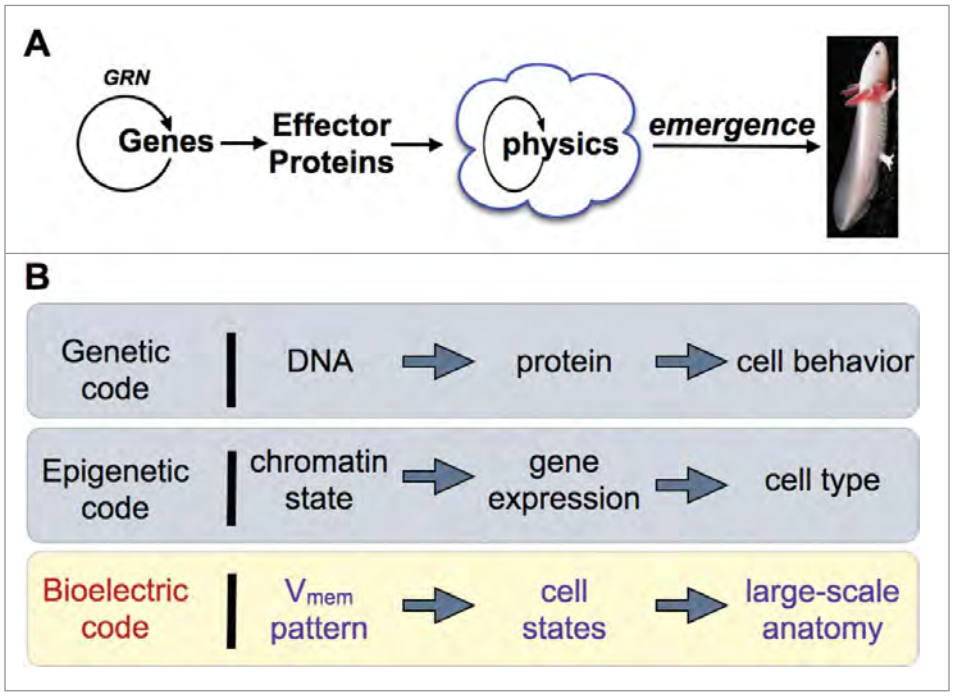

Figure 1. Interplay of genetics and physics. (A) The process of developing an organism from a fertilized egg cell involves an interplay of physics and genetics. Transcriptional gene-regulatory networks (GRNs) specify the production of effector proteins that allow coupling to specific physical processes (adhesion, tension, electric propagation, diffusion, etc.). It is the emergent order in those physical processes that ultimately results in a specific 3-dimensional shape of the body and its internal organs. (B) At least 3 codes participate in this process. The genetic code, which maps DNA sequence to protein sequence, controls cell behavior. The Epigenetic code, which maps chromatin state to expression of specific genes, regulates cell type and physiological properties. The bioelectric code maps the distribution of endogenous voltage gradients and electric fields *in vivo*, and appears to regulate large-scale anatomical patterning.[54,55,75,108]

between essential physical processes: the chiral elements that initiate asymmetry and orient it with the other 2 axes,[29-31] and the physical forces that actually execute the bending of asymmetric organs such as the heart and gut.[32,33]

More generally, GRNs have to be "painted" onto a pre-existing anatomical structure and integrated with physical effectors of genetic information. Even this picture is likely too simple, as shape is not hardcoded but can be edited by interactions with the environment.[34] Trophic memory in deer antlers occurs when damage made to one point in the branched antler structure will cause an ectopic tine in that same location next year, after the antler racks are discarded and re-grown anew.[35] We currently lack the conceptual apparatus to propose a plausible model by which genomes and physics allow the growth plate at the scalp to ascertain the 3D location of a wound, remember it for months, and use it to alter the genome-default cell growth decisions during subsequent antler re-growth.

While most of the above-mentioned models have not been addressed with molecular tools, recent work has begun to unravel genetic factors that mediate the differences among species. There are numerous incidences in which simple genetic changes drastically alter the morphology of populations as they diverge from their evolutionary ancestors. Darwin's finches, a group of 14 closely related finch species, are one such example. They display an impressive variety of beak shapes and sizes, and therefore diets, despite having evolved from the same ancestral species. This variation is due, at least in part, to differential expression levels of the genes calmodulin and Bmp4, which define beak length and width, respectively.[36,37] Another compelling example is the evolution of the bat's wing from a mouse-like ancestral arm. Increased expression levels of Bmp2 and Prx1, regulators of bone development, are responsible for the remarkable elongation of the bat forelimb digits and long bones respectively, rendering these animals capable of flight.[38,39]

Likewise, a number of epigenetic factors have been shown to make significant input into species-specific pattern outcomes. The long-term persistence of epigenetic state has been an area of focus in modern molecular and developmental biology, and work done in this field has added to models of cellular differentiation and stemness.[40] Of especial interest to evolutionary developmental biologists and ecologists are epigenetic modifications that both inform phenotype in an appreciable manner, and are transmitted to offspring through the germ line.[41,42]

Early botanist Linneaus was the first to describe the puzzling diversity in shape of the toadflax flower (*Linaria vulgaris*).[43] Despite being genetically identical, one morphological variant displays a rounded flower with lobed petals and bilateral symmetry, and the other an acute, starburst flower shape with radial symmetry. Cubas et al. found that the 2 varied only in the heritable methylation status of a single gene (*Lcyc*), demonstrating that large-scale morphological variance, eligible for natural selection, can occur solely as the result of epigenetic state.[44] Similarly, the alligator weed (*Alternatnthera philoxeroides*) has a remarkable variety of anatomical configurations, as it is able to dramatically restructure its root system, stem diameter, and internode length given development in a variable environment.[44] Other examples of environmental influence on morphology include species of reptile and fish, where incubation temperatures alter sex ratios of offspring.[45] Perhaps of even more interest are heritable epigenetic modifications[46-48] in organisms that segregate the germ line early in development. Methylation at a retrotransposon site within the Axin-fused allele produces a distinct kinked-tail phenotype in mice, which can be inherited both maternally and paternally.[49] For a more detailed account of proposed models and evolutionary significance of epigenetic heritability see refs. 50, 51 and ref. 52.

In addition to genetic sequence, chromatin marking, and environmental stimuli, it is becoming clear that biological pattern is regulated by another key set of processes: physiological signaling among bioelectrical networks.[53] In this Perspective, we discuss recent data in the frog and planaria model systems that shed light on the question: how much of the difference among species' morphologies comes from the physiological layer of control?

