MULTILEVEL ORGANIZATION AND FUNCTIONAL INTEGRATION IN ORGANISMS

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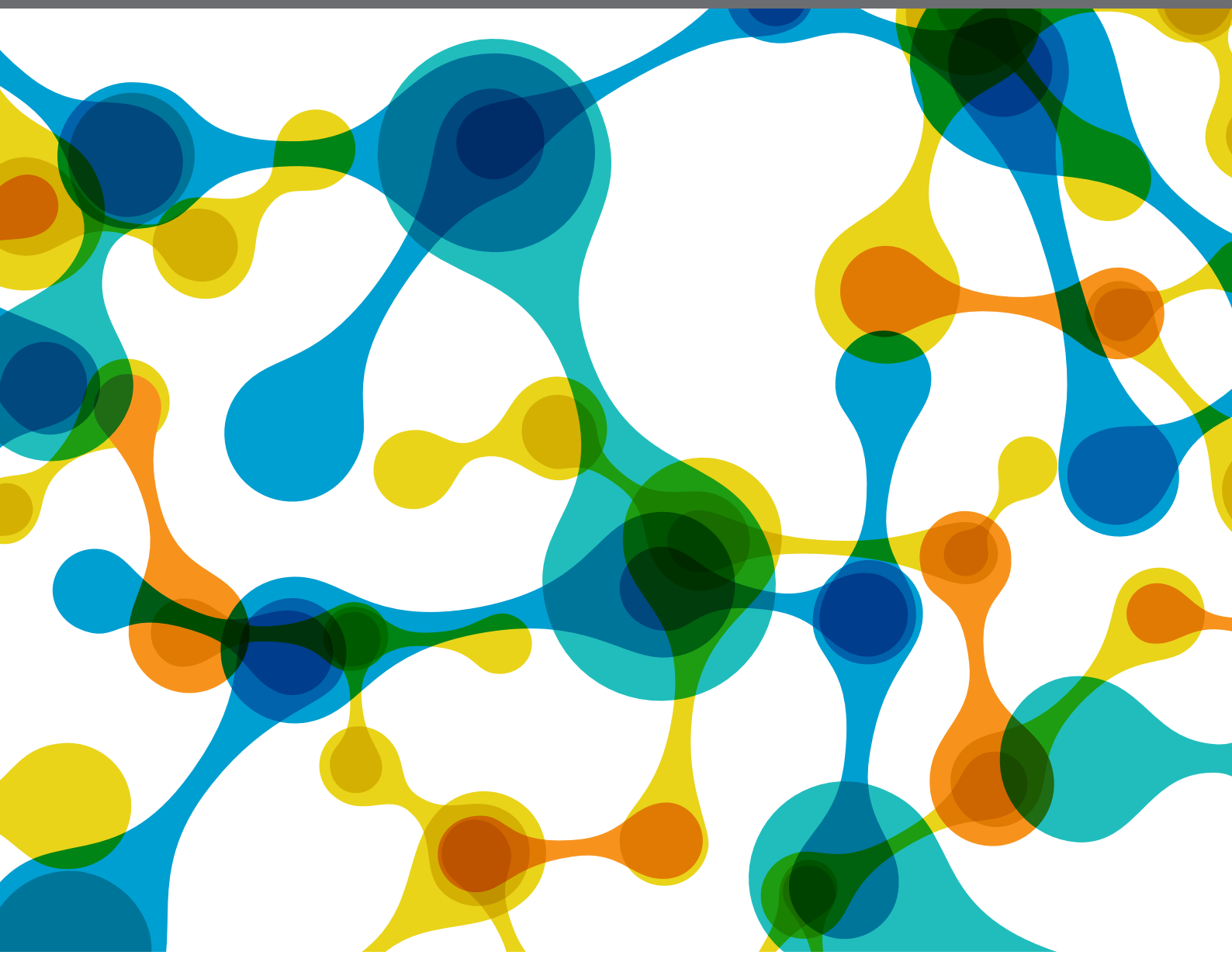

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MULTILEVEL ORGANIZATION AND FUNCTIONAL INTEGRATION IN ORGANISMS

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EDITORIAL
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Editorial: Multilevel Organization and Functional Integration in Organisms

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Editorial on the Research Topic

Multilevel Organization and Functional Integration in Organisms

Organisms are self-maintained and self-regulated entities, hierarchically organized at different levels of complexity. The investigation and understanding of multilevel organization and functional integration in organisms require not only experimental work but also biophysical and computational models. Moreover, while additional knowledge is being continuously obtained, many theoretical and philosophical issues are still open. This Research Topic proposes a multidisciplinary approach to these issues, with contributions from biologists, physicists, and philosophers of biology. They cover a broad range of topics, such as the origin of life, the emergence of animal multicellularity and its organizational integration, the existence of physiological regulations, as well as the more general question of causality in biology.

In the origins of life, biogenesis is the progressive constitution of spatial and temporal systems beyond individual proto-metabolic organizations, which requires a minimal form of reproduction. Moreno proposes a conceptual analysis of how prebiotic evolution can be viewed as the emergence of autocatalytic reaction loops between molecules leading to compartmentalized self-maintained and self-reproducing networks. This corresponds to a new form of circular causality, which integrates individual organisms and ecosystems at different spatial and temporal scales. In this view, the notion of biological type is not purely abstract but corresponds to the self-maintenance of an organization through space and time including intergenerational continuity and similarity by iteration of reproducing cycles.

The emergence of animal multicellularity from unicellular organisms is an important issue in evolutionary biology. According to Newman, the origin of the structural motifs of animal morphology such as multilayered, hollow, elongated structures, etc., is the consequence of fundamental cellular functions (contraction, excitability…) that were present in ancestral unicellular organisms, combined with the generic physical forces acting in mesoscale aggregate of cells (10^{-3}–10^{-2}m). Combination of mesoscale physics and genetic toolkits such as morphogens that mediate cell association and behavior generate reproducible dynamical patterning modules responsible for the organizational properties of animals. The emergence of Metazoan morphology can hence be understood as the consequence of physico-genetic effects specific to the multicellular context. Animals, in most of the cases large free-moving entities, are characterized by a specific kind of multicellularity. Arnellos and Keijzer propose a theoretical approach of bodily complexity in animals grounded on transitions from cilia-based to contraction/muscle-based motility, in which muscle-based and myoepithelial-based tissue contraction plays a critical role. The subsequent paper addresses the fact that multicellularity results not only from the spatial organization of cells but also from the organization of the intercellular space itself. Developing the concept of spatial

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control, Bich, Pradeu et al. analyze how the extracellular space organization, particularly the extracellular matrix (ECM), controls cell differentiation, and the emergence of some specialized functions like immunity. The authors also explain how the notion of spatial control is useful for understanding aging and cancer. The importance of the interactions between the ECM and different cell types in cancer development is evidenced by Pally et al. Combining 3D experimental and computational approaches, the authors show how the interplay between the ECM, the malignant epithelial cells, and the unaffected stroma cells is responsible for the remodeling processes that determine the progression of breast carcinomatosis.

Organisms are characterized by the existence of multiple regulatory processes that are critical for their self-maintenance. One of the most well-known and studied phenomena is the endocrine regulation of glycemia by the pancreatic hormones. In beta-cells of the pancreatic islets, insulin release is triggered by the calcium signal, whose critical determinants include the spatial and temporal interactions among the cells. To grasp the complexity of the calcium signals recorded on isolated pancreatic islets, Korošak and Rupnik have used the Random Matrix Theory (RMT), a fitting mathematical framework, to separate cell-cell interactions happening by chance from those occurring by specific interactions. Considering the same biological system, StoŽer et al. have developed a computational model of interconnected beta cells predicting the calcium signaling in pancreatic islets. The model highlights the importance of the cellular network's heterogeneity and the time lag in the cell-cell interactions generating an integrated calcium signal. Insulin release by pancreatic islets depends on the self-organized critical dynamics of the beta cell networks, which provides a well-controlled glycemia. In their conceptual analysis, Bich, Mossio et al. argue for the abandon of the classical view and language of glycemia regulation in terms of feedback loops centered on set-point and error monitoring. Instead, they propose a theoretical framework according to which glycemia regulation is the consequence of the mutual dependence among a set of functional structures acting as constraints, the so-called organizational closure. Another example of physiological regulation is the sensing by the endothelial cells of the shear stress exerted by the blood flow on the walls of the vessels (WSS). Roux et al. argue that cellular tensional pre-stress, or biotensegrity, is a relevant conceptual framework to understand how endothelial cells are sensitive to the spatial and temporal characteristics of the WSS. By its consequence on blood flow via vascular morphogenesis and remodeling and vasoreactivity, WSS sensing generates a local-global causal loop that determines the ability of the vascular system to ensure the perfusion of the tissues through the maintenance of stable local WSS value. The authors show that the classical set point theory is unable to account for these regulatory processes that should be viewed as dynamical, and not algorithmic, ones acting in a self-organized way.

Ellis and Kopel argue for a fundamental difference in causation between physical vs. biological processes. Though grounded on physical processes, biological systems share some specific properties such as goal-directedness, organization, and information flow. These properties are linked through a specific kind of branching causation. In quantum physics, branching causation is related with the irreducible randomness of quantum outcomes, whereas in biology it corresponds to a branching logic operating at each hierarchical level of biological organization. Using the example of ion channels, the authors argue that a digital logic of ON and OFF processes at the biomolecular level underlies the emergence of macroscale branching dynamics. Noble et al. develop and specify the concept of biological relativity, according to which there is no privileged level of causality in biology. The authors argue that biological systems are characterized by a multiscale network of interactions, in which any part of the network may affect every other part. The authors defend the concept of conditioned causation, i.e., a state of a system where it would be misleading to attribute causation to any particular element. In biological systems, causal loops are at play between different levels of organization, even though they are asymmetric. Upward causation is the fact that lower interacting elements produce change at higher levels, whereas downward causation, or downward determination, is the sets of initial and boundary conditions imposed by higher levels of organization. According to Bizzarri et al., the fact that organisms are complex systems requires a specific conceptual framework for the investigation and explanation of biological systems. In biology, phenomena are processes rather than material objects, meaning that biological entities have relational properties only. The authors propose a "mesoscopic way of thinking" to understand the emergence of relational organizations, their self-maintenance, and, as in pathology, their possible collapse. At the mesoscopic levels, the stochastic fluctuations that characterize the microscopic level turn into ordered behavior and the emergence of regularities in the reciprocal interactions of the parts.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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The Dynamical Emergence of Biology From Physics: Branching Causation via Biomolecules

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Biology differs fundamentally from the physics that underlies it. This paper[1] proposes that the essential difference is that while physics at its fundamental level is Hamiltonian, in biology, once life has come into existence, causation of a contextual branching nature occurs at every level of the hierarchy of emergence at each time. The key feature allowing this to happen is the way biomolecules such as voltage-gated ion channels can act to enable branching logic to arise from the underlying physics, despite that physics *per se* being of a deterministic nature. Much randomness occurs at the molecular level, which enables higher level functions to select lower level outcomes according to higher level needs. Intelligent causation occurs when organisms engage in deduction, enabling prediction and planning. This is possible because ion channels enable action potentials to propagate in axons. The further key feature is that such branching biological behavior acts down to cause the underlying physical interactions to also exhibit a contextual branching behavior.

Keywords: hierarchy of emergence, bio-molecules, top-down causation, branching logic, natural selection, voltage-gated ion channels

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1. BIOLOGY VS. PHYSICS

Biology arises out of the underlying physics, but living systems have an essentially different nature than natural systems because *inter alia* they involve purpose or function (Hartwell et al., 1999), information (Nurse, 2008), organization (Mossio et al., 2016), and variation (Montévil et al., 2016). How do they arise from the underlying physics, which has none of these characteristics? Physics and biology are essentially different, even though physics underlies biology. We will identify the physics-biology difference, once life has come into existence, as being due to the fact that biological causation is based at the cellular level in logical branching shaped by context, enabled in physical terms by the nature of particular proteins. Because this branching is controlled in a top down way by physiological conditions (Noble, 2008, 2012, 2016) this leads to contextual emergence (Atmanspacher and beim Graben, 2009), which is a form of strong emergence, enabling branching behavior to also emerge at the higher levels.

[1] An abbreviated version of the proposal made here appears in a book chapter Ellis and Kopel (2018). This version is extensively revised and extended to consider further areas in integrative physiology.

1.1. The Nature of Physics

Physics deals with laws expressing the inevitable interactions of matter and fields according to boundary and initial conditions, and their consequences for emergent physical systems such as gases, liquids, crystals, rocks, planets, stars, and galaxies.

Classical physics proceeds in a deterministic fashion, described by Hamiltonian dynamics (section 2.1). The interactions proceed in a remorseless impersonal way as described by these laws, with no hint of function or purpose. They can exhibit branching behavior in phase changes, as discussed in section 2.2 below, but there is again no trace of purpose or choice in that behavior. Quantum physics has a branching behavior, but that again is nothing to do with choice or function: it is to do with irreducible randomness of quantum outcomes (section 2.3).

When applied to large collections of particles, statistical physics emerges from these interactions and describes how ensembles of particles behave (Penrose, 1979; Blundell and Blundell, 2008). This gives constraints on biology (England, 2013; Perunov et al., 2016) which are necessary, but are not sufficient in themselves to explain function or purpose as in section 1.2.

1.2. The Nature of Biology

Many characterizations of life have been given. They include,

- All life exhibits function or purpose (Hartwell et al., 1999), as discussed in the next section.
- In order that this can arise, there must be organization (Solms and Friston, 2018) in the form of adaptive modular hierarchical structures (Ellis, 2016).
- As well as bottom up emergence of higher level structures and function in that hierarchy, there must be top-down realization of higher level processes (Noble, 2012, 2016; Ellis, 2016; Flack, 2017), enabling same level causation at each level (Noble, 2012) and closure of constraints (Mossio and Moreno, 2010; Montévil and Mossio, 2015), with processes thereby generating their own constraints with a mutual dependence such that they both depend on and contribute to maintaining each other.
- This is all enabled by information flows (Nurse, 2008) and associated cell signaling (Berridge, 2014).
- Adaptation to context is taking place all the time at all levels of the hierarchy through variation and selection (Ellis, 2016; Solms and Friston, 2018)
- In particular it is through evo-devo processes (Carroll, 2005; Müller, 2007; Gilbert and Epel, 2009) that all levels of physiological systems come into being, once life has begun[2].
- These processes have a very noisy and contingent nature at the lower levels (Montévil et al., 2016), despite which reliable physiological functioning emerges at higher levels (Rhoades and Pflanzer, 1989; Randall et al., 2002).

[2]We do not attempt to deal in this article with the vexed issue of how life started in the first place. Thus we do not discuss how compartmentalization, metabolism, or adaptive selection and associated genetic information came into being. We assume that they are already in place, and propose that our discussion is then a valid representation of the difference between physics and biology in that context.

As summarized by Hartwell et al. (1999):

> "*Although living systems obey the laws of physics and chemistry, the notion of function or purpose differentiates biology from other natural sciences. Organisms exist to reproduce, whereas, outside religious belief, rocks and stars have no purpose. Selection for function has produced the living cell, with a unique set of properties that distinguish it from inanimate systems of interacting molecules. Cells exist far from thermal equilibrium by harvesting energy from their environment. They are composed of thousands of different types of molecule. They contain information for their survival and reproduction, in the form of their DNA*."

To make this happens involves *inter alia* multiple interactions and non-linearities, the coupling of self-assembly and self-organization processes with chemical/metabolic reactions, existence of cyclic networks, modular/hierarchical substructures, compartmentalization, and cellular individualization.

Finally, what is life? Our view will be (cf. Hartwell et al., 1999) that a living system is a material system that exhibits all the characteristics just listed. From now on we will take that for granted.

1.3. The Concept of Function

Functional talk is a contested area in the philosophy of biology (Millikan, 1989; Neander, 1991; Amundson and Lauder, 1994; Godfrey-Smith, 1994).It is discussed in depth by Mossio et al. (2009). One cannot sensibly talk about physiology of living systems without talking about function or purpose (Hartwell et al., 1999): the heart exists in order to circulate blood (Randall et al., 2002, p. 476–510), pacemaking cells exist in order to determine the rhythm of the heart, blood exists in order to transport oxygen, mitochondria in eukaryotes provide energy for cell processes by converting sugars to ATP (Randall et al., 2002, p. 74), and so on Rhoades and Pflanzer (1989). This crucial role of many functions is taken for granted by working biologists, as in the Hartwell et al. quote above. We will have in mind below functions that are indeed crucial in enabling survival (e.g., the pumping of blood by the heart), and not just incidental byproducts (e.g., the sound the heart makes while pumping).

This amounts to a physiological definition however, another tradition exists that relates function to its evolutionary origin. Mossio et al. Mossio et al. (2009) state

> "*A first tradition, usually labeled 'etiological', has tried to justify and naturalize the teleological dimension of functions by appealing to a scientifically acceptable causal explanation. In the mainstream formulation, etiological approaches appeal to a historical selective causal process, through which the existence of current functional traits is the consequence of the selection exerted on the effects of previous occurrences of the trait. A second tradition, called 'systemic' or 'dispositional', discards the teleological dimension of functional attributions as a relevant explanandum by interpreting functions as causal means-end relations at work in a system. From this second perspective, functions do not explain the existence of the bearer; they refer to current contributions of functional traits to some capacity of the system to which they belong.*"

In our view it is crucial to define function in terms of physiological concepts (the dispositional view) rather than evolutionary ones (the etiological view), because if one goes the latter route it is not easily possible to discuss the issue of drift raised in Kimura (1983) and discussed in depth in Nei (2005). We return to this in section 5.2.

After discussing the options in depth, Mossio et al. (2009) in effect go this route. They propose an organizational account (OA) of functions, as follows:

> *"According to the OA, a trait type T has a function if, and only if, it is submitted to organizational closure C in a differentiated self-maintaining system S. This definition implies the fulfillment of three different conditions. Accordingly, a trait T has a function if and only if:*
>
> *C1. T contributes to the maintenance of the organization O of S;*
>
> *C2. T is produced and maintained under some constraints exerted by O;*
>
> *C3. S is organizationally differentiated."*

If such a trait exists, its function will tend to lead to evolutionary success and hence to selection for this trait, which will explain its existence (up to the issue of drift).

We will adopt this account of functions in what follows. Three further points arise: First, it is crucial that function exists at each level of the hierarchy in interrelated ways, as discussed by Farnsworth et al. (2017). They consider a *function* to describe a process (an action) and a *trait* to be a property of a biological system at one level which may enable a function to be performed in relation to another level. This is consistent with the above. Second, the organizational closure mentioned is conditional on top-down constraint or realization occurring as well as bottom-up emergence in the modular hierarchy (Noble, 2012, 2016; Ellis, 2016)[3]This is again implicit in the above.

Finally, the above does not necessarily imply consciousness or intention. However, intention does indeed come into play in the case of conscious animals, when purposive behavior (Mayr, 2004, p. 57), perhaps including deductive causation (section 6), occurs. Its emergence is based on the reliable functioning of the underlying physiological systems in the brain (Randall et al., 2002; beim Graben, 2016). We discuss this in section 6.

1.4. The Key Problem

The issue we address in this paper is thus, how does purpose or function emerge from purposeless physics on developmental and functional timescales? How does deterministic physics lead to logical branching enabling function?

At the macro level, this occurs through plastic neural networks (Kandel et al., 2013) and physiological systems (Rhoades and Pflanzer, 1989). At the micro level, it occurs through epigenetic effects (Pigliucci and Müller, 2000; Gilbert and Epel, 2009) mediated by gene regulatory networks (Gilbert and Epel, 2009) and signal transduction networks (Janes and Yaffe, 2006; Berridge, 2014), and synaptic interactions (Kandel et al., 2013). But at the underlying physical level, dynamics is Hamiltonian and does not allow a branching evolution depending on context. How are these compatible with each other? The theme of this paper is that biomolecules are the key enabling these branching processes to happen. They enable turning molecular processes ON or OFF depending on cell signals, (Berridge, 2014), which is determined by the context in which they exist (Noble, 2008, 2011, 2016). As described by Berridge in the Introduction to *Cell Signaling Biology* Berridge (2014):

> *"The basic principle of cell signaling pathways are that stimuli (e.g., hormones, neurotransmitters or growth factors) acting on cell-surface receptors relay information through intracellular signaling pathways that can have a number of components. They usually begin with the activation of transducers that use amplifiers to generate internal messengers that either act locally or can diffuse throughout the cell. These messengers then engage sensors that are coupled to the effectors that are responsible for activating cellular responses. .. cell signaling is a dynamic process consisting of ON mechanisms during which information flows down the pathway, opposed by the OFF mechanisms that switch off the different steps of the signaling pathway"* (See Module 1: Figure cell signaling mechanism.)

This is an example of the kind of contextual branching that takes place in biology (section 3.1) and distinguishes it from physics.

Note that this is not the same as saying that biological processes can be considered as computational processes, because it is not implying there is a computation or program of some kind determining the branching choices that are made[4]. It is saying that the branching processes which take place at the lower levels, controlled by a large number of cell signaling processes discussed in depth in Berridge's magisterial text (Berridge, 2014), can be regarded to a very good approximation as Boolean (digital) choice processes governed in a top-down contextual way according to functional need. Thus the core of his discussion is how signals turn a large variety of processes ON and OFF. This is a digital logic, emerging from the underlying physics, that underlies all the higher level processes discussed above (section 1.2); they could not be contextually branching processes (which they are) unless there was the possibility of such branching processes at the underlying molecular and cellular levels. To be sure in practice they are not precisely digital processes, for example ion channels do not precisely behave as ON/OFF channels but rather have are a sigmoidal approximation to such behavior [5]. Nevertheless that description gives an excellent encapsulation of what occurs, as Berridge discusses, and is used for example by Davies and Walker (2016) and Walker et al. (2016) in Boolean network models of gene regulation in yeast.

However, there is also a major random element at the molecular level introducing statistical variation in happenings at

[3]It will in some cases be appropriate to call this top-down causation. That usage is controversial: it will be defended in a forthcoming paper (Ellis and Gabriel, 2019).

[4]Although this is effectively true in some specific contexts in developmental biology, where pre-determined developmental stages occur at specific times in an organisms developmental history Gilbert (2006); Wolpert (2002) via specific mechanisms whereby "*groups of cells are progressively apportioned distinct fates through a process of cell specification*" (Berridge, 2014: Module 8). For example, gastrulation occurs at a specific stage of development (Wolpert, 2002).

[5]There may also be intermediate states of channel opening. Then the logic is (10) rather than (9).

that level. It is then remarkable that these lower level processes produce reliable physiological outcomes at higher levels, such as regular heartbeats and breathing (Rhoades and Pflanzer, 1989), as well as evolutionary convergence to produce physiological function (Natarajan et al., 2016). The view here will be, in accordance with Noble and Noble (2018) that it is precisely this variation at the lower level that allows higher level processes to determine what occurs at the lower levels in order to adapt them to higher level needs (section 5.4). Thus despite this variation one can usefully analyse gene regulation via the above mentioned Boolean network models (Davies and Walker, 2016; Walker et al., 2016), which rely on the kind of branching logic discussed in this paper. Indeed the key point is that

> **The lower level basis of higher level contextual functioning:** *None of the complex higher level biological features mentioned in section 1.2 would be possible if there was not a possibility of contextual branching function at the molecular level, which can often be well described by digital (Boolean) logic, despite the statistical nature of molecular processes.*

How that happens is the concern of this paper.

This paper focuses initially on the voltage gated ion channels that underlie neuronal functioning, although the same applies for example to the active sites of enzyme molecules which are complementary to the shape of the substrate. We first consider the difference between the logic of physics (section 2) and the logic of biology (section 3), then the biomolecules that make this difference possible (section 4), and finally how such molecules have come into being (section 5). The processes of deductive causation are discussed in section (6). The conclusion (section 7) clarifies first the three general kinds of causation that occur in biology, and second how contextual biological dynamics causes branching behavior at the underlying physical level. Overall, this is a view of how physics underlies integrative physiology (where everything occurs in a contextual way Noble, 2012, 2016; Ellis, 2016). We take it for granted that living systems are open non-equilibrium systems (Friston and Stephan, 2007). However, that by itself does not suffice to characterize life: a burning candle satisfies those criteria. More is required (section 1.2).

2. LOGIC OF PHYSICS

Basic physics evolution is Hamiltonian (section 2.1), and so does not display any branching behavior. However, two aspects of physical laws do exhibit branching: phase changes (section 2.2) and quantum wave function collapse (section 2.3); but neither of these relate to function as characterized above, enabled by branching dynamics. How then does physics enable such branching to emerge? Through symmetry breaking (section 2.4), which is how quite different behavior can emerge from the underlying physics. In a biological context where higher level branching dynamics occurs, that leads to branching physical behavior at the electron level, as discussed in section 7.2.

2.1. Classical Dynamics

Classical physics determines the evolution of a physical system by energy and momentum conservation equations (Arnold, 1989, p. 15–27), a force law (Arnold, 1989, p. 28–50), a Lagrangian (Arnold, 1989, p. 55–61), or a Hamiltonian (Arnold, 1989, p. 65–70,165–266). The context C consists of boundary and constraint conditions. The dynamical law uniquely determines later states of the relevant variable $\mathbf{X}$ from suitable initial conditions $\mathbf{X}(t_1)$ (Arnold, 1989):

$$\begin{aligned}\texttt{IF at time } t_1, \ \mathbf{X} &= \mathbf{X}(t_1), \texttt{ THEN at time } t_2,\\ \mathbf{X} &= H(C, \mathbf{X}(t_1), t_2).\end{aligned} \tag{1}$$

Here the context C is expressed via constraint equations

$$C(c, \mathbf{X}) = C_0, \ dC_0/dt = 0 \tag{2}$$

on the possible values of the variables, with control parameters c affecting the form of those constraints. Examples are the dynamics of a classical pendulum (Arnold, 1989), and the gravitational dynamics of celestial objects (Binney and Tremaine, 2008). The dynamic equations have unique solutions, as shown by Arnold (Arnold, 1989, p. 8) (this is a result of $dC/dt = dC_0/dt = 0$). Thus there is a specific unique outcome: no branching takes place as in (9).

2.1.1. Invariance of Physics

The basic point is that we cannot alter the physical laws that govern what happens. We can however shape outcomes by determining what they act on, for example a pendulum or a digital computer; mathematically this is expressed through the constraints C. The physical laws relevant to daily life on Earth are Newton's laws of motion together with Galileo's equations for a falling body and Maxwell's equations for electromagnetism:

$$\nabla \cdot \mathbf{E} = 4\pi\rho, \qquad \nabla \times \mathbf{E} = -\tfrac{1}{c}\tfrac{\partial \mathbf{B}}{\partial t}, \tag{3}$$

$$\nabla \cdot \mathbf{B} = 0, \quad \nabla \times \mathbf{B} = \frac{1}{c}\left(4\pi\mathbf{J} + \frac{\partial \mathbf{E}}{\partial t}\right) \tag{4}$$

where $\mathbf{E}$ is the electric field, $\mathbf{B}$ the magnetic field, ρ the charge, and $\mathbf{J}$ the current. Nothing can change those interactions. The motion of a particle with charge e, mass m, and velocity $\mathbf{v}$ is determined by

$$\mathbf{F} = m\frac{d\mathbf{v}}{dt} = e\{\mathbf{E} + \mathbf{v} \times \mathbf{B}\} + m\mathbf{g}. \tag{5}$$

where $\mathbf{g}$ is the gravitational field. Equation (1) represents the solutions that necessarily follow from (3–5), proceeding purposelessly on the basis of the context C. These equations are time symmetric and imply energy conservation. Bifurcations can occur in some cases when a small change in a contextual parameter or initial data occurs, but the outcomes are still determined uniquely by the dynamical equations (Arnold, 1989), even though the outcomes may be unpredictable in practical terms in the case of chaotic dynamics.

Statistical physics laws for aggregates of particles follow from the fundamental physics laws (Penrose, 1979; Blundell and

Blundell, 2008), which emergent laws by their nature determine probabilistic outcomes $P(q)$ for states q. They may also have stochastic elements due to random environmental effects, leading to stochastical dynamics represented by coupling deterministic equations of motion to "noise" that mimics the effect of many unknown variables. Then a stochastic term $\eta(t)$ must be added to (5) (see Longtin, 2010). The outcome will then not be determinate, but it will not relate in any way to function or purpose.

2.2. Phase Changes

One might suggest that bifurcations as proposed below (Equation 9) happen in physics when phase changes takes place, for example solid/liquid/gas transitions for a substance S (Blundell and Blundell, 2008). These generically have a form like

$$\begin{aligned}&\texttt{GIVEN pressure } P \texttt{ and temperature T},\\ &\qquad \texttt{IF } \{P,T\} \in S_{P,V} \texttt{ THEN S is solid},\\ &\texttt{ELSE IF } \{P,T\} \in L_{P,V} \texttt{ THEN S is liquid},\\ &\qquad \texttt{ELSE S is gaseous.}\end{aligned} \tag{6}$$

Here the context is represented by the pressure P and temperature T, and $S_{P,V}$, $L_{P,V}$ and $G_{P,V}$ are the subsets of the (P, V) plane for solids, liquids, and gases respectively. At first glance this looks like it has the biological branching form (9). However, the regions $S_{P,V}$, $L_{P,V}$, and $G_{P,V}$ are fixed by the physics of the substance. Thus this is physical logic, determined purely by the laws of physics; no historical or evolutionary factor enters. Note for example the contrast with the homeostatic process governing core body temperature, where the setpoint of 98.4°F is not determined by physical laws; it was determined through evolutionary processes related to physiological optimization.

2.3. Quantum Physics

The Schrödinger evolution is Hamiltonian, but wave function collapse, as occurs when a measurement takes place, is a branching operation. However, such wave function collapse of a wave function $|\Psi(t_1)\rangle$ (an "event") is not deterministic. It has the logic

$$\begin{aligned}&\texttt{IF } |\Psi(t_1)\rangle = c_1|u_1\rangle + c_2|u_2\rangle + ... + c_n|u_n\rangle,\\ &\texttt{THEN } |\Psi(t_2)\rangle = \texttt{EITHER } a_1|u_1\rangle \texttt{ OR } a_2|u_2\rangle \texttt{ ....OR } a_N|u_N\rangle\\ &\texttt{with probabilities } |c_1|^2, |c_2|^2, ..., |c_N|^2 \texttt{ respectively.}\end{aligned} \tag{7}$$

where a_i is the eigenvalue associated with the basis vector $|u_i\rangle$. Thus branching takes place, but the outcome that occurs is not fixed by the initial state, although the statistics of such outcomes is. It is a contextual process (Drossel and Ellis, 2018), but the logic (7) is not directly related to function. In the end all the processes we discuss in this paper are underlain by such contextual quantum-to-classical transitions.

2.4. Symmetry Breaking

The key physical effect enabling the existence of the biomolecules discussed here, with their functional properties arising out of complex molecular structures, is the existence of *broken symmetries* (Longo et al., 2012). These are what allow quite different kinds of behavior to emerge at higher levels out of the underlying physical laws, with all their symmetry properties, as explained by Anderson in his foundational paper "More is Different" (Anderson, 1972). Thus the underlying standard model of particle physics is Lorentz invariant, but the emergent biomolecules (such as shown in **Figures 3**, **4**) are not. Contextless physics is Hamiltonian, but physics in a biomolecular context is not (section 7.2). Hence in the end this is what enables the difference between physics and biology.

Again the underlying physics relevant to biological functioning is time symmetric, but biological effects such as cell signaling (Berridge, 2014) and adaptive selection (18) are not. The contextual process of wave function collapse in quantum physics (7) breaks the time symmetric of the Hamiltonian evolution of the wave function, and this underlies the way the cosmological arrow of time leads to the arrows of time in quantum physics and thermodynamics (Drossel and Ellis, 2018), and so underlies the crucial feature of the emergence of the arrow of time in biology. We will not comment further on this issue here.

3. LOGIC OF LIFE

Life of course obeys the laws of physics, so at each level whatever constraints are implied by physics are obeyed (Cockell, 2018). However, additionally living systems behave according to biological logic, leading to what Mayr characterizes as goal directed behavior (Mayr, 2004, p. 52) furthering function (section 1.3). Living systems collect and analyse information (Nurse, 2008), using it to predict probabilities and thereby use it to execute functional actions in the light of both genetic heritage and acquired information (Hartwell et al., 1999; Campbell and Reece, 2005). This involves a branching logic where outcomes are selected on the basis of context, as revealed by incoming information.

3.1. Dynamical Branching

The dynamics followed at each level of biological hierarchies is based on contextually informed dynamical branching L that support the functions α of a trait T. Thus biological dynamics can be functionally-directed rather than driven by inevitability or chance:

$$\begin{aligned}&\texttt{Biological dynamics tends to further}\\ &\texttt{the function } \alpha \texttt{ of a trait } T\\ &\texttt{through contextually informed branching}\\ &\texttt{dynamics } L\end{aligned} \tag{8}$$

where function is defined as in section 1.3, and in its simplest form L is branching logic of the form

$$\begin{aligned}&\texttt{L: given context } C, \texttt{ IF } T(\mathbf{X}) \texttt{ THEN } F1(\mathbf{Y}),\\ &\qquad\qquad\qquad\qquad \texttt{ELSE } F2(\mathbf{Z}).\end{aligned} \tag{9}$$

Here $\mathbf{X}$ is a contextual variable which can have many dimensions, and $\mathbf{Y}$ and $\mathbf{Z}$ are also variables that may have many dimensions;

they may be the same variables or not. $T(\mathbf{X})$ is the truth value of arbitrary evaluative statements depending on $\mathbf{X}$. It can arise from any combination of Boolean logical operations (NOT, AND, OR, NOR, etc.), perhaps combined with mathematical operations, while $F1(\mathbf{Y})$ and $F2(\mathbf{Z})$ are outcomes tending to further the function α. Thus they might be the homeostatic response"If blood sugar levels are too high, release insulin," or the conscious "If the calculated range of the aircraft as presently fueled is $<$500 km, add more fuel" (a default unstated "ELSE" is always to leave the status quo).

Together with (8), the crucial point is

Independence of physics: *The evaluative function $T(\mathbf{X})$ and the outcome options $F1(\mathbf{Y})$ and $F2(\mathbf{Z})$ are not determined by the underlying physical laws, despite being enabled by them.*

Thus these branching processes are not determined by Newton's laws of motion, Maxwell's equations, Newton's or Einstein's theory of gravity, the fundamental theory of particle physics, or statistical physics. Rather they are shaped by evolutionary or developmental processes (Gilbert, 2006; Gilbert and Epel, 2009) to give highly complex outcomes (Rhoades and Pflanzer, 1989; Campbell and Reece, 2005) resulting from plant or animal phsyiology or animal behavior, or can be conceived by human thought so as to result in planned outcomes (Bronowski, 1973; Harford, 2017). In many cases at the molecular level this branching logic is to a very good approximation of a discrete (digital) nature: this is clear for example in Berridge's discussion (Berridge, 2014) of cell signaling systems. There will in practice be noise and time lags in real situations, leading to more complex contextual dynamics. However, a discrete description such as given by Berridge will adequately capture the causal essence of what is going on at a molecular level from a biological viewpoint (if that were not the case, his magisterial book would not make sense).

In more complex cases, there will be multidimensional spaces of options and responses:

$$\texttt{L: given context } C, \quad \texttt{IF } B_N(\mathbf{X}) \texttt{ THEN } F_N(\mathbf{Y}) \tag{10}$$

where B_N is the Nth truth function and F_N is the Nth response function. The key point is the same: there is an evaluation function B_N independent of the underlying physics, and a branching dynamics F_N that is followed depending on that function. In principle one can take a limit where evaluation outcome is continuous but in practice that is unrealistic: there will always be sensitivity limits to detection or response processes, so that in fact responses will be discrete responses to discrete ranges of input variables. In any case we will give a number of key cases below where the biological dynamics is well represented by (9) and it is the higher level dynamics emerging out of combinations of such operations that need description as in (10). In particular (9) is true for the cell signaling networks described by Berridge Berridge (2014), which are at the heart of much molecular biology.

One can suggest that trivially any dynamics of a physical system can be programmed in terms of branching logic equivalent to (10), so (10) is really not different from (1), but as discussed in detail in Binder and Ellis (2016), physical laws are not the same as programs: a physical law is not an algorithm (it is Newton's *Law of Gravity*, not Newton's *Algorithm for Gravity*). Furthermore, there is no Hamiltonian or Lagrangian that leads to (10), and in the physics case there is no function α associated with the dynamics, as in (8). Physics *per se* is not teleonomic and does not show branching behavior related to function (section 2). That is the import of the plethora of existence and uniqueness theorems for fundamental physics (for the gravitational case, see Hawking and Ellis, 1973) whereby initial data determines a unique outcome in a specific spacetime domain (therefore the dynamics does not have a branching nature). Unlike the case of physical laws, where the relevant interactions cannot be changed or chosen because they are given by Nature and are invariable, the branching interactions (10) can fulfill widely varying biological or social or mental functions or purposes and can be selected for those purposes. Once one has this basic logical branching enabled at the molecular level, it is possible for complex emergence to take place where branching dynamics is possible at higher levels, and information can be causally effective (Nurse, 2008; Walker et al., 2017)[6].

It is of course not intended here to imply that this kind of causation is deterministic: that is why the word "tends" is used in (8); probabilities may be the best description of the branching logic at play. In particular, chance plays a key role in evolutionary theory (Glymour, 2001; Mayr, 2002) and molecular interactions. Nevertheless such causation is often reliable (Rhoades and Pflanzer, 1989; Randall et al., 2002), for example in the case of the developmental programs which underlie developmental biology (Wolpert, 2002; Gilbert, 2006; Berridge, 2014: Module 8), in the case of molecular machines (Hoffmann, 2012), the systems underlying heart function described by Noble (Fink and Noble, 2008), and the metabolic networks and gene regulatory networks described by Wagner (Wagner, 2017). We take that issue up in section 5.4. In the next sections, we look at various forms the branching logic (9) can take, always taking (8) for granted. Key cases are homeostasis (11) and adaptive selection (18).

3.2. Homeostasis

A crucial form of branching logic in biology is implemented in feedback control circuits that are the foundations of *homeostasis* (Ashby, 1956; Rhoades and Pflanzer, 1989; Randall et al., 2002; Campbell and Reece, 2005, p. 8–10). These are basically of the form (Randall et al., 2002, p. 11, Modell et al., 2015)

$$\begin{aligned}\texttt{IF } X < X_{MIN}(C) \texttt{ THEN } X_{INC}(\mathbf{Y}), \quad \texttt{ELSE IF } X > X_{MAX}(C)\\ \texttt{THEN } X_{DEC}(\mathbf{Z})\end{aligned} \tag{11}$$

where $X_{INC}(\mathbf{Y})$ is some operation that increases the value of the target variable X through changing the value of the control

[6]The concept of information is contentious in biology (Godfrey-Smith and Sterelny, 2016; Koonin, 2016). However, signaling is not (Berridge, 2014). We will take the pragmatic view that signals convey information.

variable **Y**, and $X_{DEC}(\mathbf{Z})$ is some operation that decreases the value of X through changing the value of **Z** (which may or may not be the same as **Y**). The default is to leave the situation as is. Note that this is not a simple ON/OFF effect (Modell et al., 2015): it is a mechanism which will tend to correct the value of X over time to lie between $X_{MIN}(C)$ and $X_{MAX}(C)$, with dynamics described by the equations of feedback control systems (Di Steffano et al., 1967; Sauro, 2017), using Laplace transforms to model the system and signals, in contrast to the physics Equations (3–5). The triggering values $X_{MIN}(C)$ and $X_{MAX}(C)$ are in general dependent on the context (e.g., if the organism is sleeping as against running).

This is a particular case of (9). Note that this is just one part of the complex interacting processes generating their own constraints, immersed in many dimensional interactions. However, (11) undoubtedly occurs at both macro and micro levels as part of this larger set of interactions. Thus such processes control blood pressure and core body temperature at the macro level, and potassium and sodium levels in axons and glucose concentration in extracellular fluid at the micro level [7]. Because biological homeostatic systems have been tuned through evolutionary processes, they are less subject to instabilities that afflict feedback control systems in general.

3.3. The Physical Hierarchy

The structural hierarchy of life (Ellis, 2016) is indicated in **Figure 1**. Networks of interactions between lower level modules lead to emergence of higher levels, which in turn act down on the lower levels to shape their interactions (Noble, 2008, 2016; Ellis, 2016). This leads to adaptive same level causation at each level of the hierarchy Noble (2012).

3.4. Building the Hierarchy: Black Boxing

Branching dynamics occurs at the molecular and cellular level (Berridge, 2014). When built into cell signaling networks, gene regulatory networks, metabolic networks, and neural networks, this bifurcating dynamics at the lower levels enable emergence of higher order operations such as occur in physiology and the brain, with branching logic (9) or (10) occurring at each level. However, the function of the lower levels is in turn contextually controlled by higher level elements (Noble, 2012), resulting in contextual emergence (Atmanspacher and beim Graben, 2009) where lower level logical choices are set so as to fulfill higher level purpose or function (Noble, 2008, 2012; Ellis, 2016). The combination of bottom-up and top-down effects enables the closure of constraints (Montévil and Mossio, 2015).

Figure 2 from Goelzer et al. (2008) illustrates how branching operations at molecular level in a metabolic pathway can be regulated by higher order circuits through transcription factors that control the transcription of genes. They may be ON (that is, able to bind to DNA) or OFF (Berridge, 2014), in this way controlling transcription of DNA to messenger RNA.

[7] See for example "*Regulation of Ca^{2+} homoeostasis by multiple hormonal and organ effector systems*" in Berridge (2014): Module 7, p. 76, and "*Hormonal regulation of blood Na^{+} levels*" in Berridge (2014): Module 7 , p. 105.

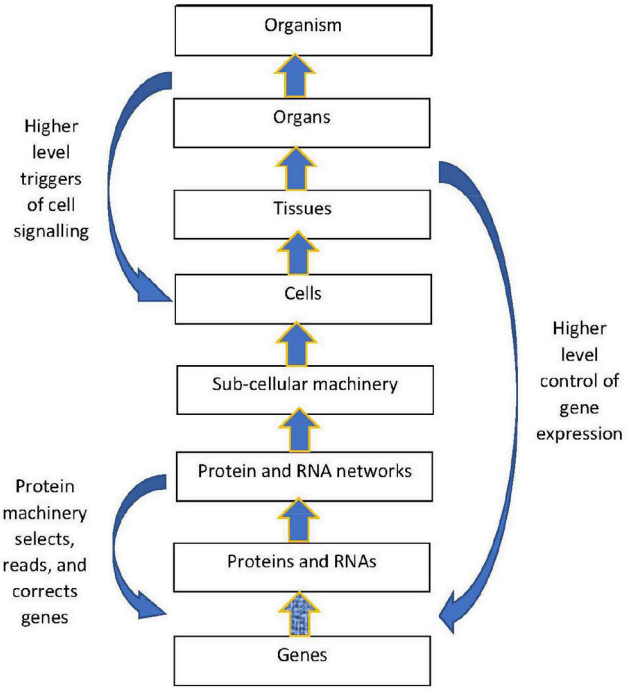

FIGURE 1 | Contextual control in the hierarchy. There is epigenetic control of lower level biological processes by higher level physiological states. These higher states determine what branching will take place at the cellular level by switching genes ON and OFF on the basis of higher level needs. Adapted from Noble (2012), with permission.

The transcription factor TF_2 is a local variable that is responsive to an intermediate metabolite X_n. It modulates synthesis of enzymes in the pathway, embodying branching logic of the form

$$IF\ TF_2\ on,\ THEN\ X_2 \rightarrow X_3,\ ELSE\ NOT \tag{12}$$

which is of the form (9). This is local branching within the module. However, the higher level regulator TF_1, sensitive to variables such as blood pressure or heart rate, modulates the synthesis of both intermediate enzymes and the local transcription factor TF_2. Thus the internal branching of the module results in a "black box" whereby conversion of metabolite X_1 to X_n is determined by the higher level variable TF_1:

$$IF\ TF_1\ on,\ THEN\ X_1 \rightarrow X_n,\ ELSE\ NOT \tag{13}$$

The outcome is again of the branching form (9), but occurring at a higher level (because TF_1 is a higher level variable). The function is production of X_n when and only when it is needed. Thus lower level branching circuits such as (12) can be used to build up higher level branching logic such as (13). This is how *abstraction* occurs in a modular hierarchy (Booch, 1994), so that internal workings of a module are hidden [in this case TF_2, E_2, X_2, and X_3 are internal variables that do not occur in the higher level relation (13)]. From the system view, what

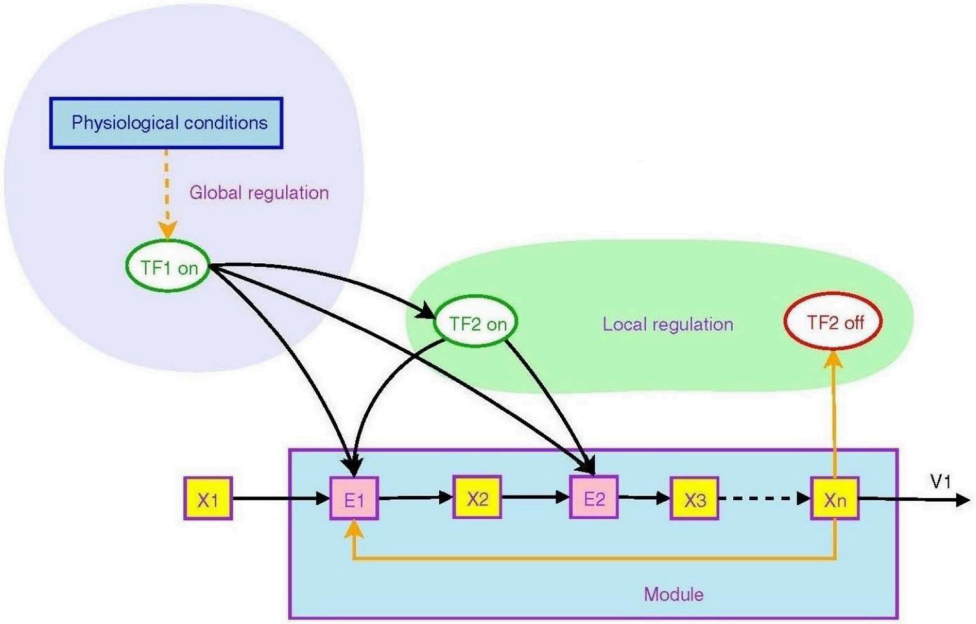

FIGURE 2 | Non-local and local regulation in the context of a metabolic pathway. Depicted is the overall logical structure of a metabolic pathway module that converts food into energy and proteins, with binding of various factors serving as ON/OFF switches. This enables the top-down effect of the physiological environment. Transcription factors are TF's, enzyme pools are E's, metabolic pools are X's. From Goelzer et al. (2008) (open access) .

matters is the emerging logic (13) where transcription factor $TF1$ controls conversion of metabolite X_1 to X_n. Regulation of lower levels through higher level conditions is possible between any adjacent levels in the hierarchy. Through it, metabolic regulation can control gene expression in a top-down way (Alam, 2016), as in Figure 1. The underlying assumption is that there is a suitable cellular context for this to happen (Hofmeyr, 2017).

3.4.1. Black Boxing

As just demonstrated, in the case of a complex logical system, you do not get the higher level behavior by coarse graining, as in the case of determining density and pressure from statistical physics (Penrose, 1979). Instead, you get it by *black boxing* and *logical combination*, involving information hiding and abstraction to characterize the exterior behavior of a module (Ashby, 1960; Oizumi et al., 2014). This is particularly clear in the case of digital computer systems, with their explicit apparatus of abstraction, information hiding, and carefully specified module interfaces, see Grady Booch's book *Object Oriented Analysis* (Booch, 1994). Even though biological systems are not running logical programs, they use the same basic principles of modularity and abstraction in cell signaling systems.

3.5. Multiple Realization

A key feature in the emergence of higher level structure and functions is the multiple realization of higher level structures and functions at lower levels. This is central to the way modularity and black boxing works: the function of a module can be realized by many different internal variables and causal networks. Thus in **Figure 2**, it does not matter what the internal dynamics of the module is provided it leads to the emergent result (13). This degeneracy occurs in all biology in relation to the underlying microbiology and physics: many different lower level realizations of the needed higher level functions can occur. Such multiple realization occurs *inter alia* in the metabolic networks in a cell, gene regulatory networks,and neural networks.

The key underlying analytic concept is existence of *functional equivalence classes* of lower level structures and functions (Auletta et al., 2008; Ellis, 2016) corresponding to a specific emergent structure or function. Equivalence classes at a lower level collect elements whose differences are irrelevant for the emergent target feature at the higher level; it does not matter which one is used to realize the higher level feature. Existence of such functional equivalence classes is an indication of top-down causation (Auletta et al., 2008). An important example is the relation of developmental systems to the genome: a huge number of different genotypes (a *genotype network*) can result in the same phenotype (Wagner, 2017). Any one of these genotypes can be selected for through evolutionary processes in order to lead to a particular emergent function that promotes survival. As far as the higher level function is concerned, it is irrelevant which specific genotype is selected, so it is membership of the equivalence class at the lower level that is the key to what genotype gets selected when adaptation takes place. The huge size of these equivalence classes is what enables adaptive selection to find the needed biomolecules and interaction networks on geological timescales (Wagner, 2017).

4. LINKING PHYSICS AND BIOLOGY: THE PHYSICAL BASIS

All these branching operations emerge from the underlying physics, but are of a quite different nature than the deterministic function of physical laws *per se* (section 2). So how is it possible that they can be realized through the functioning of the underlying physical levels? We will now focus on the brain to give the discussion a specific biological context.

4.1. The Nervous System

The operations of brains is based in the functioning of neurons that are linked together by synapses, thereby being structured as neural networks (Kandel et al., 2013) enabling neoronal signaling (Berridge, 2014): (Module 10). Spike trains proceed via dendrites to the neuron soma where a summation operation is performed. Spike trains then proceed from the cell body down axons to synapses, where another summation process occurs; signals are passed on to other neurons if the sum is above an activation threshold (Kandel et al., 2013). The function is to underlie the processes of the nervous system that enable an animal to anticipate and counter threats to its existence, thus enhancing its chances of survival.

The flow of currents in the dendrites and axons is determined by the underlying physics, described by equations (3–5) plus statistical relations and diffusion equations. In a neuronal context, these lead to the Hodgkin-Huxley equations (Hodgkin and Huxley, 1952) which characterize how ion flows underlie the existence of action potential spike trains (Randall et al., 2002, p. 132–1139). These equations result from the physical structure of ion channels (Catterall, 2000; Randall et al., 2002, p. 141–150) which control flow of ions in and out of the cell membranes. The constants occurring in these equations are not universal physical constants, but rather are constants that characterize the membrane structure. It is not possible to deduce them from the laws of physics *per se* (Scott, 1995).

4.2. Linking Physics to Logic: The Molecular Basis

The branching logical function (10) that emerges is enabled by particular proteins: namely voltage gated ion channels imbedded in axon and dendrite membranes (Catterall, 2000; Randall et al., 2002; Magleby, 2017, p. 146–151) (see **Figures 3**, **4**). They control the flow of potassium, sodium, and chloride ions, leading to action potential spike chain propagation along the axons and dendrites. Their molecular structure and function is discussed in (Randall et al., 2002, p. 139–147).

The ion channels result in branching dynamics with the following logical structure:[8]

$$\texttt{IF voltage difference } V > V_0 \texttt{ THEN allow ion flow, ELSE not} \quad (14)$$

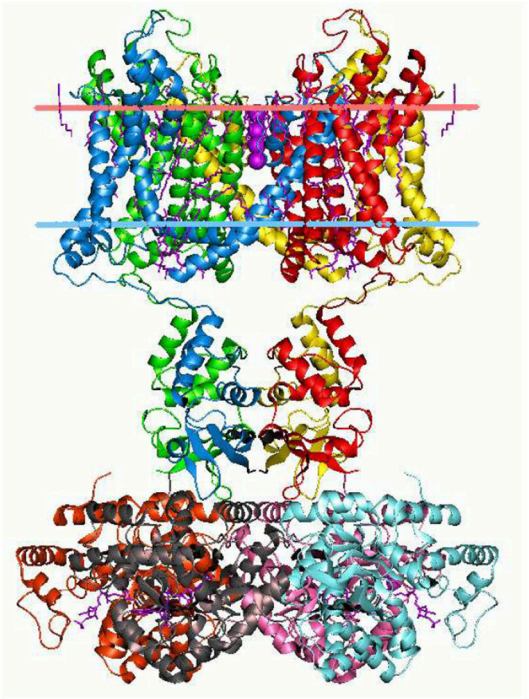

FIGURE 3 | Potassium ion channel structure in a membrane-like environment. This 3-dimensional structure alters according to the voltage difference across the membrane, hence allowing or impeding ion passage. Diagram by Andrei Lomize. From the Open Membranes (OPM) database, with permission.

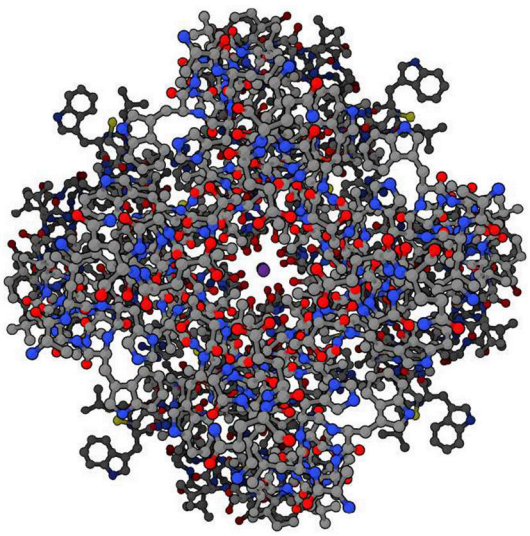

FIGURE 4 | Potassium ion channel functioning. Top view of potassium ion (purple, at center) moving through potassium channel when channel is open. (Protein Data Bank:1BL8, open access).

which is a specific case of the form (9). The function is to facilitate the propagation of action potentials in axons, and so enable functioning of the nervous system (Randall et al., 2002). It is

[8]In practice, the response function is not discontinuous as in this representation, but is a a logistic curve linking 'ON' and 'OFF' states. The principle remains the same: but one now uses a more complex response function. Equation (14) is a good first approximation (cf. Berridge, 2014.)

the detailed 3-dimensional structural form of the ion channels, specifically its tertiary and quaternary structure (see **Figures 3**, **4**), that enables conformational changes in response to local conditions that controls the flow of ions in and out of the cell. This is what enables branching dynamics to emerge from the underlying physics (Farnsworth et al., 2017, p. 313; Kandel et al., 2013; beim Graben, 2016). Similar issues arise via synapses (Kandel et al., 2013; Berridge, 2014: Module 10, p. 28–41), where a branching logic.

$$\texttt{IF summed input voltage } V > V_0 \texttt{ THEN fire action potential, ELSE not} \quad (15)$$

holds, enabled by voltage-gated Ca^{++} channels in conjunction with pre- and post-synaptic neurotransmitter transporters and post-synaptic receptors.

Once physical implementation of logical processes have been achieved at the lower levels, this provides the building blocks for implementing logical processes at higher levels, enabling emergence of branching function in cortical networks. ON/OFF logical units can be used to give the basic operations AND, OR, NOT, and can then be combined in neural networks with thousands of synaptic connections per neuron, and with both upward and downward connections. This enables the coordinated neural dynamics involved in higher level cognitive functioning. Thus the relevant low level physical structure enabling lower level branching function that then enables emergence of higher level branching function is that of proteins (Petsko and Ringe, 2009) imbedded in the cell membrane.

In summary, Given the right cellular context (Hofmeyr, 2017), biomolecules such as ion channels (Catterall, 2000; Magleby, 2017) can act as logic gates, underlying the emergence of complex life processes where branching logic occurs at the higher levels of physiological systems (Rhoades and Pflanzer, 1989; Campbell and Reece, 2005; Goelzer et al., 2008; Kandel et al., 2013).

4.3. More General Biological Contexts

The basic branching logic discussed here occurs also in the metabolic processes, cell signaling networks, and gene expression (controlled by gene regulatory networks) which underlie the functioning of all cells (Berridge, 2014; Hofmeyr, 2017, 2018; Wagner, 2017).

4.3.1. Metabolism

The purpose of metabolism (Krebs, 1993; Berridge, 2014: Module 7; Hofmeyr, 2017) is to produce molecules and free energy needed by the cell in usable form, which are crucial for its function and survival. Enzymes and ribosomes catalyse metaboliism, providing the building blocks of life. This is only possible because of the presence of extremely efficient catalysts, particularly enzymes, that are highly specific with respect to the substrates they recognize and so the reactions they catalyze. The branching logic is (cf. section 3.4),

$$\texttt{IF catalyst for reaction R1 present THEN R1 proceeds, ELSE not.} \quad (16)$$

Its molecular basis is the relevant lock and key recognition mechanism (Lehn, 1995, 2007; Alberts et al., 2007).

4.3.2. Cell Signaling Networks

These are discussed in depth in Berridge (2014). They are again based in the lock and key recognition mechanism, which at a functional level can be well-described in terms of digital logic as an ON/OFF mechanism (Berridge, 2014). At the molecular level, it is based in complementary molecular shapes (Alberts et al., 2007; Watson, 2013).

4.3.3. Gene Expression and Gene Regulatory Networks

The purpose of the genetic code is to specify the sequence of amino acids that will lead to existence of proteins with crucial cellular functions (Alberts et al., 2007; Watson, 2013). Given the cellular context $\mathcal{C}$ (without which no reading of the genetic code would take place Hofmeyr, 2017), the branching logic is

$$\texttt{IF triplet GGU THEN Gly ELSEIF triplet GGC THEN Gly ELSEIF ...,} \quad (17)$$

with a unique mapping specified for each of the 64 codon triplets. Again it is based in complementary molecular shapes that lead to molecular recognition (Watson, 2013). This particular highly degenerate mapping (Watson, 2013; Wagner, 2017) implemented by cellular processes (Alberts et al., 2007) has been determined by the specific historical events of the evolutionary history of life on Earth (Campbell and Reece, 2005; Godfrey-Smith, 2017): many other mappings are chemically possible. Physics by itself does not determine the specific mapping that in fact has occurred (Watson, 2013), represented by the logic (17).

Which sections of DNA are read where and when is under epigenetic control (Carroll, 2005; Gilbert and Epel, 2009), enabled by cell signaling networks (Berridge, 2014) and gene regulatory networks (Wagner, 2017). A key feature of DNA expression is alternative splicing, whereby a single gene codes for multiple proteins, and overlapping genes, where an expressable nucleotide sequence for one gene is also an expressable nucleotide sequence for another. Given epigenetic control that determines these aspects, readout from nucleotide sequences to amino acids takes place as in (17). Furthermore, the epigenetic systems are themselves made up of interacting molecules that arise through the kind of branching logic we discuss here through gene regulatory networks that can be described in a Boolean way to a good approximation (e.g., Wagner, 2011; Davies and Walker, 2016).

5. EXISTENCE OF THE RELEVANT PROTEINS

Two issues arise here: the possibility of existence of the biomolecules needed, for example those that comprise ion channels, and how they come into being.

5.1. The Possibility of Their Existence

Given the nature of physics as we know it (with particular values for the fundamental constants of nature such as the fine structure constant Uzan, 2003), the nature of possible physical structures at the molecular level is controlled by electromagnetism together with quantum physics. Thus the possibility of the existence of biomolecules, and specifically the proteins controlling biological activity (Petsko and Ringe, 2009), is a result of covalent bonds, hydrogen bonds, and van der Waals forces (Watson, 2013).

The result is a space of possible proteins (Petsko and Ringe, 2009) of vast dimensions: an unchanging space of all possible molecular structures (Wagner, 2017). However, their possible existence is not by itself enough: there must be viable mechanisms that can bring them into being.

5.2. Their Coming Into Being: Development and Evolution

Given this vast possibility space, how have the specific proteins that actually exist come into existence? This question has developmental and evolutionary aspects.

5.2.1. Developmental and Epigenetic Aspects

The relevant proteins come into being through molecular processes transcribing genetic information coded in DNA (Alberts et al., 2007; Watson, 2013) into amino acid chains, which then fold to create biologically active proteins. This reading of the genotype occurs in a contextual way (Wolpert, 2002; Gilbert, 2006; Noble, 2012) because epigenetic processes (Pigliucci and Müller, 2000; Gilbert and Epel, 2009), controlled by gene regulatory networks, determine which gene segment gets read at a specific time and place, thereby shaping developmental processes according to the local environment (Oyama et al., 2001; Gilbert and Epel, 2009). Epigenetic effects even allow genetic rewriting (Lee et al., 2018) so that "genes are more followers than promoters of evolution" (West-Eberhard, 2003). As stated by Noble and Noble (2017),

> "*Organisms and their interacting populations have evolved mechanisms by which they can harness blind stochasticity and so generate rapid functional responses to environmental challenges. They can achieve this by re-organizing their genomes and/or their regulatory networks. Epigenetic as well as DNA changes are involved. Evolution may have no foresight, but it is at least partially directed by organisms themselves and by the populations of which they form part.*"

Nevertheless the reading of the DNA still takes place as above (section 4.3), once epigenetic processes have selected which specific DNA segments will be read in what order.

5.2.2. Evolutionary Aspects

The question then is, how did that genetic information get written? As stated before, we do not enter here into the discussion of how life started: we assume here that somehow cells came into existence, allowing metabolism and the existence and reading of genetic information. In that context, how was it that the genotype for the specific proteins that actually occur (Petsko and Ringe, 2009) come to be written, given that there is a vast space of possible proteins that might have existed (Wagner, 2017)? What about the origin of the gene regulatory networks controlling body plan development (Peter and Davidson, 2011)?

The relevant proteins are extraordinary complex biomolecules (Petsko and Ringe, 2009) with specific functions that are essential for survival, where function is as characterized in section 1.3. For example, hemoglobin transports oxygen in our blood stream; chlorophyll enables plants to harvest solar energy, and so on. Thus they will have been strongly subject to selection pressure because of these vital functions, and so arguably cannot have come into being through genetic mutation, drift, or recombination alone (Morris and Lundberg, 2011, p. 21) without selection playing a decisive role (Farnsworth et al., 2017, p. 313). The natural hypothesis is that they were selected through the process of Darwinian adaptive selection (Darwin, 1872; Mayr, 2002; Campbell and Reece, 2005; Morris and Lundberg, 2011) occurring at the organism level, with these selective outcomes chaining down to the genotype level within a functional cellular context (Hofmeyr, 2017). The genotype-phenotype map has massive degeneracy that would have played a crucial role in enabling new phenotypes and hence associated genotypes to have come into being in the available time (Wagner, 2011), and doing so in such a way that the organism remains viable at each step. The process is contextually driven and hence is an example of top-down causation (Campbell, 1974; Ellis, 2016).

However caution is warranted. Genetic drift, leading to neutral selection (Kimura, 1983; Lynch and Hill, 1986; Nei, 2005) can explain some aspects of human physiology (Ackermann and Cheverud, 2004; Schroeder and Ackermann, 2017). How do we prove it was selection rather than drift that lead to existence of specific proteins? In the case of phenotypes, one can sometimes determine which features are due to selection pressure and which due to drift, thus a detailed study shows "*during the early evolution of the genus Homo, [...] genetic drift was probably the primary force responsible for facial diversification*" (Ackermann and Cheverud, 2004).[9].

How to determine this for proteins or gene regulatory networks is a fascinating challenge. They obviously play a key physiological role but, particularly given the existence of vast equivalence classes of genotypes that can produce acceptable phenotypes (Wagner, 2011), it is far from clear how to determine what aspects of the proteins are selectively determined and what are due to drift. We simply comment, in agreement with (Wagner, 2017) that there has to have been a major selective aspect underlying their evolutionary development, as otherwise they would not exist able to play the functional roles they do.

5.3. The Generic Selection Process

Darwinian adaptive selection is a special case of the generic selection process that is ubiquitous in biology. The basic nature of this process is that there is a random input ensemble of entities which is is filtered so as to produce an output ensemble that fulfills some environmentally dependent selection criteria (**Figure 5**), and so is more ordered than the input ensemble. The

[9]We note here that positive sexual selection may also have played a role

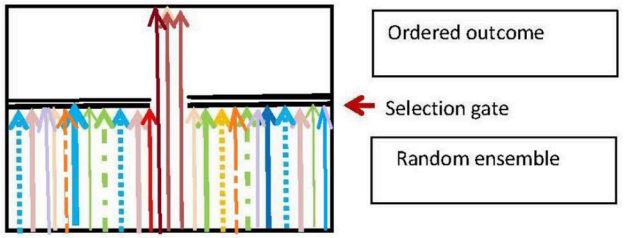

FIGURE 5 | The generic selection process. Adapted from Ellis and Kopel (2018).

branching logic of the process is:

$$\Pi_S(X) : \{\texttt{IF } X \notin S(C,\mathcal{E}) \texttt{ THEN delete } X\} \tag{18}$$

Here S is the subset of elements that is selected to survive on the basis of the selection criterion C, and the environmental context is $\mathcal{E}$. The resulting effect on the input ensemble $\{E(X)\}$ is a projection operation Π_S that gives the output ensemble $\{\hat{E}(X)$:

$$\Pi_S : \{E(X)\} \rightarrow \{\hat{E}(X) : X \in S(C,\mathcal{E})\}. \tag{19}$$

The function of the process is to produce a population of entities that fulfill the selection criterion C. The basic physics case is Maxwell's Demon (Von Baeyer, 1998), where the criterion C for allowing a molecule to pass the trapdoor is $|\mathbf{v}| > v_0$ where $|\mathbf{v}|$ is molecular speed. A biological case is the immune system, deleting invading pathogens (Rhoades and Pflanzer, 1989; Randall et al., 2002). A logical case is the deletion of emails or files on a computer, in accord with some relevance criterion C.

Darwinian selection (Godfrey-Smith, 2001; Mayr, 2002; Campbell and Reece, 2005) has the overall structure (18) where C is a measure of inclusive fitness (West and Gardner, 2013) in the context of the environment, and the input ensemble at each time t_2 is a randomized variant of the output of the previous process at time t_1:

$$\{E(X)\}(t_2) = R\{\hat{E}(X)(t_1)\}. \tag{20}$$

At the genotype level, R is randomization based in recombination, mutations, and horizontal gene transfer. This results in variation at the phenotype level, which is where the selection (survival of an animal or plant until reproduction can take place) actually occurs[10]; that selection then chains down to the genotype level. Thus the process is a continually repeated multilevel 2-step process (Mayr, 2002: p. 130–133): *reproduction with variation* (20), which is where directed sexual selection and differential reproductive success enters, followed by *elimination* (18), which is where differential survival rates matter (this only requires selection of individuals who are "good enough" (Mayr, 2002, p. 130–131); they don't have to be the fittest, which is partly why drift is possible). It is the elimination phase (18) that leads on average, in suitable circumstances, to selection of individuals with traits that are better fit to the environment. The combination of these two processes leads to inclusive fitness (West and Gardner, 2013). Thus this adaptive selection process (Morris and Lundberg, 2011) functions to produce individuals fit to survive in a specific environmental context through their physiology and functioning even though the process has no intentional "purpose" (Mayr, 2004, p. 58). It thereby leads *inter alia* to existence of the molecules we discuss in this paper (Wagner, 2017).

[10]Or perhaps at the group level; this is the contentious issue of levels of selection (Okasha, 2010)

5.4. What Role Does Chance Play?

Biological processes display a great deal of randomness, particularly at the molecular level where there occurs a "molecular storm" (Hoffmann, 2012). The occurrence of this noise does not mean the outcome is random: reliable physiological function emerges at higher levels (Rhoades and Pflanzer, 1989; Randall et al., 2002). In fact microbiology thrives on randomness (Hoffmann, 2012; Noble, 2017), and this is also the case for brain function (Glimcher, 2005; Rolls and Deco, 2010). Furthermore, randomness plays a key role in evolution (see Glymour, 2001; Mayr, 2002, p. 252–254, Kampourakis, 2014, p. 184–191), underlying that vast variety of life on Earth by providing a very varied set of genotypes on which selection can operate, for example leading to predictable convergence in hemoglobin function (Natarajan et al., 2016).

We propose, in agreement with Noble and Noble (2018), that randomness plays a key role at the molecular level by providing an ensemble of variants from which higher level selection processes can choose what happens through selection of outcomes according to higher level selection criteria (18), thus creating order out of disorder in a reliable way (Noble and Noble, 2018), as represented by (19) . As stated by Noble and Noble Noble and Noble (2018),

> *"Choice in the behavior of organisms involves novelty, which may be unpredictable. Yet in retrospect, we can usually provide a rationale for the choice. A deterministic view of life cannot explain this. The solution to this paradox is that organisms can harness stochasticity through which they can generate many possible solutions to environmental challenges. They must then employ a comparator to find the solution that fits the challenge. What therefore is unpredictable in prospect can become comprehensible in retrospect. Harnessing stochastic and/or chaotic processes is essential to the ability of organisms to have agency and to make choices"*

For example, molecular binding processes depend on random presence of the appropriate substrate for a binding site, and the adaptive immune system depends on random generation of antibodies to find the one that works against a particular pathogen. This is also the essential feature of Edelman's Neural Group Selection (Edelman, 1987), which envisages initial random neuronal connections (Wolpert, 2002) being pruned and strengthened according to selection criteria provided by an innate 'value system' in the brain (which in psychological terms can be associated with innate primary emotional systems; Toronchuk and Ellis, 2013; Ellis and Solms, 2017). Furthermore,

this underlies the possibiity of real mental emergence, as proposed by Mitchell Mitchell (2018):

> "*I argue here that physical indeterminacy provides room for the information entailed in patterns of neuronal firing—the mental content of beliefs, goals, and intentions—to have real causal power in decision-making.*"

6. DEDUCTIVE CAUSATION

Deductive causation takes place when effects are the outcome of explicit logical processes, as contrasted to the biological cases discussed so far, where they are processes that are indeed carrying out what can be characterized as logical operations, but these are implicit in the biology rather than explicit.

Deductive causation requires mental processes that explicitly consider alternative logical inevitabilities or probabilities and decide outcomes on this basis, for example, "If I wait till 10am I will miss the bus, so I'd better leave now". This requires conscious intelligence[11], and certainly occurs in the case of humans. It may also occur to some degree in animals, but we will not enter that debate here: the essential point is that it does indeed occur in the real world, as evidenced by the existence of books, aircraft, digital computers, and all the other products of conscious design (Harford, 2017). It is made possible by the existence of brains (at the macro scale) (Kandel et al., 2013) and their underlying biomolecules such as voltage gated ion channels (at the micro scale) (Scott, 1995; Kandel et al., 2013), as discussed in section 4, enabling information to be causally effective (Walker et al., 2017).

We look in section 6.1 at deductive argumentation $\mathcal{D}$, whose truth is valid independent of contingent facts, in section 6.2 at evidence based deduction $\mathcal{DE}$, where the addition of empirical data E leads to conclusions that follow from that evidence via logical deduction $\mathcal{D}$, and in section 6.3 at deductively based predictions of outcomes $\mathcal{DEO}$, which are used to decide on best choices of actions $\mathcal{DEOC}$ on the basis of logical predictions of outcomes O following from the data E together with choice criteria C.

6.1. Deductive Argumentation

Deductive argumentation can be definite or probabilistic. *Definite deductive arguments* deal with inevitable outcomes of abstract relationships between variables:[12] thus[13]

$$\mathcal{D}\text{: IF } T1(\mathbf{X}) \text{ THEN necessarily } T2(\mathbf{Z}), \tag{21}$$

where $T1(\mathbf{X})$ may involve logical operations AND, OR, NOT, and their combinations, or mathematical equalities or inequalities, or both logical and mathematical relations in any combination. Thus one might have a conjunction of conditions

$$\mathcal{D}2\text{: IF } T1(\mathbf{X}) \text{ AND } T2(\mathbf{Y}), \text{ THEN necessarily } T3(\mathbf{Z}), \tag{22}$$

where **X**, **Y** and **Z** may or may not be the same variables. These are of the same logical form as (9), but the key difference is that in that case, the context was the logic implicitly embodied in biological processes, whereas here the relations refer to explicit logical thought patterns. They may be realized at some moment in a brain, or written down on paper, or recorded in some other way (such as on a black board or a computer screen), but the patterns themselves are abstract relations with their own internal logic that is independent of whatever specific realization may occur.

Mathematical examples are the relations

$$\text{IF } \{\text{X=2}\} \text{ THEN } \{\sqrt{X} \text{ is irrational}\} \tag{23}$$

which is proved by algebraic argumentation, and the partial differential equation result

$$\begin{gathered}\text{IF } \{\text{Eqns.(3),(4) hold with } \mathbf{J} = \rho = 0\},\\ \text{THEN } \{\text{wave solutions } u(x,t) = F(x-ct) + G(x+ct) \text{ exist}\}\end{gathered} \tag{24}$$

(which mathematical fact underlies the existence of radios, TV, cellphones, etc).

Logical examples are the relations

$$IF\ \{A \Rightarrow B\}\ AND\ \{B \Rightarrow C\}\ THEN\ \{A \Rightarrow C\} \tag{25}$$

and the combinatorial rules of Boolean logic involving AND, OR, NOT, and so on.

Probabilistic logical arguments deal with likely outcomes on the basis of statistical evidence, for example:

$$\text{IF } T1(\mathbf{X,P1}) \text{ AND } T2(\mathbf{Y,P2}), \text{ THEN probably } T3(\mathbf{Z,P3}), \tag{26}$$

where $T1(\mathbf{X,P1})$ means $T1$ is valid with probability $P1$, and so on. A key example is Bayes' Theorem (Stone, 2015):

$$\text{IF } \{P(A) \text{ AND } P(B|A) \text{ AND } P(B)\} \text{ THEN } P(A|B) = \frac{P(B|A)\,P(A)}{P(B)}, \tag{27}$$

where $P(A)$ and $P(B)$ are the probabilities of observing events A and B independent of each other, $P(A|B)$ is the conditional probability of observing event A given that B is true, and $P(B|A)$ is the conditional probability of observing event B given that A is true. This relation, which is of the form (26), underlies the learning processes of the predictive brain (Huang and Rao, 2011; Clark, 2013; Hohwy, 2013), enabled by suitable neural structures (Hawkins, 2004; Bogacz, 2017, section 2.3–2.5) built from biomolecules (Scott, 1995). This topic is developed further in section 6.5.

[11] We note here that these processes can become automated after much practice so that they are intuitive rather than the result of directed mental effort. Nevertheless the nature of the causation is the same.

[12] We are not giving a formal definition of logic here, but rather a sketch of how it works. It can be any form of logic that has been discovered by the human mind.

[13] Strictly speaking, the word "necessarily" is superfluous. We add it for emphasis here and below. A similar remark applies to "probably" in (26).

6.2. The Link to Data: Evidence Based Deduction

It may well be that we know that the antecedents in some of these arguments are either true, or are highly probable, in which case we can move to evidence based deduction: (21) becomes

$$\mathcal{DE}\text{: SINCE T1(X) THEN necessarily T2(Z)}, \tag{28}$$

where $T2(Z)$ necessarily follows from $T1(X)$, and we know $T1(X)$ to be true either because we have seen it to be true (there is a dog in the room), or it is common knowledge (England is near France), or it is an established scientific fact (DNA is a key molecule underlying genetic inheritance), or at least it is a best explanation (established by abduction, i.e., inference to best explanation from observations). For example

$$\text{SINCE } E = mc^2 \text{ THEN binding energy can be made available via nuclear fission of heavy atoms}, \tag{29}$$

In other words, because we know special relativity is true, we know we can in principle make nuclear power stations and nuclear bombs. Thus reliable data (the experimental verification of the logically deduced relation $E = mc^2$) relates deductive argumentation to real world possibilities. Similarly an extension of a simple case of (26) becomes

$$\text{SINCE } T1(\mathbf{X,P1}) \text{ THEN probably } T2(\mathbf{Z,P3}), \tag{30}$$

in the probabilistic case, for example

SINCE there are dark clouds in the sky
THEN it will probably rain today.

The deduction leads to the conclusion that a specific outcome is likely to actually occur.

6.3. Deductively Based Action

Following on (28) and (30), we can deductively determine that specific actions will inevitably or probably have specific outcomes:

$$\mathcal{DEO}\text{: SINCE } T1 \text{ is true THEN action A will lead to outcome O .} \tag{31}$$

This leads to the basis of deductive choice of best actions:

$$\mathcal{DEOC}\text{: WHEN } T1 \text{ is true THEN DO A}(\mathbf{V}) \text{ TO } \mathcal{C}\text{-optimize O} \tag{32}$$

where $\mathcal{C}$ is a selection criterion for the best outcome O_*, and $A(\mathbf{V})$ is some action chosen to alter O via a control variable V. The purpose is to produce an optimal outcome O on the basis of a representation of the situation founded on the best available evidence (Papineau, 2016). An example is

$$WHEN\,\{T > T_0\} \text{ THEN } \{\text{set V ON}\} \text{ SO THAT } \mathcal{C}\text{:}\{T_1 < T_0\} \tag{33}$$

which might be part of a computer program implementing feedback control (14) to ensure that temperature T is kept below a critical level T_0 via the cooling control variable V. In the probabilistic case it might be

SINCE {there is 60% chance of rain}
THEN {take an umbrella} TO {keep dry}.

When we carry out such deductive argumentation, the abstract logic of the argument $\mathcal{D}$ [see (21)] is the causal element determining the nature of the resulting outcomes. The aircraft flies well because we have used explicit deductive mathematical logic $\mathcal{D}$, together with our knowledge of the laws of fluid dynamics $T1$, to optimize its design O by running computer aided design packages $A(V)$ representing the aircraft design via variables V. We call $\mathcal{D}$ a "causal element" because of the counter-factual argument (Menzies, 2014) that if this abstract logic were different, the outcome would be different. The same applies to $\mathcal{C}$: if the decision criteria are changed the outcome changes, for example the wing design will be different if the plane is a fighter or an Airbus. This kind of argument is a key part of planning (Epstude and Roese, 2008).

In practice (e.g., in economic planning) the argument is often probabilistic because we can never be absolutely certain of the outcome, due to uncertainty concerning the contextual effects C. Overall, the import of this section is that

> **Deductive causation**: *Logical deductions about scientific, engineering, and social issues can lead to action plans that are causally effective in terms of altering the world. In these cases it is explicit abstract logic $\mathcal{D}$ realized in brains and/or computers that guides and shapes what happens in highly productive ways (Harford, 2017) and hence may be said to be the essential cause of what happens.*

This is all possible because of the properties of brains as prediction machines that are also able to make choices between alternatives. The logical operations of deduction $\mathcal{D}$ and prediction $\mathcal{DEO}$ take place at the psychological level in the brain (Ellis, 2016), while being realized at the neural network level through spike chains, at the axon level through ion flows, and at the electronic level through electron movements (Scott, 1995). Each level does work appropriate to the logic at that level, but it is the high level deductive logic $\mathcal{D}$ that determines what happens in terms of specific outcomes through logically based choices $\mathcal{DEOC}$ (Ellis, 2016).

6.4. The Creative Element

Deductive causation depends on being able to choose between options, which is where imagination comes in. There must be a process in the brain that generates the options that are taken into account when a choice between various options is made:

$$IF\,\{\textit{The situation is } S\} \text{ THEN } \{\text{options are } O_1, O_2, ..., O_n\} \tag{34}$$

Given this ensemble of choices, one can choose between them using selection criteria as above (section 6.3): a process of adaptive selection takes place whereby an option is chosen, whether it be physical (going to a bus stop, changing a light bulb) or mental (choosing between theories, making a plan). This generation of options to choose from takes place at the psychological level (Byrne, 2005), assisted by the PLAY primary emotional system (Toronchuk and Ellis, 2013; Ellis and Solms, 2017) which is a key source of creativity. There may be an element of randomness in the options available for consideration at

the psychological level due to the underlying stochasticity at the neural level (Glimcher, 2005; Rolls and Deco, 2010), in turn due to molecular randomness (section 5.4).

6.5. The Adaptive Bayesian Brain

The deductive processes of section 6.1 are determined as valid by the brain through adaptive learning processes leading to logical understanding (Churchland, 2013), enabled by underlying brain plasticity. How does the predictive brain (Hawkins, 2004; Clark, 2013) emerge, whereby the brain estimates prediction errors leading to the Bayesian processes of Equation (27) that then enable learning (Friston, 2018) and prediction (Hohwy, 2013)? This is developed in Friston and Stephan (2007), Buckley et al. (2017), and Bogacz (2017).

which show the mechanism whereby such processes can arise in the brain through neural circuits such as shown in Bogacz (2017). Overall, this all emerges from a network of neurons connected by synapses (Kandel et al., 2013), enabled at the microlevel by the branching operation of biomolecules (section 4.2).

7. BIOLOGICAL EMERGENCE AND PHYSICAL BRANCHING

How is it possible that goal-oriented systems and deductive logic arise out of the goal-free underlying physics? The context is the hierarchy of emergence and causation, where all the complexities of biology as outlined in section 1.2, occur. Each level of the hierarchy is equally real (Noble, 2012), and branching causation takes place at each level via complex networks of interactions which, through a combination of bottom-up and top down causation, allow organizational closure. Despite the stochasticity of what occurs, the essential core of interactions at the molecular level can be well represented as binary ON/OFF choices (Berridge, 2014). It is at the network level that these individual choices become immensely complex and able to generate the processes of life (section 1.2). How can such branching dynamics emerge from physics which by its nature does not show such branching properties (section 2)? Our main conclusion is,

> *Biomolecules, and specifically proteins (Petsko and Ringe, 2009), provide the physical link between physics and biological causation by allowing branching dynamics at the molecular level, which can then underlie emergence of macro-scale branching dynamics and even deductive causation when incorporated in adaptive modular hierarchical networks. Both the networks and the proteins must have been shaped through processes of adaptive selection; however some of their aspects (that do not hinder their proper function) may be due to drift.*

Ion channels have been our main example, because they enable functioning of the brain, but many other biomolecules in cell signaling networks also carry out logical operations (Berridge, 2014), as do excitatory or inhibitory receptors in neurons (Kandel et al., 2013) with their synaptic thresholds. These branching functions are based in the lock and key mechanism of supra-molecular biology which enables molecular recognition (Lehn, 1995, 2007).

TABLE 1 | The three major forms of causation: physical, biological, and deductive. Each relies on the previous one to enable its emergence.

	Causation	Agency	Outcome	References
C1	Physical	Physical laws	Determinist	Equation (1)
C2	Biological	Goal-seeking, Selection	Adaptive	Equations (11,18)
C3	Deductive	Logical argument	Planned outcomes	Equation (32)

7.1. The Major Distinctions: Three Kinds of Causation

The major difference between physics and life has been characterized above as due to the difference between the immutable impersonal logic of physical causation (1) and the branching functional logic of biological causation (9), enabled by biomolecules in general and proteins in particular (section 4.2).

The progression of emergence is illustrated in **Table 1**. Inanimate systems are subject only to causation C1. In all life from cells to organisms to populations to ecosystems, as well as causation C1, causation C2 occurs, involving logically based branching (9) such as homeostasis (11) and adaptive selection (18). Thus causation C2 characterizes life in general (Hartwell et al., 1999) as opposed to inanimate systems. Hence there is a major difference between these two kinds of emergence out of the same basic physical elements (Ellis, 2016). What enabled causation C2 to emerge in historical terms was the origin of life out of a physical substratum, when both metabolic and adaptive evolutionary processes first came into being. We do not know how that occurred.