Bioelectricity: A novel epigenetic layer regulating pattern formation

Bioelectric networks (Fig. 2) consist of 2 basic components.[54,55] The first is ion channels (and pumps), which set the resting potentials of cells; these implement bioelectric circuits whose activity regulates the time-dependent changes of spatial gradients of resting potential (V_{mem}) across anatomical areas. Downstream of these bioelectric dynamics lie a number of targets, including the movement of neurotransmitters like serotonin,[56-59] and changes in gene expression[60] and chromatin state.[61-63] Endogenous, and experimenter-induced changes in V_{mem} implement intrinsic plasticity; recent functional approaches showed that these dynamics normally implement, and can be used to control, eye induction, organ size, craniofacial patterning, neoplastic transformation, axial polarity, and regenerative capacity.[64-71]

An even more important element of bioelectric networks consists of gap junctions (GJs, electrical

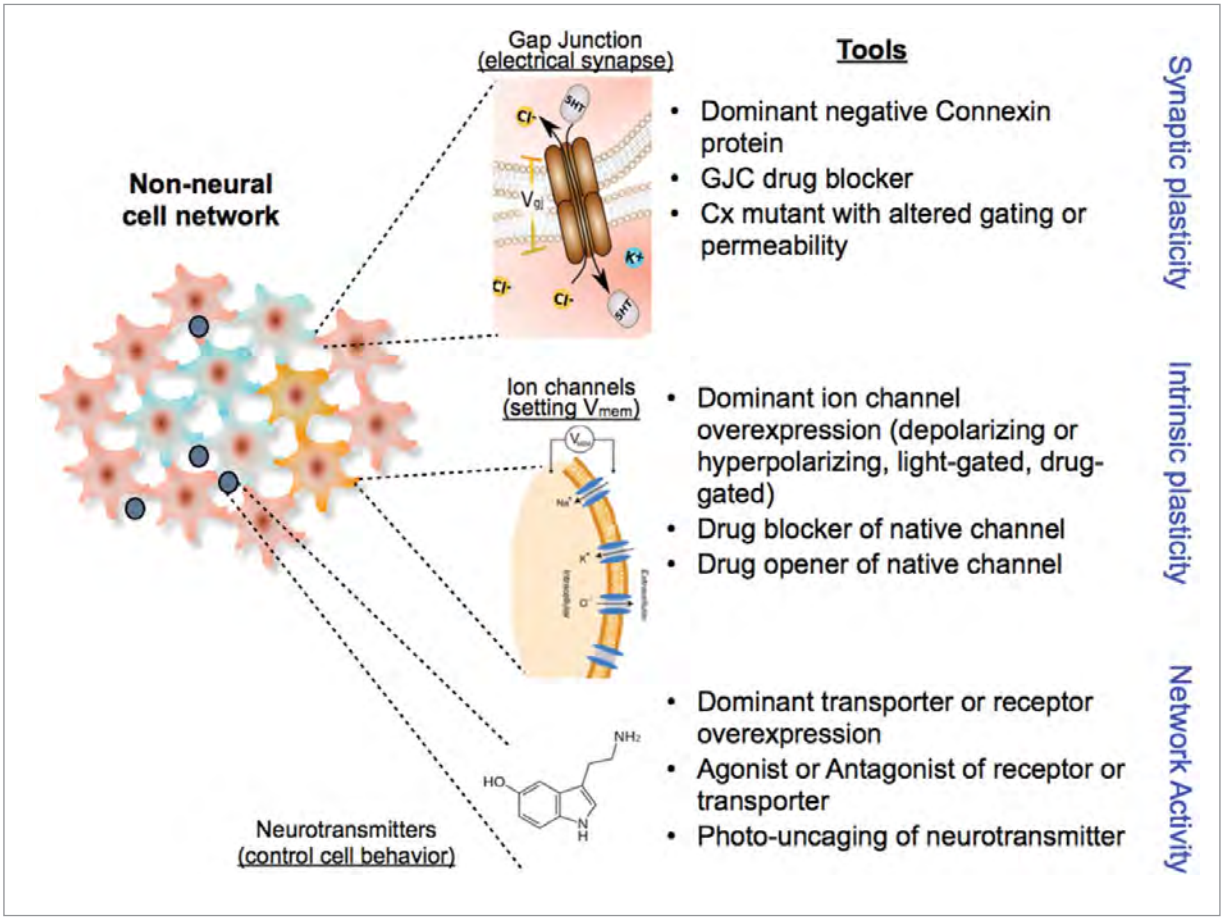

Figure 2. Bioelectric networks and their modulation. Many tissues, not only the nervous system, maintain active electrical communication among cells. Developmental bioelectricity, as in neurons, relies on ion channels and pumps to produce resting potential gradients across their cell membranes, and gap junctional channels to spread those potentials to neighboring cells. Signals within this network are mediated by the transfer of current and small molecules such as neurotransmitters. Because many ion channels and gap junctions are themselves voltage-sensitive, this system can support complex dynamics; the output of these dynamics includes changes in gene expression and cell behavior, mediated by transduction machinery such as neurotransmitter flux. Experimental (and endogenous) modulation of these dynamics can occur via regulation of gap junctional connectivity within the network (targeting gap junctions to alter plasticity of the electrical synapses), changes in ion channel activity (editing of resting potentials as a kind of intrinsic plasticity), or direct effects on the resulting gradients of neurotransmitters. A wide variety of genetic, pharmacological, and optical tools are now available to manipulate physiological networks in developmental or regenerative contexts *in vivo*. Graphics by Alexis Pietak.

synapses), which allow cells to share their voltage with neighbors, as well as communicate via small molecules such as neurotransmitters. Importantly, gap junctions are themselves voltage-sensitive, allowing them to serve as highly versatile gates for bioelectric circuits that underlie plasticity and computation.[72,73] Gap junction dependent patterns of cell:cell connectivity represent synaptic plasticity – the basis of information storage in the electrical networks of the brain and in non-neural tissues. Disruption of GJ signaling underlies numerous developmental disorders as well as the patterning defect known as cancer.[74]

Brains capitalized on the transistor-like properties of voltage-gated gap junctions (electrical synapses) and ion channels for memory and integrated decision-making, in the control of animal behavior. However, brains did not invent these tricks *de novo*: they speed-optimized computational properties of slower, "developmental" ionic signaling that cells were using to organize embryogenesis, wound healing, and adaptive physiology long before central nervous systems appeared.[75-80] Evolution capitalized on the unique suitability of bioelectric circuits for guiding complex, flexible, robust outcomes early on; ion channels and neurotransmitter molecules (which move as a result of bioelectric gradients) are present in all somatic cell types, not just excitable nerve and muscle. They are also present in unicellular organisms and predate multicellularity.[81-83] The extensive use of rapid bioelectrical signaling in the CNS likely offers a hint of more fundamental somatic functions from which neural dynamics evolved.

Mechanistically, bioelectric circuits in the brain and body use the same, highly conserved set of molecules and pathways. But they use them for very different purposes. The brain harnesses bioelectricity for the control of muscles, moving a body through 3-dimensional space. Developmental bioelectricity controls cell functions such as proliferation and differentiation,[84,85] moving the configuration of a body through morphospace, the abstract mathematical space in which an organism's shape is defined. As the chromatin code helps set cell identity and physiology, and physical forces are exploited by genetic circuits to control morphogenesis, bioelectricity enables the genome to utilize unique computational capabilities and long-range information integration.[86-90] Recently we posed the hypothesis that evolution exploits plasticity of non-neural (body-wide) bioelectric networks to process epigenetic information, as does the brain, but whose output is pattern regulation.[53,91] This hypothesis makes a number of specific predictions which have been borne out by recent results.