However, a higher form of causation C3 occurs in intelligent life, when deductively based action (32) occurs, enabling deductive logic *per se* to have causal powers. Emergence of this kind of causation is a major transition in evolution (Maynard Smith and Szathm, 1995); we also do not know how that occurred. Intelligent organisms are those that can engage in deductive causation C3, which enables transcending the physical limitations of bodies through the power of abstract thought, prediction, planning, and imagination, enabling technology to develop (so that for example they can fly through the sky or make computer systems). It is this kind of causation (made possible by symbolic systems such as language and mathematics) that underlies the rise of civilisation and the domination of humans over the planet (Bronowski, 1973; Harford, 2017): we are no longer limited by the strength of our bodies but by the limits of our imagination and understanding.

Note that we are able to say this without having to make any specific comments on the relation between the brain and consciousness. What is indisputable is that deductive causation does indeed take place in the real world, as demonstrated by many examples (such as the existence of aircraft and computers), and is crucially different than the kind of causation characteristic of physics (section 2), although it is enabled by that kind of causation (which allows the brain to function as it does; Scott, 1995; Kandel et al., 2013).

7.2. Branching Physical Causation in a Biological Context

There is however a key underlying question: it is clear that branching dynamics takes place at the biomolecular level, so how then does the underlying physics allow this branching to take place? The physics *per se* does not show such branching dynamics (section 1.1); but physics in a biological context must do so, in order to allow the biological branching processes discussed here to emerge.

The solution (**Figure 6**) is that top-down causation takes place (Ellis, 2016) whereby the local biomolecular context causes bifurcation of the underlying physical dynamics. Firstly, the structural constraint caused by biomolecular shape channels causation at the electron and ion level. Thus for example when a photon releases an electron in a chlorophyll molecule, that is a non-Hamiltonian process that took place because of the biological context of existence of a chlorophyll

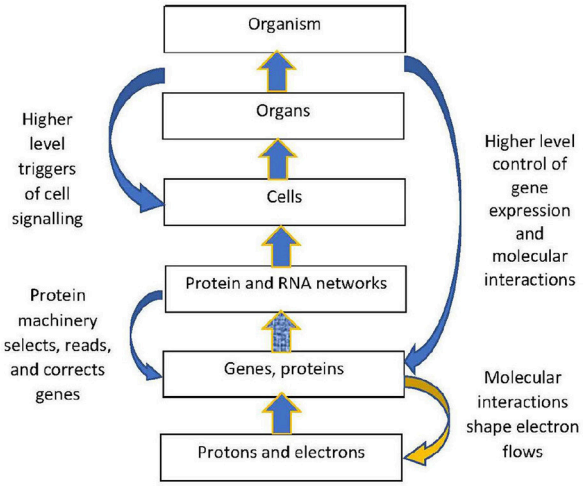

FIGURE 6 | Branching physics in a biological context. Branching biological logic chains down to cause the underlying physical dynamics to branch.

molecule in a leaf. This is underlain at the quantum level by contextual wavefunction collapse (Drossel and Ellis, 2018). Secondly, the cell signaling processes at the molecular and cellular level discussed by Berridge (Berridge, 2014) shape how electron flows take place at the underlying physical level, because when a messenger in a signaling pathway turns a process ON, that causes electrons in component molecules to flow in a structured way that would not otherwise have occurred.

In particular, such top down processes take place in the brain (Ellis, 2016; Ellis G., 2018), for example underlying the formation of memory. Eric Kandel states (Kandel, 2001), "*One of the most remarkable aspects of an animal's behavior is the ability to modify that behavior by learning*". He then identifies how this happens at the molecular level as what he calls "*A dialogue between genes and synapses*" (Kandel, 2001). A specific event, say seeing a car crash, results in gene expression that alters synaptic strengths, which is enabled by underlying flows of electrons as indicated in **Figure 6**. The physics acts in such a way as to instantiate the neural connections at the neuron level needed for that memory to be stored and then available for recall at the psychological level. Neural mechanisms such as those discussed by Kandel Kandel (2001); Kandel et al. (2013) and molecular mechanisms such as discussed by Berridge Berridge (2014) enable this to happen, so what happens at the electron level is determined (up to equivalence classes) by the overall social, psychological, and mental context in a top-down way (**Figure 6** omits those higher levels, but they are key parts of the causal context; Ellis, 2016).

In this way, branching physical dynamics at the bottom level emerges from the higher level branching biological dynamics (you might have seen the crash, or not; the outcomes at the electron level are affected by this contingent situation at the psychological level). Physical outcomes are determined by context, which break the symmetries of the underlying physical laws (Anderson, 1972). In the cases we consider, the relevant constraining context is the physical structure of bio-molecules in their cellular context (Hofmeyr, 2017, 2018).

> **Biologically generated branching of physical outcomes:** *Biomolecules and cells shape electron flows at the physical level firstly by setting constraints on possible electron flows through their geometric shapes and dispositions (Gray and Winkler, 2009). Second, though signaling processes (Berridge, 2014) originating from higher levels (Noble, 2012) that shape (up to equivalence classes) what electron flows actually take place. This enables branching dynamics occurring in these signaling networks to cause branching outcomes at the electron level.*

This enables physiological processes such as those occurring in the heart (Fink and Noble, 2008) to influence electron flows at the micro-physical level through the top-down influences[14] in physiology described by Noble (2012). Mental processes such as learning (Kandel, 2001) and deductive causation (section 6.3) can do the same, enabled by the ON/OFF operations of cell signaling networks (Berridge, 2014). The way this works during deductive argumentation (section 6.1) is similar to the way algorithms control the flow of electrons in transistors in digital computers. The branching logic of an algorithm, realized in a digital computer program, controls branching electron dynamics (which transistors are ON, allowing electron flows, or OFF, at what time) at the physical level. In that case the physical structure enabling this branching logic at the electron level is the junctions between different layers in transistors[15].

> **Biology-physics closure of constraints.** *Extension of the needed functional closure of constraints in biology (section 1.2, Mossio and Moreno, 2010; Montévil and Mossio, 2015) to the underlying physics level is provided by the fact that the branching biological logic at higher levels, including cellular (Rhoades and Pflanzer, 1989; Randall et al., 2002; Berridge, 2014), and mental (Kandel, 2001) functioning, induces congruent branching dynamics at the underlying physical level by changing constraints at that level.*

Equation (2) has to be replaced by

$$C(c(t), \mathbf{X}, t) = C(t) \tag{35}$$

where the time-dependent nature of the physics constraints derives from the time-dependent biological context, and means that the physics evolution is no longer subject to the uniqueness theorems mentioned in sections 2.1, 3.1. This has to be so in order that the biology-physics relation be consistent.

A physics analogy is a pendulum made of a bob of mass m that is constrained to move on a circular arc by a string of length $L(t)$ that varies with time (this is the constraint $C(t)$ governing possible motions of the bob) (Feldman, 2007), see the **Appendix**. The evolution is determined by the macroscopic constraint $C(t)$, which controls outcomes at both macro and micro levels in a way that cannot be predicted from a knowledge of the initial data (starting position $\mathbf{X}_0$ and speed $\mathbf{v}_0$) alone. The dynamics can be controlled by an experimental protocol for $L(t)$ designed by a scientist (which is top down causation $\mathcal{DEOC}$ from the mental level as in section 6.3), or can be unpredictable even in principle, when $L(t)$ is controlled by a computer receiving signals from a detector of particles emitted by decay of a radioactive element (cf. section 2.3).

AUTHOR CONTRIBUTIONS

GE provided the main idea and drafted the main text. JK contributed further ideas and helped develop the text.

[14] Philosophical objections to this possibility based in the idea of supervenience are countered in (Ellis, 2016) and will be fully refuted in a forthcoming paper (Ellis and Gabriel, 2019).

[15] See for example *Bipolar junction transistor* in Wikipedia.

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APPENDIX

Pendulum With Varying Length

The dynamic equations for a pendulum of varying length are set out clearly in Feldman (2007). A bob on an idealised massless rod swings back and forth about a hinge; the rod angle from the vertical at the hinge is $\theta(t)$. The bob mass is M. The bob can slide up or down the rod, so the length $L(t)$ from the hinge to the bob in general varies: $\dot{L}(t) \neq 0$ for some time t. The bob position $(x(t), y(t))$ at time t relative to the hinge is

$$x(t) = L(t)\sin\theta(t), \quad y(t) = -L(t)\cos\theta(t), \tag{A1}$$

giving the constraint equation (cf. Eqn.(35))

$$L(t) = \sqrt{x(t)^2 + y(t)^2}. \tag{A2}$$

The kinetic energy $T(t)$ and potential energy $U(t)$ are

$$T(t) = \frac{1}{2}M[\dot{L}(t)^2 + L(t)^2\dot{\theta}(t)^2], \quad V(t) = -MgL(t)\cos\theta(t) \tag{A3}$$

The Lagrangian is

$$\mathcal{L}(t) = T(t) - U(t) = M[\frac{1}{2}\{\dot{L}(t)^2 + L(t)^2\dot{\theta}^2(t)\} + gL(t)\cos\theta(t)] \tag{A4}$$

and the Lagrange equation of motion

$$\frac{d}{dt}\left(\frac{\partial\mathcal{L}}{\partial\dot{\theta}}\right) - \frac{\partial\mathcal{L}}{\partial\theta} = 0 \tag{A5}$$

shows that

$$\frac{d^2\theta}{dt^2}(t) + 2\frac{\dot{L}(t)}{L(t)}\dot{\theta}(t) + \frac{g}{L(t)}\sin\theta(t) = 0, \tag{A6}$$

reducing to the standard pendulum equation when $L(t) = L_0 \Leftrightarrow \dot{L}(t) = 0$. The initial data $(\theta(t_0), \dot{\theta}(t_0))$ does not determine the solution $\theta(t)$ for $t > t_1$ if $\dot{L}(t) \neq 0$ at any time $t_1 > t_0$, because of this time-variation of the constraint $L(t)$.

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REVIEW
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Inherency of Form and Function in Animal Development and Evolution

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I discuss recent work on the origins of morphology and cell-type diversification in Metazoa – collectively the animals – and propose a scenario for how these two properties became integrated, with the help of a third set of processes, cellular pattern formation, into the developmental programs seen in present-day metazoans. Inherent propensities to generate familiar forms and cell types, in essence a parts kit for the animals, are exhibited by present-day organisms and were likely more prominent in primitive ones. The structural motifs of animal bodies and organs, e.g., multilayered, hollow, elongated and segmented tissues, internal and external appendages, branched tubes, and modular endoskeletons, can be accounted for by the properties of mesoscale masses of metazoan cells. These material properties, in turn, resulted from the recruitment of "generic" physical forces and mechanisms – adhesion, contraction, polarity, chemical oscillation, diffusion – by toolkit molecules that were partly conserved from unicellular holozoan antecedents and partly novel, distributed in the different metazoan phyla in a fashion correlated with morphological complexity. The specialized functions of the terminally differentiated cell types in animals, e.g., contraction, excitability, barrier function, detoxification, excretion, were already present in ancestral unicellular organisms. These functions were implemented in metazoan differentiation in some cases using the same transcription factors as in single-celled ancestors, although controlled by regulatory mechanisms that were hybrids between earlier-evolved processes and regulatory innovations, such as enhancers. Cellular pattern formation, mediated by released morphogens interacting with biochemically responsive and excitable tissues, drew on inherent self-organizing processes in proto-metazoans to transform clusters of holozoan cells into animal embryos and organs.

Keywords: biogeneric, morphogenesis, pattern formation, cell differentiation, self-organization, phase transition, reaction-diffusion

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INTRODUCTION

Organization and functional integration are characteristics of multicellular organisms that distinguish them from mere associations of cells. Some organisms such as the social amoebae (e.g., *Dictyostelium discoideum*) (Bonner, 2009) and certain of the holozoans, a clade that includes both animal and nonanimal species (Sebé-Pedrós et al., 2017), exhibit both unicellular and multicellular life-cycle phases with a small range of differentiated cell types. But, it is in the animals or Metazoa, with up to hundreds of cell types constituting multifunctional, phenotypically plastic tissues, that organization and integration are most prominent. This review will focus on this

group and the biological properties that distinguish it from its nearest relatives, the nonmetazoan holozoans.

There has been a wealth of approaches to the physiological relationships between, and evolutionary bases of, organization and integration in multicellular forms (see Arnellos et al., 2014 for a review). The main treatments have been within an evolutionary adaptationist perspective. These emphasize (1) the suppression of potential intercellular conflicts of genetically heterogenous cells leading to a "unicellular bottleneck" in development, (2) functional division of labor among cell types arrived at through cycles of natural selection for improved fitness, and (3) continuing evolution for integration among subsystems. Concepts that have been advanced to describe these processes include "alignment of fitness," "export of fitness," and "goal-directedness" (at the level of the organism as a whole), all of which, in most treatments, are proposed to be products of natural selection (Buss, 1987; Ruiz-Mirazo et al., 2000; Queller and Strassmann, 2009; Folse and Roughgarden, 2010). To this Arnellos et al. (2014) add the idea of "autonomy," a concept related to function and goal-directedness, which has developed alongside the discourse on organization and integration (Ruiz-Mirazo et al., 2000; Ruiz-Mirazo and Moreno, 2004) and has been explored subsequently, in depth, in a monograph by Moreno and Mossio (2015). This property is seen as foundational to the possibility of Darwinian selection.

Following this line of thought, Keijzer and Arnellos (2017) have questioned whether animal-type autonomy can itself be accounted for by selectionist and adaptationist accounts and suggest that it might not. What is required is a type of organization, characteristic of animal bodies and organs, that distinguishes metazoans from other multicellular organisms like plants and fungi, and also from the unicellular populations that were their direct ancestors, some of which have extant forms with multicellular stages. In his foreword to the Moreno and Mossio volume, Cliff Hooker problematizes the kind of organization that is found in its most intensive form in animals:

> Organisation is not the same as order; pure crystals are highly ordered, so uniform they cannot show any organisation. Neither can gases, because they are too random to be organized. Organisation lies between the crystal and gas extremes but we don't have a good theory that tells us exactly where and why. (Moreno and Mossio, 2015, p. vii)

I suggest that recent work on the mesoscale physics (i.e., processes at the 10^{-3}–10^{-2} m scale) of cell collectives and on the products of the genetic toolkit that mediate these cell associations in animal tissues permit us to formulate a theory of the organizational properties of metazoan organisms. Specifically, I draw on earlier work on "dynamical patterning modules" (Newman and Bhat, 2008, 2009), developmental units in which the products of certain conserved genes mobilize physical forces and processes at the multicellular scale, and on "biogeneric materials" (Newman, 2016a). The latter are condensed forms of matter (in Hooker's formulation, "lying between the crystal and gas extremes") that, while arising from collections of living cells, exhibit predictable morphological motifs due to their having properties in common with nonliving materials such as liquids and elastic sheets. These concepts can help us understand how and why certain developmental forms and patterns of animal bodies and organs (multilayering, lumens, segmentation, appendages) have appeared recurrently throughout evolution.

In addition to their morphological characters, animal bodies exhibit coordinated and integrated functioning of distinct cell types. These alternative states of cellular activity, which depend on, but are not entirely reducible to differential gene expression, are partly conserved across the animal phyla, and the means of their progressive emergence from unicellular ancestors of the metazoans are only now becoming known (Brunet and King, 2017; Sebé-Pedrós et al., 2018). While the range and stereotypical character of the cell types of animals are not inherent to multicellular matter in the same way that the morphological motifs of these organisms are, their functions are intrinsic to the biology of their ancestral cells. The partitioning of these functions among up to several hundred different cell types depended on certain innovations in gene transcriptional regulation. The relations between the mechanisms of gene expression employed in the animals' immediate unicellular ancestors and those regulatory mechanisms that distinguish them from other kinds of organisms are key to their ability to allocate inherent functions to specialized cells and will therefore be described in detail.

Beyond the inherency of tissue morphology and cell function described above, the development and evolution of complex bodies and organs involves a third category of processes that depends on, but is not a mere combination of, these two: the reliable spatial arrangement of different cell types within three-dimensional tissues (Salazar-Ciudad et al., 2003). These processes, collectively termed cellular *pattern formation*, are also inherent to animal systems, but in a way still different from morphogenesis and differentiation. They arise from the fact that biogeneric materials, having subunits that are living cells, are dynamically more complex than their nonliving counterparts. In multicellular systems, short- and long-range cell-cell communication *via* contact, chemical and mechanical signaling or electrical coupling, along with positive and negative feedback effects, produce lateral inhibitory effects, oscillations, synchrony, and repeated structures. Together, the animal-specific set of morphogenetic, differentiative, and patterning inherencies appear to be the organizational preconditions of subsequent metazoan evolution.

For each of the processes I discuss I will describe the main genes and molecular mechanisms involved in distinguishing animals in general from their inferred unicellular ancestors and some of the major organizational differences within Metazoa from one another. This has led to the identification of biological properties dependent on (1) novel genes or regulatory motifs coincident with emergence of Metazoa, (2) novel genes acquired after metazoan origins, and (3) ancestral genes repurposed to novel functions when they came to operate on the multicellular scale (**Table 1**).

If, as I suggest, inherency of form, function, and pattern make key features of animal body plans [and, as we propose elsewhere, those of plants (Benítez et al., 2018)] highly constrained and readily achieved, the role of natural selection for incrementally improved fitness becomes less decisive as the driving force of

TABLE 1 | Novel inherent properties in animal development and evolution.

Property	Gene or molecular motif	Character
1. Properties dependent on novel genes or regulatory motifs coincident with emergence of Metazoa		
Liquid-tissue state	Classical cadherins	Multicellularity; layering
Regulated cell polarity	Wnt	Lumens; elongated tissues
Capacity to exaggerate intrinsic cell functions	Enhancers; PcGl proteins	Differentiation
Morphogen gradients	Hedgehog, BMPs	Simple cell patterns
2. Properties dependent on novel genes acquired after metazoan origins		
Liquid-crystalline-tissue state	Vang/Stbm	Tissue elongation
Wettable substrata (basal lamina)	Peroxidasin	Appendages, glands
Lateral inhibition; oscillation of gene expression	Notch, Hes1	Complex cell patterns
Multiple alternative cell types	MyoD, PPARγ, SMAD	Complex tissues, organs
3. Properties dependent on ancestral genes repurposed into DPMs in the multicellular context		
Cell-cell cohesion in liquid tissues	Grainyhead	E-M Transformation
Apicobasal cell polarization	β-catenin	Epithelia and lumens
Nonliquid cellular assemblages *via* matrices	Collagen IV	Mesenchymal tissues
Cell-cell electrical coupling	Voltage-gated channels	Bioelectrical integration

Each list is nonexhaustive but contains the most important examples of its respective category.

organismal diversification and complexification than generally believed. In the concluding section, I will discuss the relationship between these evolutionary perspectives.

INHERENT FORMS IN ANIMAL ORIGINATION AND EVOLUTION

In this section, I discuss the structural motifs that would inescapably characterize the metazoans once they emerged from an ancestral population of unicellular organisms. These characters were due to animal tissues being constituted as a new form of *mesoscale matter* by the innovation of molecular links between cells that also harnessed the cells' motile activity. The physics of these living materials generated a specific array of morphological motifs with a constrained range of geometric and topological relationships among them. Later, with the appearance of additional novel genes (some of them phylogenetically unprecedented), new physical forces and constraints were mobilized in some of these multicellular entities, leading to successively more complex forms. The morphological properties described, which can be identified with phylotypic bodies and organs, were generally predictable properties of these materials and therefore inherent to animal tissues.

The Liquid-Tissue State: The Defining Character of Metazoa

The animals or metazoans arose around 700 million years ago within an evolutionary lineage of eukaryotic cells – the holozoans – which were also ancestral to present-day unicellular choanoflagellates (reviewed in Newman, 2016b). The animals are multicellular, and in all metazoan phyla cell-cell attachment is mediated by members of the cadherin family of cell adhesion molecules (CAMs). Organisms of all these phyla express cadherins with a cytoplasmic domain that links to the actin cytoskeleton and permits cells to remain tightly bound to their neighbors, while they move past one another (reviewed in Newman, 2016a). With one exception [the ctenophores – "comb jellies" – a group that is an outlier of the metazoans in several respects (Lanna, 2015; King and Rokas, 2017; Whelan et al., 2017)], cell-cell attachment during development is mediated by the so-called "classical" cadherins (reviewed in Newman, 2016). These proteins are present in the morphologically simplest, and earliest diverging, animals, the sponges (Porifera) and the single extant species of Placozoa, *Trichoplax adhaerens*, the so-called *basal metazoans*. The cytoplasmic domain of the classical cadherins is not found in any nonmetazoan holozoans and has no counterpart in any other sequenced organisms. [The cadherins of ctenophores have a different cytoplasmic domain (Belahbib et al., 2018).] Such unprecedented coding sequences have been termed "*de novo*" or "orphan" genes (Bornberg-Bauer et al., 2015; Schmitz et al., 2018).

Materials with subunits that cohere but can nonetheless perambulate in a random fashion are, by definition, liquids. They exhibit surface tension and the propensity (like oil and water, for example) to phase-separate from other such materials with different cohesive properties. The liquid properties of metazoan cell clusters are based on biological functions rather than purely physical attributes. For example, in nonliving liquids, cohesion of molecular subunits is mediated by electrical forces, and the random movement of those subunits is due to Brownian motion driven by thermal energy (March and Tosi, 2002). In animal tissues the subunits are cells, whose cohesion is mediated by classical cadherins and whose random movement results from surface protrusive activity and amoeboid motion driven by cytoskeletal mechanics. Nevertheless, the physical laws that describe these two categories of liquid are sufficiently similar, so that many properties of the biological materials are predictably "generic" (Newman and Comper, 1990; Forgacs and Newman, 2005). Liquid tissues and other cell collectives with biologically based generic physical properties are thus termed *biogeneric* (Newman, 2016a).

The "liquid-tissue" state enabled by metazoan cadherins was a primitive defining condition of animal life. Liquid tissues have several emergent features that appeared independently of natural selection in the transition between ancestral colonial holozoans and ancestral metazoans. Liquid droplets, for thermodynamic reasons, assume the geometry with smallest surface-to-volume ratio, a sphere. Embryos and newly formed organ primordia are therefore spherical by default (Steinberg and Poole, 1982). In liquids that contain two different kinds of subunits (molecular species, in most physical examples), one of which has greater affinity for its own type than the other (due, in liquid-tissues, to different amounts or types of cadherins on cell surfaces), there is a separation of phases. The interface between the phases can be flat or curved, depending on the balance of self- and nonself-affinities, and in extreme cases, one liquid phase will completely engulf the other. Such configurations are common in cell mixing and sorting experiments (Forgacs and Newman, 2005). Gastrulation, the organization of a coherent mass of cells into closely apposed but nonmixing layers, which is the first step in body plan formation during development, is directly attributable to phase separation in some animal systems (Krieg et al., 2008). In other organisms, where the connection to phase separation is not as direct, more elaborate mechanisms of gastrulation may have built on morphological templates arising from this inherent tissue behavior early in their evolution (Newman, 2016a).

Phase separation of cell populations in a liquid tissue mass illustrates the concept of dynamical patterning modules (DPMs) mentioned above (Newman and Bhat, 2008; Newman et al., 2009). In this example, cells with different homotypic and heterotypic adhesive strengths undergo spontaneous spatial redistribution to a predictable pattern by mobilizing, in multicellular clusters, a physical force (cell-cell adhesion) that is irrelevant to individual cells. The molecules that mobilize the physical effect are classical cadherins, which arose concomitantly with the metazoans. Other such examples of DPMs co-originating with Metazoa will be described in what follows. But in still other cases, proteins that evolved in unicellular ancestors took on novel DPM roles in the multicellular context, or novel gene products acquired later in animal evolution took on developmental functions by harnessing new physical processes in the form of DPMs.

In addition to having cytoskeletally linked classical cadherins, the metazoans are distinguished from all other life forms by the protein Wnt, which is similarly specified by an orphan gene unique to this group of organisms (reviewed in Newman and Bhat, 2009)[1]. Wnt induces reorganization of the cytoskeleton, acting *via* surface receptors (e.g., Frizzled, LRP5/6), β-catenin [itself unique to Metazoa among its close unicellular relatives, but with a homolog in the more distantly related *D. discoideum* (Miller et al., 2013; Gul et al., 2017)], and the PAR complex of adapters and protein kinases (Lang and Munro, 2017). The PAR complex predated the metazoans and serves to make the surfaces of cells nonuniform by mediating the insertion of different sets of integral membrane proteins on different portions (e.g., apical, basolateral) of the plasma membrane. In a manner that is physically analogous to polar molecules spontaneously organizing into micelles in water, liquid tissues containing apicobasally (A/B) polarized cells spontaneously form lumens and interior spaces (Forgacs and Newman, 2005).

Planar Liquid-Crystalline Tissues and the Eumetazoans

To move beyond the simple forms of sponges and placozoans, two additional features were required, *planar cell polarization* (PCP) and the capacity to form a *basal lamina*. These effects, in turn, required several molecular innovations, using genes not present in the basal metazoans. One of these effects is a "noncanonical" Wnt-activated signaling pathway that reorganizes the cytoskeleton *via* the PAR complex but does so (in contrast to the canonical pathway described above) in a β-catenin-independent fashion. This pathway is facilitated by the inhibitory effect of the β-catenin antagonist Vang/Stbm, still another orphan gene, in this case unique to the *eumetazoans*, animals that develop from two or three distinct embryonic layers (diploblasts and triploblasts). The PCP pathway may have arisen in the common multicellular ancestor of the metazoans, but key components were lost in most sponge groups and placozoans, and it is incompletely present in ctenophores (Schenkelaars et al., 2016).

Unlike the canonical Wnt pathway, the PCP pathway causes cells to be polarized in their shapes or spatial orientation rather than over their surfaces. In a multicellular context, cells that elongate due to PCP align and intercalate, which causes the tissue mass to narrow in the direction of intercalation and elongate orthogonally to it. This tissue reorganization, termed *convergent extension* (reviewed in Forgacs and Newman, 2005), is the biogeneric counterpart of the deviation, in liquid crystals, from the spherical default shape of a liquid droplet due to alignment of polymers or anisotropic nanoparticles (reviewed in Newman, 2016).

The evolutionary appearance of these "liquid-crystalline tissues" was accompanied by the capacity to form *epithelia*, planar tissues whose cells reside on a stiff extracellular layer, the *basal lamina*[2]. Direct cell-cell attachment in such tissues continues to be mediated by cadherins, as in the *epithelioid* mode of tissue organization that characterizes some nonplanar tissues in all metazoans. But the basal lamina constitutes a substratum upon which the liquid-tissue can adhere and spread, a physical process referred to as "wetting" (Jensen et al., 2015). Depending on the relative degree of affinity between the basal regions of the apicobasally polarized cells that produce the basal lamina and the substratum itself, and their lateral affinity to one another, the epithelium will be columnar, cuboidal, or squamous.

[1]Glass sponges, of the poriferan class Hexactinellida, have syncytial tissues. Although they contain and utilize the "canonical" Wnt pathway (see infra), they lack the extracellularly acting Wnt morphogen itself (Windsor Reid et al., 2018).

[2]There are differences in the literature on what constitutes a "true" epithelium. Here, since my focus is on physical distinctions among tissue types and their role in phyletic transitions, I reserve this term for planar tissues underlain by a basal lamina, which are present in all eumetazoans and in the homoscleromorph sponges. Others use the term more broadly for any cell layer with junctional complexes that permit it to serve a barrier function, a feature found in all sponge groups (Leys and Riesgo, 2012).

Basal laminae are absent in the placozoan and most sponge species but are present in all eumetazoans (reviewed in Newman, 2016). The basal lamina is predominantly composed of the extracellular matrix (ECM) protein type IV collagen, which is produced by all metazoans, but only in the more morphologically complex, eumetazoans and the homoscleromorph sponges are its molecular subunits chemically crosslinked into a basal lamina (Fidler et al., 2017). This crosslinking is catalyzed by the enzyme peroxidasin, which is specified by an orphan gene unique to this group of organisms (Fidler et al., 2014)[3]. The capacity to form epithelia, in coordination with ability of the main body mass and subsidiary buds to undergo convergent extension, permitted diploblastic, and eventually triploblastic, eumetazoans to generate elongated bodies, appendages, tentacles, and tissue ridges, folds, and clefts. These morphological motifs were not only enabled by these new tissue organizational properties, but they were also made readily achievable.

Epithelial-Mesenchymal Transformation, Mesenchymal Condensation, and Triploblasty

With the evolutionary emergence of a third tissue layer, which came to be sandwiched between the two epithelial germ layers in one or more diploblastic ancestors, the *triploblasts*, the most ecologically successful of the metazoan groups, came into existence (reviewed in Newman, 2016). The middle layer – mesoderm – enabled generation a new array of morphological motifs, which were correspondingly inherent to these new organisms. These intrinsic organizational propensities led to triploblasts having more complex body plans than diploblasts, and, in contrast to the latter, geometrically complex organs containing multiple cell types. An important reason for this was that the surface and enteric epithelia in triploblasts could continue to serve (as in diploblasts) as a boundary between the organism and its environment, while the mesodermal layer, having no analogous protective role, could disaggregate into a new physical state consisting of cells dispersed in a matrix, a *mesenchyme*. Mesenchymal tissues, on their own, or in interaction with an adjacent epithelium, could form structures that were impossible in simpler animals, but predictable, based on the additional physical effects to which they were susceptible (reviewed in Newman, 2016b).

The morphologically simplest triploblasts develop with a single body cavity, the digestive tube. Such organisms, including flatworms like planaria, have ovaries and testes, and clusters of neurons, termed ganglia. Most triploblasts, however, such as arthropods, mollusks, and chordates, have a second body cavity, the coelom, within which digestive tube is suspended. In these coelomate triploblast lineages, organ complexity took a dramatic leap. This is because instead of their being just two epithelial-mesenchymal interfaces, as in acoelomates, there were now four. The interaction of the epithelium of the body surface (the ectoderm) with its underlying mesenchyme produced, in various species, bristles, hairs, feathers, teeth, and limbs, while the interaction of body lining epithelium (the endoderm) with its overlying mesenchyme produces intrinsic and extrinsic elaborations of the digestive tube, respectively, villi and crypts, and the liver and pancreas. The blood vessels and heart, lungs, and urogenital organs, as well as various glands are generated by invagination and evagination, folding and branching, and thickening and thinning of composite epithelial-mesenchymal layers in other regions of the developing embryo (reviewed in Newman, 2016a).

Similar to the rise of the animals as a whole, and in their turn, the eumetazoan subgroup, the appearance of the triploblasts was accompanied and facilitated by novel gene products. These included structural ECM molecules such as fibronectin and tenascin (Hynes, 2012), glycans bearing unusual sugar moieties, and enzymes such as Has2 [which may have had a viral origin (Blackburn et al., 2018)] that generate ECM polysaccharides such as hyaluronan, which induce cell motility (Spicer and Tien, 2004). These molecules led to the ingression, typically accompanied by disaggregation of an epithelial layer (*epithelial-mesenchymal transformation*; EMT), in one or more diploblastic ancestors. Because of the heterogeneity of the mesoderm-inducing components, it is possible that triploblasts might not be monophyletic (Burton, 2008).

Mesenchymes and their mature counterparts, connective tissues, lack direct integration of cell-cell attachment with cytoskeletally driven motility and are therefore not liquid tissues by the definition above. They are nonetheless biogeneric materials, variously viscous, viscoelastic, or solid, depending on the composition of their ECMs or (as described below) the state of mechanical stress of the contained cells. As with liquid- and liquid-crystalline tissues, mesenchymal tissues have characteristic inherent forms. One prominent example is *cell condensation*, wherein groups of cells embedded in ECM are drawn closer to each other (a structural motif unavailable in epithelioid tissues), forming transient clusters with epithelial-type contacts. Condensations serve as the primordia of hairs, teeth, and feathers, as well as the bones of vertebrate fins and limbs (reviewed in Forgacs and Newman, 2005).

Cnidarians, which are diploblasts, can form solid-phase exoskeletons consisting of calcium-based mineral or the polysaccharide chitin. In later diverging triploblastic forms such as bivalve mollusks and arthropods, exoskeletons continue to be produced by epithelia. But the presence of mesoderm enables some triploblasts, such as echinoderms and chordates to also produce endoskeletons (reviewed in Brown, 2011). These are typically based on patterned arrays of mesenchymal condensations (Hall and Miyake, 2000) and become solid by the production of collagenous ECMs, containing a high density of proteoglycans in cartilage, or hydroxyapatite mineral in bone and the dentin of teeth. (Tooth enamel is a mineralized exoskeletal tissue.)[4]

[3]The placozoan *Trichoplax* has a peroxidasin gene but lacks a basal lamina (Fidler et al., 2017), possibly due to the absence of the requisite crosslinkable amino acid residues in its type IV collagen molecules (Fidler et al., 2014).

[4]The tissue layers of most sponge groups exhibit morphological plasticity that allows them to assume both epithelium- and mesenchyme-like configurations, though these are transient and interconvertible, and thus physically different from the germ layers of triploblasts. Within the quasi-mesenchyme consisting of the extracellular matrix (mesohyl) and its resident cells, however, some sponges species form a spicule-containing endoskeleton (Leys and Hill, 2012).

Mesenchymal tissues can also undergo a different rigidifying transformation termed "fluid-to-solid jamming" (Mongera et al., 2018). This is a phase transition between the liquid-tissue state and a solid state [analogous to glass transitions in supercooled fluids (Sadati et al., 2014)], in which persistent cytoskeletally mediated stresses at a supracellular scale solidify the tissue. Jamming is employed as a mechanism (alternative to convergent extension) of elongation of the primary body axis in zebrafish embryos. It is impaired by knockdown of a cadherin, suggesting that it depends on a shift from mesenchymal ECM-based tissue cohesion to an epithelioid mode of cohesion, like mesenchymal condensation. The nominally "solid" phase after jamming therefore exhibits some liquid-tissue properties. Specifically, cell-scale stresses fluctuate rapidly, enabling cell rearrangements, thus effectively melting the tissue at the growing end and permitting longer-term supracellular-scale stresses to guide morphogenetic flows in fluid-like tissue regions.

Metazoan tissues thus have inherent morphogenetic propensities due to their liquid, liquid-crystalline, planar, and solidifying nature. These comprise mutlilayering and lumen formation (which is seen in all groups, even the most basally diverging ones), body elongation and appendage formation (which are seen in all eumetazoans), and organ formation (found in all triploblasts). The bodies of triploblasts can also become segmented by the deployment of oscillatory processes intrinsic to cells and tissues (see below, "Pattern Formation: Inherency in the Integration of Form and Function"). The structural motifs of organs, tissue multilayers, folds, ridges and projections, straight and branched tubes, segments and lobes resemble those that produce the phyletic body plans and employ the same conserved set of developmental toolkit genes. Since the relevant biogeneric properties, the genes that mobilize them, and the inherent forms they facilitate, arose very early in the history of the metazoans, gradualist natural selection did not bear the exclusive burden of producing the major structural features of animal bodies and organs.

Morphological motifs are not cell types, however, and it is through the functional specializations of their cells that the physiology of animal life is realized. I describe next how ancestral and derived inherency rather than classical gradualist trial-and-error scenarios plausibly accounted as well for the evolution of cell differentiation in metazoans.

INHERENT CELL FUNCTIONS IN ANIMAL ORIGINATION AND EVOLUTION

Just as the structural motifs of animal bodies (e.g., layers, lumens, segments) are inherent to the materials that constitute them, so are the functional capabilities (e.g., contractility, excitability, detoxification) that came to be reflected in differentiated cell types, tissues, and organs. The reasons are not the same, however. Notwithstanding speculations based on the dynamical multistability of gene regulatory networks (GRNs; Kauffman, 1969; Furusawa and Kaneko, 2002), the full range of different cell types compatible with an organism's genome does not seem to be the global state-space attractors of any unitary physical system. Here I do not mean to suggest that the nonlinear dynamics of GRNs and transitions between adjacent attractors of such systems are not suitable accounts of progression along developmental lineages, e.g., Huang et al. (2007). Such steps take place in functionally delimited domains of the full gene expression state space, with modest combinatorial differences in transcriptional determinants. There are persuasive examples of such phenomena (Mojtahedi et al., 2016; Corson and Siggia, 2017; Verd et al., 2017). However, the differential equation or Boolean representations of GRNs usually employed in the global models have long been criticized on conceptual grounds (Rosen, 1991) and have been further called into question by new findings on transcription factor disorder and condensed state chemistry reviewed in this section (see Niklas et al., 2015).

Instead, I suggest that the inherency of the specialized functions of multicellular organisms, such as animals and plants, resides in the fact that they are exaggerated counterparts of life-sustaining activities of their ancestral unicellular forms. Cellular functions in general have a history that goes back to the transition between the nonliving and living state (Arnellos and Moreno, 2012) but are not entirely shared in all clades. Some differentiated cell types amplify what are universal cell functions, but others draw on more directly ancestral physiology.

In this section, I show how cell types likely arose through the recruitment and partitioning of ready-made ancestral cellular functions by a highly plastic metazoan-specific gene regulatory apparatus. Although based on the "write and read" histone modification language that had evolved in the earliest eukaryotes (Prohaska et al., 2010), this apparatus is unlike that of any extant unicellular relatives. It appeared coincidentally with the metazoans, incorporating elements found in present-day holozoan and more distantly related fungi, as well as some unique elements not found in any other known life-forms. The new gene regulatory mode came to operate in a multicellular context that (as I will show below in relation to the role of the ancestral grainyhead transcription factor) was brought about by some of the same effects. Once metazoan lineage-defining transcription factors appeared (an evolutionary step that is currently enigmatic), partitioning of ancestral functions into differentiated cell types followed in a straightforward and potentially rapid fashion.

To reiterate, although metazoan-type mechanisms of cell differentiation were not inherent in unicellular progenitors, most cellular functions that came to be specialized in differentiated cell types were. Furthermore, once the enhancer-based, developmentally hierarchical means of exaggerating gene activity were in place, the evolution of developmental lineages of initially related, but progressively divergent, cell types was readily achieved.

Enhancers, Silencing Complexes, and the Gene Regulatory Ground State of the Metazoans

While the genes of all categories of organisms are regulated by nearby upstream cis-acting promoters, the additional effects of enhancer sequences, which can be located upstream or downstream from their targets, or in introns, are, with the apparent exception of Placozoa, hallmarks of metazoan gene control. Enhancers are novelties of Metazoa or its direct

antecedents: they are absent in extant unicellular holozoans (Sebé-Pedrós et al., 2016). There are more enhancers than regulated genes [as many as 50,000 in mammalian genomes, for example (Heinz et al., 2015)], and while their TF-binding motifs are similar to those of promoters (Arenas-Mena, 2017), they function differently. Enhancers mediate the high levels of expression of the characteristic genes of terminally differentiated cells, as well as the precise spatiotemporal generation of cell and tissue types during development due to their responsiveness to patterning cues (Lenhard et al., 2012; Zabidi and Stark, 2016) (see section "Pattern Formation: Inherency in the Integration of Form and Function").

Enhancers show not only evidence of conservation but also divergent usage over evolution and between species (Chen et al., 2018). They are employed in sponges and all eumetazoans, with an indication of their presence in ctenophores, although not the placozoan *T. adhaerens*, which appears to rely solely on promoters to generate its few cell types (Sebé-Pedrós et al., 2018). Enhancers regulating stage- and cell type-specific "developmental" and "terminal differentiation" genes are often located at significant linear distances from the respective promoters and interact with a partly different set of TFs from the more proximal enhancers regulating ubiquitously expressed "housekeeping" genes (Zabidi et al., 2015).

The origin of enhancers may reside in the duality of promoter functional architecture in ancestral cells. Unicellular holozoans have inducible promoters that respond to external cues as well as promoters that regulate constitutive genes, with the latter often exhibiting distal cooperative interactions. Arenas-Mena (2017) has proposed a scenario by which inducible promoters were converted in few steps to a distally acting enhancer mode of developmental gene regulation in metazoans.

In metazoans, chromatin loops containing cell-type regulated DNA sequences enter or exit distinct liquid-phase proteinaceous condensates along with other loops containing enhancers and a large multi-subunit obligatory regulatory complex called Mediator, forming "topologically associating domains" (TADs) within the interphase nucleus (Galupa and Heard, 2017; Furlong and Levine, 2018; Plys and Kingston, 2018). Thousands of enhancers can be recruited to a given gene by lineage-determining transcription factors (LDTFs) (Heinz and Glass, 2012; Link et al., 2015) such as the muscle "master regulator" MyoD (Blum and Dynlacht, 2013). This occurs in a highly cooperative and synergistic fashion, with the number of enhancers recruited being responsive to developmental cues external to the differentiating cells.

Transcription in animal cells is initiated by the action of the master coactivators p300 and CBP (Chan and La Thangue, 2001). These highly homologous multidomain proteins contain histone acetyltransferase domains that relax the structure of nucleosomes at promoters and enhancers of all or most transcribed genes, enabling the recruitment of RNA polymerase II and TFs to those sites. Mediator, p300/CBP, and TFs all contain intrinsically disordered regions (IDRs) that assume different 3D structures under different conditions (Liu et al., 2006; Minezaki et al., 2006). The IDRs are required for the formation of nuclear TADs and are the basis for p300/CBP's role as an interaction hub with a multitude of TFs and coregulators [most prominently nuclear receptors (NRs)] that mediate tissue responses to developmental and physiological cues (Dyson and Wright, 2016). Expression of a specific gene is caused by p300/CBP binding to transactivating protein domains (also abbreviated as TADs) of the TFs and NRs targeting the gene's promoter, followed (based on evidence from some "super enhancers") by induction by TF TADs of phase separation of Mediator into activating droplets (Boija et al., 2018).