Switching species-specific head morphology in planaria

The planarian flatworm is an excellent model system for the study of the generation of form.[92,93] Planarians have extraordinary regenerative abilities, allowing them to reproduce any tissue or organ system given traumatic injury.[94] In addition, they are amenable to a variety of molecular biology techniques, and show a diverse array of head shapes across species.[95,96] Alteration of bioelectrical homeostasis in the planarian during regeneration has been shown to lead to dramatic patterning defects, including duplication of anterior polarity,[97] altered scaling of head structures,[98] and most recently, alteration of species-specific head shape.[99] These data implicate bioelectrical signaling in the maintenance of rational regenerative programs, and indicate an interesting modality with which large-scale morphology may be perturbed experimentally in the laboratory, or on evolutionary timescales.

When the electrical coupling of large populations of cells in the planarian species *G. dorotocephala* is transiently perturbed using the non-mutagenic long chain alcohol octanol, regenerating fragments grow heads that resemble entirely different species of planarian (Fig. 3A–D, Ai–Di). While the appropriate head morphology of *G. dorotocephala* is triangular with pointed auricles, octanol-treated fragments stochastically regenerate as several discrete shapes belonging to other species. Observed head shapes include those that are rounded, like *S. mediterranea*, triangular with no auricles like *D. japonica*, or flattened with anterior auricles like *P. felina*,[99] and require multiple coordinated shape changes that are not explained by simple scaling along 1 dimension (Supplement 1). Importantly, this head shape change was not only skin deep – it included coherent, multi-tissue anatomical change, not only editing of the external morphology. Immunostaining against synapsin and phosphorylated histone 3 (a marker of proliferation) revealed that morphology of the brain and the distribution of the adult stem cell population are also altered to resemble that of the corresponding other species (Fig. 3Aii–iv, Bii–iv, Cii–iv, Dii–iv). The significance of shape alteration was validated using geometric morphometrics, and the shape space defined by canonical variate analysis (CVA) allowed for characterization of morphological transformations (Fig. 3E).

These data indicate that some of the difference among planarian species could be due to the dynamics of signaling among their bioelectric networks, revealing a new epigenetic layer that could have important implications for evolution. Indeed, the frequency of appearance of the various planarian species' heads from random bioelectric network perturbation was proportional to the evolutionary distance among species, suggesting a relationship between morphological speciation and modification of bioelectrical dynamics. The evolutionary implications of bioelectrical controls of patterning have been discussed elsewhere,[53] and offer rich opportunities for future study of the bi-directional interplay between mutations of ion channel / gap junction genes and intrinsic (self-organizing) dynamics of bioelectric circuits.[100-103] One possibility is that the frameworks used to analyze evolutionary dynamics of memory and behavior such as Baldwin-like effects[46,104] (also mediated by a set of bioelectric circuits, those in the CNS) could be fruitfully applied to pattern regulation in embryogenesis and regeneration, and the variability of developmental outcomes from a specific genome.[105,106]

The voltage reporter dye DiBAC4(3) was used to show that bioelectric coupling among cells in worms with inappropriate head morphologies is altered long after octanol is washed out of the body of the worm, revealing long-term changes to the physiological network among cells (plasticity akin to the synaptic plasticity observed in electrical synapses in the CNS[72,99]). Efforts to build quantitative models of specific head shape outcomes stored in stable bioelectrically-mediated pattern memories in tissue are ongoing in our lab. Importantly, unlike the permanent morphological change (double headedness) induced in *D. japonica*,[53,107] in the time required for complete cellular turnover in the planarian (roughly