Extant unicellular holozoans such as *Capsaspora owczarzaki* contain p300/CBP, and their promoter nucleosomes exhibit p300/CBP-specific histone acetylation marks. This indicates that the gene regulatory role of p300/CBP is phylogenetically older than that of enhancers (Grau-Bové et al., 2017). These coregulators contain bromodomains, which in metazoans promote enhancer-dependent expression of developmental and cell-type specific genes (Wei et al., 2008). Metazoans also express the bromodomain-containing protein Brd4, which co-localizes with LDTFs on active enhancers, where it recruits Mediator and RNA polymerase II (Lee et al., 2017).

Suppressing some genes can be as important as activating others in shaping cell type identity. In metazoan development and terminal cell differentiation, this is accomplished by "silencing" complexes such as the Polycomb-group (PcG) proteins (Khan et al., 2015) and other lysine methyltransferases such as SUV39H (Jih et al., 2017). These inhibit gene function by methylating histone 3 in the nucleosomes on both promoters and coding regions of genes that are inactivated at developmental stages and in terminally differentiated cell types, but not in the nucleosomes of housekeeping genes (El-Sharnouby et al., 2017). Silencing mechanisms based on PcGII proteins evolved in a common ancestor of holozoans and fungi and were retained in yeast and presumably the direct holozoan progenitors of Metazoa (Kingston and Tamkun, 2014; Steffen and Ringrose, 2014), though they were lost in the unicellular holozoan *C. owczarzaki* (Grau-Bové et al., 2017). Silenced sequences are converted into stably inactive heterochromatin by PcGI proteins, which are metazoan innovations (Grossniklaus and Paro, 2014; Steffen and Ringrose, 2014). Like transcription hubs, silencing complexes are phase separated into biomolecular condensates (Tatavosian et al., 2019), and their association with the latter appears to be regulated by short-lived noncoding RNAs (eRNAs) transcribed from enhancers (Chan et al., 2018).

To summarize, gene activation mechanisms based on p300/CBP activating and PcGII and SUV-type gene silencing mechanisms were conveyed to metazoans from ancestral unicellular holozoans, while enhancers and PcGI gene heterochromatin sequestration mechanisms accompanied the origination of these organisms. Understanding the emergence of cell differentiation, then, requires focusing on an inferred population of holozoan organisms, which, unlike other members of this group, contained enhancers, which are promiscuous and condition dependent in their gene activating activities, and silencing complexes, which since they depend on enhancers, are also subject to microenvironmental input. These organisms also contained β-catenin, a component integral to metazoan cell adhesion (see section "Inherent Forms in Animal Origination and Evolution") that is also a key element of externally regulated gene expression. This was the biological "ground state" of metazoan evolution.

Grainyhead and the Origin and Stabilization of Metazoan Multicellularity

It is unknown whether multicellularity preceded or followed the appearance of the described metazoan-specific modes of gene regulation in the ancestors of animals. But for a single organism's cells to diversify into functionally specialized types, multicellular clusters must already exist. At the level of gene regulation, this requires factors that promote cell-cell adhesion and inhibit cell-cell detachment. The metazoan innovations of the classical cadherin cell attachment molecules and their associated cytoskeletal adaptors have been discussed above. This is one side of the multicellularity dyad. Cell division is commonly followed by cell separation, however, and this function must be suppressed for organismal identity to be determinate.

The grainyhead (Grh) family of TFs has a deep evolutionary history in the opisthokonts, i.e., the fungi and holozoans, including all metazoans, where they are intimately involved in epithelial organ development and epithelial barrier repair after tissue damage (Wang and Samakovlis, 2012). In the fungus Neurospora, the TF GRHL has a DNA-binding specificity close to that of animal grainyhead proteins (Pare et al., 2012). Moreover, Neurospora grhl mutants [allelic with conidial separation-2 (csp-2) mutants] are defective in spore dispersal. A conserved role for Grh-like TFs in the development and remodeling of cell-cell adhesion connections thus appears to have been already in place at the origin of the metazoans.

We can speculate on how Grh may have promoted metazoan multicellularity in light of other components we know to have been present at the metazoan ground state by referring to experiments on extant organisms. In the mouse, the Grh gene Grhl2 is crucial for the expression of the classical E-cadherin as well as claudin4, a junctional protein with homologs in sponges (Werth et al., 2010; Riesgo et al., 2014). E-cadherin promoter activity depends on an intronic enhancer, which binds Grhl2, and the absence of the TF leads to a specific decrease in acetylation and other activating histone modifications (Werth et al., 2010). In addition, Grhl2 has a nearly unique suppressive activity on gene activation by p300, exerted at the histone acetylase domain of the master coregulator. It performs this role in the negative regulation of several metalloproteinases, thereby inhibiting EMT and cell detachment events that lead to tubulogenesis (Pifer et al., 2016). Grainyhead TFs therefore have synergistic, reciprocal effects on promoting cohesion of cell clusters. They may have acted constitutively during early metazoan evolution, only to have become subject to downregulation in later-emerging complex forms in which EMT is a mode of development.

As noted, Grh can reduce expression of certain genes, but it can simultaneously increase expression of others. It shares this property with just a minority of TFs, referred to as "pioneer" regulators, which act at upper levels of enhancer hierarchies to prime target regions for the binding of activating or inhibitory cofactors (Jacobs et al., 2018; Mayran and Drouin, 2018; Gehrke et al., 2019). On this basis, it is possible that the repurposing of Grh was an originating step not only in metazoan multicellularity but also in metazoan developmental gene regulation. Once constituted with components inherited from unicellular ancestors (e.g., p300/CBP transactivating hubs, Grh factors, β-catenin, PcGII proteins) or emerging at the unicellular holozoan-metazoan interface (e.g., enhancers; classical cadherins and their cytoskeletal adaptors, including PcGI proteins), a new category of organism, a "proto-metazoan" could proceed to create a multiplicity of differentiated cell types.

Cell Differentiation as the Amplification of Inherent Cell Functions

Opisthokont progenitors and ancestral holozoans likely exhibited alternative cell phenotypes and states. While some of these may have been antecedents of differentiated cell types in metazoans (Brunet and King, 2017), there are more than 250 cell types that characterize complex animals such as the vertebrates, and they all could not have arisen from ancestral ones. Furthermore, while GRNs for specific cell types (e.g., neurons, skeletal myoblasts) exhibit a fair degree of conservation across animal phyla (Arendt et al., 2016), regulatory mechanisms for the "same" cell types, and even for homologous genes show great variability across, phyla, species, and even in individuals within a species, often making assignment of similarly acting or appearing cell types as homologs ambiguous (Halfon, 2017). It has been suggested, for example, that neurons of ctenophores and cnidarians were independently evolved (Moroz and Kohn, 2016), although this remains controversial. As mentioned above, the mesoderm in triploblastic phyla might be polyphyletic (Burton, 2008), and within the deuterostomes, skeletogenesis is regulated by different TFs in chordates and echinoderms (Rafiq et al., 2012; Burke, 2018; Taylor and Heyland, 2018).

Cell-type homologies based on descent-with-modification exist because early-radiating members of a phylogenetic lineage can transmit their cell-type regulatory modes to their descendants. However, several phenomena, partly overlapping, contribute to "developmental system drift" that can disconnect cell types from their originating GRNs (True and Haag, 2001; Halfon, 2017). These include, as mentioned above, variable enhancer use across species, the fact that enhancers and promoters do not have entirely disjoint roles and can substitute for one another, the fact that developmental TFs have high concentrations of IDP domains and consequently operate at different cis-acting elements in different contexts, including different stages in the establishment of a cell-type lineage (Niklas et al., 2015; Masoudi et al., 2018), and the fact that a TF can act in entirely opposite fashions depending on the context (Murgan et al., 2015; Lukoseviciute et al., 2018).

Cell differentiation proceeds according to relatively routinized programs in present-day metazoans, but it has likely been less programmed earlier in evolution. The pan-metazoan secreted molecule Wnt, acting at the cell surface, typically initiates gene expression changes during early animal development utilizing a conserved ("canonical") signal transduction pathway. However, the key transcriptional regulatory component of this pathway, β-catenin, which resides at cadherin-cytoskeletal linkages at the inner surface at the cell membrane until it is mobilized for this function, can also be activated at other pathway nodes by environmental influences, such as mechanical stress (Zhang et al., 2016) and hyperoxia (Popova et al., 2012). The most common

co-transcriptional activators of β-catenin, the TCF/LEF family of transcription factors, can also be independently modulated, with numerous identified regulators of its transcription including the Wnt pathway itself (Cadigan and Waterman, 2012). Further, both β-catenin and TCF are highly promiscuous regarding the TFs they partner with, due to the intrinsically disordered (protein) TADs at the N- and C-terminal regions of β-catenin (Zhao and Xue, 2016). Moreover, β-catenin can also function independently of TCF/LEF (Doumpas et al., 2019). In ctenophores, deployment of β-catenin and TCF/LEF appears to entirely circumvent Wnt during early development (Pang et al., 2010).

Single-cell transcriptomic analysis of developmental systems shows a remarkably variable set of regulatory modes. In early *Drosophila* embryogenesis, for example, where the complex interaction of about 1,000 enhancers defines the activity of a few segmentation genes, some genes express across the field of cells in random bursts, others in broad, progressively refined patterns, and still others in sharp synchrony, to eventually achieve precise and reliable levels of expression (Vera et al., 2016; Bothma et al., 2018). Spatiotemporal precision, as discussed in the following section, is probably imposed at a different level of organization from that on which cell type identity is determined.

A highly plastic but ultimately globally controllable transcriptional regulation apparatus that distinguished among housekeeping, developmental, and specialty genes thus arose coordinately with Metazoa and was in operation in basal metazoans like sponges (though apparently not fully in placozoans). The subsequent appearance of 200 or so cell types was the result of recruitment of this metazoan ground state system of gene control for the enhancement and elaboration of preexisting (and thus inherent) unicellular physiological functions. Whether one or more of the cells in a multicellular entity performed any of these intrinsic functions, e.g., motility, extracellular matrix production, light or nociception, oxygen capture, fuel storage, detoxification, and so forth, may or may not have contributed to the survival of the novel differentiated form. This was decided by natural selection (based on possibilities afforded by the new feature) or construction and occupation of previously unexploited niches.

"Proto-cell types," providing, for example, evolutionary starting points for the evolution of muscle, neurons, connective tissue, blood, glands, and liver, need not have originated *via* germline encoding (in this scenario, the germline itself may have arisen by this same "plasticity first" process), or in a reproducible spatiotemporal or even quantitatively regulated fashion. That is, the novel cellular phenotypes could have appeared in a cell mass randomly or reversibly. More programmed modes of appearance could have followed through the self-organizational properties of a critical mass cells (see the following section) and by genetic assimilation, once a germline was in place (Newman, 2011).

The most enigmatic ingredient in this scenario for the transition from the metazoan ground state to complex animals with scores or hundreds of cell types was the appearance of LDTFs and related TFs. These initiate the expression of suites of genes that start an embryo or organ primordium on a progressive developmental trajectory leading, in a hierarchical cascade, to one or more terminally differentiated states (Obier and Bonifer, 2016). The LDTFs include such proteins as MyoD, Nkx3-1, neurogenin, Sox9, and PPAR-γ, which specify (with accessory factors), skeletal muscle, heart muscle, neurons, cartilage, and fat, respectively (reviewed in Newman et al., 2009). Included among these are pioneer TFs, like Grh. Some LDTFs act in a mutually antagonistic fashion ensuring, in concert with silencing effectors like PcGI proteins, that mixed-identity cell types are not produced (Sunadome et al., 2014). Although proteins related to certain LDTFs are present in the genomes of unicellular holozoans or yeast, most are novelties of Metazoa (Grau-Bové et al., 2017). They are therefore orphan genes like many of those involved in the morphogenetic innovations described above in "Inherent Forms in Animal Origination and Evolution." Although there are speculations on the origins of such genes (Bornberg-Bauer et al., 2015; Neme and Tautz, 2016; Schmitz et al., 2018; Werner et al., 2018), there are no generally accepted accounts for the connection between their appearance and their roles in the recruitment of ancestral cellular functions for cell differentiation.

Finally, it is important to recognize that while cell types in mature metazoans are typically functionally stable, wound repair and regeneration require cell type plasticity. The mechanisms described above are reversible under certain conditions, consistent with this need (Wenger et al., 2016; Gehrke et al., 2019), a phenomenon also manifested in the experimental production of stem cells (Sardina et al., 2018).

PATTERN FORMATION: INHERENCY IN THE INTEGRATION OF FORM AND FUNCTION

We have seen how the innovations in form and function that accompanied the transition from unicellular holozoans to the metazoans were fundamentally expressions of latent, inherent properties of this new category of organism. To reiterate, hollow, multilayered, elongated, appendage-bearing diploblastic and triploblastic forms were direct consequences of the mobilization of mesoscale physical forces and effects in liquid and liquid-crystalline cell aggregates. The existence of such multicellular aggregates and the presence within them of cell types specialized to perform exaggerated versions of ancestral unicellular functions were direct consequences of the new type of enhancer- and silencer-based gene regulatory systems that emerged simultaneously with the metazoans. While inherent to metazoans, morphogenesis and differentiation were not present in their unicellular ancestors. New genes proteins, and regulatory motifs (classical cadherins, Wnt, enhancers), several of which with no known predecessors, were needed to create these inherencies.

The capacity to arrange differentiated cell types in reproducible geometries to produce functionally adequate organs and appendages, that is, cellular *pattern formation*, is still another category of phenomena with inherent and therefore predictable outcomes. Once the basic ingredients, i.e., pan-metazoan toolkit genes and their products, and the physics of permeable, active, and excitable media to which animal tissues are intrinsically

susceptible, were in place, many of the shared features of animal functional anatomy emerged with relative facility.

Morphogens and Lateral Inhibition

The simplest way cells in a tissue mass can be induced to differentiate in a spatially dependent fashion is by *morphogens*, secreted diffusible proteins. Wnt, as noted above, is the most fundamental and ubiquitous of these in the metazoans, but there are more than a dozen widely shared ones, including those of the Hedgehog family, such as Shh and Ihh, Bmps, and Fgfs (reviewed in Newman and Bhat, 2009). Any molecule of this type produced in a cluster of ancestral cells could easily have become nonuniformly distributed by leakage from the surface of the mass, or possibly due to poor nutrient supply in the interior leading to lower expression of its gene. Since such conditions would automatically have been reproduced each time a cell cluster was reconstituted, the morphogen and its physically determined spatial arrangement, combined with its concentration-dependent effects in eliciting changes in gene expression or other aspects of cell phenotype (Loh et al., 2016), may have constituted a primitive developmental program. Subsequent natural selection would have reinforced the accuracy and reliability of such pattern-forming modalities if the resulting organisms proved to be survivable. Eventually the transport of morphogens has come to be not only due to diffusion but to active processes mediated by cells along the distribution routes (Sagner and Briscoe, 2017). In the perspective of the present analysis, however, a key feature of these mechanisms is their origins in inherent properties of collectives of metazoan cells.

In addition to morphogen-based global patterning, the phenomenon of *lateral inhibition* enforces a choice between alternative cell fates at the local level. This is accomplished by early differentiating cells signaling to cells adjacent to them to take on a fate different from them and is typically mediated by the Notch signal transduction pathway, which is found in all metazoan groups with the exception of Placozoa (reviewed in Newman, 2016b). The activity of this pathway depends on the binding of a member of the Notch family of cell surface receptors, with an integral membrane protein ligand from among the Delta, Serrate (or Jagged), and Lag-2 (DSL) class of proteins (Ehebauer et al., 2006). This interaction results in an intracellular portion of Notch translocating to the nucleus where, as a transcriptional co-regulator of dual-action transcription factors of the CSL (CBF1, Su(H), and Lag-1) class (Lai, 2002), changes them from repressors to activators of key target genes. Since Notch's effects on cell phenotype depend on which of the dual-action factors are present in a given cell type, the pathway does not determine the cell's fate, but rather causes it to choose a potential fate different from the one it would have it assumed without the juxtacrine (i.e., direct cell-to-cell) signal.

The Notch pathway, like many of the metazoan morphogenesis and differentiation mediators discussed above, is unique to this group, although some components or portions of them can be recognized in earlier emerging forms. Protein modules of Notch receptors are present in the choanoflagellate holozoan *Monosiga brevicollis* (King et al., 2008) and a Notch receptor, ligands, and an intracellular mediator are present in the filasterean holozoan *Ministeria vibrans* (Shalchian-Tabrizi et al., 2008). Studies on CSL homologs in yeast indicate that their role in determining alternative cell states is evolutionary deeper than the holozoans (Prevorovsky et al., 2015).

The physical behaviors of the biogeneric materials discussed earlier in this article – rounding up, phase separation, lumen formation, elongation, wetting, condensation – notwithstanding their dependence on living cells – are analogs of those that result from passive arrangements and rearrangements of subunits in nonliving liquids. Here, however, with the effects of morphogen induction and lateral inhibition in eliciting alternative cell states, we see analogs to chemical transformations. Like chemistry, these are active (i.e., energy consuming) rather than passive processes, in which one kind of entity (a molecule or a cell type) is changed into another. The simplest of these effects is unidirectional, a morphogen choosing the fate of a cell from among the organism's repertoire of types, or a cell imposing a choice on a second cell. When there are reciprocal influences – positive or negative feedbacks, or synchronization – among the components, the systems can become not merely active, but excitable (i.e., energy storing and releasing; self-organizing (Süel et al., 2006; Sinha and Sridhar, 2015)], and more complex patterns can emerge.

Oscillations and Morphogenetic Fields

Lateral inhibition mediated by the Notch pathway is well suited to generating fine-grained salt-and-pepper-like patterns in which adjacent cells are of different types. Organogenesis in complex animals, however, depends on large-scale coordination of patterns of cell differentiation in which patches and domains of cells of a given type—tissues—associate in specific geometric arrangements with other such domains in the architecture of body layers, appendages, and primordia arising from the morphogenetic processes described above. Morphogen gradients, mentioned above, can mediate long-range control of differentiation, but in order to effect accurate pattern formation, the cells in the targeted regions need to be in similar states of receptivity. This might appear to be a property that is not intrinsic to cells in developing tissue primordia and must be a product of adaptive selection. But, it can in fact arise spontaneously from mechanisms and ingredients that have evolved for other reasons.

A main target gene of the CSL transcription factors at the end of the Notch signal transduction pathway is Hes1 (called hairy1 in Drosophila). Hes1, itself a transcriptional regulator, has the unusual property of regulating its own gene expression through a negative feedback mechanism. Due to this feedback control, the level of Hes1 protein in expressing cells will tend to undergo temporal oscillation (Lewis, 2003). Then, through a well-described physical phenomenon of synchronization of weakly coupled oscillators (Garcia-Ojalvo et al., 2004), the cells in a spatially extended domain of tissue will find themselves in identical though periodically changing states of Hes1 concentration and of any other biochemical factor Hes1 controls. This poises them for concerted response to an external regulatory factor such as a morphogen. The Notch pathway and its downstream effectors, therefore, not only enable cells to directly influence the fates of their nearest neighbors through lateral inhibition, but facilitate the emergence of a "morphogenetic

field" (Gilbert et al., 1996; Bhat et al., 2019). This phenomenon was described by early embryologists as "a system of order such that the positions taken up by unstable entities in one part of the system bear a definite relation to the position taken up by other unstable entities in other parts of the system" (Needham, 1937, p. 71).

The cell state-coordinating effect of synchronized oscillations of Hes1 expression or potentially other factors with similar feedback dynamics has a propensity (not invariably realized) of producing spatially periodic tissue structures. Hes1 oscillations are synchronized, for example, in the lateral plate mesoderm of vertebrate embryos, so that cells in any band of tissue along the cranial-caudal axis have approximately the same (periodically varying) Hes1 concentration (Özbudak and Lewis, 2008). The cells will aggregate, due to upregulation of adhesive molecules, when a certain Hes1 level is reached, but only when they are not inhibited from doing so by a graded suppressive activity, a morphogen, that emanates from the tail tip. As the embryo elongates, successive bands of tissue become distant enough from the caudal end to escape from the morphogen's effect. When the Hes1 oscillation brings the factor's concentration into the adhesion-inducing range in the newly competent cells (uniformly, because of synchrony), a mesenchymal-epithelial transformation takes place, and discrete blocks of tissue, "somites," form to either side of the central axis (Hubaud and Pourquie, 2014). This process continues as long as the embryo elongates, generating a species-characteristic number of somites, which depends on both the rate of elongation and the period of the oscillator. While the somites give rise to various spatially periodic (the vertebrae and the ribs) and nonperiodic (the dermis of the back skin, and the muscles of the back, body wall, and limbs) structures, the manner in which they are generated suggests that the emergence of tandem tissue blocks is the side effect of various inherent processes (feedback regulation, morphogen action) that only incidentally, under certain mutually tuned conditions, lead to a repetitive spatial pattern.

In a similar fashion, Hes1 oscillations across the digital plate in the embryonic avian limb become synchronized during skeletal pattern formation. This enables spatial cues for skeletogenesis (see below) to act in a coordinate fashion over larger distances across the cellular field than the natural wavelength of their generating mechanisms (Bhat et al., 2019).

Reaction-Diffusion Systems

Morphogens do not only function in the form of simple gradients to induce patterns of cell differentiation. By interacting with biosynthetic processes (including gene expression) in target cells and tissues, they can assume more elaborate geometries, which lead to more complex cell arrangements. Alan Turing, in a paper titled "The chemical basis of morphogenesis," showed that a balance of positive and negative feedbacks in an open chemical system, when coupled with differences in the rates of diffusion of the key reactive molecules, could lead (contrary to the expectation that diffusion evens things out) to stable, nonuniform concentration patterns (Turing, 1952). These are referred to as "reaction-diffusion" mechanisms, and in developmental biology, "Turing-type" systems, but when living tissues are concerned neither "reaction" (actually cell-based production of, and response to, molecular factors) nor "diffusion" (or more generally, transport of released molecules through and between cells, with the latter's active participation), are typically like those seen in purely chemical systems. Moreover, the core processes may differ substantially from Turing's, lacking some of the original features, e.g., the difference in diffusivities of the chemical species involved, and containing others, e.g., chemotaxis and advection (see, for example, Glimm et al., 2014; Madzvamuse et al., 2015; Nesterenko et al., 2017).

Turing-type mechanisms pattern cell types in a variety of embryonic systems, including spatially nonperiodic ones such as the generation of left-right asymmetry (Muller et al., 2012) and the formation of individual tooth cusps (Salazar-Ciudad, 2012). But, it is in the production of repetitive or quasi-repetitive structures such as hairs and feather germs (Glover et al., 2017; Painter et al., 2018), and pigment patterns in skin (Haupaix et al., 2018), that this category of self-organizing process has found its broadest applications. The paired appendages – fins or limbs – of the jawed vertebrates, for example, are characterized by arrangements of cartilaginous and/or bony elements that appear to have arisen evolutionarily by and continue, to some extent, to be mediated by such mechanisms. In cartilaginous fishes such as sharks, skates, and rays, the fin skeleton consists of one or more cartilage rods or plates to which are appended as many as several dozen parallel, jointed cartilage rods. In ray-finned fishes (carp, zebrafish, and so forth), the endoskeleton is made of plates, rods, and nodules of cartilage and bone. Finally, in the lobe-finned fish like the coelacanth, and tetrapods, such as amphibians, birds, and mammals, the cartilaginous or bony skeleton is comprised of increasing numbers of parallel elements along the proximodistal axis originating at the body wall (Newman et al., 2018).

Evidence from experimental embryology and molecular genetics, phylogenomics, and mathematical and computational simulations has provided plausible scenarios for transitions, often abrupt, between these patterns over the course of evolution of the paired appendages resulting from subtle changes in transcription factor-cis-regulatory element binding within conserved GRNs, and protein-protein interactions at the cell surface. In these cases, and in others which have been explored in detail, active and excitable mechanisms of cellular differentiation and pattern formation, in coordination with more passive morphogenetic mechanisms of the liquid- and liquid crystalline-tissue states, each utilizing genes, molecules, and regulatory motifs particular to metazoan life, lead to the formation of recognizably animal-type tissues and organs with the capacity to perform functions carried over from ancestral cells.

CONCLUSION: PREDICTABILITY OF ANIMAL ORGANIZATION AND INTEGRATION

This review has described how three main properties of animal bodies and organs, their forms, their differentiated cell types,

and the patterned arrangement of cells of various types, are determined by inherent properties of aggregates of metazoan cells. These inherent properties are not all of the same type. The only such properties that were actually latent in directly ancestral unicellular organisms are those manifested as the specialized functions of cell types. As indicated above, unicellular holozoans are variously capable of adhering to one another, absorbing molecules and ingesting particulate nutrients, exhibiting contractility and electrical excitability, surrounding themselves with semisolid or solid matrices, binding oxygen, neutralizing toxins, and excreting waste products. All of these functions appear in exaggerated form in one or more of the 200–300 animal cell types. Functions inherent to the ancestors of other multicellular lineages, such as the capture of light and the storage of its energy in sugars in vascular plants, appear in differentiated cells of those organisms, but not the animals.

As noted above, the recruitment of inherent cell functions for cell differentiation sometimes employed transcription factors which controlled the relevant function-related genes in the unicellular ancestor. Most often, however, the differentiation and other developmental TFs are not found outside the metazoans (Grau-Bové et al., 2017). In all cases, however, the TFs involved in cell differentiation became incorporated into hierarchies, based on the novel and unique gene regulatory apparatus of Metazoa that distinguished their roles in establishing cell types from those in maintaining them, and set both of these apart from regulation of gene expression in nonspecialized "housekeeping" activities.

The relevant organizational properties for morphogenesis and pattern formation are those characteristic of mesoscopic materials (e.g., liquid- and liquid-crystalline tissues) or extended excitable media (e.g., "diffusion" and "reaction," synchronization of oscillations). While the respective physical effects are therefore generic to such materials, the concept of inherency was only applicable once the materials came into existence as multicellular masses. It is notable that gene innovations that are specific, or now uniquely confined, to Metazoa were required for such materials to be constituted and thus for the inherent properties of their physical state to be realized.

The fact that body and organ forms, cell and tissue functions, and cellular patterns are inherent to animal systems has major consequences for understanding evolution (Newman, 2017). (See Webster and Goodwin, 1996 and Amundson, 2005 for accounts of the structuralist tradition in evolutionary theory, in which inherency is an important theme.) Inherency clearly does much of the work attributed in the standard model to trial-and-error-based natural selection. But inherency of form and function does not mean simultaneous emergence of all possibilities. The main animal types do not appear in the fossil record prior to strata deposited tens of millions of years after the first signs of metazoan-type organisms, more than 600 million years ago. With the exception of placozoans and ctenophores, which, respectively, implement the developmental gene-regulatory and liquid-tissue constituting activities of enhancers and classical cadherins differently, the capability to mobilize pre-existing holozoan cell functions in morphologically varied, spatially patterned assemblages was present from the start and carried forward in all subsequent evolution of this group. Additional complexity was achieved mainly by the addition of new genes specifying ECM components.

Finally, the remarkable phenomenon of electrical integration of multicellular patterns must be mentioned. Although thus far not demonstrated to be a primary form-generating or patterning mechanism like differential adhesion, morphogen gradients, gene expression oscillation, and reaction-diffusion coupling, bioelectric establishment of voltage gradients across tissues serves to reinforce developmental patterns and restore them if perturbed during embryogenesis or wounding (McLaughlin and Levin, 2018; Pietak and Levin, 2018). Voltage-gated channels with single-cell functions are inferred to have existed in unicellular holozoan ancestors of the animals (Cai, 2012), but their developmental role in the multicellular context is entirely different from any function they evolved to perform in individual cells. Along with canalizing evolution that serves to stabilize the phenotype of an evolutionary lineage against mutation and developmental noise once it has become ecologically established, the dynamically responsive encoding of morphology by bioelectricity is a key factor in organismal autonomy (Arnellos et al., 2014; Moreno and Mossio, 2015).

Major steps in animal evolution not only occurred by such repurposing of ancestral genes (as we also saw in the case of the grainyhead transcription factor), but also involved in the appearance, often without precedent, of novel genes whose products set into play forces and effects, which though having predictable outcomes, were unavailable up to the points at which they appeared (**Table 1**).

Natural selection can act as a sieve for organisms with novel combinations of inherent characters, or characters that become inherent to a lineage after genetic augmentation, but it is not responsible for producing those traits. The characters' origination, whatever the source of the associated genes, can often only be understood on the basis of physico-genetic effects specific to the multicellular context. Characters produced in this fashion are constrained in their nature and may therefore appear abruptly (McGhee, 2011). If they enable organisms to survive in new ways in existing ecological niches, or to occupy new niches (Laland et al., 2017), their roles in enhancing fitness will be "after-the-fact" (Gould and Lewontin, 1979; Müller, 1990; West-Eberhard, 2003) and thus do not require elaborate or farfetched adaptationist narratives to account for their existence.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Corrigendum: Inherency of Form and Function in Animal Development and Evolution

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Keywords: biogeneric, morphogenesis, pattern formation, cell differentiation, self-organization, phase transition, reaction-diffusion

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A Corrigendum on

Inherency of Form and Function in Animal Development and Evolution
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In the original article, there was a mistake in **Table 1** as published. Because of an editing error, the lines for "Apicobasal cell polarization" and "Nonliquid cellular assemblages *via* matrices" were transposed. The corrected **Table 1** appears below.

The author apologizes for this error and states that this does not change the scientific conclusions of the article in any way. The original article has been updated.

TABLE 1 | Novel inherent properties in animal development and evolution.

Property	Gene or molecular motif	Character
1. Properties dependent on novel genes or regulatory motifs coincident with emergence of Metazoa		
Liquid-tissue state	Classical cadherins	Multicellularity; layering
Regulated cell polarity	Wnt	Lumens; elongated tissues
Capacity to exaggerate intrinsic cell functions	Enhancers; PcGl proteins	Differentiation
Morphogen gradients	Hedgehog, BMPs	Simple cell patterns
2. Properties dependent on novel genes acquired after metazoan origins		
Liquid-crystalline-tissue state	Vang/Stbm	Tissue elongation
Wettable substrata (basal lamina)	Peroxidasin	Appendages, glands
Lateral inhibition; oscillation of gene expression	Notch, Hes1	Complex cell patterns
Multiple alternative cell types	MyoD, PPARy, SMAD	Complex tissues, organs
3. Properties dependent on ancestral genes repurposed into DPMs in the multicellular context		
Cell-cell cohesion in liquid tissues	Grainyhead	E-M transformation
Apicobasal cell polarization	β-catenin	Epithelia and lumens
Nonliquid cellular assemblages *via* matrices	Collagen IV	Mesenchymal tissues
Cell-cell electrical coupling	Voltage-gated channels	Bioelectrical integration

Each list is nonexhaustive but contains the most important examples of its respective category.

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ORIGINAL RESEARCH
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Heterogeneity and Delayed Activation as Hallmarks of Self-Organization and Criticality in Excitable Tissue

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Self-organized critical dynamics is assumed to be an attractive mode of functioning for several real-life systems and entails an emergent activity in which the extent of observables follows a power-law distribution. The hallmarks of criticality have recently been observed in a plethora of biological systems, including beta cell populations within pancreatic islets of Langerhans. In the present study, we systematically explored the mechanisms that drive the critical and supercritical behavior in networks of coupled beta cells under different circumstances by means of experimental and computational approaches. Experimentally, we employed high-speed functional multicellular calcium imaging of fluorescently labeled acute mouse pancreas tissue slices to record calcium signals in a large number of beta cells simultaneously, and with a high spatiotemporal resolution. Our experimental results revealed that the cellular responses to stimulation with glucose are biphasic and glucose-dependent. Under physiological as well as under supraphysiological levels of stimulation, an initial activation phase was followed by a supercritical plateau phase with a high number of global intercellular calcium waves. However, the activation phase displayed fingerprints of critical behavior under lower stimulation levels, with a progressive recruitment of cells and a power-law distribution of calcium wave sizes. On the other hand, the activation phase provoked by pathophysiologically high glucose concentrations, differed considerably and was more rapid, less continuous, and supercritical. To gain a deeper insight into the experimentally observed complex dynamical patterns, we built up a phenomenological model of coupled excitable cells and explored empirically the model's necessities that ensured a good overlap between computational and experimental results. It turned out that such a good agreement between experimental and computational findings was attained when both heterogeneous and stimulus-dependent time lags, variability in excitability levels, as well as a heterogeneous cell-cell coupling were included into the model. Most

importantly, since our phenomenological approach involved only a few parameters, it naturally lends itself not only for determining key mechanisms of self-organized criticality at the tissue level, but also points out various features for comprehensive and realistic modeling of different excitable systems in nature.

Keywords: excitable cells, self-organized criticality, beta cells, calcium imaging, computational model, cellular heterogeneity, activation delay

INTRODUCTION

Self-organized collective dynamics is a remarkable phenomenon observed in various natural and man-made systems, in which collective behavior emerges from local interactions between individual elements (Bak, 1996; Marković and Gros, 2014; Ellis and Kopel, 2019). Regardless of the specific mechanisms responsible for self-organization, the resulting coherent global structures or dynamics are characterized by scale-invariant properties and a power-law distribution of systems' observables (Khaluf et al., 2017; Muñoz, 2018). Such emergent behavior is often associated with critical dynamics and is assumed to be particularly beneficial for the functioning of several living systems, from the microscopic to the macroscopic (Tamayo et al., 1999; Nykter et al., 2008; Chialvo, 2010; Bialek et al., 2012; Furusawa and Kaneko, 2012; Sasai, 2013; Allegrini et al., 2015; Muñoz, 2018). Criticality has been argued to originate from the fact that many biological systems operate in the vicinity of a critical point of a phase transition between an ordered and disordered phase, which ensures a balance between robustness against perturbations and flexibility to adapt to a changing environment. However, the exact reasons why signatures of criticality can be conjectured to emerge in living systems are still under debate and the underlying principles are incompletely understood (Lovecchio et al., 2012; Moretti and Muñoz, 2013; Nonnenmacher et al., 2017). Most importantly, despite some skepticism and limitations, the evidently increasing amount of empirical evidence, fostered also by technological and computational advances, is nowadays inspiring more and more researchers to investigate the complexity of biological systems through the lens of phase transition behavior and criticality.

In the domain of biological networks, the concepts of self-organization and criticality have received the most attention in the field of neuroscience. On the smallest scales, patterns of activity in neuronal populations have been found to be very heterogeneous, with sizes of so-called neuronal avalanches following a power law distribution (Beggs and Plenz, 2003; Pasquale et al., 2008; Timme et al., 2016). Empirical evidence for criticality has been reported in both different *in vivo* preparations and on larges scales of whole-brain imaging (Plenz and Thiagarajan, 2007; Haimovici et al., 2013; Hesse and Gross, 2014). The presence of emergent critical dynamics in the nervous system is theoretically appealing and consequently computational models and tools from the realms of statistical physics have been utilized to unveil the mechanisms and correlations between phase transition behavior and the occurrence of scale-invariant neuronal avalanches (Plenz and Thiagarajan, 2007; Rubinov et al., 2011; Friedman et al., 2012; Zare and Grigolini, 2013; Tkačik et al., 2015; Brochini et al., 2016; di Santo et al., 2018). Importantly, several studies have underscored the emergence of critical dynamics in neuronal networks as one of the key pillars for their optimal operational abilities (Kinouchi and Copelli, 2006; Shew and Plenz, 2013; Shew et al., 2015; Stoop and Gomez, 2016). Moreover, complex and hierarchically organized network structures along with neuronal plasticity were identified as the main neurophysiological determinants that ensure robust critical behavior (Levina et al., 2007; Rubinov et al., 2011; Moretti and Muñoz, 2013; Hutt et al., 2014; Massobrio et al., 2015b). It should be noted that deviations from critical behavior occur in neuronal networks during development (Tetzlaff et al., 2010; Massobrio et al., 2015b) and under pathological conditions (Massobrio et al., 2015a; Tagliazucchi et al., 2016; Hahn et al., 2017). Especially during epileptic seizures (Hobbs et al., 2010; Meisel et al., 2012) or by pharmacological disruptions of the excitation-inhibition balance (Barral and D Reyes, 2016), an excess of large system-spanning avalanches occur, as is characteristic for supercritical dynamical states. Consequently, it has been hypothesized that the healthy brain resides near a critical or even slightly subcritical state, thereby ensuring a safety margin from supercriticality, which has been linked to some pathophysiological disorders (Priesemann et al., 2014; Tomen et al., 2014; Massobrio et al., 2015a).

Notably, recent research indicates that the concept of critical dynamics and power-law scaling in living beings applies well beyond the spatiotemporal activity patterns of neurons. At the (sub)cellular level, mitochondrial network of heart myocytes was reported to operate at the edge of dynamic instability characterized by a fractal scaling of depolarized mitochondrial clusters (Aon et al., 2004). In this regime, constancy in terms of a steady supply of ATP is provided in combination with flexibility, which ensures the adaptation of energy production in accordance with metabolic demands (Aon et al., 2006). Moreover, hallmarks of self-organized criticality have also been observed in the spatiotemporal organization of Ca^{2+} waves. Jung et al. (1998) reported a power law distribution of noise-induced spiral Ca^{2+} wave sizes in cultured networks of astrocytes. Heavy-tailed distributions and an avalanche-like behavior have also been observed in intracellular Ca^{2+} signalization in cardiac myocytes (Nivala et al., 2012) and in immature oocytes (Lopez et al., 2012). In both studies Ca^{2+} waves in individual cells resulted from random local Ca^{2+} events, reflecting small Ca^{2+} release events from individual channels or a cluster of channels, which can occasionally integrate to global events reflecting a whole-cell Ca^{2+} signal (Berridge et al., 2000). As the localized subcellular Ca^{2+} events interact, e.g., via diffusion, they can self-organize and lead to avalanches of activity that propagate through the cell.

The concept does not only assert that the extent of such events is characterized by scale invariance, but also makes the global Ca^{2+} signals appear rather deterministic in spite of their stochastic origin (Skupin et al., 2008).

Even though information processing in living organisms is often performed by large networks of interacting cells, little attention has been devoted to the principles underlying critical dynamics on multicellular and tissue levels of organization. Recently, we have empirically shown that fingerprints of criticality are also found in the spatiotemporal dynamics of interconnected beta cells from islets of Langerhans (Gosak et al., 2017). These endocrine cells synthesize and release insulin, the anabolic hormone which promotes postprandial storage of nutrients, and thus serves a crucial role in homeostasis of energy that becomes disrupted in diabesity (Kahn et al., 2014). Insulin concentration in the blood displays inherent multimodal oscillations (Satin et al., 2015), and several studies have attempted to reveal the underlying mechanism by providing links to oscillations in beta cells metabolism and to a feedback between ion channels and electrical activity. This was further corroborated by modeling the interplay of the two signaling aspects (Bertram et al., 2007). Moreover, recent theoretical studies emphasized the role of biphasic feedback circuits in controlling functional beta cell mass (Karin et al., 2016) and progression of diabetes mellitus (Karin and Alon, 2017). On the organizational level of a single islet, beta cells respond to nutrient stimulation with an initial transient depolarization, followed by fast oscillations in membrane potential that are superimposed on a plateau phase (Gilon and Henquin, 1992; Rorsman and Braun, 2013; Skelin Klemen et al., 2017). Intracellular Ca^{2+} concentration ($[Ca^{2+}]_{IC}$) closely follows changes in membrane potential due to tight coupling between electrical and calcium dynamics in beta cells (Gilon and Henquin, 1992; Dolenšek et al., 2013). However, all beta cells within an islet do not show identical electrical or $[Ca^{2+}]_{IC}$ activity and can therefore not be regarded as uniformly and strongly coupled identical units or even as a single supercell. Rather, the collective activity of beta cells is characterized by a phase shift between individual cellular oscillations, ultimately resulting in heterogeneous waves of membrane potential and $[Ca^{2+}]_{IC}$ changes. These waves spread repetitively over an islet, but not always from the same source and not always throughout the whole syncytium (Benninger et al., 2008; Dolenšek et al., 2013; Stožer et al., 2013a). They are thought to originate in specific sub-regions with elevated excitability (Benninger et al., 2014) or higher intrinsic oscillation frequency (Westacott et al., 2017). A plethora of evidence demonstrates that an essential prerequisite for the coordinated beta cell activity and formation of waves is intact intercellular connectivity mediated via gap junctions (Calabrese et al., 2003; Ravier et al., 2005; Bavamian et al., 2007; Bosco et al., 2011; Benninger et al., 2014) and probably other modes of communication, such as paracrine, contact-dependent, and ciliary signaling (Squires et al., 2002; Konstantinova et al., 2007; Yang et al., 2011; Gerdes et al., 2014). Most importantly, intercellular connectivity is not only necessary for normal islet function, its perturbations were also linked to metabolic diseases and impaired insulin secretion (Hamelin et al., 2009; Carvalho et al., 2012; Head et al., 2012; Hodson et al., 2013; Benninger and Piston, 2014; Benninger et al., 2018; Nasteska and Hodson, 2018).

Moreover, individual beta cells are intrinsically highly heterogeneous (Gutierrez et al., 2017). Several different approaches have demonstrated relatively large differences in the extent of coupling between beta cells (Pérez-Armendariz et al., 1991; Farnsworth et al., 2014), as well as different levels of excitability (Jonkers and Henquin, 2001; Benninger et al., 2011) and rates of glucose metabolism (Benninger et al., 2014; Benninger and Hodson, 2018; Nasteska and Hodson, 2018). Because of these features, the spatiotemporal responses of beta cells are very complex and to understand how a population of these heterogeneous and heterogeneously coupled cells activate to work in synchrony is a hot topic in islet physiology research (Pedersen et al., 2013; Benninger and Piston, 2014; Benninger et al., 2014; Markovič et al., 2015; Cappon and Pedersen, 2016). Motivated by complexity science approaches, several studies have investigated beta cell responses in terms of phase transition behavior (Hraha et al., 2014; Loppini et al., 2014; Stamper et al., 2014). In this vein, in our recent study we demonstrated that under physiological circumstances, the initial response to glucose is characterized by a power-law probability distribution of Ca^{2+} wave sizes, which can be maintained in the long run by periodic stimulation, but changes to supercriticality upon constant stimulation, thereby demonstrating empirically the fingerprints and basic preconditions of critical behavior (Gosak et al., 2017).

In the present work, we extend our preceding research to supraphysiological glucose concentrations that are usually used in experiments, but accompany pathophysiological states *in vivo*. It turns out that higher glucose levels evoke more rapid and qualitatively different beta cell responses when compared to physiological levels of stimulation. To assess the measured non-trivial and rich dynamical patterns, we propose a phenomenological model of coupled excitable cells that accounts for the observed physiological as well as pathophysiological behavior and encompasses both beta cell signaling specifics and heterogeneity. Moreover, in contrast to the exhaustive computational models with many parameters, our phenomenological modeling approach made it easier to empirically explore the necessary ingredients and physiological determinants that ensure a good overlap between experimental and computational results.

MATERIALS AND METHODS

Multicellular Calcium Imaging in Pancreatic Tissue Slices

Acute pancreatic tissue slices were prepared as described previously (Speier and Rupnik, 2003; Stožer et al., 2013a). Briefly, low-melting point agarose (1.9% V/V) was injected into the proximal common bile duct that was clamped distally at the major duodenal papilla. Retrograde inflow of the agarose served, once cooled, as a scaffold for subsequent tissue cutting into 140 μm thick slices on a vibratome (VT 1000 S, Leica). Staining with the calcium sensitive dye Oregon Green 488 BAPTA-1 AM [6 μM final concentration, 0.03% Pluronic F-127 (w/v), and

0.12% dimethylsulphoxide (v/v) dissolved in HEPES-buffered saline at RT, consisting of (in mM) 150 NaCl, 10 HEPES, 6 glucose, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$; titrated to pH = 7.4 using 1 M NaOH], was performed for 50 min at RT. Confocal imaging of calcium dynamics was performed on the Leica TCS SP5 AOBS Tandem II upright confocal system (20x HCX APO L water immersion objective, NA 1.0) and Leica TCS SP5 DMI6000 CS inverted confocal system (20X HC PL APO water/oil immersion objective, NA 0.7), utilizing a perifusion system filled with extracellular solution, consisting of (in mM) 125 NaCl, 26 $NaHCO_3$, 6 lactic acid, 3 myo-inositol, 2.5 KCl, 2 Na-pyruvate, 2 $CaCl_2$, 1.25 NaH_2PO_4, 1 $MgCl_2$, 0.5 ascorbic acid and added either substimulatory 6 mM or stimulatory 8 or 12 mM glucose. The calcium dye was excited at 488 nm via an argon laser line and the emitted fluoresces was detected in the range of 500–700 nm by a Leica HyD detector. Time series were acquired at 10 Hz (512 × 512 pixels). Further analysis was done off-line by manually selecting ROIs corresponding to beta cells. The exported time series of the F/F_0 ratio were then further processed as explained in the continuation.

Computational Model

Phenomenological Single Cell Model

We utilized a phenomenological model to describe the dynamics of the electrically excitable beta cells. In particular, we made use of a two-dimensional iterated map proposed by Rulkov (2002):

$$u_i(t+1) = \alpha_i(t)/(1+u_i(t)^2) + v_i(t) + g_i \sum_j \varepsilon_{ij}(u_j - u_i) + \beta\xi_i(t), \quad (1)$$

$$v_i(t+1) = v_i(t) - \sigma u_i(t) - \chi, \quad (2)$$

where $u_i(t)$ and $v_i(t)$ are the slow and the fast dynamical variables for the i-th cell, respectively, and are considered as dimensionless variables, t is the discrete time index, α_i, χ, and σ are systems parameters, and $\beta = 0.0045$ defines the strength of Gaussian noise with zero mean and unit variance that accounts for stochasticity in beta cell dynamics. The fast variable $u_i(t)$ describes the dynamics of the membrane potential of the cell, whereas the slow variable $v_i(t)$ reflects the gating variable. Although this is an abstract and simple mathematical model, it mimics well the basic principles of more complex cellular behaviors that are observed in different cell types, including beta cells.

More specifically, in terms of metabolic changes, electrical activity, $[Ca^{2+}]_{IC}$ dynamics, and insulin secretion, beta cells within islets respond to stimulation by glucose in two phases. During the first phase which is transient, they show elevated levels of intracellular ATP and NAD(P)H, followed electrically by very fast bursting or continuous bursting. Bursts are periods of very fast depolarizations called spikes that last a few seconds and continuous bursting consists of an uninterrupted set of spikes at a frequency around 10 Hz. At the level of $[Ca^{2+}]_{IC}$ dynamics, this first phase consists of a transient increase in $[Ca^{2+}]_{IC}$, during the ascending part of which a few fast $[Ca^{2+}]_{IC}$ oscillations may be present, reflecting fast burst before continuous bursting. It should be pointed out that at present the very fast spikes cannot be resolved in $[Ca^{2+}]_{IC}$ imaging. This first phase lasts a few minutes and at the level of hormone output overlaps with the first phase of insulin secretion. During the second phase, continuous bursting and the accompanying transient increase in $[Ca^{2+}]_{IC}$ change to regular bursting and corresponding fast $[Ca^{2+}]_{IC}$ oscillations. At the level of metabolism and hormone secretion, NADPH and ATP are elevated during this period and insulin secretion shows a stable second phase (Henquin and Meissner, 1984; Gilon and Henquin, 1992; Li et al., 2013, 2014; Gilon et al., 2014; Skelin Klemen et al., 2017; Rorsman and Ashcroft, 2018). It should be noted that during this second phase, insulin is also secreted in bursts synchronized with electrical bursts and fast $[Ca^{2+}]_{IC}$ oscillations (Gilon et al., 1993; Bergsten et al., 1994). This fast electrical, $[Ca^{2+}]_{IC}$, and secretory pattern is superimposed on a slower set of oscillations in ATP, membrane potential, $[Ca^{2+}]_{IC}$, insulin secretion, and some other parameters, which has been reviewed in detailed elsewhere (Satin et al., 2015). A further layer of complexity to this behavior comes from the fact that it is glucose-dependent. In higher glucose, the frequency or duration of bursts and correspondingly the fast $[Ca^{2+}]_{IC}$ oscillations increase, such that the active time and insulin secretion increase. Noteworthy, it seems that the underlying slow pattern is not glucose-dependent (Satin et al., 2015; Gosak et al., 2017; Skelin Klemen et al., 2017).

Most importantly, modeling all of the above aspects of beta cell responses to glucose requires the use of realistic biophysical models. However, in this study we focused only on fast $[Ca^{2+}]_{IC}$ dynamics which can be satisfactorily captured by the phenomenological model employed here. Most importantly, since we wanted to study the effects of various types of heterogeneities in a network of coupled beta cells, in comparison with a genuine biophysical model, a phenomenological description of the complex cellular dynamics is not only beneficial in terms of numerical efficiency, but also enables exploration of the system with very few free parameters.

The Rulkov map displays a variety of dynamics depending on the parameter choice, as extensively investigated in the past (Rulkov, 2002; Ibarz et al., 2011; Markovič et al., 2012). To better understand the dynamical phases that occur in our study, we performed a stability analysis. For $\chi = \sigma$ the fixed point equals $u^* = -1$ and $v^* = -1 - \alpha/2$. If $\alpha < 2$ the steady state is stable and for $\alpha < 1.86$ the fixed point is asymptotically stable, since the both eigenvalues, λ_1 and λ_2 have only real parts and their absolute value is less than 1 (see **Figure 1A**). For the values $1.86 < \alpha < 2$, the fixed point is still stable ($|Re(\lambda_1)| < 1$ and $|Re(\lambda_2)| < 1$), but the eigenvalues are complex making the fixed point a spiral sink. For the values of bifurcation parameter $2 < \alpha \leq 4$, the solution (u^*, v^*) becomes unstable and the system exhibits sustained periodic pulses, chaotic bursts of pulses and sustained chaotic pulsing. In our study we focused on the region $1.86 < \alpha < 2$, where the steady state is excitable and oscillations can be induced by noise and/or heterogeneity. This is shown in the bifurcation diagram in **Figure 1B**. For the chosen noise level, oscillations occur at $\alpha > 1.93$. Noteworthy, with increasing α the excitability level and the cellular activity increase as well. Therefore, increasing α in our model emulates the decrease in

glucose-induced K_{ATP}-channel conductance, the main trigger of beta cells in realistic models (Stamper and Wang, 2019). The temporal behavior of our single-cell phenomenological model is visualized in **Figure 1C**, for different values the bifurcation parameter α.

Intercellular Coupling Model

The sum in Eq. (1) signifies the electrical coupling and it runs over all cells, whereby $\varepsilon_{ij} = 1$ if the unit i is coupled to unit j, whilst otherwise $\varepsilon_{ij} = 0$. g_i is the coupling constant. The structure of the intercellular coupling between beta cells was modeled by the random geometric graph model (Penrose, 2007). First, all $N = 200$ cells were arranged randomly in a unit square with a prescribed minimal possible distance (0.04) to ensure a more homogeneous and realistic spatial distribution of cells. Then, the i-th and the j-th cell were considered to be connected, i.e., $\varepsilon_{ij} = 1$, if their physical distance was less than $r_{ij} = \sqrt{\langle k \rangle / (N\pi)}$, where $\langle k \rangle = 6$ signifies the average number of connections per cell. A typical intercellular network structure is shown in **Figure 2A**.

Modeling the Temporal Responses to Stimulation

To simulate the progressive recruitment of beta cells after switching from substimulatory to stimulatory or suprastimulatory glucose concentrations, we introduced a time-dependent function for the parameter α_i that reflects the cellular excitability level:

$$\alpha_i(t) = \alpha_0 + \Delta\alpha_i \left[A\,(t - t_s)\,Be^{-B(t-t_s)+1} + \frac{(t - t_s)^2}{(t - t_s)^2 + \left(T_{\mathrm{m},i} - t_s\right)^2} \right], \quad (3)$$

In this manner, we took into account the delay due to glucose metabolism. In Eq. (3) $\alpha_0 = 1.90$ is the basal substimulatory level of excitability with no activity, $\Delta\alpha$ is the amplitude of the increased excitability for the i-th cell provoked by increased glucose concentration, and t_s is the initial time before the cells respond to stimulation. The first term within the brackets on the right side of Eq. (3) stands for the initial and the second term for the successive beta cell activations. We implemented such a biphasic and glucose-dependent response to account for the previously observed biphasic and glucose-dependent behavior of beta cells in terms of their metabolic, electrical, $[Ca^{2+}]_{IC}$, and secretory response described above. The parameters $A \in [0, 1]$ and B signify the glucose-dependent amplitude and decay rate of the first activation. The parameter $T_{\mathrm{m},i}$ specifies the temporal scale of the final activation, i.e., elevation in the level of excitability. On the basis of experimental results we hypothesized that under lower and physiological stimulatory conditions the amplitude of the first response and the decay rate are lower ($A = 0.45$ and $B = 0.0004$) than under high and supraphysiological stimulatory levels ($A = 0.7$ and $B = 0.0008$). Moreover, to account for the longer activation phase observed under 8 mM glucose in comparison to 12 mM stimulation, we set the half-activation times to $T_{\mathrm{m},i}^{(8)} = 35000$ and $T_{\mathrm{m},i}^{(12)} = 20000$. Finally, to resemble a higher intrinsic beta cell activity under higher stimulation, we set the parameters when emulating the behavior under 8 mM glucose to $\Delta\alpha = 0.08$ and $\sigma = \chi = 0.001$ and to $\Delta\alpha = 0.09$ and $\sigma = \chi = 0.0012$ when emulating the behavior under 12 mM glucose. It should be noted that these small changes in the parameters σand χ have an insignificant effect on bifurcation behavior reported in **Figure 1**. Temporal traces of simulated excitability rates when switching to stimulatory and suprastimulatory conditions are shown in **Figure 2B**. Since the parameter α regulates the cellular activity (see **Figure 1**), by this means a stimulation-specific temporal recruitment of beta cells is modeled.

Heterogeneity of Beta Cells

Previous studies have suggested an extensive heterogeneity among β cells due to differences in topography, cell sizes, functional maturity, channel densities, intercellular coupling, rates of glucose metabolism, membrane potential changes, $[Ca^{2+}]_{IC}$ oscillations, granule content, and secretory capacity, to name only a few examples (MacDonald and Rorsman, 2006; Benninger and Piston, 2014; Bader et al., 2016; Roscioni et al., 2016; Pipeleers et al., 2017; Skelin Klemen et al., 2017; Benninger and Hodson, 2018; Nasteska and Hodson, 2018). To robustly account for the abovementioned differences in glucose sensitivity and metabolism, electrical excitability and $[Ca^{2+}]_{IC}$ signals, as well as intercellular coupling strength, we introduced in our phenomenological model heterogeneity three crucial aspects of cellular signaling: (i) stimulation-induced temporal change in excitability (parameter $T_{\mathrm{m},i}$), (ii)stimulation-dependent increase in excitability (parameter $\Delta\alpha_i$), and (iii) intercellular coupling strength (parameter g_i). All three parameters were assumed to follow a truncated normal distribution with a relative standard deviation of 30% and a cut-off of 90%. The three types of cellular heterogeneity are schematically visualized in **Figure 3**.

Processing of Time Series and Activity Pattern Classification

Time series of individual cells obtained from experimental recordings were first accordingly processed to achieve a coherent and accurate binarization. The main task of this pre-processing step is to level and smooth the individual time series, remove noise, and firmly extract the fast component of Ca^{2+} oscillations. To this purpose, we utilized a band pass filter, whereby the frequency band of interest was determined by visual assessment. The filtered signal was then additionally smoothed with standard sliding window algorithm, with a window size of four frames (Yaroslavsky et al., 2001). Preprocessing of computationally obtained traces was not required. In continuation the time series from experiments and simulations will be referred to as x and the corresponding value at (discrete) time t as $x(t)$. The following binarization procedure was based on the: (i) standard deviation std $[x]$, (ii) first derivative of time series $x'(t)$, and (iii) standard deviations of its the first derivative std $\left[x'\right]$. First, we have defined the potential onset and ending times of individual Ca^{2+} spikes by searching for local extremes in the first derivative. More precisely, our algorithm searches for local maxima's, which satisfy

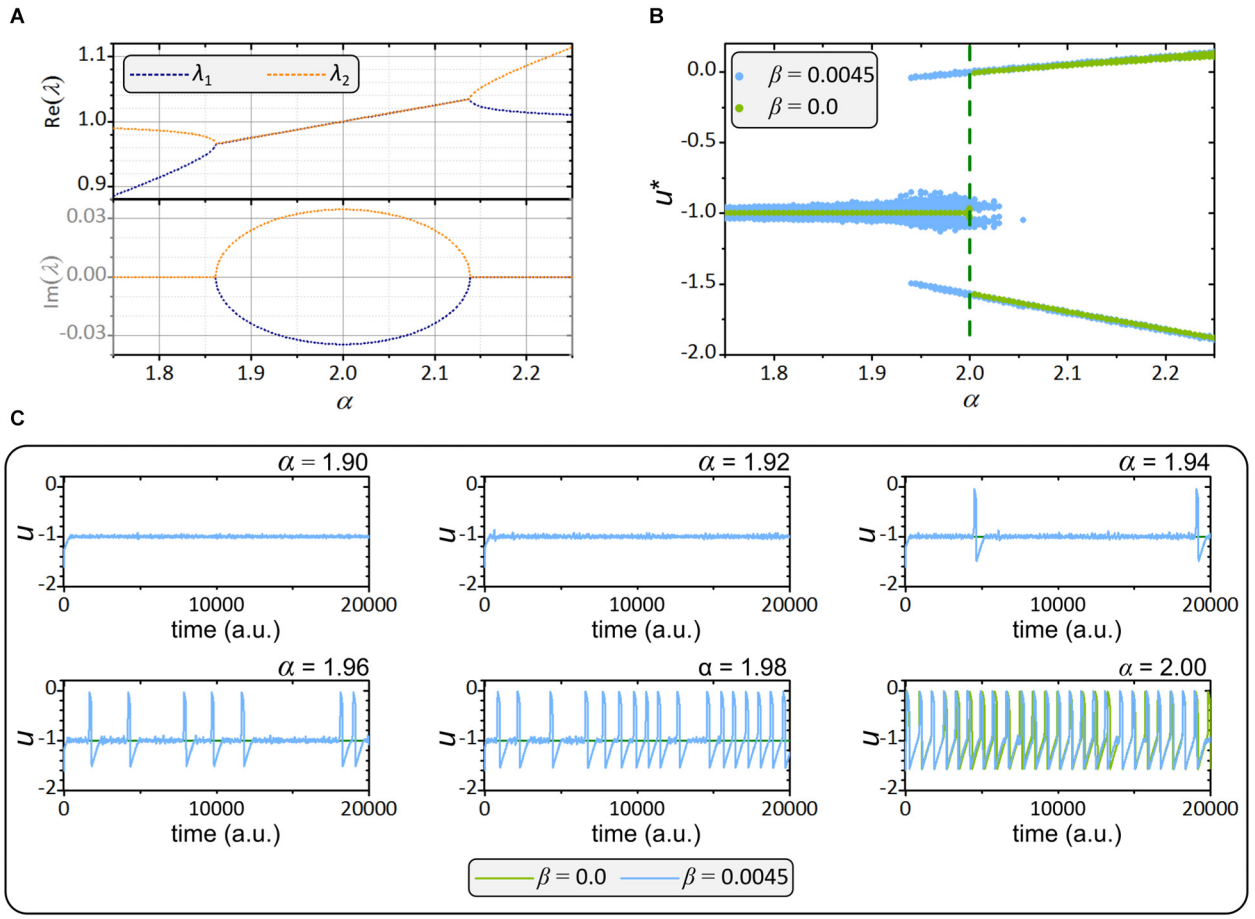

FIGURE 1 | Dynamical features of the Rulkov map. **(A)** Real and imaginary eigenvalues λ_1 and λ_2 for different values of the control parameter α. **(B)** Bifurcation diagram of the fast variable with (blue) and without (green) added noise. **(C)** Traces of the fast variable with (blue) and without (green) noise for different values of excitability levels α.

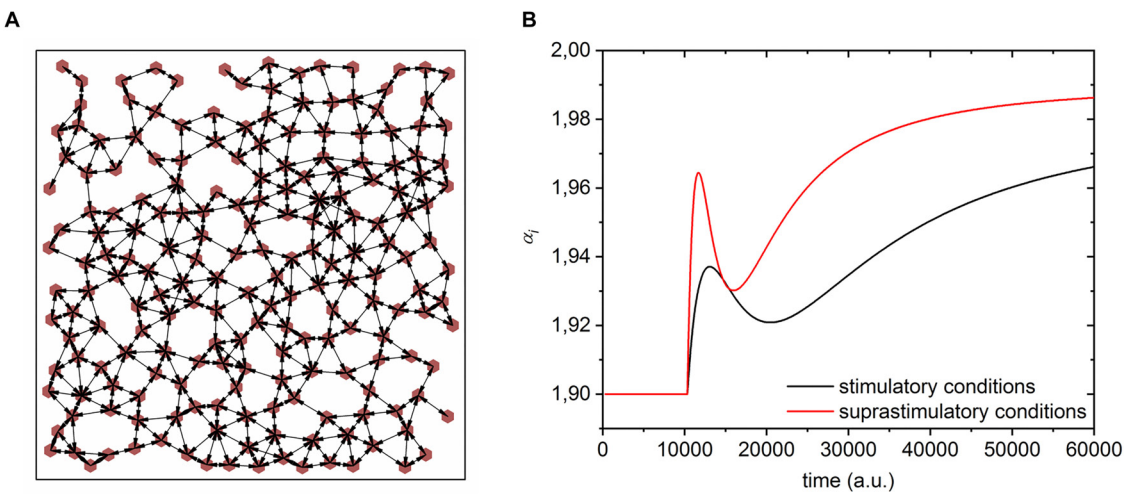

FIGURE 2 | Features of the phenomenological model of beta cell population. **(A)** A typical simulated beta cell network architecture. Red dots denote individual cells and the arrows depict intercellular electrical coupling. **(B)** Simulated time course of beta cell excitability rate after switching from substimulatory to stimulatory ($\Delta\alpha = 0.08$, $A = 0.45$, $B = 0.0004$, $T^{(8)}_{m,i} = 35000$, $t_s = 10000$) and suprastimulatory ($\Delta\alpha = 0.09$, $A = 0.70$, $B = 0.0008$, $T^{(12)}_{m,i} = 20000$, $t_s = 10000$) conditions.

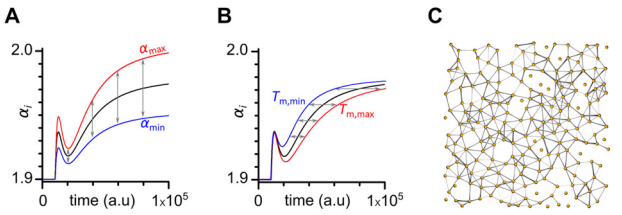

FIGURE 3 | A schematic representation of all three types of heterogeneities in the model. **(A)** electrical excitability $\Delta\alpha_i$, **(B)** beta cell metabolism $T_{m,i}$, **(C)** electrical coupling g_i. Widths of connections reflect the coupling strength.

the condition $x^{'}(t) > 1.5std\left[x^{'}\right]$. The time, at which the local maxima is found is then the potential onset of Ca^{2+} spikes, t_{START}. In a time forward direction, we than seek the first local minima that satisfies the condition $x^{'}(t) < -1.5std\left[x^{'}\right]$ and store its time of occurrence, t_{END}. Lastly we test, if the local maximum of the Ca^{2+} signal within the time interval $t \in [t_{START}, t_{END}]$ satisfies the condition $x(t) > 1.5std[x]$. The corresponding binary time series takes on a value of 1 in all time intervals $t \in [t_{START}, t_{END}]$, where the three conditions are satisfied, whilst otherwise the value is 0.

Afterward, we used binarized time series and the physical positions of individual cells to merge spatially and temporally synchronized events into clusters by performing the space-time cluster analysis, as proposed by Jung (1997) and Jung et al. (1998). In brief, we combined the information about the positions of cells and their binary traces into a space-time cube (STC). In this STC we defined a cubic region of interest (STC-ROI) in which we search for active cells. If two cells in neighboring STC were simultaneously active, they were considered to belong to the same cluster. In other words, we traced the course of the wave from cell-to-cell and if the nearby cells became activated within a short time period and if they were close enough, the given activation was considered as one individual cluster with size p. By this means, an individual STC contains the information about the number of cells that were activated in a given excitation wave, as well as about the temporal extent of the given event, as described previously (Gosak et al., 2017). The spatial side-length of the STC was determined as the average distance to 6 closest neighbors (∼25 μm in experiments and ∼0.12 in simulations). The temporal side-length was determined empirically, so that a firm of discrimination of individual waves was attained. To quantify the spatiotemporal activity patterns in experiments and simulations, we calculated the distribution of cluster sizes $N(p)$ for different stimulation protocols and activity phases. Finally, the results were fitted with a power-law function to qualitatively evaluate the nature of the distribution, i.e., critical vs. supercritical behavior. In particular, by visually assessing deviations from the power-law distribution in the form of an excess of global events we determine the supercritical nature of the spatio-temporal activity, whereas a close-to-power-law behavior implies critical-like behavior, as suggested previously (Levina et al., 2007; Friedman et al., 2012).

RESULTS

First, we present experimentally measured beta cells activity after switching from substimulatory to stimulatory and suprastimulatory levels of glucose. Then, we show the results of our computational model of interconnected excitable cells, which was designed to mimic the activity patterns observed in experiments under physiological as well as under supraphysiological levels of stimulation. Data either from experiments or from simulations were handled in the same manner, in order to provide foundation for further characterization of the spatiotemporal activity.

Experimental Results

To record beta cell responses to glucose stimulation, we used multicellular confocal imaging on acute tissue slices as described in Materials and methods. We stimulated islets with two glucose concentrations: one commonly observed *in vivo*, i.e., 8 mmol/l, and one measured in conditions of stress or glucose intolerance, i.e., 12 mmol/l. We termed the former physiological and the later supraphysiological concentration. Following either stimulus, beta cells exhibited a two-phase response: (i) an activation phase, characterized by a transient increase in $[Ca^{2+}]_{IC}$ and presence of fast oscillations, during which beta cells were gradually recruited, and (ii) a subsequent plateau phase, characterized by repeated and more regular oscillations of now fully recruited beta cells (**Figures 4A**, **5A**). Heterogeneity of beta cells responses was reflected in the time window during which cells activate within an islet. These intervals differ for the two stimulatory concentrations: about 600 s (100 s < t < 700 s, **Figure 4A**) for the physiological and about 300 s (150 s < t < 450 s, **Figure 5A**) for the supraphysiological concentration. To surpass the qualitative description of the two phases, we looked for collective spatiotemporal behavior of beta cells. To this aim, we meticulously detected Ca^{2+} waves during both phases, and plotted them as individual events in space-time for better visualization. While being stimulated with the physiological concentration, the activation phase exhibited very heterogeneous spatiotemporal behavior, one that resulted in calcium waves of very different sizes (**Figure 4B**). The following plateau phase evoked a more regular pattern of $[Ca^{2+}]_{IC}$ oscillations, with prevailing global intercellular calcium waves that encompassed often the majority of the cells within an islet (**Figure 4D**). However, the activation/plateau pattern changed during supraphysiological stimulation. Majority of the cells responded with a rapid burst of oscillatory activity followed by brief refractory period during the activation phase (**Figure 5B**). The subsequent plateau phase was dominated by global intercellular calcium waves (**Figure 5D**). To be able to quantify the former description, we determined the distribution $P(s)$ of relative wave sizes s and plotted it on log-log scale (**Figures 4C,E**, **5C,E**). While comparing $P(s)$ for the two concentrations in question, we observed that in the lower concentration the $P(s)$ in the activation phase followed the power law, whereas the plateau phase was again dominated by global waves. Such switching in behavior from the critical to the supercritical was not observed in the higher stimulatory

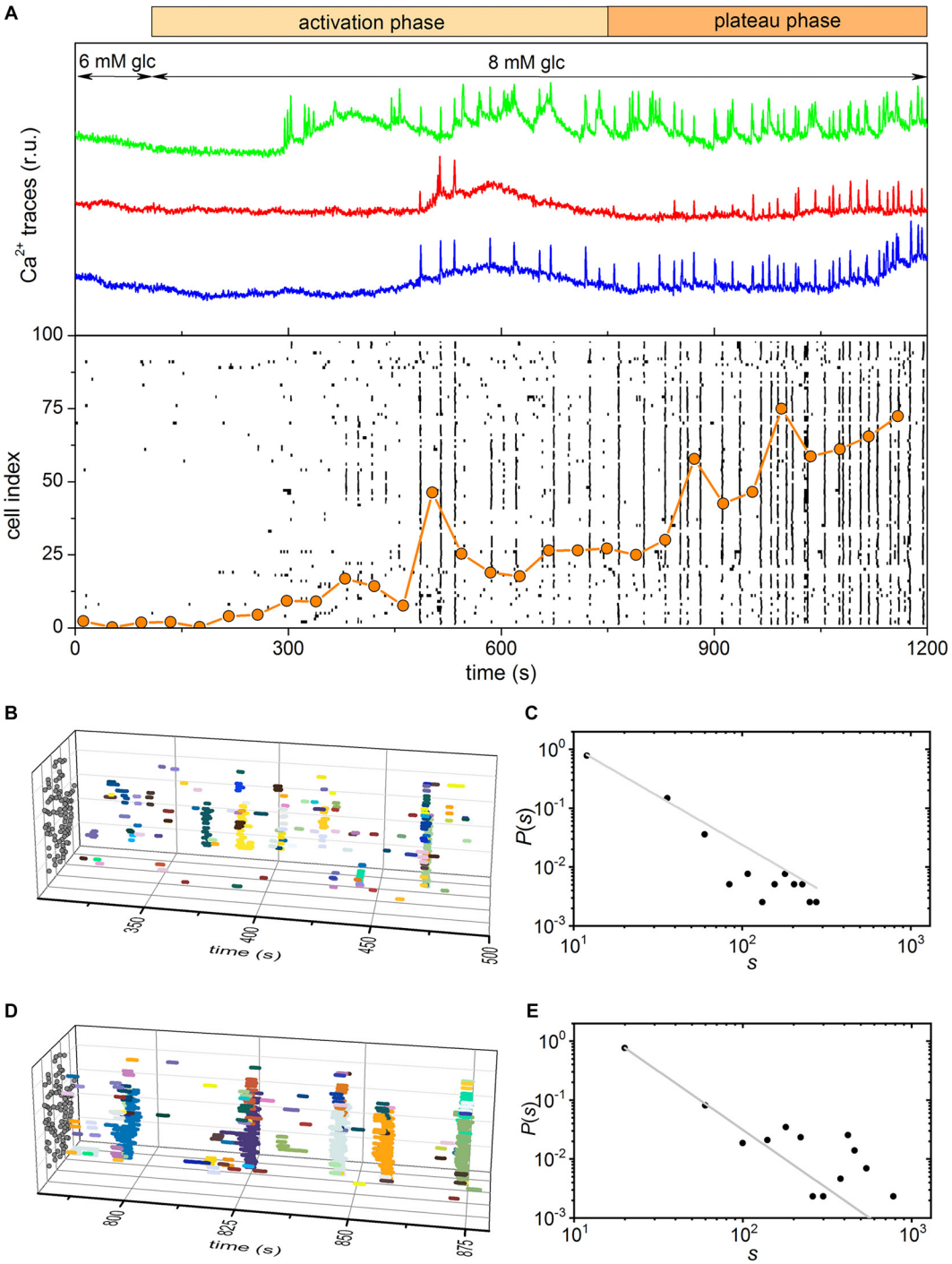

FIGURE 4 | Experimentally measured beta cell responses after stimulation with 8 mM glucose. **(A)** Three characteristic Ca^{2+} traces and the raster plot of binarized Ca^{2+} activity of all cells in the islet. The orange dotted line indicates the fraction of active cells within the given time-window that was slid throughout the recording. **(B,D)** 3D raster plots showing the Ca^{2+} activity waveforms for selected intervals for the activation **(B)** and plateau **(D)** phase. Colors denote specific Ca^{2+} events. Gray dots on the *y*, *z* plane stand for coordinates of cells. **(C,E)** The distributions of Ca^{2+} wave sizes for the activation **(C)** and plateau **(E)** phase. The gray dashed line indicates the power-law fit. The slope in the critical-like activation phase is –1.69.

concentration, during which the behavior was locked to the supercritical during both phases. Namely, the activation phase under supraphysiological concentrations was too rapid, exhibited a huge activation burst, and lacked the progressive recruitment of cells that featured an emergent behavior with very heterogeneous Ca^{2+} waves.

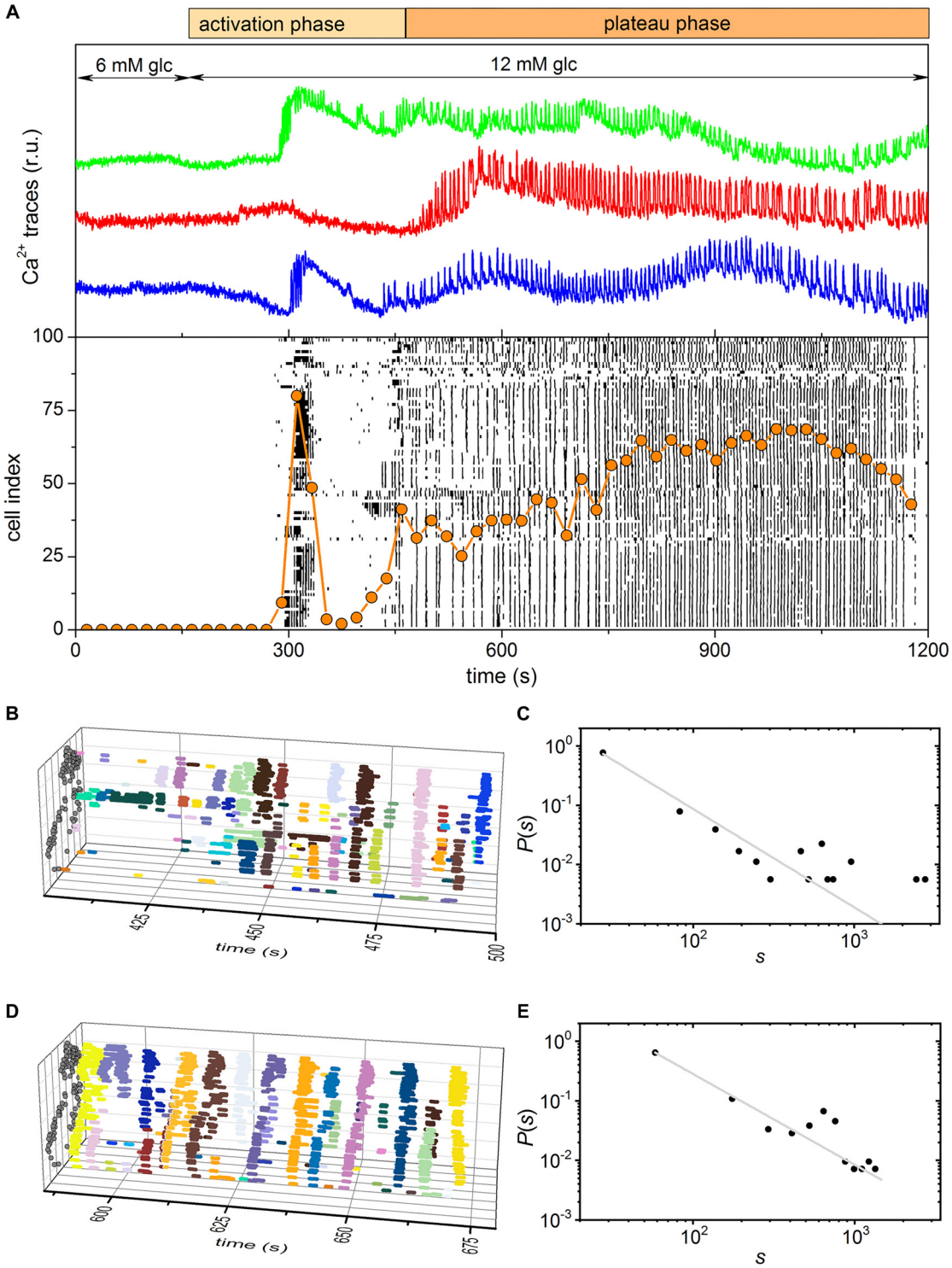

FIGURE 5 | Experimentally measured beta cell responses after stimulation with 12 mM glucose. **(A)** Three characteristic Ca^{2+} traces and the raster plot of binarized signals of Ca^{2+} oscillations in all cells in the islet. The orange dotted line indicates the fraction of active cells within the given time-window that was slid throughout the recording. **(B,D)** 3D raster plots showing the Ca^{2+} waves for selected intervals for the activation **(B)** and plateau **(D)** phase. Colors denote specific Ca^{2+} events. Gray dots on the *y*, *z* plane stand for coordinates of cells. **(C,E)** The distributions of Ca^{2+} wave sizes for the activation **(C)** and plateau **(E)** phase. The gray dashed line indicates the power-law fit.

Computational Results

We developed a phenomenological model of coupled excitable cells with the aim to explore the prerequisites and mechanisms that lead to complex dynamical behavior observed in experiments. The minimalistic map-based description of excitable dynamics mimics the activity of beta cells. The stimulation was modeled as a heterogeneous and delayed increase in the excitability level, whereby a higher increase was used when supraphysiological stimulation was simulated (see section "Materials and Methods"). To further account for beta cell heterogeneity, we additionally included cell-to-cell variability in the absolute levels of excitability and in the intercellular coupling strength. In this case, we obtained a good qualitative agreement with experimental findings. The results are presented in **Figures 6**, **7** for the simulation of physiological and supraphysiological stimulations, respectively.

Regardless of the stimulation level, we observed a biphasic response. Most importantly, the activation phase under lower stimulation levels was rather long and exhibited waves of various sizes and many of them were confined to small sub-regions of the islet. This is a result of very heterogeneous responses to stimulation, which in turn led to non-trivial and self-organized dynamical patterns. As in experimental measurement, the distribution of spatiotemporal cluster sizes was found to roughly follow a power-law (**Figure 6C**), which pinpoints toward a transient phase of critical dynamics. In contrast, the activation phase under supraphysiological conditions was shorter and the wave sizes were larger and more homogeneous, similarly as in the experiment. Consequently, the distribution deviates from the pure power-law behavior, mostly on account of an excess of larger excitation events (**Figure 7C**). The second plateau phase was qualitatively very similar in both scenarios. In both cases the spatiotemporal activity was dominated by global waves, thereby indicating supercritical behavior (**Figures 6E**, **7E**). However, the waves were found to be more frequent and coherent under higher stimulation levels. This resulted to a large extent due to higher excitability levels, which made the cells operate in an even more ordered regime in which stochasticity is less pronounced.

A good agreement between experimental and computational results was obtained only if all three types of heterogeneities, i.e., in excitability level, in the delayed responses to stimulation, and in intercellular coupling strengths were implemented simultaneously. To test the necessity of such a multilayered heterogeneity, we systematically performed simulations with physiological and supraphysiological stimulations without considering one of the particular heterogeneities. Results are presented in **Figure 8**. It can be seen qualitatively that without any of the heterogeneities the simulations do not match well with experimental results. The most obvious difference occurs in the activation phase after the initial activation, where especially in the case of physiological stimulation diverse wave sizes were observed if all three types of heterogeneities were considered. Here, on the other hand, the dynamics after the initial activation and before the system shifts to the plateau phase, is very inactive and lacks on an emergent transitory phase with progressive recruitment of cells. On the contrary, the plateau phase seems to be weakly affected by the lack of any type of variability and even if one of the heterogeneities is missing, the system behavior very similar as in control simulations. This behavior is somehow expected, since after (probably unphysiological) prolonged stimulation all cells get very excitable and placed in the supercritical regime. Also, the heterogeneity imposed by variability in metabolism diminishes. For a more quantitative evaluation we present in **Table 1** the relative activity time in both phases for the experimental data, for the control simulations with 30% variability in all three types cellular heterogeneity, and for simulations without one particular aspect of heterogeneity. The results indicate that indeed the interplay between all three types of heterogeneities is necessary to firmly reproduce the experimentally observed behavior, although it seems that variability in metabolism is the most important determinant, whereas the heterogeneity in the coupling appears to be the least important.

Finally, after determining that all three types of heterogeneities are required, we explored the impact of their level on the spatio-temporal activity. **Figure 9** features the results. It can be observed that no or low degrees of heterogeneities fail to firmly reproduce experimental findings. In case of physiological stimulation, the initial activation of cells is missing and the cells respond much later without a progressive recruitment characterized by excitation waves of different sizes. Moreover, also the emulated supraphysiological stimulation differs if the level of heterogeneity is too low. Especially the dynamical phase after the initial activation is in this case very quiet, in contrast to the experiment and simulations with a higher degree of cellular variability, where a certain fraction of cells oscillates in this intermediate regime before the switch to the plateau phase. A quantitative assessment of this observation is presented in **Table 1**. It can be seen that with increasing levels of cell-to-cell variability the behavior in simulations becomes more similar to experimental results, although especially in the case with physiological stimulation 20% heterogeneity is not sufficient to achieve good consistency.