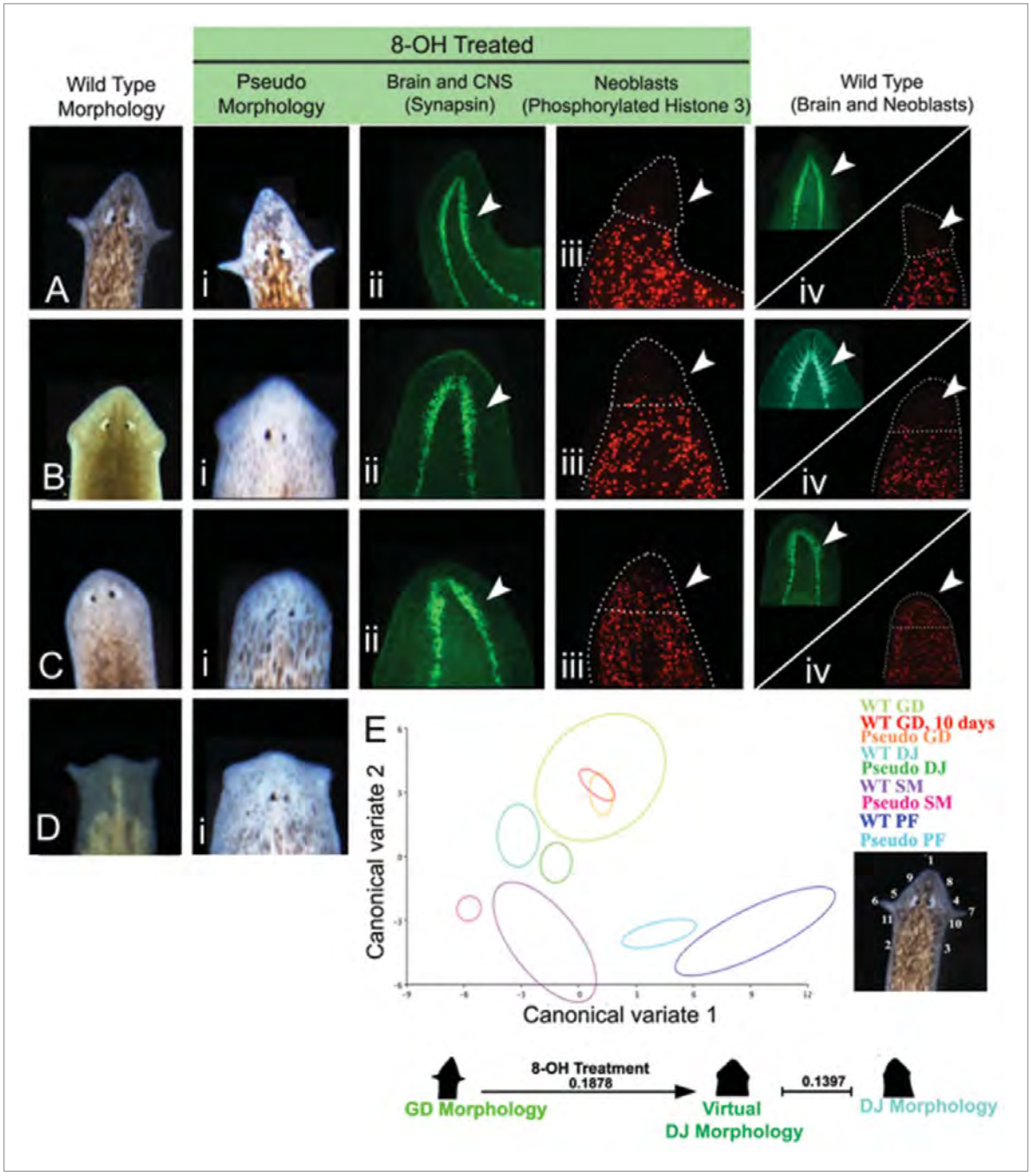

Figure 3. Changes to species-specific head shape in planaria. Physiological determination of species-specific head anatomy in the planarian flatworm. Transient exposure to octanol after amputation in *G. dorotocephala* results in regeneration of head anatomies resembling other species of planarian. Brain shape and distribution of neoblasts is also altered. Shape change can be quantified using geometric morphometrics, and used to produce a shape space accounting for much of the variation in shape between species. (A) Wild type *G. dorotocephala* morphology. (Ai) pseudo *G. dorotocephala* morphotype after octanol treatment. (Aii) brain morphology of pseudo *G. dorocephala* morphology by anti-synapsin immunostaining. (Aiii) neoblast distribution of pseudo *G. dorotocephala* morphology by anti-phosphorylated histone 3 immunostaining. (Aiv) wild type *G. dorotocephala* brain morphology and neoblast distribution. (B) Wild type *D. japonica* morphology. (Bi) pseudo *D. japonica* morphotype after octanol treatment. (Bii) brain morphology of pseudo *D. japonica* morphology by anti-synapsin immunostaining. (Biii) neoblast distribution of pseudo *D. japonica* morphology by anti-phosphorylated histone 3 immunostaining. (Biv) wild type *D. japonica* brain morphology and neoblast distribution. (C) Wild type *S. mediterranea* morphology. (Ci) pseudo *S. mediterranea* morphotype after octanol treatment. (Cii) brain morphology of pseudo *S. mediterranea* morphology by anti-synapsin immunostaining. (Ciii) neoblast distribution of pseudo *S. mediterranea* morphology by anti-phosphorylated histone 3 immunostaining. (Civ) wild type *S. mediterranea* brain morphology and neoblast distribution. (D) wild type *P. felina* morphology. (Di) pseudo *P. felina* morphotype after octanol treatment. (E) CVA analysis of wild type morphologies and pseudo morphologies, with morphometric landmarks shown on a wild type *G. dorotocephala* head. Figures used with permission from ref. 163.

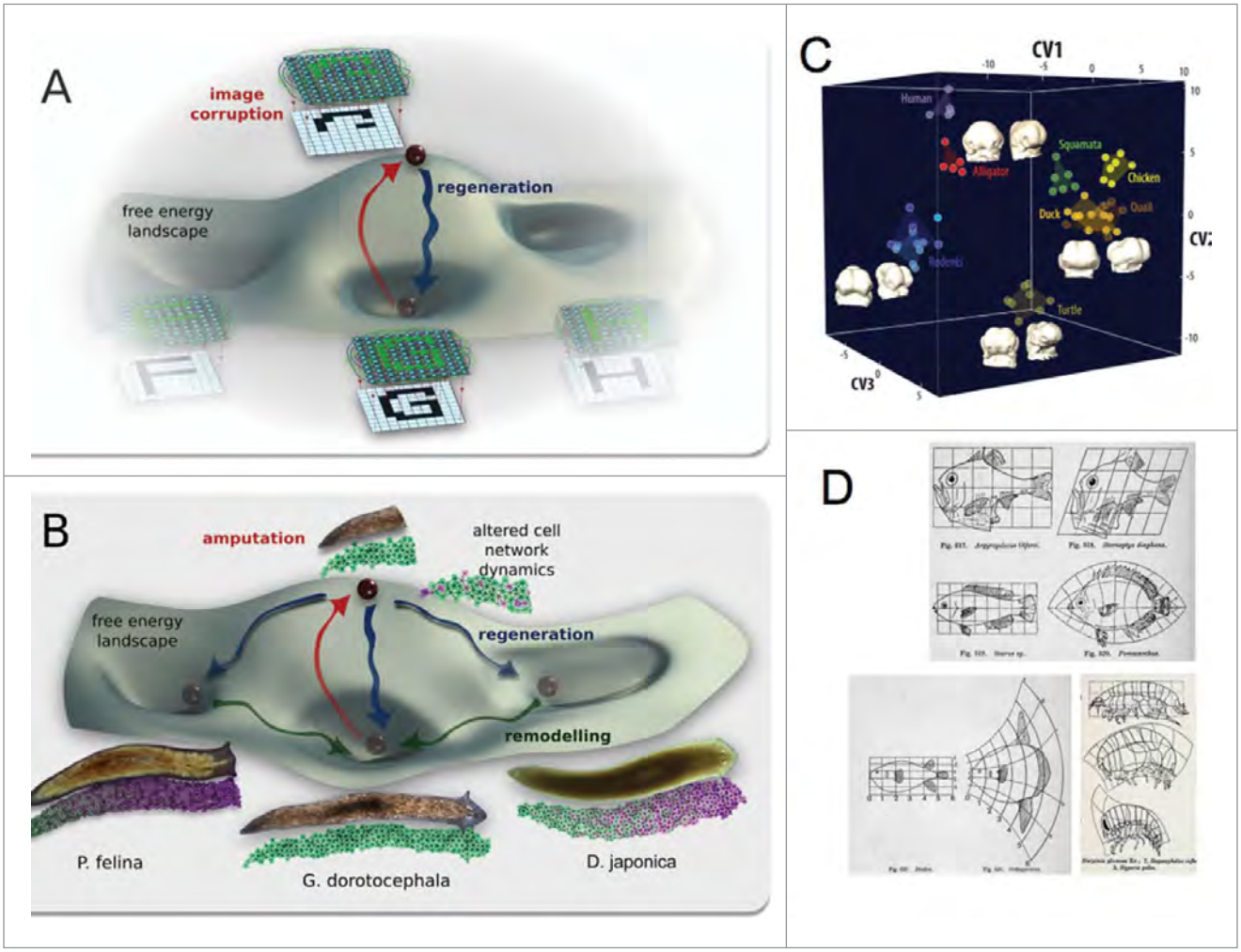

Figure 4. Morphogenetic state spaces. A conceptual model of shape change driven by physiological network dynamics. Planaria regeneration parallels classical neural network behavior; both can be described in terms of free energy landscapes with multiple attractor states. (A) Behavior of a classical Hopfield neural network trained to reproduce 3 types of patterns, in this case shapes of the letters 'F', 'H', or 'G', which are the 3 stable states of the network's free energy landscape. The state of the Hopfield network's nodes directly relate to the brightness of pixels on a display, generating output. Perturbation of the network from a stable state (red arrow) by deleting (damaging) part of the pattern is akin to moving a ball to an unstable point on the free energy landscape, and leads to regeneration of the closest learned attractor state (blue arrow). In this, such networks' well-known ability to implement memory is analogous to regenerating organisms restoring a specific target morphology upon damage. (B) The parallel concept of planaria regeneration into head shapes of one of 3 different species, which are attractor states of the free energy landscape. Outcome morphology is driven by the dynamic outputs of physiological cellular network. Amputation (red arrow) is akin to moving the system to an unstable point on the free energy landscape. Normal regeneration would return the system to its main attractor, but altering cell network dynamics via gap junction blockade allows for regenerative transitions (blue arrow) to alternative local minima, corresponding to morphospace regions normally occupied by *P. felina* and *S. mediterranea* worms. In time, remodeling (green arrow) transfers these morphologies to the global minimum of the wild-type state (*G. dorotocephala*). (C) Morphospaces are conceptual structures within which distances along specific metrics can represent the differences among species' morphologies. This panel illustrates how variants in the shape of a structure (skull shapes in this case) can be represented in a virtual space describing several orthogonal control parameters. We suggest that the physiologically-induced conversion of an animal with a normal genome into a different species-specific morphology could be modeled by an appropriate bioelectric circuit model whose measured states control relevant parameters forming the axes of a morphospace. Such spaces often include stable attractors corresponding to anatomical configurations that stable to small perturbations of the key parameters. (D) It is possible that the global coordinate axes that facilitate the Thompson transformations are mediated by bioelectric field properties across the organism. Images used with permission as follows: A,B,[163] C,[113] D.[115]