DISCUSSION

Information processing in living organisms is orchestrated by large networks of interacting cells. In many cases, the dynamics of these networks is guided by the activation of one or a few elements, which in turn provokes the triggering of other elements, thereby leading to avalanches of activity that propagate through the system. Such emergent behavior is associated with self-organization and very often with critical dynamics resulting in a power-law distribution of the spatial and/or temporal extent of activity profiles. This scenario is particularly appealing for excitable systems, such as neuronal networks (Plenz and Thiagarajan, 2007; Hesse and Gross, 2014; Muñoz, 2018) or excitable cells and tissues (Lopez et al., 2012; Nivala et al., 2012; Gosak et al., 2017). Typically, critical dynamics emerges at the transition between randomness (subcritical dynamics) and order (supercritical dynamics). Variations of dynamical regimes can be induced by changes of global parameters, such as the

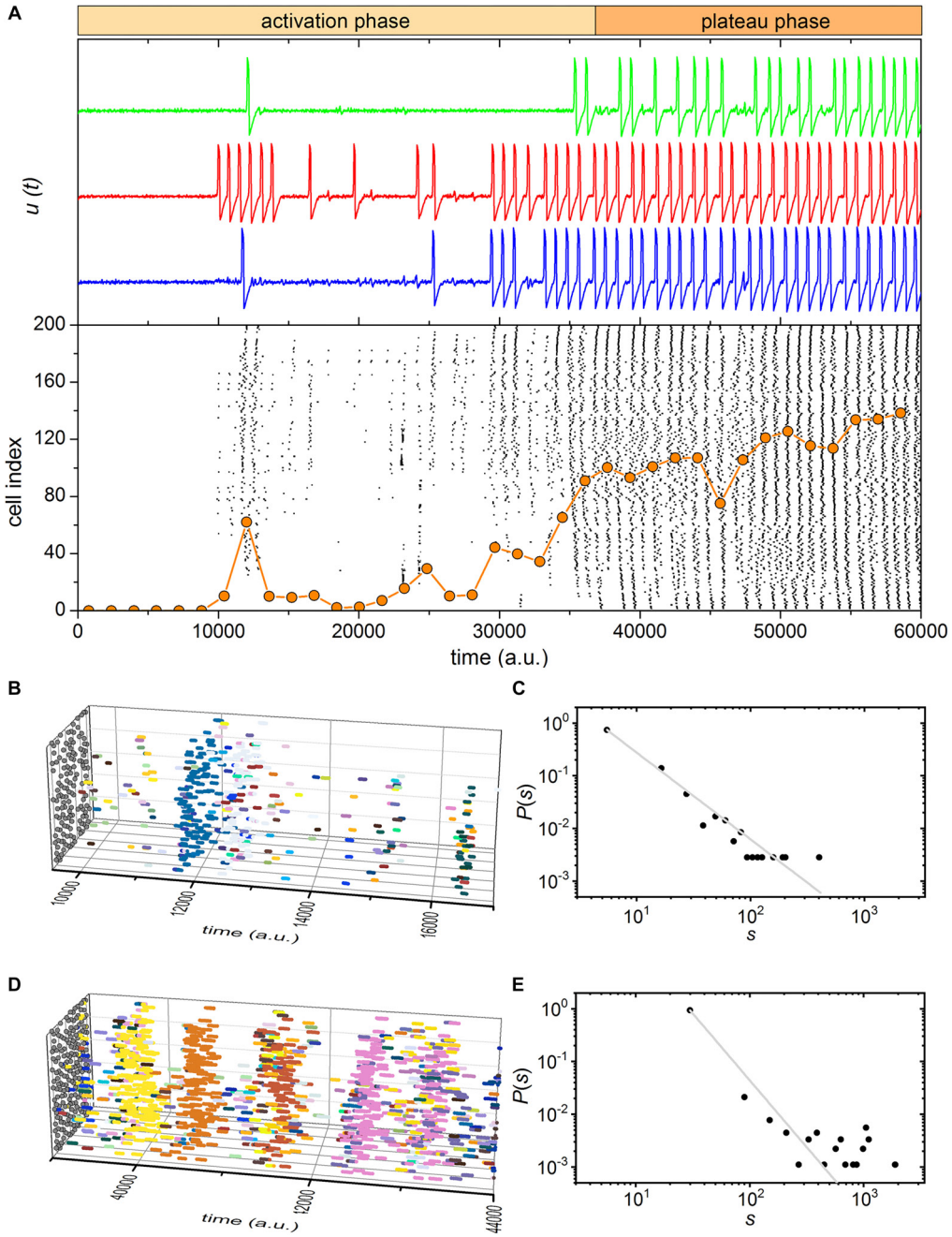

FIGURE 6 | Simulated beta cell responses after switching from a substimulatory to stimulatory levels of stimulation, i.e., from 6 to 8 mM glucose. **(A)** Three characteristic traces of simulated cellular dynamics and the raster plot of binarized cellular activity. The orange dotted line indicates the fraction of active cells within the given time-window that was slid throughout the simulation. **(B,D)** 3D raster plots showing the excitation waves for selected intervals for the activation **(B)** and plateau **(D)** phase. Colors denote individual waves. **(C,E)** The distributions of excitation wave sizes for the activation **(C)** and plateau **(E)** phase. The gray dashed line indicates the power-law fit. The slope in the critical-like activation phase is –1.64.

excitability level, which reflects stimulus intensity. However, in this case a power-law behavior would only be expected in a narrow parameter space in the proximity of a phase transition point. Previous research has underlined the activity-dependent synaptic plasticity, heterogeneity, and hierarchical network organization as plausible mechanisms to overwhelm this drawback. Namely, these biological determinants were found to facilitate scale-free behavior and drive neuronal networks toward the critical state (Levina et al., 2007; Rubinov et al., 2011; Moretti and Muñoz, 2013). These self-organization mechanisms make the oscillators hover around the critical point, which are therefore able to generate effective scale invariance across quite

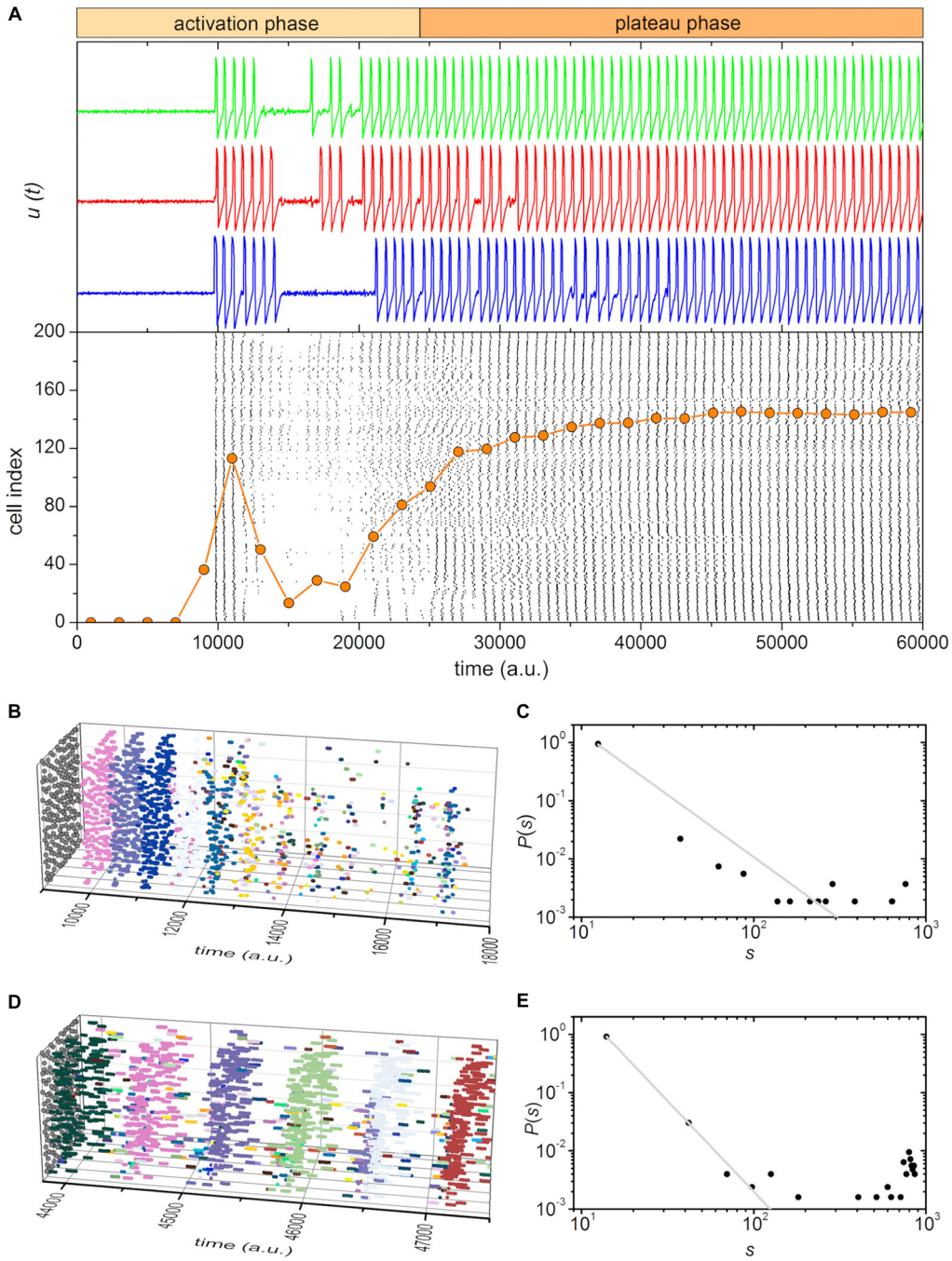

FIGURE 7 | Simulated beta cell responses after switching from a substimulatory to a suprastimulatory level of stimulation, i.e., from 6 to 12 mM glucose. **(A)** Three characteristic traces of simulated cellular dynamics and the raster plot of binarized cellular activity. The orange dotted line indicates the fraction of active cells within the given time-window that was slid throughout the simulation. **(B,D)** 3D raster plots showing the excitation waves for selected intervals for the activation **(B)** and plateau **(D)** phase. Colors denote individual waves. **(C,E)** The distributions of excitation wave sizes for the activation **(C)** and plateau **(E)** phase. The gray dashed line indicates the power-law fit.

a few scales. The phenomenon is often termed as self-organized quasi-criticality (Muñoz, 2018).

In the present study, we suggest a new mechanism that realistic excitable systems might exploit for expanding the operation in a critical-like regime. As the level of cellular excitability (parameter α) increases with time, the spatio-temporal activity switches from an inactive to an active phase (Osipov et al., 2007). If excitable oscillators are homogeneous, critical behavior is expected only at the phase transition point. In our setting, where the control parameter increases with time, criticality would therefore be

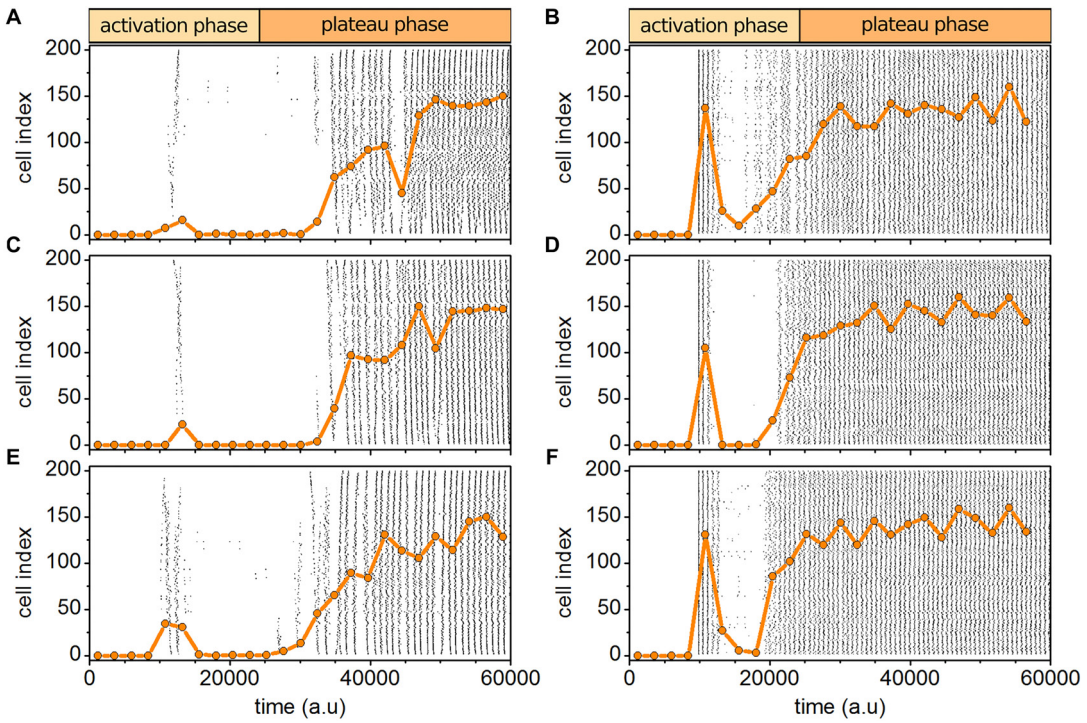

FIGURE 8 | Simulated beta cell responses without particular types of cellular heterogeneities. Behavior without heterogeneity in the intrinsic excitability level **(A,B)**, without heterogeneity in the delayed responses to stimulation **(C,D)**, and without heterogeneity in intercellular coupling **(E,F)**, for both physiological **(A,C,E)** and supraphysiological **(B,D,F)** stimulation levels.

observed for a very short transient period of time. However, the combination of cell-to-cell variability and heterogeneously delayed increases in excitability levels substantially broaden this regime of critical-like behavior. As a result, a rather long transient dynamical phase emerges with heterogeneous wave sizes, the distribution of which closely follows a power law. Even though this critical phase is only temporary and later followed by a supercritical phase dominated by global excitatory events, critical-like behavior persists for substantial periods of time. It should be emphasized that due to the transient nature of the scale-invariant activation phase, the observed power law is only an estimation, since much higher number of events would be required to confirm a pure power-law behavior. Moreover, the present study only provides an empirical observation of transient critical-like dynamical state, and further theoretical efforts are needed to uncover the exact mechanisms for the emergence of critical dynamics in such heterogeneous systems with delayed feedbacks.

TABLE 1 | Activity time during physiological and supraphysiological stimulations.

	Physiological stimulation (8 mM glc)			Supraphysiological stimulation (12 mM glc)		
	Act. ph.	**Plat. ph.**	**Plat./Act.**	**Act. ph.**	**Plat. ph.**	**Act./Plat.**
Exp	**3.0**	**12.0**	**4.0**	**3.9**	**16.7**	**4.3**
Sim (30% het)	**3.2**	**13.4**	**4.2**	**3.3**	**14.4**	**4.4**
No het cpl	2.0	12.6	6.3	2.1	15.3	7.3
No het exc	1.1	13.2	12.0	2.5	14.6	5.8
No het met	0.7	13.4	19.1	1.0	15.5	15.5
Het 0%	0.0	3.1	/	0.5	13.9	27.8
Het 10%	0.0	6.2	/	1.0	14.5	14.5
Het 20%	1.1	12.1	11	2.2	15.0	6.8

Shown are percent of active time during activation phase of the response (Act. ph.), plateau phase of the response (Plat. ph.), and the relative ratio of the two (Plat./Act.), calculated for experimental data (Exp), and for the model with 30% heterogeneity in all three aspects [Sim (30% het)]. Additionally, values are depicted for the model with no heterogeneity in the coupling parameter (No het cpl), in the excitation parameter (No het exc), and in the metabolism parameter (No het met), as well as the values for no heterogeneity (Het 0%), for 10% (Het 10%), and for 20% (Het 20%) variability in all three aspects of heterogeneity. Bold values refer to original main results.

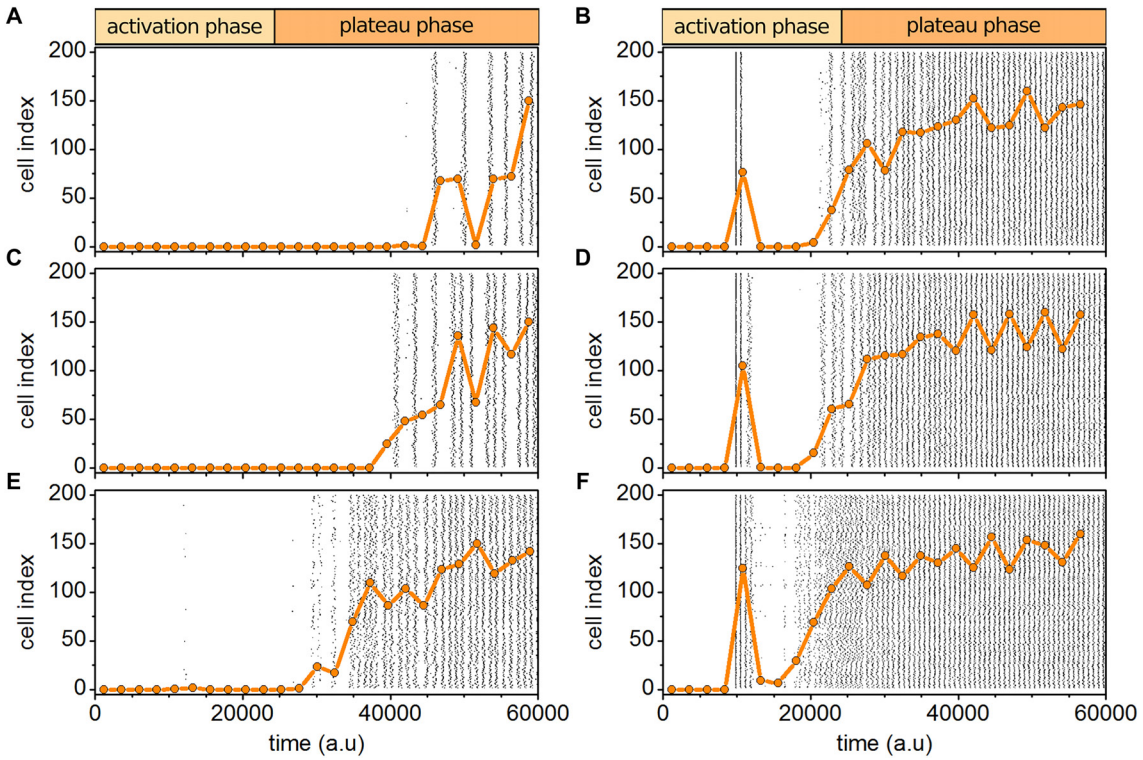

FIGURE 9 | Simulated beta cell responses for different degrees of heterogeneities. Simulating the behavior without **(A,B)**, with 10% **(C,D)**, and 20% **(E,F)** of the used degree of heterogeneities for physiological **(A,C,E)** and supraphysiological **(B,D,F)** stimulation levels.

In real-life settings, it is quite common to have transient or oscillatory stimulation patterns instead of a long-lasting permanent stimulation. In neurons (Buzsaki, 2004; Schroeder and Lakatos, 2009), in pancreatic islets (Pedersen et al., 2013; Satin et al., 2015), and in cardiac myocytes (O'Rourke et al., 1994), the excitation dynamics is governed by basal variations in intrinsic excitability, for instance due to oscillations in hormone or nutrient concentrations. Notably, in our previous study we have shown that such an oscillatory entraining might be a key toward persisting criticality in pancreatic beta cells (Gosak et al., 2017). Apparently, providing proper transitory conditions for heterogeneous excitable elements that exhibit delayed and variable responses to stimulation is a viable route to scale-free behavior. Because of these heterogeneities, changeable and confined regions with elevated excitability emerge from which the excitation waves are triggered. In the activation phase, the waves are typically triggered from cells whose excitability level increased faster, whereas in the plateau phase no specific patterns can be inferred. Moreover, the range of waves in the activation phase depends on the coupling and on the variable excitable state of surrounding elements. This in turn leads to emergent behavior with very heterogeneous spatiotemporal patterns. Most importantly, the critical-like activation phase is only possible to achieve if the stimulation level is not too high. Namely, in case of supraphysiological stimulation levels, the transition to the supercritical state is too abrupt and possibly accompanied by processes that do not occur under physiological conditions, at least not to a notable extent. Consequently, excitable cells are not able to self-organize into a scale-invariant dynamical state, as is the case in physiological stimulation conditions. Cell-to-cell differences are always present to some degree in any cell population, impacting the signaling processes in various tissues and settings (Muotri and Gage, 2006; Marhl et al., 2010; Paszek et al., 2010). Notably, cellular heterogeneity is more than a nuisance and often serves a biological function or contains meaningful information (Altschuler and Wu, 2010). In islet research, beta cell heterogeneity has been one of the key issues for decades and is becoming increasingly popular, particularly in the context of subpopulations (Bader et al., 2016; Avrahami et al., 2017). Thus, the central concept of our model, i.e., multiform beta cell heterogeneity, has a long tradition. In 1987, Pipeleers defined it on grounds of structural, functional, and replicative differences between beta cells. More specifically, he pointed out differences between beta cells in terms of contact with other types of endocrine cells, in gap junctional coupling, and cellular hormone content, in their ability and sensitivity to mount a response to glucose, and in their replicative potential. Ahead of time, he argued that altered beta cell heterogeneity may turn out to be important in development of diabetes and in islet transplantation (Pipeleers, 1987). In two updates shortly thereafter, he provided evidence for functional differences between beta cells in their rates of glucose-induced insulin synthesis and secretion that were attributed to differences in thresholds for glucose utilization and oxidation. Notably, already

at that time the idea was put forward that cellular heterogeneity crucially determines dose-dependence of the beta cell response to glucose due to recruitment of beta cells into an active state by increasing glucose concentrations and that elevated levels of glucose can decrease the extent of heterogeneity. Additionally, he proposed that heterogeneity is not just an experimental artifact observed in dispersed beta cells, but at work also in intact tissue (Pipeleers, 1992; Pipeleers et al., 1994). Following the advent of new molecular markers and the omics approaches, in the last decades the concept of beta cell heterogeneity has been further supported at the transcriptomic and proteomic level, together with novel findings that heterogeneity affects beta cell proliferation and survival, as well as their stimulus-secretion coupling, from glucose metabolism and Ca^{2+} signaling to insulin secretion (Benninger and Piston, 2014; Roscioni et al., 2016; Avrahami et al., 2017; Gutierrez et al., 2017; Pipeleers et al., 2017; Benninger and Hodson, 2018). Finally, it has been proposed that the lack of beta cell heterogeneity may importantly contribute to islet failure in diabetes (Johnston et al., 2016; Pipeleers et al., 2017; Skelin Klemen et al., 2017; Benninger and Hodson, 2018; Nasteska and Hodson, 2018). One major drawback, common to most recent work, is the use of dispersed beta cells. It therefore remains to be investigated to what extent the heterogeneity described thus far is translationally relevant in the tissue context or even *in vivo* (Carrano et al., 2017; Benninger and Hodson, 2018; Gosak et al., 2018; Nasteska and Hodson, 2018).

By employing the tissue slice approach, we studied a large number of coupled beta cells in their normal tissue environment. Our experimental and modeling results intersect with both the original and more recent findings on beta cell heterogeneity at several points and provide some new ideas. First, during the activation phase, differences in glucose sensitivity were observed between different cells within the same islet and these differences were larger in lower glucose (8 mM). In other words, in lower glucose, gradual recruitment of beta cells into an active state, brought about by local $[Ca^{2+}]_{IC}$ waves displaying critical behavior seems to be a major feature of the islet response to constant stimulation. In contrast, in high glucose (12 mM), recruitment is less well pronounced due to early global $[Ca^{2+}]_{IC}$ waves showing supercritical behavior. We wish to speculate that recruitment (Stožer et al., 2013a; Gosak et al., 2017) and local $[Ca^{2+}]_{IC}$ waves (Benninger et al., 2014; Westacott et al., 2017) in islets, as opposed to dispersed cells or clusters of cells (Jonkers and Henquin, 2001), have received less attention in the scientific community due to use of high stimulatory glucose concentrations. Second, further aspects of adaptation in the response to higher glucose are the shorter average delay to activation, shorter activation phase, and higher activity during the plateau phase. Since it has been shown recently that in addition to different pools of granules, the triggering Ca^{2+} signal importantly shapes the biphasic insulin secretion in response to constant stimulation by glucose (Pedersen et al., 2019), our findings shall importantly inform future models of beta cell insulin secretion. Additionally, the behavior of beta cells during the activation phase, when they are not functioning in synchrony with other cells, could be compared with their properties during the plateau phase to more precisely establish the relationships between their different roles. More specifically, such comparison could help answer the question whether the cells that activate first are also the ones that initiate global $[Ca^{2+}]_{IC}$ waves and possess the most functional connections, i.e., function as hubs, during the plateau phase (Stožer et al., 2013b; Johnston et al., 2016; Westacott et al., 2017). Third, using our phenomenological model, we found that all three types of heterogeneities, the choice of which is further substantiated in the following section, are necessary and sufficient to reproduce the experimentally observed behavior. This of course does not exclude the possibility that additional aspects of heterogeneity exist in reality and further modulate beta cell responses.

In comparison with our previous study, we used here a very simple phenomenological model to reproduce the experimentally observed non-trivial activity patterns in islets (Gosak et al., 2017). Such minimalistic modeling approaches have of course limitations, since they do not allow for any mechanistic insights into physiological processes and signaling pathways. At the same time, they offer several advantages. They are numerically very efficient, and most importantly, they contain a small number of parameters whose roles are rather clear, which makes it easier to explicitly study particular aspects of cellular heterogeneity. In contrast, realistic and multi-component cellular models exhibit many parameters that in general affect several aspects of signalization, which hinders a systematic and definitive examination of their particular influences on cellular behavior. Finally, it should be noted that the majority of existing comprehensive beta cell models were mainly focused on the activity on the plateau phase, whereas modeling of collective cellular activations after switching from substimulatory to (supra)stimulatory glucose received very little attention. The mechanisms that govern such stimulus-dependent activation are also understood incompletely. Phenomenological modeling is therefore beneficial in this respect, as long as the empirical description of the processes ensures good agreement between modeling and experimental results. In particular, the time lags and temporal evolution of the excitability level [see eq. (3)] are plausible processes that can be qualitatively linked with previous experimental observations, such as differences in metabolic sensitivity to glucose and the following electrical and $[Ca^{2+}]_{IC}$ responses (Stožer et al., 2013a,b; Benninger et al., 2014; Farnsworth and Benninger, 2014; Johnston et al., 2016). Moreover, we decided to include heterogeneity in intercellular coupling due to the extensive experimental support demonstrating its importance in both normal and pathological islet functioning (Hodson et al., 2013; Farnsworth et al., 2014, 2016; Johnston et al., 2016; Skelin Klemen et al., 2017). However, here we focused only on the fast $[Ca^{2+}]_{IC}$ oscillations and future studies will conceivably need to include additional aspects of heterogeneity to provide a comprehensive and realistic beta cell model capable of describing other components of the $[Ca^{2+}]_{IC}$ pattern, other parameters in the stimulus-secretion cascade, as well as responses to different levels of stimulation and different secretagogues.

Multiscale and multidimensional heterogeneity represent a viable route to critical-like behavior for a substantial period of time. Specifically, the dynamical transition between the inactive

and active state occurs in a rather broad temporal interval, especially in the case of physiological levels of stimulation. In other words, as the glucose level increases the activation of cells is not abrupt. Before switching to the dynamical state with global Ca^{2+} events, a transient period of very heterogeneous wave sizes is observed, which implies a critical-like behavior, since the system as a whole bypasses the critical point rather slowly. On the other hand, supraphysiological high stimulation levels lack on such progressive recruitment of cells and lead to a rapid transition to a fully active state. This might be crucial for healthy physiological functioning of pancreatic islets and potentially of other biological tissues as well. Namely, for other biological tissues, there is a large body of evidence indicating severe pathophysiological consequences of an abrupt collective transitions to hyper-regulated synchronous tissue responses. An overview was given by Trefois et al. (2015), showing that critical transitions are identified as early warning signals for the onset of different pathologies ranging from microbiome dysregulations to irritable bowel syndrome, asthma, pulmonary disease, depression, type 1 and type 2 diabetes, inflammation, start and termination of epileptic seizures, cancer, and cardiovascular events.

Further investigations are needed to understand the onset of pathological supercritical behavior in more detail. The molecular and cellular mechanisms are still obscure; however, the results of our study, although only empirical, give at least a hint to an improved methodology, opening a new dimension in studying the (premature) onset of supercriticality by looking at the extent of cell heterogeneity. Some preliminary studies in our lab show that beta cell responses in terms of $[Ca^{2+}]_{IC}$ signals shall also be correlated with other aspects of heterogeneity to get a more complete picture about the mechanisms that make some cells more responsive to glucose and to find out whether this is a stable property or something that changes with time and on which temporal scale. In addition, different concentrations of glucose and additional stimulation protocols, as well other secretagogues shall be used in future studies. Moreover, the general extent of heterogeneity in the islets, as well as the properties of individual cells shall be investigated in mouse models of diabetes and in human islets from normal and diabetic donors to more clearly define the changes under pathological conditions and suggest targets for treatment (Benninger and Hodson, 2018; Stožer et al., 2019). In general, from the viewpoint of clinical approaches, the understanding of critical transitions might help us develop therapies that are more effective. From the viewpoint of preventive health care, an improved understanding of the pathological premature transitions to supercriticality could help us identify and characterize some early warning signals predicting the upcoming pathological transitions. Finally, beyond the preventive and therapeutic role Bargaje et al. (2017) showed that an increase in cell heterogeneity of stem cells just before the critical transition correlates with a branching point on the trajectory of cell fate. This represents a useful tool for forecasting the cell fate outcomes and can be used for optimizing the differentiation protocols in order to obtain desired cell populations, which opens a completely new dimension of bioengineering in the future.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

The study was conducted in strict accordance with all national and the European recommendations pertaining to care and work with experimental animals, and all efforts were made to minimize suffering of animals. The protocol was approved by the Veterinary Administration of the Republic of Slovenia (Permit Number: U34401-12/2015/3).

AUTHOR CONTRIBUTIONS

AS, RM, JD, MP, MSR, MM, and MG designed the study, carried out the research, and wrote the manuscript.

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REVIEW
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Biological Relativity Requires Circular Causality but Not Symmetry of Causation: So, Where, What and When Are the Boundaries?

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Since the Principle of Biological Relativity was formulated and developed there have been many implementations in a wide range of biological fields. The purpose of this article is to assess the status of the applications of the principle and to clarify some misunderstandings. The principle requires circular causality between levels of organization. But the forms of causality are also necessarily different. They contribute in asymmetric ways. Upward causation can be represented by the differential or similar equations describing the mechanics of lower level processes. Downward causation is then best represented as determining initial and boundary conditions. The questions tackled in this article are: (1) where and when do these boundaries exist? and (2) how do they convey the influences between levels? We show that not all boundary conditions arise from higher-level organization. It is important to distinguish those that do from those that don't. Both forms play functional roles in organisms, particularly in their responses to novel challenges. The forms of causation also change according to the levels concerned. These principles are illustrated with specific examples.

Keywords: biological relativity, downward causation, circular causality, entangled causation, boundaries in physiology

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INTRODUCTION

The principle of Biological Relativity is that, *a priori*, i.e., before performing the relevant experiments, there is no privileged level of causality (Noble, 2012). In multi-scale networks of interactions, as found everywhere in organisms, any parts of a network at any level might affect every other part.

The principle is based on mathematical approaches to understanding biological processes. While the differential (or equivalent) equations represent the dynamics of the components of the system, the initial and boundary conditions represent the historical and contextual (environmental) factors without which no specific solutions to the equations would be possible.

The principle has found many applications in physiology and in other fields of biology. This is not surprising since the mathematical point being made is a necessary one, regardless of whether the components are molecular (genes, proteins, and metabolites), networks (at all levels), cells, tissues, organs, or any other kind of component. Moreover, in practice the principle has been applied many times in physiology even before it was formulated as a mathematical principle. All forms of feedback

between levels in biological systems inherently assume the principle. It can therefore be seen as formalizing an idea that has been inherent in physiology, at least since Claude Bernard in the 19th century (Bernard, 1878, 1984; Noble, 2008, 2013), and Walter Cannon in the 20th century (Cannon, 1932) formulated the ideas of homeostasis. Nevertheless, the principle is not limited to the usual interpretations of homeostasis as linear circularity. The regulatory systems in organisms do much more than act like sophisticated thermostats. There are no fixed set-points. There are sets of set-points each of which can vary as the organism seeks to maintain itself. Buiatti and Longo (2013) express this point by using the word homeorhesis in place of homeostasis:

> "Biological objects are, as discussed by Waddington, "homeorhetic," as opposed to homeo-static, in the sense that, during their cycles, they keep changing. Moreover, their onto-phylogenetic path is largely unpredictable, though preserving, as long as possible, the internal coherence of an organism and its relations to the ecosystem. It is unpredictable because of the random effects at each level and of the bio-resonance effects between different levels."

As our article will make clear, the various levels communicate both randomness and order between each other. We agree therefore with Rosen in *Life Itself* (Rosen, 1991, 2000), that it is the *organization of the organism itself* that constrains the component parts, not the other way round. That organization forms the basis of active agency in organisms (Noble and Noble, 2017; Noble, 2018). One of the aims of this article is to interpret the principle of biological relativity in a more radical way.

The principle also raises many other questions. The aim of this paper is to formulate those questions and attempt to resolve them. Foremost amongst those are questions concerning what is meant by a boundary.

As physiologists we might think that question has an obvious answer. Cells have membranes, tissues have surfaces, organs have shapes with anatomical boundaries, the organism has its outer structure, skin. But where are such boundaries of the great systems of the body, the immune, nervous, circulatory, digestive, respiratory, reproductive, and hormonal systems? Merely to ask the question shows that the answer is not obvious. Anatomy is not necessarily the best basis for defining a functional boundary. To varying degrees, the boundaries used in models are somewhat arbitrary. And even when we can identify an anatomical boundary it is not necessarily the mathematical computational boundary.

As an example of the kind of problem we will address consider the problem faced in modeling the electrophysiology of the heart during the 1980s when processes involving changes in ion concentrations were added to the existing equations for the gating of ionic channels (McAllister et al., 1975). Prior to the DiFrancesco-Noble equations (DiFrancesco and Noble, 1985) this had not been done in any systematic way. Yet it was necessary to incorporate changes in K^+ concentration in intercellular spaces to understand how these could make a non-specific cation channel conducting both Na^+ and K^+ behave like a pure K^+ channel. The new model was completely successful in achieving this aim. But that was not possible without changing the boundaries of the model. One of us explained this boundary problem in 2012:

> "The obvious next step was to develop the McAllister–Noble–Tsien model of 1975 to replace i_{K2} by i_f. But that was much easier said than done. It took a full 5 years of development. This was because it was not just a matter of replacing one ionic channel mechanism by another. It also involved modeling global ion concentration changes for the first time in an electrophysiological model of the heart, including the intracellular calcium signaling. Dario and I did that because it was necessary to explore fully what we had discovered. We did not know then that we would be creating the seminal model from which virtually all subsequent cardiac cell models would be developed. There are now over a hundred such models for various parts of the heart and many different species[1]."
>
> Extending biological models is often like tumbling a row of dominoes. Once one has fallen, many others do too. The reason is that all models are necessarily partial representations of reality. The influence of the parts that are not modeled must either be assumed to be negligible or to be represented, invisibly as it were, in the assumed boundary conditions and other fixed parameters of the model. Once one of those boundaries is removed, by extending out to a different boundary, other boundaries become deformed too. In this case, modeling external potassium changes required modeling of the influence of those changes not only on the ion channels already in the model, but also on exchange mechanisms, like Na-K-ATPase (sodium pump) and the Na-Ca exchanger. That, in turn, required the model to extend to modeling internal sodium concentration changes, which in turn required modeling of intracellular calcium changes, which then required modeling of the sarcoplasmic reticulum uptake and release mechanisms. For a year or two it was hard to know where to stop and where to stake out the new boundaries" (Noble et al., 2012) (Page 58).

Even more difficult is the fact that physiological boundaries can be dynamic. When and why they occur are also important questions since it is at boundaries that many of the vital functional processes occur. Recall that the nervous system develops from the embryonic "boundary," the ectoderm, and in single cell organisms the surface membrane can be regarded as its nervous system. Organisms are open systems, so their boundaries are necessarily where much of the action occurs.

DEFINITIONS

Biological Relativity

Biological relativity is the principle that there is, *a priori*, no privileged level of causation. The necessary mathematical basis of the principle was first proposed in 2012 (Noble, 2012) when it was categorized as a "theory." It is better viewed as a principle since it expresses the conceptual point that there is no empirical justification for privileging any particular level.

Upward Causation

Upward causation is the set of processes by which the lower elements in a system interact and produce changes at higher levels. In differential equation models these processes are

[1] www.cellml.org

described by the dynamics represented by the differential equations themselves.

Downward Causation

Downward causation is the set of constraints imposed by the higher levels on the dynamics at lower levels through determining many of the initial and boundary conditions. El-Hani and Queiroz (2005) use the term, "Downward Determination", but they agree that what is involved is something that "can be understood in terms of constraints that the condition of belonging to a system-token of a given kind imposes on the behavior of the components." The sense of cause we are using includes that of determination. We agree that there are different kinds of causation (Noble, 2016) (pp 176–181). Mossio et al. (2013) also emphasize the role of higher level constraints when they refer to "emergent causal powers exerted as constraints, and we claim that biological systems crucially differ from other natural systems in that they realize a closure of constraints."

Initial Conditions

Initial conditions are the initial values of each dynamic element at lower levels. They are determined by the history of development of the system, including stochastic variation as well as previous states of the system. The upward and downward forms of causation interact (**Figure 1**).

Boundary Conditions

Boundary conditions are the conditions attributable to interaction with the environment. In partial differential equation models these conditions are represented by the state of the spatial boundary of the system. In ordinary differential equation simplifications in which spatial changes are assumed to be instantaneous these conditions are represented by the constant coefficients at any moment in time.

Structure

Structure is also a condition that could be regarded as initial or boundary according to the modeling chosen.

Conditioned Causation

Conditioned causation is a state of a system where it would be misleading to attribute causation to any particular element.

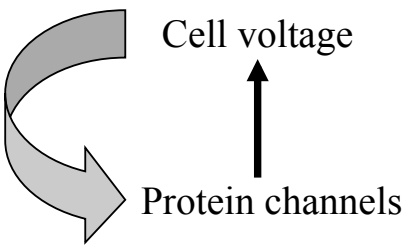

FIGURE 1 | An example of circular causality in physiology. The Hodgkin cycle represents the fact that global cell properties, such as electric potential, control molecular level properties, such as ion channel proteins, which in turn determine changes in cell properties.

MAIN SECTIONS

How Do Upward and Downward Forms of Causation Differ?

The existence of both upward and downward forms of causation is often represented as circular causality. While obviously correct in the sense that both forms exist and, in many ways, must influence each other, such diagrams hide the fact that there is an important difference. The upward and downward forms are necessarily different, just as the initial and boundary conditions of differential equation models are clearly not the differential equations themselves.

It is also important to distinguish conceptual questions about how we see things from what nature does. Nature is a continuum on which we impose somewhat arbitrary boundaries which are dependent on the models we use to understand nature. This point should be borne in mind throughout this article.

Upward Causation

Lower levels influence higher levels through the dynamic changes represented by the differential equations. These will result in global changes, for example in concentrations of ions, metabolites, proteins in cells, tissues and organs and these may in turn trigger further changes at any or all of the higher levels.

As an example, consider the processes involved in calcium movements in the various kinds of muscle in an athlete during vigorous exercise. Too much intracellular free calcium may cause maintained serious problems in the athlete's heart, skeletal muscles or smooth muscles. Any of these, such as a sudden heart attack, may cause severe pain, in turn leading the athlete to collapse. Then the influences become wider and wider as the team coach and physiotherapist enter the scene, which further leads to social interactions. This is an example of unintended effects at a lower level triggering many other events at higher and higher levels.

Downward Causation

Now let's consider how the athlete became an athlete in the first place. He spent hours a day training. This was his decision. It wasn't a decision of the calcium ions in his muscles, nor of the gene sequences in his DNA. Molecules and ions are not causes in that sense (Noble, 2016). It was a high-level choice that he made (Noble and Noble, 2018) and it resulted in many changes in his musculoskeletal, respiratory and cardiovascular systems, all becoming more powerful. Many of these changes came about through exercise influencing gene expression of the proteins in muscles, the lungs and the cardiovascular system. This in turn changes the innumerable boundary and initial conditions under which all the muscles in the athlete's body behave. The changes influence how much muscular, breathing and cardiovascular capacity the athlete has. Although the differential equations for each of his muscle fibers will still be much the same, the changed initial and boundary conditions now ensure that the athlete can do the same or even more vigorous exercise without experiencing disabling fatigue and cramp. This is an undeniable physical effect at the molecular level arising from the athlete's choice of lifestyle.

It doesn't alter the laws of molecular behavior. It alters the solution to the equations for those laws.

Identical Twin Athletes

At this point a rigorous genetic reductionist (Comfort, 2018; Plomin, 2018) might want to argue that no downward causation was involved. The athlete was simply born with the right genes to develop as an athlete. While that must be true – someone suffering from a genetic disease like muscular dystrophy, for example, could not do what the athlete does – it is far from being the complete story. Studies of identical twins who chose very different kinds of sports and exercise training show that very clearly. **Figure 2** is taken from such a study (Keul et al., 1981). The runner and the weightlifter showed completely different effects on their body physique. Bathgate et al. (2018) have recently published a more extensive study of many differences in muscle and cardiovascular health and performance in monozygotic twins. They conclude that "the cardiovascular and skeletal muscle systems exhibit greater plasticity than previously thought." Furthermore they have identified precisely which RNA levels of control are changed by the lifestyle choices.

Genome-Wide Association Studies

Genome sequence studies have failed to find just a single or a very few genes that are strongly correlated with athletic performance. A literature search on publications in the period 1997–2014 showed at least 120 genes show correlations with athletic performance, many of the correlations being very small (Ahmetov and Fedotovskaya, 2015). That number of correlated genes is likely to grow as even more extensive GWAS results appear. So much so that some GWAS scientists have come to the conclusion that virtually the whole genome may be correlated with most phenotypes, the so-called omnigenic hypothesis (Boyle et al., 2017). A study of 1520 endurance athletes and 2760 controls "did not identify a panel of genomic variants common to these elite endurance athlete groups" (Rankinen et al., 2016), and see their earlier studies (Rankinen et al., 2000, 2005). One recent study comparing the impact of genes and environment concluded "that the traditional argument of nature versus nurture is no longer relevant, as it has been clearly established that both are important factors in the road to becoming an elite athlete." (Yan et al., 2016) In a review of elite athletic performance Joyner and Coyle concluded "finding genetic markers that are strongly predictive of either success in endurance athletic performance or somehow preclude it is likely to be a daunting task because of the many cultural and environmental factors that contribute to success in sport, the many physiological factors that interact as determinants of performance, and the heroic nature of the training required" (Joyner and Coyle, 2008).

Epigenetic Control

The main reason for the failure to explain athletic performance from genetics alone is that the genome is controlled by the organism and its life-style experiences through extensive epigenetic control.

As an example, athletes have lower heart rates than non-athletes, which was once attributed to greater vagal tone. The changes have now been traced to microRNAs that downregulate expression of the HCN gene, so that the depolarizing current (I_f) produced in the sinus node cells is reduced by as much as 50% (D'Souza et al., 2017). Moreover, that changes in autonomic tone could not be the explanation was shown as long ago as 1967, but the authors could not at that time identify the mechanism (Sutton et al., 1967). The advent of modern techniques for identifying epigenetic control has transformed this field of study.

The interface between DNA and epigenetic control is therefore another important boundary. It is one of the means by which the organism controls its genome as a "highly sensitive organ of the cell" (McClintock, 1984). This boundary was first identified by Waddington (1957), who was the

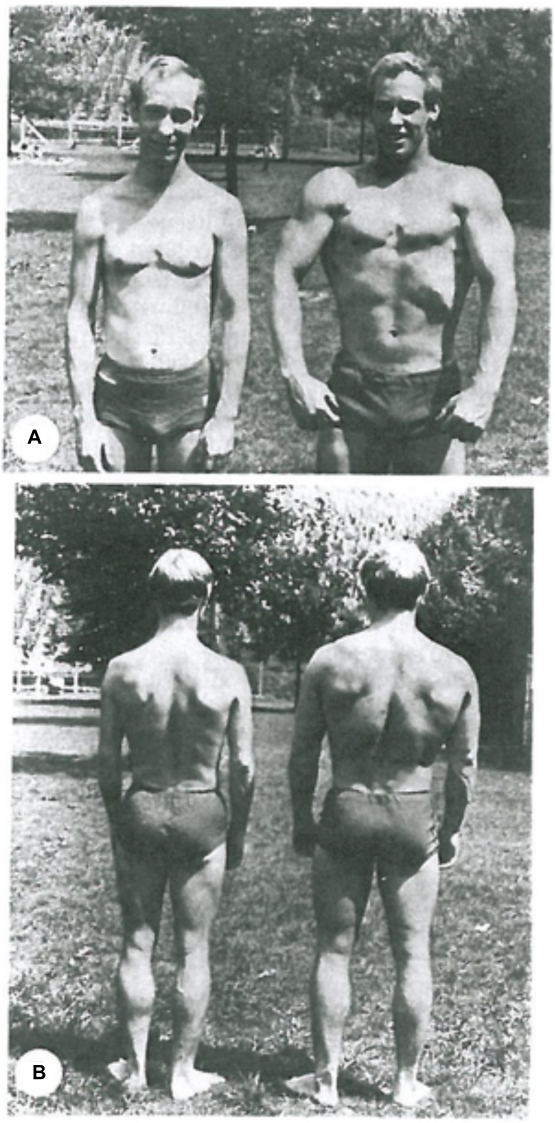

FIGURE 2 | Identical twins. **(A)** Long-distance endurance runner. **(B)** Weightlifter. Notice the highly developed calf muscles in the runner and the contrast with the highly developed arm and chest muscles of the weightlifter. Reproduced with permission from the publisher of Keul et al. (1981).

originator of the term epigenetics. Since then many forms of epigenetic control have been discovered. This control is so effective in transmitting the adaptive properties of the networks that most gene knock-outs have very little effect. The exceptions are, of course, the rare genetic diseases, such as cystic fibrosis, where the networks do not have sufficient plasticity to cope with a knock-out. But, in general, plasticity is common. In yeast, for example, 80% of gene knock-outs are silent in the sense that they produce no phenotypic effect when the yeast is well-nourished (Hillenmeyer et al., 2008). That result has been broadly confirmed by Galardini et al. (2018) who have shown the extent to which the effect of a gene deletion depends on the genetic background. They conclude that "interpretation of the impact of genetic variants on the phenotypes of individuals would likely need detailed gene-phenotype information in more genetic backgrounds than that of a model individual." We would add that the phenotype background must also be relevant. The boundary between regulatory networks and DNA is necessarily a two-way boundary. The regulatory networks can filter genetic changes, acting as what we have characterized as a "cloud" at the boundary (Noble and Noble, 2017; Galardini et al., 2018).

The downward forms of causation represented by the choices made by the individual organism and the influences of its environment must therefore be widespread and necessary.

Open Systems and Their Boundaries

One reason why boundaries are important is that all organisms are open systems. The interaction with the environment is an essential process of being alive. It is across the boundary between the organism and its environment that all the exchanges of energy and matter occur. The same principle applies within the organism. There are boundaries between cell components, between cells, tissues, organs, . . .all the way up. Downward causation can be seen to be traversing a cascade of boundaries. Each level of organization provides the boundary and initial conditions for solutions to the dynamic equations for the level below.

Are All Forms of Downward Causation Functional?

So far, we have established why downward causation is effective and that its necessary effectiveness is mathematically demonstrable. Now let's look at those initial and boundary conditions more carefully. When we inspect the most complete of the mathematical models of skeletal, cardiac and smooth muscles we can identify more than 100 constants in the equations (DiFrancesco and Noble, 1985; Yang et al., 2003; Shorten et al., 2007). Each of those, alone or in combination, reflects an initial or boundary condition. So, there are at least that many parameters that might be sensitive to causative action from higher levels. These parameters are determined by the state of the boundaries between higher and lower levels. In reality there will be many more. The model is just a partial abstraction of reality.

Could all parameter changes in the initial and boundary conditions be attributable to downward causation? There are several reasons why that cannot be true.

The lowest boundary: molecular stochasticity

As Robert Brown showed in 1827, fine particles suspended in water show stochastic movement which was eventually shown by Einstein to be produced by random bombardment by individual water molecules. The molecules in cells are an aqueous suspension and must also be subject to Brownian motion. Water, and all molecules, will also be subject to quantum mechanical randomness. On some interpretations of quantum mechanics, all objects are subject to such randomness (Becker, 2018), although it becomes negligible at a large enough scale.

This is a boundary *within* the system. In a sense it is a boundary between levels or scales. Later in this article we will discuss how organisms use this and other boundaries between levels. But here it is sufficient to note that the boundary is fuzzy. There is no precise cut-off scale at which molecular stochasticity, whether quantal or not, becomes negligible. This is a major issue in the interpretation of quantum mechanics (Becker, 2018), but it need not detain us here. We note that it is a good example of a boundary that cannot be given a precise anatomical location. In a sense the boundary is everywhere. It is a boundary between levels of organization.

Functional and non-functional initial and boundary conditions

Influences on a system from its environment and higher scales can be of at least two kinds. Some will be contingent and even apparently random. These will provide opportunities for novelty in the organism's behavior, in much the same way as we have described in related articles (Noble and Noble, 2017, 2018). Stochasticity can be used by organisms to generate novelty. That can happen whatever the origin of the stochasticity, whether molecular within the organism or environmental without the organism.

But what is usually meant by downward causation are influences that arise from the regulatory *organization* at higher levels. Organization is what defines a level as distinct from a scale. Cellular organization defines the level of cells, organ organization defines the level of organs, and so on through the levels.

What do we mean here by organization? What precisely is homeostasis? Yet again, the common diagrams of upward and downward causation can be misleading. Regulatory processes in the body are rarely simple feedback loops maintaining a specific parameter, like blood pressure or temperature, constant. Nor is the circularity a simple feedback loop that can be described as a linear sequence of causation: A leads to B which leads to C and so on. This way of thinking leads to the need to specify the *direction* of the causation, in turn leading to the idea of emergence, usually interpreted to mean that the higher-level organization emerges *from* the lower level activity. But how can that be? At the lower level we can't even *see* the organization. Low-levels do not possess such organization. The constraints of higher-level organization will be represented by a seemingly disorganized set of initial and boundary conditions. We don't for example "see" the organization of bird haemoglobins as they vary according to different altitudes by sequencing their genomes. At that level, the different species have used different molecular level solutions to evolve haemoglobins for high and low altitudes. At

the functional level, the haemoglobins can be characterized as functional for the altitude at which they live so that all high-altitude birds show higher affinity for oxygen even though the DNA sequences are different (Natarajan et al., 2016). Only at the higher level of organization is the function of the genome changes evident.

We have elaborated on this problem in a previous article (Noble and Noble, 2017). From the molecular level of DNA, RNA, proteins, metabolites, ions etc., we will not be able to see the organization. As we noted earlier, it was not the athlete's calcium ions that caused his decision to be an athlete.

Emergence – a-mergence?

For these reasons, we have argued elsewhere for replacing the term e-mergence (suggesting privileging upward causation) with the neutral term a-mergence (Noble and Noble, 2019). In terms of causation, this requires replacing the linear sequence A causes B which causes C etc., with the existence of the state X, the occurrence of which means that A, B, and C etc., will also occur. This is the characteristic of high-level attractors. Once they occur, they take over the organization of the system. This fact becomes hidden when we insist on a linear causation viewpoint. Yet it is implicit when we solve model differential equations numerically since all factors are taken into account at each integration step. In a cell model we don't, for example, first calculate the influence of all the global cell parameters (such as potentials and concentrations) and then calculate the influence of the microscopic elements (such as transporter and enzyme states) separately.

This issue of simultaneity of action is fundamental. Another way of expressing it is to ask whether circular causality can be said to have a direction. Diagrams often strongly imply that they do, by giving the impression that, if one could be a nano-level observer, one would see one stream of causation running upward and another flowing downward. That picture is far from the reality. This is where the mathematical interpretation of circular causality is so useful in providing a totally different picture of the situation, since the integration procedures must proceed simultaneously (Noble, 2012). A nano-level observer would surely see something more like a cloud of happenings, which would not be resolvable into separate streams of happenings[2].

In this respect, the Biological Relativity interpretation of multi-level causality resembles wave theories of quantum mechanics. Electrons circling a nucleus, for example, are referred to as a cloud because the wave interpretation does not, and cannot, identify where any particular electron may be. The cloud exists as a quantum mechanical state that is precisely and quantitatively described by quantum mechanical wave equations. What matters is the existence of that state, not where any particular electron may be.

[2]In any programming of the integration procedure the precise algorithm used depends on the integration formula used. Usually this consists in successive iterations until a preset level of accuracy is achieved. It would not make sense to divide the integration step up into parts. The step itself is just an approximation to an infinitesimally small step. From the viewpoint of this article everything computed in each step can be regarded as an approximation to true simultaneity.

Similarly, it is the *state* of a multi-level biological system that matters, not just its breakdown into any particular separate sequences of causation. In any case everything else depends on the existence of the combined state of the system, which is unresolvable into two streams of causation. Not only would there not be two separate streams of causation, what is happening would not be evident in a single slice in time. The attractor or any other organizational property would only be apparent in a phase space representation within which the organizational pattern can be appreciated in an extended time period.

Purely reductionist thinking tends to avoid such language, which is usually criticized as being somehow fuzzy. But it is no more so than quantum mechanics. The analogy is quite close, since the breakdown of an attractor state can be viewed in much the same way as the collapse of a QM wave function. The same criterion for success is also applicable: is the resulting theory empirically predictive? Multi-scale physiological modeling is increasingly successful by this criterion. Vecchi et al. (2018) have introduced the term Entangled Causation to represent their conclusion that "there is no biological rationale for assuming that every switch point should be regulated by a single causal factor and that development generally involves interactive causation in the form of multiple simultaneously contributing difference-making causes to the regulation of the threshold mechanism at every switch point." The resemblance of their conclusions to ours is clear.

Representing organisms as high-level attractors and similarly organized states therefore corresponds much better to what we know experimentally. Most changes at the level of DNA are buffered by the high-level attractors. As Baverstock and Rönkko have shown, the phenotype can best be "represented by high dimensional attractors, evolutionarily conditioned for stability and robustness" (Annila and Baverstock, 2014; Baverstock and Rönkkö, 2014).

Further Physiological examples

We have already used a specific example, that of muscular exercise, to illustrate some of the main points of this article. We will now give further physiological examples. These will illustrate the variety of the forms of boundaries in physiology. It will be through understanding this variety that we will be able to summarize some general principles in See Sections "Delayed differential equations" and "Boundaries between levels: how do they differ?"

Anatomical and functional boundaries in the heart. The heart as an organ has many anatomical boundaries within it since the cells from the sinus node, the atrium, the AV node, the ventricular conducting system, and the ventricle all have different electrophysiological properties, which reflect different protein expression patterns. These in turn are susceptible to different dynamic states within the regulatory networks. The anatomical boundaries between these parts of the heart will therefore experience different magnitudes and direction of ion current flow between them.

These differences also occur within each area. Ventricular cells, for example, differ between epicardial cells and endocardial cells and between the base and apex of the ventricle. These

differences are very important in the interpretation of the electrocardiogram. Cells within the sinus node also differ in a graded way. Cells from the periphery have a higher natural frequency than cells near the center.

These differences led to a surprising result when multicellular models of the sinus node became possible, as a result of the increase in computer power offered by the first parallel computers in the 1990s. Using a 64,000 parallel array with each computer processor representing a single cell model, it was found that the origin of the heartbeat, defined as the first cells to depolarize, occurred at the periphery of the model node, creating a wave that spreads inward toward the center (Winslow et al., 1993). This is surprising since in a real heart the beat originates near the center and spreads outward toward the periphery.

The solution to this puzzle was given by the experimental work of Boyett et al. (2003). When the sinus node is carefully separated from the atrium by surgical dissection, the node does indeed behave like the computer model. The sinus-node/atrium boundary is therefore functionally important in creating the conditions in which the beat begins toward the center of the SA node. The high negative resting potentials of the atrial cells together with their high membrane conductance due to high expression of inwardly rectifying potassium channels create the functionality of the complete structure.

Furthermore, the shape of the boundary involved here is not a simple circle or ellipse. The regions of atrial and sinus cells interdigitate in a pattern that enables the weak sinus cells to succeed in depolarizing the stronger atrial cells by almost entirely surrounding cells at the tips of the interdigitations. The impedance-matching process at this boundary is critical in enabling the SA node signal to succeed in spreading through every part and so exciting the whole heart in a functionally important sequence. This functionality is clearly constrained by the high-level geometric structures (Boyett et al., 2003).

Intercellular potassium waves generate oscillatory growth patterns in bacterial films and in vertebrate circulations. Not all bacteria are free swimming single cell organisms. Many form multicellular colonies in the form of films, strings and various matted structures. In their patterns of growth these colonies can behave as intercommunicating networks resembling those of multicellular organisms. Thus, a bacterial film may not grow at a constant speed. It may instead display oscillations in growth rate. These oscillations have been shown to be produced by communications between the cells involving intercellular potassium waves. In effect the cells at the center of the colony are informing those at the periphery when to divide since the release of potassium ions is linked to metabolic activity which in turn enables division to occur (Prindle et al., 2015). Prindle et al. (2015) conclude: "The ensuing "bucket brigade" of potassium release allows cells to rapidly communicate their metabolic state, taking advantage of a link between membrane potential and metabolic activity. This form of electrical communication can thus enhance the previously described long-range metabolic co-dependence in biofilms" (Liu et al., 2015).

Intercellular communication is widespread even in nominally single cell organisms. Potassium wave communication occurs in many organisms, particularly in the circulation in vertebrates, where it is responsible for functionally important phenomena like retrograde vasodilation (Longden et al., 2017). The evolutionary origin of such communication between cells and tissues is clearly very ancient.

Such boundaries can be maladaptive. In the brain, the phenomenon known as spreading depression is due to the generation of a wave of potassium efflux arising principally from glial cells that leads to the depolarization of neurons, resulting in their refractoriness to the nerve impulse with consequent loss of neural activity.

In such forms of communication, the boundaries are fuzzy and distributed. What is a component from some levels may be a boundary at others. Functional boundaries can come and go according to the state of the whole system. Boundaries are themselves therefore interactive. Thus, in the life history of Amoeba *Dictostylium* (?), intercellular boundaries exist at some phases of the cycle and not at others since the organism can function either as an integrated well-ordered colony or as single cells or spores.

Cancer formation and suppression. The standard theory of cancer formation is the somatic mutation theory according to which the accumulation of mutations cause some cells to proliferate abnormally to develop the cancerous tissue. A competing theory is the tissue organization field theory which attributes the cause of cancerous development to properties at a tissue rather than cell or genetic level (Soto and Sonnenschein, 2011). This theory locates the main action at the boundaries between individual cells and the state of the surrounding tissue. A key prediction of this explanation of cancer is that cancers may be "normalized" by changing the boundary, i.e., by transplanting the cancerous or precancerous tissue into normal tissue. This has been shown to happen (Mintz and Ilmensee, 1975; McCullough et al., 1997; Maffini et al., 2005; Kasemeier-Kulesa et al., 2008).

Sponges. All multicellular organisms and colonies of unicellular organisms face the problem of the open boundary requiring exchange with the environment. If the cells are packed too close together some will not be able to exchange nutrients and waste rapidly enough. In See Section ("Intercellular Potassium Waves Generate Oscillatory Growth Patterns in Bacterial Films and in Vertebrate Circulations") above we saw that bacterial colonies solve this problem by signaling when parts of the colony experience metabolic stress. Sponges solve this problem in a different way: the organism is structured using collagen forming open networks of spaces through which freshwater or seawater can flow. Water is wafted through the channels by flagella on the lining of cells, so enabling all cells to exchange freely with the environment. This movement of fluid is the sponge's equivalent of a circulation. There is experimental evidence that this slow-moving aqueous boundary enabled the earliest animal sponges to survive in very low oxygen levels and therefore to evolve before the general oxygenation of the environment around 580 million years ago (Mills et al., 2014).

Delayed differential equations

Equations of this form are sometimes used to represent situations in which there is a significant delay in the action of a part or level of the system on its components (Bocharov and Rihan, 2000). These are important because they also show that chaotic behavior can arise from deterministic equations (Ikeda and Matsumoto, 1987). This form of mathematical representation may seem to contradict our earlier claim of simultaneity of upward and downward causation. That this is not so can be understood by noting that such equations represent an *ordinary* differential equation simplification of any real system, where a full representation would require *partial* differential equations in which the delay would be modeled as a diffusion process in space. This more complete representation would then satisfy the simultaneity condition, with the delay being properly computed in time at each point in space. At each point in space there would be no delay.

Boundaries between levels: how do they differ?

Figure 3 shows the original diagram of multi-level causation (Noble, 2006). The downward arrows were drawn as large and as separate arrows to emphasize the importance of downward causation [see also (Tasaki, 2013)]. These are the forms of causation that constrain the lower levels and which are necessary for an organism to be alive.

However, there are two aspects of this diagram that could be misleading.

First, both the upward and downward forms of causation differ in their details as we move between the levels. We have discussed examples of these differences in the present paper. An important difference that we will highlight here is the difference between the downward forms of causation onto the genome. The arrow between *Protein and DNA Networks* and *Genes* (the smaller left downward arrow) will consist of molecular details concerning the set of transcription factors, regulatory RNAs and methylation by which molecular events at the network level control gene expression. The higher level causation of the same process (right downward arrow) will include properties at the highest levels of the organism that would enable these controls of the genome to be understood functionally, for example why some cells are constrained to produce the patterns of expression for bone cells while others are constrained to become heart cells, albeit from the same genome. Comparable differences occur between the upward arrows. The arrow from *Genes* to *Proteins and RNAs* consists in the transcription and translation machinery of cells. That between *Cells* and *Tissues* consists in the processes that bind cells together to form tissues. The causation at the different levels depends on all the other forms of causation between lower and higher levels. There is a form of nesting of causation, both upward and downward.

Second, as we have already shown, it would be a mistake to think of the upward and downward causations between any levels as sequential, with one occurring before the other. The lesson we learn from representing these forms of causation in mathematical models is that they are necessarily simultaneous.

Figure 4 gives a different representation in which double-headed arrows are used on the left to indicate the simultaneity of action between the different levels. Yet it is still formally correct to say that each of these consists of different kinds of causation. Some will be stochastic, others are ordered constraints. We can therefore imagine these as formally separate lines, as illustrated on the right hand diagram.

The brown colored arrow between DNA and the level of proteins and RNAs is special. The upward influence is a kind of template: genes as DNA sequences act as a template for amino acid sequences in proteins. The downward influences are twofold:

Normal. Influence on expression levels of proteins and RNAs with no change in DNA sequence.

Special. Creation of new DNA by, e.g., the immune system, and other forms of targeted mutations and natural genetic engineering.

Boundaries beyond the organism

Figure 4 also illustrates the fact that, since organisms are open systems, there are necessarily levels above that of the whole organism, extending into the various forms of social interactions and, in the case of humans, the constraints of laws and ethics. Here we simply note that they also introduce different forms of causation, including constraints on behavior exerted by reasons and habits. The blue arrow at the top therefore represents the very different forms of causation that depend on reasons and contextual logic The relations and distinctions between reasons and causes are deep philosophical issues which we do not deal with here. This is part of the reason why we have represented the social and cultural factors involved all together as a single cloud. The diagram does not imply fuzziness or "ghostliness" in the actions on organisms. On the contrary, there is nothing ghostly about the fact that choice of lifestyle affected the muscles of the identical twins in **Figure 2** so differently, nor in the fact that Bathgate et al. (2018) have now identified the specific RNA changes involved at the molecular level.

This is a suitable point to comment on Craver and Bechtel (2007) case against the use of "causation" in top-down influences. Their case is that "the notion of top-down causation is incoherent or that it involves spooky forces exerted by wholes upon their components." We see nothing incoherent in the expression of top-down influences in terms of boundary and initial conditions. Open systems necessarily have boundaries. The forms of causation across those boundaries differ in the two directions, as we have shown and acknowledged throughout this article, but they are nonetheless real. Both forms are mathematically rigorous. As differential equation models show, they are both also necessary. An important clue to the substantial difference between our viewpoints is their statement that "both phrases describe *mechanistically mediated effects*" (their emphasis). We agree that setting boundary conditions is not "mechanistic" in the same sense as the dynamic role of upward causation represented in the differential terms in model equations. Moreover, processes that harness stochasticity are not well represented by the term "mechanistic." It is precisely their non-mechanistic nature that is important.

We are not the first to draw attention to the fact that the causal effects of organization at higher levels are exerted through

the boundary conditions at lower levels. The physical chemist Michael Polanyi made exactly this point as long ago as 1968 (Polanyi, 1968):

> "Therefore, if the structure of living things is a set of boundary conditions, this structure is extraneous to the laws of physics and chemistry which the organism is harnessing. Thus the morphology of living things transcends the laws of physics and chemistry."

Polanyi's article is remarkably close to our use of differential equation models to illustrate the different forms of causation in multi-level interactions. The only aspect of his work that has dated is his complete acceptance of Watson and Crick's Central Dogma. He wrote "the morphogenetic process is explained in principle by the transmission of information stored in DNA." He did not know that organisms can influence DNA sequences (the downward aspect of the brown arrow in **Figure 4**) and that much more than DNA is involved in the morphogenetic process.

It is difficult to represent all of these important theoretical distinctions in a single diagram. **Figures 1**, **3**, **4** in our article should therefore each be taken as partial guides to understanding. They each have their limits in representing the conceptual distinctions we are making.

DISCUSSION

The Questions in Our Title: What, Where and When Are Boundaries?

What?

Our paper shows that there are many kinds of boundaries in and around living organisms. Furthermore they are not usually, or ever, passive. They are an essential ingredient of functionality. The reason is that organisms are open systems, operating far from equilibrium. Boundaries are where many of those non-equilibrium processes take place. We cannot therefore understand the behavior of organisms or their parts from their composition alone, and certainly not from the genome alone. The consequences for physiological research are profound. Isolated components of organisms, whether molecules, cells, tissues or organs, do not necessarily behave in the same was as those components *in situ*. This fact is evident even at the molecular level. Proteins, for example, assume different forms in different environments (Balchin et al., 2016) and so do the processes in which they take part (Garcia-Contreras et al., 2012).

Where?

In answering this question we need to remember that it is we who decide what to study in physiological research, whether whole organisms or their components. The way in which we divide nature up determines where the boundaries lie in modeling systems. Where a boundary exists therefore depends on our choice (see the example of the DiFrancesco-Noble equations cited in the Introduction). These choices are not arbitrary, they depend on what has already been discovered. As an example, before the discovery of the variety of epigenetic controls of the genome, the idea of a boundary between the genome and its control by cellular and higher level processes would not have been conceivable. The discovery of these processes and the relevant boundary has far-reaching consequences for physiological research, including interpretations of the Central Dogma of Molecular Biology and of the Weismann Barrier (Noble, 2018).

Choice of boundary also plays a major role in the way in which multi-scale physiology discovers the relative importance of different molecular components. Examples in this article include how the extensions of heart muscle modeling in the 1980s led to the discovery of the quantitative importance of the sodium-calcium exchanger, and how the importance of this exchanger and its regulation has now been discovered using a similar shift from cell to tissue level modeling in skeletal muscle.

When?

Organisms develop, so many boundaries do not exist in the same way at the earliest, single cell, stages. Furthermore, they may differ in their ingredients from system to system even though achieving similar objectives. Boundaries between levels can obviously only arise when those levels develop.

Clarifications of the Principle of Biological Relativity

Our article clarifies several aspects of the Principle of Biological Relativity.

(1) The forms of causation involved in downward and upward causation are fundamentally different. Downward causation consists in constraints exerted by higher levels on the initial and boundary conditions within which the dynamics of lower level elements operate. By contrast, upward causation is the way in which those dynamics influence higher level states.
(2) These two forms of causation do not form a temporal sequence. They occur simultaneously.
(3) It is the state of organization of a higher level that can constrain lower levels. Causation by a state means that it does not make sense to separate out causation by any one element of the state.
(4) Conditioned causation exists in attractors since any perturbation of the state will be resisted. The strength of an attractor can be measured by the speed with which it re-establishes itself (Kaneko, 1998). The strength of downward causation in organisms is generally high since organisms are very effective at resisting changes in phenotype in response to changes at the molecular level, including changes in DNA sequences. Some authors describe conditioned causation as entangled causation (Vecchi et al., 2018). This is a term borrowed from quantum mechanical theory. The analogy is correct to the extent that the causal states involved should not be separated and the entanglement involved resembles that in quantum mechanical states. But there is also an important difference, which is that entangled states in quantum mechanics are very fragile, collapsing in a fraction of a second, whereas the attractor states in biology are often very robust.

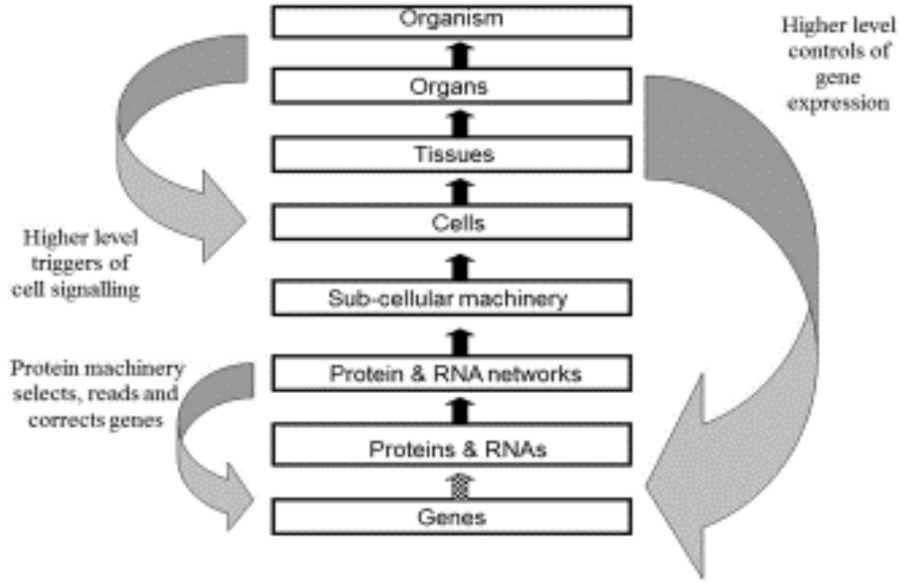

FIGURE 3 | Original multi-level causation diagram illustrating some of the forms of downward causation. Redrawn from Noble (2006), **Figure 2**.

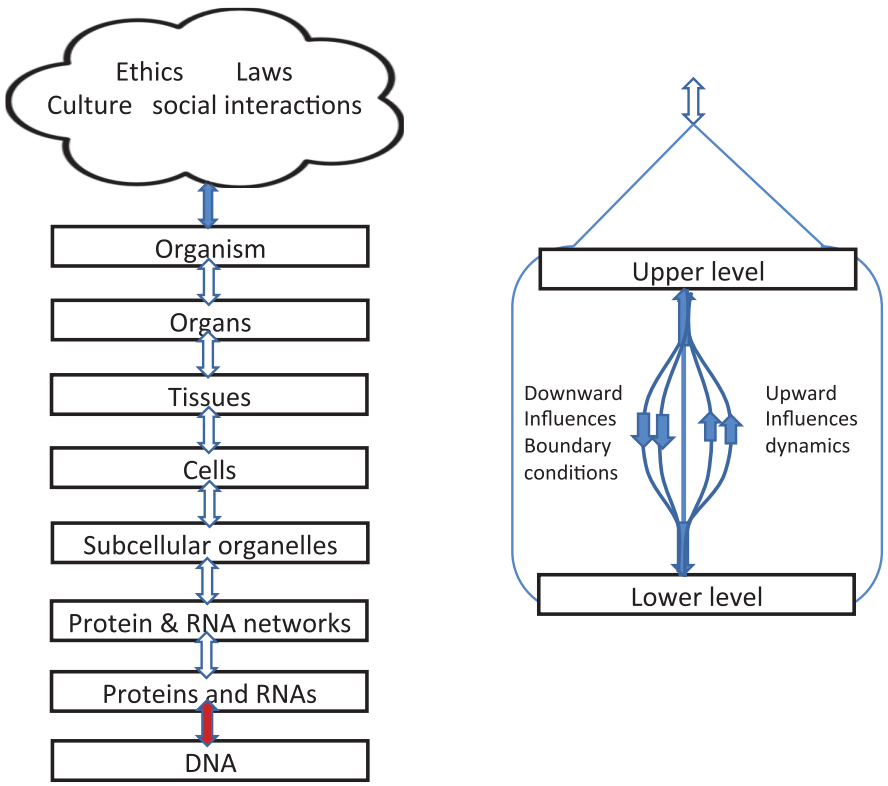

FIGURE 4 | Left: Representation of levels of interaction emphasizing that upward and downward causation operate simultaneously and are shown as double arrows. Right: diagram showing that, within each bidirectional causal arrow, there are different forms of causation, up and down.

Consequences for the Foundations of Physiology

(5) By clarifying the principle of biological relativity, and the nature of the boundaries, multi-level physiology gains rigor. We have not used specific mathematics in this article, nor are many of the points we have discussed primarily mathematical. They are points about the fundamentals of *physiology*. Expressing those fundamentals in terms of arguments drawn from mathematics simply shows that they can, in principle, be as rigorous as any form of science.

(6) What have we not explained? We believe our article opens up many further questions concerning the nature of multi-level physiology. In See Section "Boundaries Beyond the Organism" we have drawn attention to the fact that the causal relations between different levels differ in important ways. One of the most important of these is the increasing role of logic and reasons as we move up to and beyond the level of the whole organism. This is one of the most intractable problems in philosophy and clearly requires more research.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CONCEPTUAL ANALYSIS
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Co-emergence and Collapse: The Mesoscopic Approach for Conceptualizing and Investigating the Functional Integration of Organisms

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The fall of reductionist approaches to explanation leaves biology with an unescapable challenge: how to decipher complex systems. This entails a number of very critical questions, the most basic ones being: "What do we mean by 'complex'?" and "What is the system we should look for?" In complex systems, constraints belong to a higher level that the molecular one and their effect reduces and constrains the manifold of the accessible internal states of the system itself. Function is related but not deterministically imposed by the underlying structure. It is quite unlikely that such kind of complexity could be grasped by current approaches focusing on a single organization scale. The natural co-emergence of systems, parts and properties can be adopted as a hypothesis-free conceptual framework to understand functional integration of organisms, including their hierarchical or multilevel patterns, and including the way scientific practice proceeds in approaching such complexity. External, "driving" factors – order parameters and control parameters provided by the surrounding microenvironment – are always required to "push" the components' fate into well-defined developmental directions. In the negative, we see that in pathological processes such as cancer, organizational fluidity, collapse of levels and dynamic heterogeneity make it hard to even find a level of observation for a stable explanandum to persist in scientific practice. Parts and the system both lose their properties once the system is destabilized. The mesoscopic approach is our proposal to conceptualizing, investigating and explaining in biology. "Mesoscopic way of thinking" is increasingly popular in the epistemology of biology and corresponds to looking for an explanation (and possibly a prediction) where "non-trivial determinism is maximal": the "most microscopic" level of organization is not necessarily the place where "the most relevant facts do happen." A fundamental re-thinking of the concept of causality is also due for order parameters to be carefully and

correctly identified. In the biological realm, entities have relational properties only, as they depend ontologically on the context they happen to be in. The basic idea of a relational ontology is that, in our inventory of the world, relations are somehow prior to the relata (i.e., entities).

Keywords: living dynamics, systems thinking, mesoscopic way, data emergence, micro-environment, physical constraints, relational ontology, biological relationships

COMPLEXITY

Complexity as a concept emerged as a necessary stance – from both an epistemological and an ontological point of view – once the reductionist approach established since Descartes's time proved to be inadequate in explaining a number of relevant phenomena. Such inadequacy is particularly evident in biology and resonates with the concept of "unfathomable complexity," proposed by Elsasser (1987). This notion of complexity has to do with the impossibility to devise a series of experiments to clarify the way in which all properties of an organism can be *reduced* to consequences of molecular structure and dynamics, the latter being controlled and fully determined by the laws of physics. The fall of reductionist approaches to explanation leaves biology with an unescapable challenge: how to decipher complex systems. This apparently simple association of words entails a number of very critical questions, the most basic ones being: "*What do we mean by 'complex'*?" and "*What is the system we should look for*?"

Besides the existence of several excellent operational definitions of complexity elaborating on different notions of the amount of correlation among parts of a system (see for example: 28, 30, 31, 32), herewith we sketch three relevant "signatures" of complex systems:

1. A complex system includes a vast number of components (nodes), linked each other through dynamic relationships (links), enabling its representation in terms of a correlation network.
2. Such a network (independently of the chosen correlation metrics) exhibits a hierarchical structure, allowing the system to have meaningful dynamics at different spatial and temporal scales.
3. The spatial and temporal relationships among the different elements are subdued to a non-linear dynamics giving rise to both memory (hysteresis) and multi-stability (different equilibrium states) effects.

Thereby, describing the evolving system in both space and time is not trivial, as different functional states of the system can be supported by the same underlying structure. Indeed, a complex system can occupy different attractors along the paths of a hypothetical landscape, as suggested by C.H. Waddington in the 1950s. According to the topological architecture of such landscape, the system displays various degrees of robustness, i.e., resilience in respect to internal/external perturbations. As sharply observed by Elsasser (1987), "If we accept the concept of an organism as just stated, we can say that biology is a non-Cartesian science."

To be "non-Cartesian," in this context, means simply that the system looks different at different magnification scales and no privileged (and context independent) explanation layer exists.

Therefore, it is quite unlikely that such kind of complexity could be grasped by current approaches focusing on a single organization scale: complexity does not depend either on the number of genes (indeed, humans show lower values in respect to even evolutionary lowest living individuals, as strawberry!), or on their connections [the so-called Gene Regulatory Network (GRN)]. As an example, it was posited that extensive search for genome structures among animals would have come to identify those genes that actually hold the key to humanness. Yet, the result of such an effort showed that humans and chimps are basically isogenic; no specific human genes responsible for our human properties could be identified (The Chimpanzee, Sequencing, and Analysis Consortium., 2005). Similar problems were present in the "gene centric" explanations of complex human traits and diseases. As aptly remarked, "although some niche applications have been found for precision medicine, and gene therapy is now becoming a reality for a few rare diseases, the effects on public health are minuscule while the costs are astronomical" (Joyner and Paneth, 2019).

Classical molecular studies focused on the dynamics of single molecular components conceived as "drivers" of the biological process. A major drawback of such approach that it is unable in explaining the emergence of complex patterns. The emergence of a pattern (i.e., of a relatively stable configuration of n elements being them gene expression levels or amino-acid residue positions in 3D space) has to do with the energy minimization on the entire n-dimensional space and cannot be traced back to separate "optima" for each element. The among elements correlation and the presence of environmental constraints drastically "restrict" the number of patterns that the system at hand can really assume (Kitano, 2002; Chuang et al., 2010; Ma'ayan, 2011).

A case in point is provided by microgravity conditioning of living cells, where the "removal" of the gravity constraints enable the system to freely explore new – previously "inaccessible" – phenotypic attractors, by splitting a previous homogeneous population into two – morphologically and functionally – different clusters (Masiello et al., 2014). This phenomenon cannot be understood by investigating the "molecular dynamics" of the involved components, and it should be viewed as a true "emergent" property of the system, triggered by an environmental, physical cue. Constraints belong to a higher level that the molecular one and the aforementioned effect reduces and constrains the manifold of the accessible internal states of

the system itself. Conclusively, the form a molecule/a cell assume cannot be linearly derived ("reduced") only from the physical laws governing combinatorics.

This is a crucial issue separating the operational measures of complexity (mainly applied to alphabetical or numerical strings) from "semantic sensitive" functionally oriented definitions: "sequence complexity" does not mirror "structural complexity" of the organism that the sequence gives rise to. This statement applies (among the others) to Kolmogorov complexity measure, as this index is a degree of regularity (correlation in time), rather than of semantic-functional complexity (a random sequence is accorded maximum Kolmogorov complexity, yet, a biologist could hardly be interested with, given that random sequences do not give rise to organisms) (Adami, 2002). Along the suggestions fostered by Kolmogorov-dependent definition of complexity, several attempts have been made to correlate (and even to equate) complexity to entropy measures. However, entropy in dissipative systems does not increase, but eventually decreases just as negative entropy corresponds to (relative) order, certainty, and organization (Mikhailovsky and LevichEntropy, 2015). This implies that Kolmogorov measure proceeds likely in the opposite way from classical, entropy-based, order parameters.

This conundrum lies on the deceptive assumption that equalize "order" and "complexity." Yet, complex system are neither fully disorganized (like a gas), nor steadily ordered (like a crystal). In complex system order values are changing over time, showing dramatic fluctuation at points where the system undergoes critical transitions, leading to the emergence of new configurations ("phenotypes," in the biological parlance) in which order values (i.e., negentropy) is frequently uncoupled from Kolmogorov-based complexity parameters. As a result, changes in entropy may likely reflect true differences in system's order, but not necessarily in complexity. Moreover, in biological systems, complexity cannot be computed based on the number of functions they fulfill. Differentiated cells lose several capabilities when compared to their progenitor stem cells, yet it would be paradoxical to affirm that such a highly structured cell like a neuron is deprived of complexity. To make a long story short, we can state "In biology function is related but not deterministically imposed by the underlying structure."

CO-EMERGENCE AND COLLAPSE

Co-emergence of system, parts and properties can be adopted as a conceptual framework to understand functional integration of organisms, including their hierarchical or multilevel patterns, and including the way scientific practice proceeds in approaching such complexity (Bizzarri et al., 2013; Bertolaso, 2016).

Complex systems (paradigmatically those entailed by living beings) show emergent properties, which likely arise from the non-linear dynamics of the relationships established among the entities (molecular components) that make up the system. This statement implies some non-trivial consequences, at both the epistemological and ontological level.

First, as forecasted by Whitehead, biological phenomena consists of processes rather than material objects, and that processes are best defined by their relations with other processes, thus undermining the common shared belief on "bits of matter" – like genes or other molecular units – that exist and function independently of one another (Whitehead, 1967). Accordingly, molecular components are nothing more and nothing less than the sum of their relations to other entities, "its synthesis of and reaction to the world around it" (Whitehead, 1985). As "all things flow" – a philosophical stance that can be traced back to Heraclitus and that has been adopted recently in contemporary philosophy of science (Duprè and Nicholson, 2018) – it is mandatory to shift our focus from "molecules" to "relationships."

A phenomenon can in principle be studied at different levels (sub-atomic, atomic, molecular, cell, etc.), but we are mostly interested in "effective" relationships, i.e., those relations triggering "emerging properties" of the system at a higher organization layer. Effective relations live at the "mesoscopic level," as pinpointed by Laughlin and Pines (2000). This is the level where "order" can be fruitfully found, as only systems behave in a coherent fashion (even gene activity is subjected to intrinsic stochastic fluctuation (Elowitz et al., 2002). Indeed, while at the microscopic level, objects and their relationships are affected by fluctuations around the average – due to both environmental and intrinsic stochasticity – at the mesoscopic level stochastic fluctuations turn into ordered behavior, thus allowing order to emerge. A useful architectural analog of the mesoscopic level are the arches of a gothic cathedral: the arch occupies the intermediate layer between the brick and the entire building and represents the optimal level where to study the forces responsible for the stability of the cathedral as a whole.

The aforementioned reflections push to reconsider the current epistemological approach, concepts borrowed from classical mechanics – like those referring to determinism in biological reactions and causality – do not hold the same meaning when we are referring to the mesoscopic level and need to be re-framed accordingly. Going back to the architectural metaphor the statement "The arch generates (is the 'cause' of) the cathedral structure" is devoid of any sense, and must be substituted by "The presence of a given push-pull momenta configuration correspondent to arches network drives the entire cathedral toward an 'allowed' (stable) global structure." Deterministic (if-then) causation is substituted by the drastic restriction of "allowed solutions" stemming from the underlying mesoscopic configuration.

Co-emergence of systems, parts and properties are a natural (and hypothesis free) consequence that can be adopted as a conceptual framework to understand functional integration of organisms, including their hierarchical or multilevel patterns, and including the way scientific practice proceeds in approaching such complexity (Bizzarri et al., 2013). This can be crucially important in such pathological processes such as cancer, where organizational fluidity, collapse of levels and dynamic heterogeneity, make it hard to decide *a priori* a specific level of observation for a stable *explanandum* (what must be explained) to persist in scientific practice. Parts and the system both lose their properties once the system is destabilized. In the negative, we see that in pathological processes such as cancer, organizational fluidity, collapse of levels and dynamic

heterogeneity make it hard to even find a level of observation for a stable *explanandum* to persist in scientific practice. Parts and the system both lose their properties once the system is destabilized.