30 days), altered head shapes in *G. dorotocephala* are remodeled back to the species-appropriate morphology, along linear pathways in the shape space defined by CVA.[99]

The output of bioelectric circuits are spatially-extended instructive signals for cell behaviors such as proliferation, differentiation, and apoptosis.[54,108] Conceptually, we suggest[109] to represent the head shape morphologies as stable

attractors in the state space of the physiological network guiding cell behavior *in vivo* (Fig. 4A and B). This has the advantage that a significant body of work exists in the computational neuroscience and artificial neural network fields, explaining the dynamics of pattern memories in such networks.[110-112] Each head shape corresponds to a distinct low-energy state of the bioelectric circuit. Amputation temporarily raises the "energy" of the system, which normally relaxes into the standard species-specific shape attractor. However, perturbing the connectivity in the circuit can cause it to instead settle into a different attractor (Fig. 4B). We are currently working on deriving quantitative models of this process that will explain why the above-mentioned shapes are transient (belong to only local minima) while other states (the default head shape, and the 2-head morphology described below) represent permanent, or very deep, minima.

The conversion among different species-specific shapes is formalized in the notion of morphospace[18,113,114] (Fig. 4C), where specific outcomes of some system (the key canonical variates) can explain a range of observed morphologies. A very early line of thought in this direction was made by D'Arcy Thompson,[115,116] who showed that the bodyplans of distinct species can be converted to those of other species via simple geometric transformations (Fig. 4D). Biochemical gradients have been suggested for the mechanism implementing such spatial axes,[117] and it is also tempting to speculate that these coordinates can also be provided by bioelectrical aspects of large-scale physiological networks.[118,119] Specification and empirical validation of a

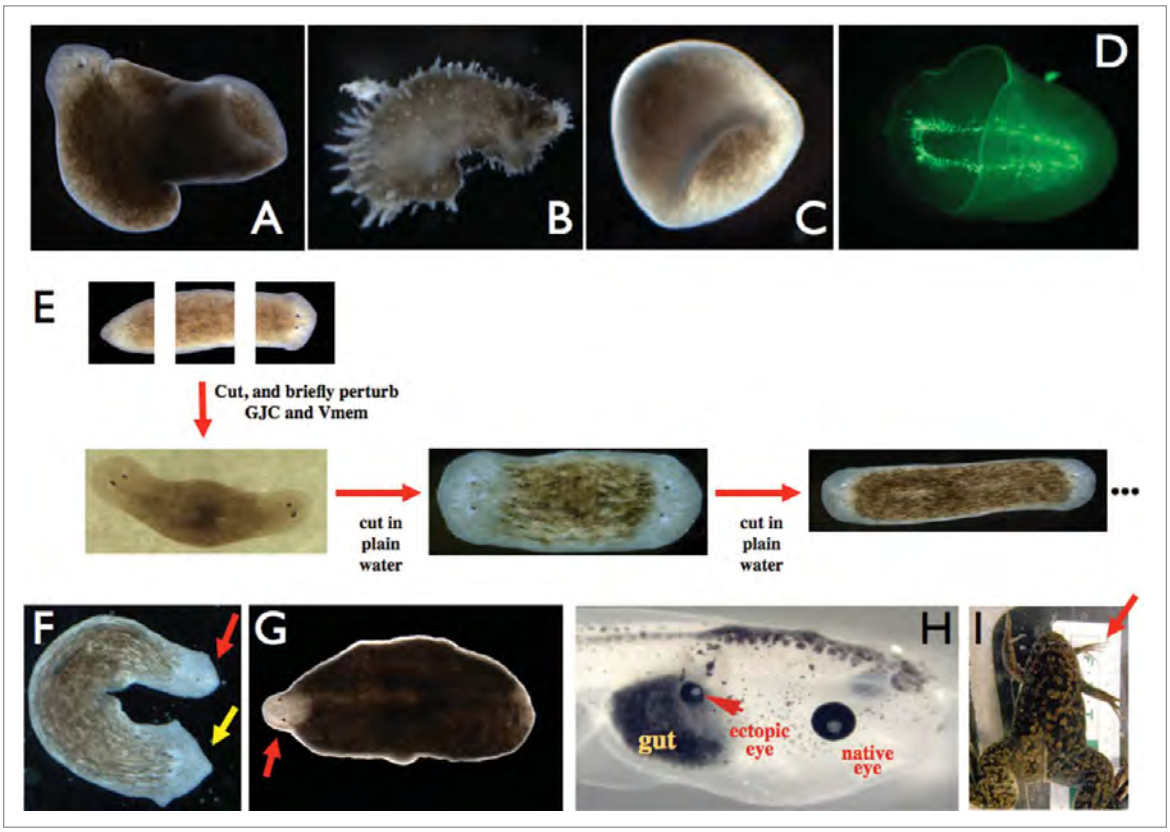

Figure 5. Unexplored regions of morphospace reached by editing bioelectric circuits. Briefly altering bioelectric circuit dynamics, despite the presence of a normal genome, can result in drastic alterations of the bodyplan, to regions not currently occupied by extant species. *D. japonica* worms treated with disruptors of gap junctional connectivity or modulators of ion channel-depenedent bioelectric signaling can acquire compound (A), spiky (B), or cup-shaped (C) morphologies instead of the genome-default flat architecture of the planarian. The specimen in panel D has been stained with an antibody to reveal the central nervous system, and cleared to highlight the pocket-like morphology. In some cases, these edits to the normal target morphology can be permanent (E): a flatworm middle fragment that regenerates 2 heads after a brief reduction of bioelectrical coupling among its cells will continue to regenerate as 2-headed in subsequent cuts made in plain water. Even if the "reprogrammed" head tissue is removed, the target morphology information in other fragments of the body have been physiologically altered so that a 2-headed form results. This new worm architecture has distinct behavioral and anatomical structures and is stable across the most common mode of reproduction in this species (fission + regeneration), stretching the definition of speciation. (F) Changes of head shape (red arrowhead) and number (yellow arrow) can occur in the same animal, as can changes in head size (G, red arrow). Editing of large-scale bodyplan can be induced in vertebrate models as well, inducing ectopic eye growth out of gut tissue (red arrow), and ectopic limb growth out of the mouth (I, red arrow). Images used with permission as follows: E,[53] G,[124] H.[67] Photo in panel I courtesy of Erin Switzer.

quantitative model of a bioelectric circuit and the morphological consequences of its stable modes represents one of the most exciting prospects in this field. A full understanding must also involve explanation of the stochastic nature of this process, perhaps couched in the language of dynamical systems theory,[120-123] to understand the origin of variability. The study of deterministic chaos has long helped analyze many systems where very small initial differences (e.g., physiological noise) can be amplified by feedback loops into distinct large-scale outcomes in a stochastic manner. Such feedback loops are certainly present in bioelectric regulation of organ-level patterning, such as between V_{mem} and the gene *Rx1* in eye induction[67] or V_{mem} and the gene *Notch* in brain patterning.[68]

Interestingly, while perturbing the topology of the physiological network results in head shapes of several extant species (Fig. 3), the same techniques can result in animals that correspond to regions of the morphospace[18,113,114] not exploited by evolution (at least in currently-known variants). For example, planaria can be deviated from their normally flat body architecture to give rise to geometrically very distinct shapes, such as pockets (Fig. 5A–D). Another example is revealed in *D. japonica*, where a 2-headed form can be produced which is stable – further cycles of amputation (in plain water) of the ectopic head result in 2-headed worms from normal gut fragments, revealing a permanent change of the encoded target morphology.[107] What is to be made of the fact that GJ inhibition induces a change in head number in *D. japonica* (Fig. 5E) but a change in head shape in *G. dorotocephala* (Fig. 3)? One possibility is that different species of planaria (which in this case differ by an estimated 100 million years) utilize the outputs of their bioelectric networks for different purposes (determination of head number in *D. japonica*, and of head shape in *G. doro-*

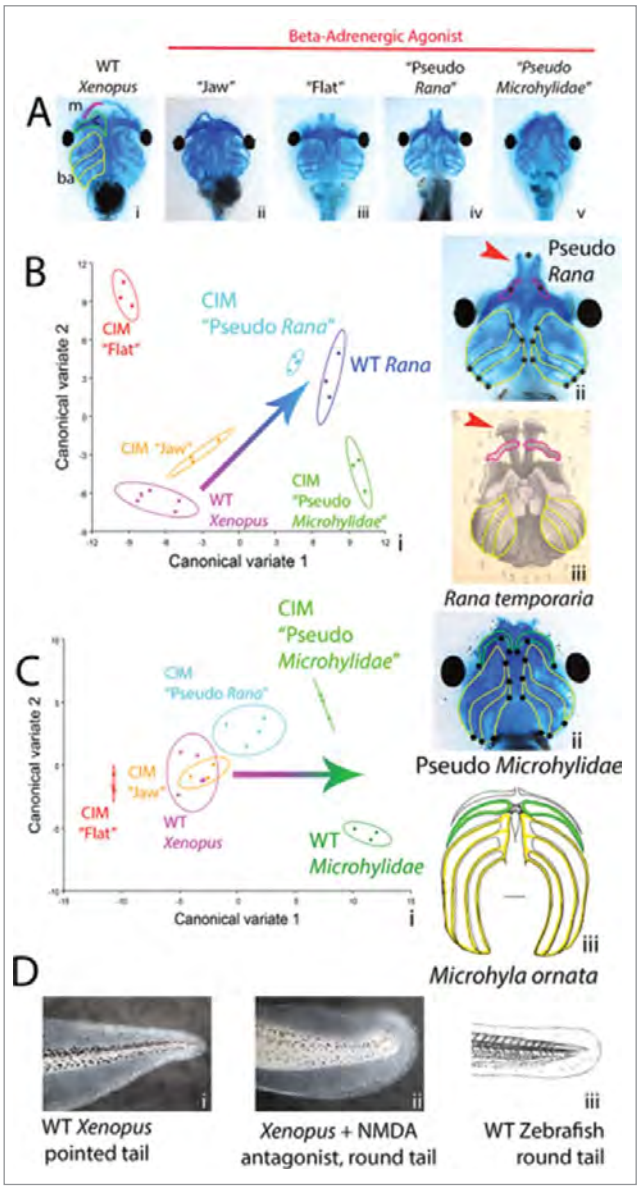

Figure 6. Altering neurotransmitter signaling induces inter-species shape changes in Xenopus tadpoles. (A) Treatment with the β-adrenergic agonist Cimaterol (CIM) stochastically induced 4 distinct head anatomies. Embryos were exposed to CIM from st. 10-45, sacrificed at st. 47 and stained with Alcian blue. (i) Wild-type *Xenopus* tadpole with major ventral craniofacial cartilages labeled on one side of the face. Meckel's cartilage (m) is outlined in Pink, ceratohyal cartilage (c) is outlined in green, and the branchial arches (ba) are outlined with yellow. (ii) Tadpole with malformed jaw, but otherwise normal craniofacial morphology. (iii) Tadpole with horizontal or "flat" branchial arches and ceratohyal cartiages. (iv) Tadpole whose head anatomy visually resembles that of *Rana* frogs. (v) Tadpole whose head anatomy resembles that of frogs belonging to the family *Microhylidae*. (B) Comparison of Pseudo *Rana* head morphology to that of wild-type Rana species. (i) Graphical output for Canonical Variate analysis of shape data in wild-type and experimentally-derived craniofacial morphologies, showing confidence ellipses for means at an 0.9 probability. Each point represents one individual's face shape data. Data for wild-type *Rana* frogs were derived from anatomical diagrams of *Rana temporaria*,[130] *Rana dalmatina*,[132] and *Rana palustris*.[131] (ii) ventral view of alcian-blue stained Pseudo rana tadpole with landmarks (black dots) used for morphometric analysis. (iii) Anatomical diagram of ventral craniofacial skeleton of Rana temporaria tadpole. For both ii and iii, protruding nasal cartilages are indicated with red arrows, Meckel's cartilages are outlined in pink, and branchial arches are outlined in yellow. (C) Comparison of Pseudo *Microhylidae* head morphology to that of wild-type *Microhylidae* species. (i) Graphical output for Canonical Variate analysis of shape data in wild-type and experimentally-derived craniofacial morphologies, showing confidence ellipses for means at an 0.9 probability. Each point represents one individual's face shape data. Data for wild-type *Microhylidae* frogs were derived from anatomical diagrams of *Microhyla ornata*,[133] *Gastrophryne carolinensis*,[134] and *Dermatonotus muelleri*.[135] (ii) ventral view of alcian-blue stained Pseudo *Microhylidae* tadpole with landmarks (black dots) used for morphometric analysis. (iii) Anatomical diagram of ventral craniofacial skeleton of a *Microhyla ornata* tadpole. For both ii and iii, ceratohyal cartilages are outlined in green, and branchial arches are outlined in yellow.

tocephala). However, this is unlikely because worms (Fig. 5F) can be created which have both an abnormal number of heads and a different species' head shape. In *S. mediterranea*, head size (Fig. 5G) is regulated by distribution of voltage gradients,[124] echoing the bioelectric control of brain size in *Xenopus*[68] and tail size in zebrafish.[70] In *Xenopus*, similar modulation of resting potential (intrinsic plasticity, as distinct from the synaptic plasticity edited by gap junctional modulators), can produce aberrant forms possessing ectopic eyes[67] (Fig. 5H) or limbs (Fig. 5I). One possible implication of these data is that, once predictive, quantitative, biophysical models that link bioelectric circuit modes to anatomical outcomes are developed, it would be possible to approach cell groups as "universal constructors", inducing pattern formation that has never before appeared in biological evolution, for purposes of creating synthetic organisms for specific functions.[2,125-127]

Frog embryogenesis and neurotransmitter controls

Continuing the theme of plasticity in pattern regulation, it was recently shown that frog development does not merely utilize a hardwired set of deformations that convert a tadpole face into the very different frog face. Rather, it includes a robust and plastic system that can make up for deformed tadpole anatomies (which begin the remodeling process in an abnormal starting state) and correct them to make largely normal froglets.[128] The best-known system that is able to reliably reach specific goal states from diverse starting conditions is the brain, where goal-satisfaction circuits make use of bioelectrical networks (composed of neural cells and non-spiking astrocytes) and the flow of neurotransmitter signals to implement flexible decision-making. We recently began to examine the roles of these neurotransmitters during frog development. In a pharmacological survey screen, we found that treatment of embryos prior to the mature development of the nervous system with drugs modulating catecholamine and glutamatergic signaling induced a wide variety of alterations in craniofacial, eye, muscle, visceral organ, and pigment patterns.[129]

Interestingly, we identified several neurotransmitter endpoints that when manipulated pharmacologically, caused the morphology of the head and tail to resemble those of other species. The β-adrenergic agonist Cimaterol altered the shape and placement of the craniofacial cartilages, stochastically inducing 4 distinct head anatomies (Fig. 6A). One such anatomy (which we label as "Pseudo-*Rana*" below) resembled the larval skull of frogs belonging to the genus *Rana* due to proboscis-like nasal cartilages, inset Meckel's cartilages, and compressed branchial arches (Fig. 6B). Another (which we term "Pseudo-*Microhylidae*") resembled the head anatomy seen in tadpoles belonging to the Microhylid family of frogs due to the dramatic arching of its ceratohyal cartilages and the steep downward slant of its branchial arches (Fig. 6C).

Geometric morphometrics was used to compare experimentally-derived head morphologies to wild-type *Xenopus*, as well as compare "pseudo" head morphologies to the craniofacial anatomy of frogs belonging to the genus or family that they resemble. Principal Component analysis and Canonical Variate analysis (CVA) were run on landmark data taken from alcian-blue images of control and experimental *Xenopus* tadpoles as well as anatomical diagrams of different frog species (Fig. 6Bi, 6Ci). The Rana species analyzed included *Rana temporaria*,[130] *Rana palustris*,[131] and *Rana dalmatina*.[132] In the Microhylid family of frogs, we analyzed *Microhyla ornata*,[133] *Gastrophryne carolinensis*,[134] and *Dermatonotus Muelleri*.[135]

In our CVA using nasal, branchial arch, and Meckel's cartilage landmarks, the Pseudo-*Rana* clustered dramatically closer in morphospace to true rana than wild-type *Xenopus* on both the CV1 and CV2 axes (Fig. 6B). Movement along the CV1 axis represents anterior movement of the inner branchial arch landmarks and posterior movement of the outer branchial arch landmarks. Movement along the CV2 axis represents movement of the Meckel's cartilages toward the midline, movement of the inner branchial arch landmarks away from the midline, and anterior projection of the nasal cartilage. We can therefore confirm that out Pseudo-*Rana* tadpoles were indeed more anatomically similar to true *Rana* tadpoles than to wild-type *Xenopus* due to the visual features we observed. In our CVA using branchial arch and ceratohyal landmarks, we found that our Pseudo-*Microhylidae* clustered dramatically closer to true Microhylids than to wild-type *Xenopus* along CV1 (Fig. 6C). On CV2, pseudo and true *Microhylidae* diverged equidistantly from wild-type *Xenopus*. In this analysis, movement along CV2 represents compression of the ceratohyal cartilages toward the midline and movement of the outer branchial arch landmarks away from the midline. Movement along CV1, however, represents anterior movement and bunching of the inner branchial arch landmarks, posterior movement and bunching of the outer branchial arch landmarks, and posterior movement of the outer-most ceratohyal cartilage landmarks. Although pseudo and true *Microhylidae* diverge on CV2, their locations along CV1 account for the easily visible traits that make them appear *Microhylidae*-like and different from wild-type, such as notably arched ceratohyal cartilages and steep downward-sloping branchial arches. Therefore, we can still confirm that Pseudo *Microhylidae* are anatomically closer to true Microhylids than they are to untreated *Xenopus*.

In addition to the craniofacial alterations caused by Cimaterol, we found another interesting inter-species phenotype during our drug screen. Treatment with the noncompetitive NMDA antagonist Norketamine induced the development of a tail that was morphologically similar to that of a zebrafish (Fig. 6D). As in zebrafish, the portion of the tail containing spinal cord and muscle was truncated and did not continue to the posterior most edge of the tailfin as it does in wild-type *Xenopus*. In addition, the tailfin was rounded as opposed to the normal pointed shape of a *Xenopus* tail. These data implicate pre-nervous (and non-neural) neurotransmitter signaling as a key functional player in diverse aspects of vertebrate embryonic development. Echoing the above-mentioned work, neurotransmitters have recently been found to modulate patterning in planaria,[136] the left-right axis in frog and chick,[56,57] cleavage and gastrulation in the sea urchin,[137,138] and craniofacial development in frogs and rodents.[139,140,141,142]

More broadly however, these data are consistent with the role of neurotransmitters as chemical cues that participate in an active and ongoing interplay with the bioelectric signals in the brain. Neurotransmitters are traditionally known to act as downstream effectors of bioelectricity, yet they also have the power to alter electrical communication between cells by coupling to signaling cascades capable of altering the connectivity of gap junctions. For example, glutamate (via the NMDA receptor[143,144]) and serotonin (which stimulates IP3 mediated calcium release[145]) raise intracellular calcium concentration, triggering CamKII-mediated phosphorylation of gap junction proteins and altering the permeability of electrical synapses. Other neurotransmitters couple to signaling cascades involving cyclic nucleotides. For example, both nitric oxide (NO) and histamine modulate gap junction coupling via cGMP signaling.[146,147] Similarly, dopamine and noradrenaline make gap junctions less permeable by triggering cAMP-dependent PKA phosphorylation of connexins.[148,149] The bi-directional relationship between neurotransmitters and GJs enable yet another set of feedback loops. Synthesizing the frog and planaria data described above and elsewhere,[89,108,150] it appears that species-specific shapes can be set, and reset, by modulating physiological network topology, specific bioelectric states, and the interconnected neurotransmitter pathways that often mediate between electrical activity and cell functions.[56,59,138,151,152]

Conclusion

Recent work has shown that species-specific shape is derived not only from regulatory gene loci and chromatin marking. Large-scale anatomical structure is also a function of physiological signaling among many cell types, and significant (coordinated) changes in morphology can be induced by altering the topology or function of physiological circuits. In some cases, these dynamics can be used to select among morphologies of existing species, although entirely novel forms can also result. Interestingly, manipulation of physiological signals results in a much wider overall range of patterning outcomes (Fig. 5) than has been seen in genetic screens targeting individual gene products.[153-156] This new set of inputs into anatomical structure has several important implications for evolution.

First, these data highlight the importance of truly epigenetic layers of influence over organism anatomy. While ion channel and gap junction proteins are specified in the genome, bioelectric circuits have their own unique and complex dynamics that derive from the fact that ion channels and GJs determine cell voltage but are also themselves regulated by voltage gradients. These feedback loops and the resulting electric circuit state transitions over time are not predictable from the rules governing genetic sequence, transcriptional networks, or chromatin state[53] because channels and GJs open and close post-translationally, implementing functional signaling that is invisible to profiling at transcriptional or translational levels. Divergence between genetic state and bioelectric state can be readily seen, as 2 cells with precisely the same complement of ion channels (i.e., identical on any molecular-genetic profile) can be in very different physiological states depending on whether their channels are open or closed. The functional signaling properties of physiological networks are determined by the electrical activity, not the mere presence or absence of specific molecules. This implies a departure from the standard molecular biological paradigm, where cell state is thought to be derivable by proteomic and transcriptomic profiling. Bioelectric information can only be read out in the living state (not in fixed, biochemically-analyzed tissue). This is analogous to the content of computer memory, which resides in the flow of energy through the circuit (and is not captured by a full account of the atoms making up the transistor hardware), and to the semantic content of a mature brain, which is neither visible from nor directly determined by the genome. These facts have clear implications for future efforts to fully profile and re-write the signaling that ultimately determines anatomical structure and its modification.

Second, bioelectric networks facilitate robustness of physiological states and the patterning they regulate (via negative feedback loops and long-range state sensing). Morphogenetic functions guided by bioelectric circuits are also robust to mutation in channels and their

transcriptional regulation. Multiple ion channels often contribute to the same V_{mem} state, with extensive compensation by other channel types (which is one factor confounding discovery of these circuits by single gene mutation screens). Moreover, bioelectric circuit modes show significant stability to some perturbations.[103] Thus, individual channel involvement can diverge evolutionarily, as long as the overall signaling dynamic remains.

Third, bioelectric states as control points tend to be powerful "master regulators", allowing the initiation of self-limiting patterning modules as subroutines from a low information content input (trigger);[66,157] this is due to the existence of positive feedback loops, which sustain and amplify bioelectric states once a threshold has been surpassed by a transient bioelectric stimulus (exploited also by action potentials in brain circuits). Such modularity is well known to improve evolvability.[158-160] All of these factors are likely to be exploited by evolution in its exploration of shape spaces. Fourth, these data suggest a complementary view to the usual perspective in which the DNA is the software implemented by a cell's hardware. An alternative view is that the DNA specifies the hardware (the complement of channels, neurotransmitters, and GJ proteins), while the resulting bioelectric activity of these circuits (with spontaneous symmetry-breaking, self-organization, and other complex dynamics) is the software. As in computer science, and as observed in the appearance of distinct animal shapes from the same genome (Figs. 3 and 5), many different types of software can be implemented on the same genetically-specified hardware.

The study of ways in which evolution exploited the computational properties of electric cell networks outside of the central nervous system is an exciting, emerging area of developmental biology. One corollary to this is the possible role of commensal species in determining growth and form, not only physiology. For example, bacteria are known to make compounds that modulate gap junctions and ion channels;[161] thus, it is possible that factors produced by other species living within a host organism's body can serve as an additional input to the organism's pattern by editing or altering its endogenous bioelectrical circuits This adds a layer of complexity to the typical "evo-devo" story, but also provides new, tractable, potent control points to be exploited by biomedicine or synthetic bioengineering through the use of novel vectors for bioelectric modulation of host tissue.[162]

The list of the molecular signals that propagate through physiological networks is likely to grow rapidly in the following years. However, several players (current, calcium, neurotransmitters) are already implicated. The molecular and algorithmic analogies between how somatic tissues and the brain utilize these same components are a fertile area for novel inquiry, and remain to be tested in specific contexts. It is likely that a mature understanding of the unique properties of bioelectrical signaling, and their integration into the molecular toolkits of developmental biology and synthetic bioengineering, will have transformative effects in a number of areas. In addition to a fuller appreciation of the interplay between genomes and physical forces[20] in evolution and development, the increased control over growth and form is sure to have numerous practical applications in biomedicine and beyond.

Glossary of unusual terms

Ectopic – in the case of an organ, located outside the area of the body where it develops during normal embryogenesis.

Target Morphology – the anatomical configuration toward which normal embryogenesis and regeneration coordinate cell behavior; the pattern which, once reached, makes further growth and remodeling stop. Living organisms seek to maintain target morphology against cell turnover, aging, cancer, and injury as a kind of pattern homeostasis.

Plasticity – flexibility that allows past events to alter future behavior of a system. Physiological plasticity enables bioelectric circuits to retain state memory or respond adaptively to future physiological inputs. Anatomical plasticity occurs when animal bodies remodel or regenerate despite diverse types of damage, reaching the same target morphology state from different starting configurations. The ability to respond to unpredictable stimuli with coherent repair programs is contrasted with hardwired responses.

Gap Junctions – Protein channels that enable cell-cell communication by directly connecting the cytoplasm of 2 adjacent cells. This allows ions and small molecules to flow from one cell to another as dictated by electrochemical gradients between the cells. Some gap junction proteins confer gating by electric and chemical signals, and thus both determine and are themselves regulated by bioelectric state. These electrical synapses are thus versatile elements that underlie physiological plasticity in neural and somatic circuits.

Axial polarity – consistent differences along a major body axis (dorsal-ventral, anterior-posterior, or left-right) of the body. Anatomical and histological polarity are preceded by, and established by, bioelectric and chemical gradients which control large-scale pattern.

Neoplastic – a state in which cells begin to ignore the normally tight patterning cues of the body and begin to change toward a "unicellular" phenotype. This developmental disorder is part of the process of carcinogenesis, where cells change shape, overproliferate, and eventually become highly motile and invade distant sites.

Tensegrity – organization of structure consisting of discrete un-stretchable components situated in a continuous web of tension that defines the overall shape of the structure. Tensegrity is one example of essentially physical (non-genetic) regulators of morphogenesis.

Morphospace – an abstract mathematical space in which shape is defined by independent shape metrics along each orthogonal axis. A point in the space represents one possible shape configuration for a biological structure, which may or may not describe an extant animal species. Movement along an axis of the space represents a particular geometric transformation.

Baldwin effect – a mechanism by which learned behavior can affect evolution. Systems which can learn from experience can thereby acquire adaptive states which facilitate evolution to novel fitness peaks. For example, non-genomic (physiological) changes to bioelectric circuits can result in new animal morphologies, which could eventually be canalized into genomic changes by selection and mutation (much as new phenotypes can eventually evolve when animals learn specific behaviors and interact in novel ways with their niche).

Dynamical Systems Theory – a set of mathematical approaches for learning to understand and rationally manipulate the behavior of complex systems. Often such systems feature recursive (feedback) components, resulting in surprising and rich behaviors.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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