This specifically holds true in carcinogenesis, were some key aspects of tumor development (metastasis, phenotypic transition, growth or dormancy) emerge from the non-linear dynamics of the interactions between cells and their microenvironment (Bizzarri and Cucina, 2014). Changes in the tissue microenvironment act as stress factors on cells causing a range of adaptive responses within the reaction norm of the genome that may at some stage also include higher mutation rates. Tissue stress factors can be changes in the ECM composition (Extra-Cellular Matrix) caused by exposure to some carcinogen (and most carcinogens are not primary mutagens), changes in mechanical tissue forces after trauma, surgery and wound healing, or a change in fundamental signaling interactions between groups of cells due to changes in pH, Oxygen balance, and metabolic conditions which are all progressively changing during the course of a tumor's evolution. More generally, the life history of the biological entity intrinsically depends on a constitutive and continuous orientation of the parts among themselves and depending on the contextual signals. The asymmetry so generated is vital in the sense that it guarantees the adequate growth of the organism, as the effects of changes in cell or tissue shape seem to show. This unity of action admits degrees, and parts-whole relationships – as long as they hold – are to be explained through a specific kind of regulation. The biology of cancer shows that the stability of constitutive elements depends on the organization, and that there is a source of regulation in the biological context: cells change their behavior depending on their functional integration in the tissue; alteration in cell communication in turn alters gene expression, and the loss of integration of cells within a functional tissue leads to genetic instability and apoptosis.

The collapse of levels, as characterized in cancer, results from the loss of the general functional integration of a biological entity. It is here that the "mesoscopic level," as pinpointed by Laughlin, is substantiated.

THE MESOSCOPIC APPROACH

The described situation highlights the importance of identifying an explanatory level that is adequate to the observed phenomenon, by finding relational structures that are able to relate microscopic elements and macroscopic phenomena and parameters. "Mesoscopic way of thinking" is an increasingly popular statement in the epistemology of biology (Noble, 2006; Bertolaso, 2009; Bertolaso, 2016) and corresponds to looking for an explanation (and possibly a prediction) based on such kind of description. "Mesoscopic" is a term that originates in physics and engineering, and very frequently adopted in Ecology, perhaps the most epistemologically conscious branch of biology (Hastings et al., 2011).

Ecologists have long recognized that the "most microscopic" level of organization is not necessarily the place where "the most relevant facts do happen." On the contrary, the most fruitful scale of investigation (most of the times) is where "non-trivial determinism is maximal" (Pascual and Levine, 1999). That is to say, the scale more "rich" in meaningful correlations between features pertinent to micro- and macro- scale or, to use an ecological term, the mesoscopic realm. Non-trivial determinism can be, in fact, defined in terms of prediction error as:

$$\text{Prediction } r^2 = 1 - E^2/S^2$$

In the above formula, E is the mean prediction error and S the standard deviation. In the case of a simple linear regression in which a dependent variable Y must be predicted by an independent variable X, non-trivial determinism is nothing else than the usual squared Pearson correlation between the two X and Y variables. The formula can be extended to any other situation in which we wish to predict a system feature Y located at a hierarchically higher layer with respect to X, moreover both X and Y do not need to represent single variables but any suitable set of information at any definition scale. Consequently, in the "many Y"/"many X" case, non-trivial determinism corresponds to the first canonical coefficient (Härdle and Simar, 2007) while in the case of a binary diagnosis it equates to the area below the ROC curve (Heagerty and Zheng, 2005).

The mesoscopic way of reasoning closely resembles the "middle-out" paradigm set forth by Laughlin et al. (2000) as the next frontier of basic science – of chemistry, quantum physics for coherent dynamic behaviors (Bertolaso et al., 2015), and, more recently, of network-based approaches in biology (Giuliani et al., 2014). Complex networks (whichever level of biological organization they belong to) allow for a natural convergence between top-down and bottom-up approaches for the simple fact the computation of network invariants encompasses the simultaneous consideration of microscopic (single node), mesoscopic (cluster of nodes) and macroscopic (entire network) features (Csermely et al., 2013). This allows the environmental effects at different levels of definition to be tracked (Masiello et al., 2018).

This implies that statements like "*Drug A provokes a drastic increase of average shortest path of protein contact network*" or "*Cancer provokes a decrease in modularity of gene regulation network*" should be accepted as meaningful explanations without the need to go in depth into specific amino-acid residues or genes. When looking at networks, functional properties of a node are inferred from its topological role in the network. However, without the right choice of mesosystems and the appropriate estimation of local/global constraints, the problem of finding elements that are causally specific with respect to the initial *explanandum* will not be solved, no matter which mechanisms at the lowest (e.g., molecular) level are found. On the other hand, empirical topology/function rules are discovered at different degrees of generalization by moving from a population of heterogeneous biological networks to a single wiring architecture (Kohestani et al., 2018).

It is worth noting the same concepts (here mainly linked to spatial organization) apply to temporal structures. Recurrence

Quantification Analysis (RQA) (Marwan et al., 2007) is a non-linear signal analysis technique focusing on the search of "non-trivial determinism" that here takes the form of "sojourn points," i.e., areas of the phase space that are visited by different system trajectories corresponding to "stable configurations" of the temporal organization.

The onset of such "deterministic islands" in a time course is instrumental to detecting emerging properties of the system as a whole: e.g., the onset of "fatigue" (a global system feature), corresponds to the observation of a drastic increase in determinism of EMG time series (a mesoscopic (muscle fibers) level feature) (Liu et al., 2004).

Conceptualizing the Mesoscopic Way

The mesosystem is an identified system that exhibits regularities where not only the peculiar relations among the parts, but also the properties of the parts themselves and their reciprocal interactions, emerge (Laughlin et al., 2000). Laughlin and Pines (2000) state: "*The emergent physical phenomena regulated by higher organizing principles have a property, namely their insensitivity to microscopic details that is directly relevant to the broad question of what is knowable in the deepest sense of the term.*"

Insensitivity to microscopic details is the core of the middle-way and stems from the possibility to establish a "network thermodynamics" (Mikulecky, 2001), building upon the existence of laws of nature only dependent on the wiring architecture of a system that co-exist (but do not interfere) with the laws typical to the material the elements are made of. That is why an electrical circuit can simulate a mechanical one and, after all, why we do not need to enter into the details of electronic properties of single constituent atoms to get onto enzyme kinetics.

The most basic mathematical construct of the mesoscopic approach is the "complex network" wherein a set of nodes are linked to each other by edges. Nodes can be any relevant element of the system at hand (genes, proteins, amino-acid residues, neurons) and edges any connection between them (correlation coefficients, physical interactions, spatial proximities, simultaneous firing). Network invariants are nothing else than descriptors of the corresponding wiring architecture such as:

1. "degree": how many links are attached to a given node, which is a local descriptor,
2. "average shortest path," corresponding to the average length of minimal paths connecting all the node pairs, which is a mesoscopic feature.
3. "connectivity" that is the density of links, which in turn is a global property.

Figure 1 depicts some of these descriptors.

It is immediate to note that the values taken by the above descriptors depend (and influence) all the different organization layers: thus a node with a high degree (microscopic level) will be traversed by many shortest paths (mesoscopic level) that in turn will influence general network connectivity (macroscopic level).

In operational terms, shortest paths spanning protein contact networks (those networks whose nodes are amino-acid residues and whose edges correspond to residues set apart along the sequence and put in contact by protein folding) correspond to the "fast lanes" along which allosteric signals travel. This creates an immediate link between a global functional property of the protein molecule (allostery) and the mesoscopic level (shortest paths) strictly resembling the architectural metaphor we introduced in section "Complexity" (arches = shortest paths, gravitational forces = allosteric signal) (Di Paola and Giuliani, 2015).

It is worth noting that this approach is by no means limited to complex networks: any meaningful representation of the correlation structure of a system can extract relevant "mesoscopic principles of organization." This is the case of a time-honored technique, principal component analysis (Pearson, 1901; Giuliani, 2017), which allows for an immediate quantitative appreciation of the degree of order and organization of a system (Soofi, 1994).

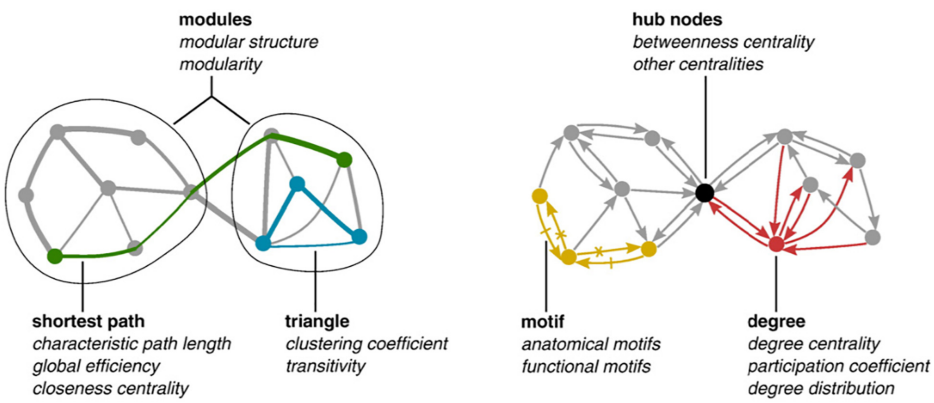

FIGURE 1 | Modules correspond to subset of nodes having much more links among them than with other nodes of the network. Measures of centrality (closeness, betweenness) describe nodes in terms of the number of shortest paths traversing them. Shortest path is the characteristic metrics for networks: they correspond to the shortest distances (in terms of number of nodes/links to be traversed) for linking pairs of nodes.

Success of the mesoscopic approach strictly depends upon choice of the correct level (and consequently the correct observables) to be investigated. Choice of the "preferable" level is dictated by the function-phenomenon we are focusing on. It is at this level – the mesoscopic one – that microscopic elements are organized in a coherent manner so to produce macroscopic features. The mesoscopic approach strives to "capture" the self-organizing process, which in turn will lead to the emergence of specific system properties.

That approach implies we should put any singularity (i.e., discrete change in a unitary molecular element) in dynamic correlation with the context it belongs to. Function and meaning of each molecular change emerges thereby within such a context, having no "ontological" meaning *per se*. This aspect is specifically epitomized when looking at the role played by those genes that can exert their effects in opposite ways, depending on the time/context in which they are activated. Conclusively, these findings imply the necessity to take into consideration, even when we concentrate on a single element (e.g., the lethal character of a specific mutation), the general functional frame in which the element is inserted.

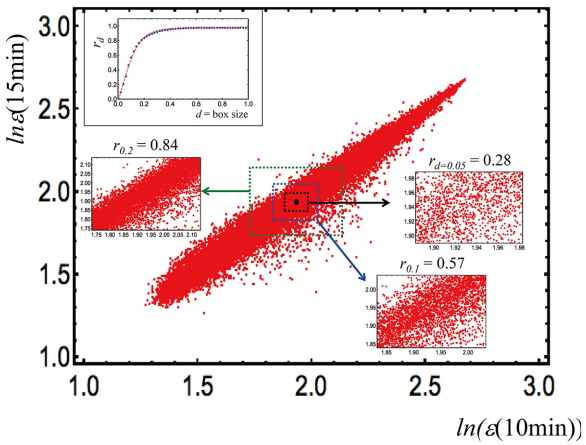

FIGURE 2 | In order to visualize the ability of this approach to identify the optimal scale of analysis, we report in this figure, the bi-dimensional plot having as axes two independent MCF7 (a breast cancer derived cell line) samples (data obtained from Tsuchiya et al., 2016). The points of the graph (around 23000, expression values in logarithm units) are the single gene expression values, the *d*-value corresponds to the range (box size) of variation, inside which the correlation (Pearson coefficient, *r*) is computed.

INVESTIGATING THE MESOSCOPIC WAY

Experimental practices both clarify the notions related to the mesoscopic approach and demonstrate its consistency and usefulness. In the previous section, we introduced the concept of non-trivial determinism as an operational guide for setting the optimal scale definition. The Pearson correlation can be substituted in the formula by any suitable correlation metrics as connectivity or average shortest path in the case of networks, amount of determinism in the case of recurrence analyses (Marwan et al., 2007). It is worth noting already that such statistical methodology allows for a quantitative check of the heuristic power of a given temporal or spatial scale of organization in terms of maximization of "non-trivial determinism" (Pascual and Levine, 1999).

In order to visualize the ability of this approach to identify the optimal scale of analysis, we report in **Figure 2** the bi-dimensional plot having as axes two independent MCF7 (a breast cancer derived cell line) samples. The points of the graph (around 23000, expression values in logarithm units) are the single gene expression values, the *d*-value corresponds to the range (box size) of variation, inside which the correlation (Pearson coefficient, *r*) is computed.

The correlation computed overall is near to unity ($r = 0.98$), and declines at decreasing range of variation; the inset on the top left corner of the figure shows that a correlation plateau is reached at $d = 0.45$. This remark outlines how correlation values are tightly dependent on the observation scale: in this case, the optimal scale is where correlation between the two samples (correspondent to the existence of an ideal gene expression profile of such cell kind) reaches its maximum and corresponds to the correlation attainable with a random choice of 50 genes. This implies that the "minimal set" for making the "specific cell kind transcriptome signature" emerge is 50 genes, which in turn corresponds to a very important property of complex systems called "percolation" (Pike and Stanley, 1981) linked to the level of perturbation needed to provoke a transition in the system at hand (Tsuchiya et al., 2016).

In this peculiar case, the scale dependence of the correlation is instrumental to maintain both tissue functionality (the specialized physiological function asks for an invariant ideal pattern of gene expression) and the flexibility required to adapt to a changing microenvironment (the specific gene expression levels need a "free motion" range to cope with environment).

As the mesoscopic level is where organizational principles act on the elementary biological units that will become altered, or constrained, by both their mutual interaction and the interaction with the surrounding environment, constraints acting at the mesoscopic level can therefore shape the activity of single elements (proteins, genes, etc.), eventually driving them into different and even opposite functions. This explains why putative so-called oncogenes can act as tumor-suppressor genes (Lee et al., 1995; Johnson, 2000; Muñoz et al., 2008; Schneller et al., 2011; Wang et al., 2011), a well-known paradox that undermines the fundamentals of the Somatic Mutation Theory of carcinogenesis (Baker and Kramer, 2007; Bizzarri and Cucina, 2016).

THE DYNAMICS OF MESOSCOPIC PARAMETERS

As we are dealing with processes – no matter the number and the specific identities of the involved molecular components – in which mesoscopic properties of the system are thought to change in space and time, we should focus on the dynamics of mesoscopic parameters. A useful way is to adopt a landscape diagram in which system's transitions from one state (attractor)

to another is portrayed by means of calculating order and control parameters, by analogy with phase-space diagrams employed in physics and chemistry. Such a model is already currently in use, albeit framed within a reductionist stance. Indeed, the Waddington's model usually adopted is featured by hills and valleys linked each other through branched pathways to portray the differentiation tree (Waddington, 1957). Both valleys and hills are determined by calculating values of state variables recognized by GRNs models, where the mean trajectory of observational data are obtained by numerically solving ordinary differential equations (ODE). This procedure usually leads to identifying several activation states, as featured by specific subsets of gene expression patterns (Wu et al., 2014). Based on this approach, a number of studies managed to demonstrate how intrinsic stochasticity in gene expression can activate a wide range of gene patterns, thus accounting for differences in phenotypes observed even among isogenic cell populations (Kupiec, 1983). The random pattern of gene expression produces probabilistic outcomes by activating switching mechanisms that select among alternative paths, ultimately leading an isogenic cell population to be partitioned into different phenotypes (McAdams and Arkin, 1997) because of the interplay between stochasticity of gene activity and non-linear dynamics of the transcriptional regulatory network (Huang, 2009). Current models posit that GRNs activity is modulated in a subtler fashion and results to be extremely sensitive to even small fluctuations occurring in the molecular dynamics in both the internal and the external microenvironment. Therefore, the GRN configuration can "capture" any kind of perturbation occurring in the system, although it is still unclear how GRNs activity could "sense" perturbations from the biophysical microenvironment. Moreover, GRNs sensitivity to external changes can hardly accommodate with one of the advantages displayed by complex systems, i.e., robustness (resilience to fluctuations).

RELATIONAL ONTOLOGY FOR BIOLOGICAL PROPERTIES

Mesoscopic models are able to capture many characteristics of a system. But then, what kind of causality is able to support biological explanations? Perturbations coupled with intrinsic genomic stochasticity can both destabilize an attractor state thus resulting in different gene expression patterns that would support independent cell "identities." The resulting presence of different transcriptional profiles at the bifurcation point is properly a "transient state" and it represents a raw substrate for cell fate switching, but in its own cannot decide about the fate the cell will choose and why a transition ends up into a unique phenotypic specification. By focusing only on the intrinsic dynamics of GRNs we could hardly find out why a cell population shall take a specific direction among many others. Namely, an unsolved problem is represented by what happens at the bifurcation points, where cell fate decisions take place (Moris et al., 2016). Is an external, "driving" factor required to "push" cell fate into one well-defined direction (Masiello et al., 2018)? Indeed, Waddington's based diagram include both control parameters, mainly provided by the surrounding microenvironment, which mostly belong to the class of physical constraints, and order parameters. In fact, ordered phenomena might only arise from global cues and constraints that superimpose their driving effects upon the local, random dynamics of molecular agents (Bizzarri et al., 2013).

A preliminary re-thinking of the concept of causality is due to allow order parameters to be carefully and correctly identified. In classical physics, the individual item is subjected to rigorous causality recognition according to the paradigm of linearity briefly mentioned in section "Complexity": effects are linearly transduced from causative factor(s) to end up into an effect in a deterministic fashion. In quantum mechanics, instead, such item is undetermined, whereas the average behavior of the including set (the "ensemble" in mathematical terms) can be defined in terms of statistical probability. That is to say, while in classical physics every individual object obeys causality, in quantum mechanics – also applying to complex systems – causality is meaningful only by considering an entire class of objects. In other words: causality applies to a class of entities, rather than to a single object. Therefore, the quantities/properties that are in common to all members of a class are the observables of the ensemble/system. As an example, consider cardiac rhythm: this is an outstanding (system) parameter, *per se* able to capture the functioning of the cardiac system and its possible pathological features. However, this rhythm does not pertain to any single myocardial cell or to single molecules either (Noble and Noble, 1984). It is a true system property, arising from the coordinated activity of the system, and it represent the parameter we must look at in order to grasp really the functioning of the cardiac muscle.

Noble (2017) emphasizes that the existence of system properties that constrain the behavior of lower-level entities is a case of downward causation – "the control of lower-level processes by higher-level processes" (p. 81). However, it is not exactly clear how this might be possible – if entities at lower levels have certain characteristics that make them behave in certain ways, how do we make sense of system constraints? By drawing on previous work (Bertolaso and Ratti, 2018), we propose to use a relational ontology to ground both the conceptual and explanatory aspects of this issue.

The basic idea of a relational ontology is that, in our inventory of the world, relations are somehow prior to the *relata* (i.e., entities). In biological terms, this would mean that the identity of biological entities should not be conceived in terms of their (internal) characteristics, but rather in terms of the relations they have with other entities – ultimately, in terms of the habitat they are embedded in. Therefore, understanding biology in terms of a relational ontology, means shifting the focus from the entities taken in isolation, to the historical context (bio-environment) of the entities themselves. Let us make these considerations more precise. An entity is a specific (i.e., biological) class of things that are subjected to the predication of properties. Simplifying, we can distinguish between intrinsic properties – properties that entities have in virtue of what they are (e.g., having a specific mass) – and relational properties – properties that entities have because of the way they interact with other entities (e.g., acidity).

The idea behind a relational ontology is that this distinction is spurious. In fact, even properties that look like they are intrinsic, in fact they are relational. A case in point is the notion of gene (Boem et al., 2016). Genes can be specified in terms of intrinsic properties (i.e., the gene x is a specific sequence of nucleotides), while in other contexts such as in networks biology a specific gene is defined as a node within a network of interactions (Barabasi and Oltvai, 2004) defined by a set of descriptors related to its connectivity features (e.g., degree, clustering coefficient, etc.). However, as Bertolaso and Ratti (2018) say "the fact that a gene has a specific sequence, and the fact that this sequence has a certain causal role (i.e., being transcribed as a blueprint for a specific protein) strictly depend on the context where the gene happens to be. Therefore, even properties that seem prominently internal are somehow relational, i.e., they depend on the context."

To specify this further, let us introduce the notion of "ontological dependency." As Wolff clarifies, "[t]o say that A ontologically depends on B is to say that both A and B exist, but that B is in some sense ontologically and explanatorily prior to A (…) A exists (at least in part) because B exists" (p. 618). Therefore, in the biological realm entities have relational properties only, as they depend ontologically (in the sense just specified) on the context they happen to be in. This view can vindicate even more the relevance of the mesoscopic approach in contemporary biology. It also supports the thesis defended elsewhere (Plutynski and Bertolaso, 2018) concerning the explanatory import of systemic models when dealing with complex biological dynamics. They deal in fact with properties of signaling networks and concern general patterns of stability and instability in the dynamics of, for example, cancer progression. "This requires placing cell intrinsic mechanisms associated with cancer initiation and progression in a larger context, at a variety of temporal and spatial scales, from the cell-signaling networks active in wound healing to evolutionary and developmental history. In this way, they integrate top-down and bottom-up perspectives on the same phenomena" (ibidem). Robust feature of networks therefore are the target explananda of mesoscopic models.

CONCLUSION

Organisms are complex entities co-emerging with their parts and properties. At the microscopic level, objects and their relationships are affected by fluctuations around the average – due to both environmental and intrinsic stochasticity – and then subject to quantum mechanics laws; at larger scales, stochastic fluctuations turn into ordered behavior, thus allowing order to emerge. The co-emergence of system, parts and properties – and the collapse thereof, as occurring in pathological processes such as cancer – can become a conceptual framework to understand the functional integration of organisms by adopting a mesoscopic approach, i.e., by focusing on the organization scale that – in network theory terms – maximizes the entity and number of correlations among the system's elements. Experimental practices both clarify the notions related to the mesoscopic approach, and demonstrate its consistency and usefulness, as relevant results can be achieved only by means of right and sensible choice of mesosystem variables and the appropriate estimation of local/global constraints. A relational ontology is necessary to ground both the conceptual and explanatory aspects of the mesoscopic approach, driving a re-thinking of the concept of biological causality.

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MBi and AP contributed to the scientific and biological sections. AG contributed to the bio-statistical aspects and statistics. MBe contributed to the overall structure of the manuscript, and philosophical and epistemological sections. ER contributed to the epistemological section.

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An Interplay Between Reaction-Diffusion and Cell-Matrix Adhesion Regulates Multiscale Invasion in Early Breast Carcinomatosis

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The progression of cancer in the breast involves multiple reciprocal interactions between malignantly transformed epithelia, surrounding untransformed but affected stromal cells, and the extracellular matrix (ECM) that is remodeled during the process. A quantitative understanding of the relative contribution of such interactions to phenotypes associated with cancer cells can be arrived at through the construction of increasingly complex experimental and computational models. Herein, we introduce a multiscale three-dimensional (3D) organo- and pathotypic experimental assay that approximates, to an unprecedented extent, the histopathological complexity of a tumor disseminating into its surrounding stromal milieu via both bulk and solitary motility dynamics. End point and time-lapse microscopic observations of this assay allow us to study the earliest steps of cancer invasion as well as the dynamical interactions between the epithelial and stromal compartments. We then simulate our experimental observations using the modeling environment Compucell3D that is based on the Glazier–Graner–Hogeweg model. The computational model, which comprises adhesion between cancer cells and the matrices, cell proliferation and apoptosis, and matrix remodeling through reaction–diffusion–based morphogen dynamics, is first trained to phenocopy controls run with the experimental model, wherein one or the other matrices have been removed. The trained computational model successfully predicts phenotypes of the experimental counterparts that are subjected to pharmacological treatments (inhibition of N-linked glycosylation and matrix metalloproteinase activity) and scaffold modulation (alteration of collagen density). Further parametric exploration-based simulations suggest that specific permissive regimes of cell–cell and cell–matrix adhesions, operating in the context of a reaction–diffusion–regulated ECM dynamics, promote multiscale invasion of breast cancer cells and determine the extent to which the latter migrate through their surrounding stroma.

Keywords: breast cancer, multiscale invasion, cell adhesion, reaction diffusion, cellular potts model (CPM)

INTRODUCTION

Within physiologically functioning tissues and organs, cells constantly interact with their surrounding extracellular matrix (ECM). This complex and continual interaction is essential for organ development and homeostasis (Bhat and Bissell, 2014; Bhat and Pally, 2017). Alterations that affect cell–ECM interactions aid in the progression of pathologies like cancer (Nelson and Bissell, 2005; Simi et al., 2018). In normal mammary glands and breasts, luminal epithelial cells are surrounded by a layer of myoepithelial cells that secrete basement membrane (BM): a sheet-like ECM rich in laminin and non-fibrillar collagens. Such mammary epithelial architectures are surrounded by stromal ECM that is rich in fibrillar matrix proteins such as collagen I (**Figure 1A**) and connective tissue cells such as fibroblasts, macrophages, and adipocytes. In breast cancer, this architecture is lost: the lumen is filled with proliferating apolar transformed epithelia, myoepithelia are absent, and the BM is ultimately breached by invading cells (Polyak and Kalluri, 2010). The stroma shows degradation of ECM, fibrosis, leucocytic infiltration, neoangiogenesis, and lymphangiogenesis (Wiseman and Werb, 2002; Orimo et al., 2005; Dumont et al., 2013). Malignant transformation results in a recalibration of existent interactions with novel constituents and interactions comprising the tumor microenvironment. Studying and quantifying the contribution of a given interaction to the progression phenotype of cancer spatiotemporally are a challenge, as our histo- and biochemical analyses are limited to distinct stages of breast cancer from various patients. Three-dimensional (3D) organotypic and pathotypic cultures of cancer cell lines and primary patient cells have helped extend our understanding of the molecular mechanisms underlying cancer (Torras et al., 2018; Weinhart et al., 2019). The 3D cultures are approximations of the histopathological complexity of *in vivo* tumor microenvironments. Current models involve embedding cancer epithelia within natural or tunable synthetic matrix scaffolds (Balachander et al., 2015; Bhat et al., 2016; Furuta et al., 2018). More complicated versions comprise efforts to mimic the perivascular and endothelial metastatic niches (Ghajar et al., 2013; Carlson et al., 2019) as well as efforts to engineer platforms consisting of multiple organs-on-a-chip reviewed by Zhao et al. (2019).

Noncancerous and malignant breast cell lines, when cultured in reconstituted BM (rBM) matrix, cluster into discrete morphologies that have been described as "round," "mass," "grape," and "stellate" in increasing order of aggressiveness (Kenny et al., 2007). The round phenotype is characteristic of untransformed cells that form growth-arrested acinar-like multicellular clusters with basoapical polarity, and a lumen. Mass and grape phenotypes are characteristic of malignantly transformed epithelia which mimic carcinoma *in situ* or indolently progressive cancers with cells that have completely lost their polarity. The stellate phenotype is typical of highly metastatic cancer cells that actively migrate, although in a collective manner, into and through rBM matrices. Using such 3D assays of cells embedded in rBM, it is possible to study the role of specific expressed proteins in regulating the adhesion between cancer cells or with ECM proteins such as laminins. However, such culture frameworks are inadequate for investigations into the spatial dynamics of cellular transitions between two matrix microenvironments that have distinct rheological properties, such as the non-fibrillar BM-like microenvironment and its fibrillar collagen-like types (**Figures S1A,B** shows scanning electron micrographs of nonfibrillar rBM and fibrillar type 1 collagen matrices; see **Supplementary File 1** for legends of all Supplementary Figures). In addition, it is infeasible to design experiments to observe the phenotypic consequences of an exhaustive exploration of interaction space within a multicomponent biological system.

The second limitation can especially be mitigated by adopting a computational approach and simulating the progression of cancer-like phenotypes for a diverse range of interactive parameter combinations. Computational models, particularly the Cellular Potts model (CPM), have been shown to be useful for such efforts (Zhang et al., 2011; Swat et al., 2012). For example, the deployment of proteolytic and non-proteolytic mode of cancer migration through collagenous scaffolds, or between solitary and collective cell invasion, has been well elucidated using *in silico* approaches (Kumar et al., 2016). However, to the best of our knowledge, no theoretical model has explicitly explored the transitioning dynamics, and the consequences thereof, of cancer cells moving between dissimilar matrix microenvironments. Moreover, while the dynamical role of individual mesoscale physicochemical processes has been well studied in cancer and development (Grant et al., 2004; Zhang et al., 2011; Pantziarka, 2016), whether their combined deployment constrains or widens the phenotypic reaction norm, the spectrum of discrete and distinct phenotypes achievable by cancer cells has not been investigated.

In this paper, we present a unified experimental–computational framework to investigate the interactions between cancer epithelia and spatially compartmentalized ECM microenvironments. The experimental model allows us to break down the phenomenon of cancer cell migration into cellular interactions with the BM, their remodeling of the same, their transition from BM to type 1 collagen, and the subsequent remodeling of, and migration within, type 1 collagen. We closely train the computational model on experimental results. The computational model successfully predicts results of the cancer epithelia upon pharmacological perturbations or scaffold modification. The trained theoretical model also predicts that emergent interplay between reaction–diffusion (R-D) and cell–matrix adhesion can explain the diversity in the extent and mode of invasion of breast cancer cells.

MATERIALS AND METHODS

Cell Culture

MDA-MB-231 cells were maintained in DMEM:F12 (1:1) (HiMedia, AT140) supplemented with 10% fetal bovine serum (Gibco, 10270). MCF-7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (HiMedia, AT007F) supplemented with 10% fetal bovine serum. Immortalized Human Mammary

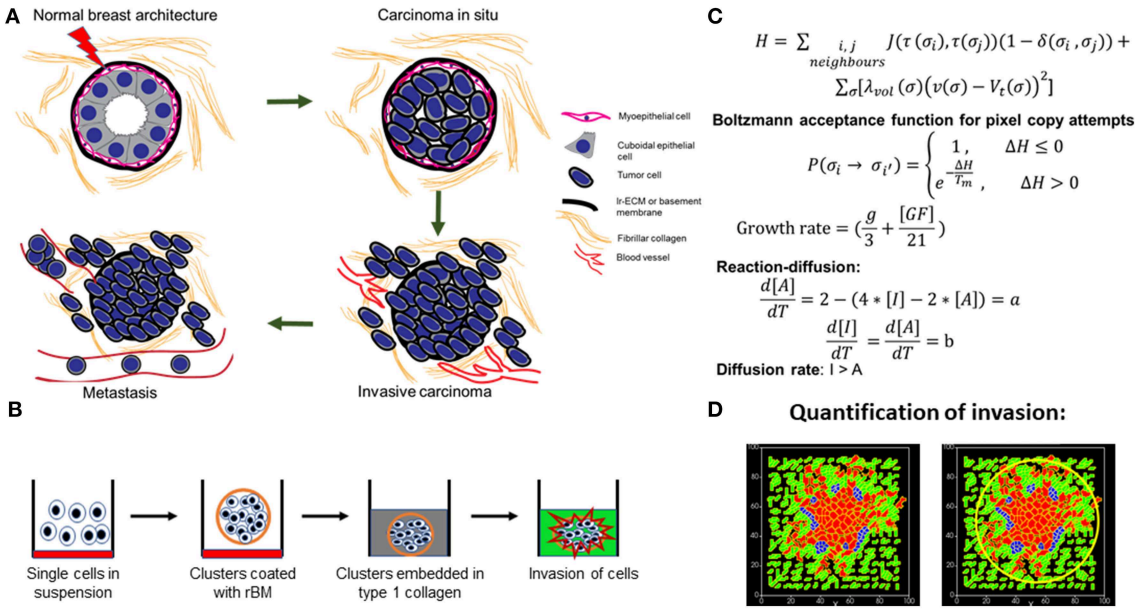

FIGURE 1 | Schematic depiction of experimental system and computational model. **(A)** Early stages of breast cancer progression: Top left denotes the normal glandular (ductal/luminal) architecture of human breast. Top right denotes the pattern of breast epithelia undergoing ductal carcinoma *in situ* where normal epithelia are malignantly transformed, lose polarity, and proliferate, resulting in filling up of ductal lumen. Bottom right shows the architecture of invasive ductal/luminal carcinoma where transformed cells breach the basement membrane (BM) and invade into collagen-rich breast stroma. Bottom left shows how invasive breast cancer cells, having traversed through stroma intravasate into blood/lymph vessels and metastasize to secondary organs. **(B)** Schematic workflow of 3D invasion assay used throughout the paper: Cells are first cultured on top of nonadhesive substrata in medium containing 4% reconstituted BM (rBM). Once cells assembled into clusters, the latter is embedded within type 1 collagen scaffolds and cultured in serum-free conditions. **(C)** Governing equations used for setting up the computational model. The first equation calculates *H*, which is Hamiltonian of the simulation: *H* determines the probability *P* associated with index-copy attempts, movements by generalized cells to minimize local effective energy through the default dynamical algorithm known as modified Metropolis dynamics. The equation for calculating growth rate shows it to be dependent on [GF], the growth factor concentration, and *g*, which denotes nutrient availability. Extracellular matrix (ECM) remodeling follows the kinetics of reaction-diffusion (please refer to the appropriate sections in Material and Methods for a more detailed description of model construction). **(D)** Quantification of invasiveness of cancer cells in the computational model is performed using the minimal enclosing circle algorithm developed using MATLAB.

Epithelial Cells (HMLE) cells were a gift from Dr. Robert Weinberg, Harvard Medical School, and Dr. Annapoorni Rangarajan, Indian Institute of Science, and were cultured in DMEM:F12 (1:1) supplemented with 1% fetal bovine serum, 0.5 μg/ml hydrocortisone (Sigma, H0888), 10 μg/ml insulin (Sigma, I6634), and 10 ng/ml recombinant human epidermal growth factor (hEGF) (HiMedia, TC228). All the cells were cultured in a 37°C humidified incubator with 5% carbon dioxide.

Preparation of Cancer Cell Clusters

Normal/cancer cells were trypsinized using 1:5 diluted 0.25% trypsin and 0.02% EDTA (HiMedia, TCL007). A total of 30,000 cells per 200 μl of defined medium (Blaschke et al., 1994) supplemented with 4% rBM (Corning, 354230) were cultured on 3% polyHEMA (Sigma, P3932)-coated 96-well plate for 48 h in a 37°C humidified incubator with 5% carbon dioxide.

3D Invasion Assay

rBM-coated clusters were collected into 1.5-ml tubes, centrifuged briefly, and then the supernatant is removed (**Figure 1B**). Acid-extracted rat tail collagen (Gibco, A1048301) was neutralized on ice in the presence of 10X DMEM with 0.1 N NaOH such that the final concentration of the collagen is 1 mg/ml. Pellet of clusters was resuspended in 50 μl of neutralized collagen and seeded in eight-well chambered cover glass (Eppendorf 0030742036) and supplemented with defined medium. 3D cultures were grown in a 37°C humidified incubator with 5% carbon dioxide.

Processing of 3D Invasive Clusters

3D invasion cultures were washed with phosphate-buffered saline (PBS) (pH 7.4) once after removing medium and fixed with 4% neutral buffered formaldehyde (Merck, 1.94989.0521) for 30 min. Glycine (2%) in PBS was used to neutralize traces of formaldehyde and was blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) (HiMedia, MB083) in PBS +0.1% Triton X-100 (HiMedia, MB031). After blocking, clusters were stained with 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen, D1306) and Alexa Flour 633-conjugated phalloidin (Invitrogen, A22284) overnight at 4°C. The next day, cultures were washed with PBS +0.1% Triton X-100 for 10 min each three times.

Laser Scanning Confocal Microscopy and Time-Lapse Imaging

Processed clusters were imaged using laser scanning confocal microscope (Zeiss LSM 880) with system-optimized Z

intervals. Images were analyzed using the Zen Lite software. Brightfield time-lapse imaging was done using Olympus IX81 equipped with stage top incubator and 5% carbon dioxide (see **Video 1**). Imaging was done at 10 min interval over 24 h. Images acquired were analyzed using the ImageJ software (Schindelin et al., 2012).

Computational Model

Modeling Environment

A multiscale modeling environment is required to simulate the spatiotemporal dynamics within a biological milieu wherein cellular growth, proliferation, invasion and morphogenesis occur simultaneously. The software package CompuCell3D (Swat et al., 2012) fulfills this purpose. Compucell3D is based on the CPM, also known as the Glazier–Graner–Hogeweg model, which was designed to model the collective behavior of active matter (Sanyal and Glazier, 2006; Chen et al., 2007). This is done by calculating an energy function called Hamiltonian at each step of the simulation. Each *cell* (we italicize this term to distinguish CC3D *cells* from biological cells in this paper) is represented by the set of all lattice sites or pixels sharing same cell ID. A rectangular lattice has been used in all our simulations. The evolution of the model happens at each Monte Carlo step (MCS), which consists of index copy attempts of each pixel in the cell lattice. Output of each MCS depends on the Hamiltonian calculation denoted by H (**Figure 1C**). The Hamiltonian in our model has two contributors which are affected by different properties sum of the *cells* and chemicals. The first contributor is the sum over all neighboring pairs of lattice sites i and j with associated contact energies (J) between the pair of cells indexed at those i and j. In this term, i, j denotes index of pixel, σ denotes *cell* index or ID, and τ denotes cell type. The δ function in this term will ensure that only the $\sigma_i \neq \sigma_j$ terms are calculated and also contact energies are symmetric. The contact energy between two *cells* can be considered to be inversely proportional to adhesion between those two *cells*. The second contributor is a function of the volume constraint on the *cell*, where for cell σ, $\lambda_{\mathrm{vol}}(\sigma)$ denotes the *inverse compressibility* of the cell, $\nu(\sigma)$ is the number of pixels in the cell (*volume*), and $V_{\mathrm{t}}(\sigma)$ is the *cell's target volume*. For each *cell*, this term is governed by its growth equation. If any change in the Hamiltonian is negative at a given MCS for any configuration with respect to its previous one [$\Delta H = (H_{\mathrm{f}} - H_{\mathrm{i}}) < 0$], then index copy attempts of pixels resulting in that configuration will be successful. Otherwise, the attempt will be accepted with probability $P = \exp(-\Delta H/T_{\mathrm{m}})$. A default dynamical algorithm known as modified Metropolis dynamics with Boltzmann acceptance function is used at each MCS to move the system toward a low-energy configuration as MCS increases. The term T_{m} can be considered as temperature or magnitude of effective membrane fluctuations. In the model, the membrane fluctuation is kept high for cancer cells compared with matrix elements in order to strike a distinction between living and dead elements. Random movements of pixels leading to different transition probabilities at each MCS mimic the stochasticity present in biological systems. We have modeled the movement of cells in metastasis as guided by differential adhesion and R-D-regulated degradation of ECM surrounding the cells. The MCS can be considered to be the natural unit of time in the model. In biological contexts, MCS and experimental time are considered to be proportional with respect to each other (Alber et al., 2004; Cickovski et al., 2007; Swat et al., 2012).

Model Components

Cell and matrix orientation

Using a 2D computational model, several aspects of cancer invasiveness and tumor-associated 3D phenomena have been studied where the property of spherical symmetry of tumor morphologies was used to obtain the minimalistic setup (Jiao and Torquato, 2011). Our 2D simulations mimic experiments in which biological cells may require 3D space to allow certain interactions, but in the computational model, only the properties associated with *cells* will play a role in determining the output irrespective of 2D or 3D. 2D simulations are computationally more efficient as it carries out an exponentially smaller number of calculations for the whole system. Our model space is 100 × 100 × 1 pixel in size where a group of cancer *cells* is initially located at the center grid surrounded by ECM. Any element of the model that is required to actively participate through MCS pixel copy attempts must be assigned a *cell* type, as instructed, the laminin ("BM") and type 1 collagen ("C1") are assigned different *cell* types along with cancer cells ("CELL"). In the setup, clusters of cancer *cells* are surrounded by blob-like two-*cell* layer of BM. The BM, in turn, is surrounded by fibrillar collagen. To mimic *in vivo* ECM architecture, BM is modeled as dense adhesive blob-like "*cells*" similar to the lamina densa of basal lamina, whereas C1 is modeled as the interconnected fibers similar to type 1 collagen. All components of the system have a depth of 1 pixel in the z direction, so there is no overlapping of objects. A *cell* cannot cross another *cell* if it does not degrade it, and without degradation, the *cell* will be trapped in a zone due to steric hindrance by its surrounding environment or find ways to squeeze through small spaces in its vicinity, which become accessible by random movement of that *cell*. In an initial configuration, cancer *cells* start as a rectangular objects of 16-unit volume (4 × 4 pixels) spanning 14 × 14 × 1 unit volume at the center (x, y = 43:57) of the simulation grid without any intercellular space. Tightly packed BM *cells* of 9 unit volume (3 × 3 pixels) is then created around the cancer mass (x, y = 37:63) having two layers of laminin *cells* separating it from C1. C1 is formulated around the cancer and BM structure throughout the whole grid with initial configuration of 4 unit volume cells (2 × 2 × 1 pixels) with two pixel gaps in between them. In order to make the C1 fibrillar, a plugin is applied on the *cells* which elongate them in the axis, random with respect to each other at 0.8-unit volume increment at each MCS until 5. The length scale of the components of ECM (BM and C1) is kept relatively smaller than the cancer cells (Das et al., 2017). The lattices with no assigned *cell* type or, in other words, the gaps or the free spaces are assigned *cell* type "medium" as a default of the Compucell3D syntax.

Contact energies (differential adhesion)

Compucell3D requires setting interactions between all *cell* types in terms of contact energies. Higher contact energy values between two *cell* types signifies lower adhesion or higher

TABLE 1 | Contact energies for simulations of discrete cancer morphologies.

Morphology-wise contact energies	Cell-cell	Collagen-cell	Laminin-cell
Carcinoma-*in-situ* phenotype	3	24	20
Apolar clusters	30	24	30
Multiscale invasive	35	24	45

mechanical hindrance between them. This is denoted by the term J in the Hamiltonian (H). The contact energies are set in our simulation by qualitatively considering interactions between pairs of components of the experimental setup in terms of adhesion or repulsion. After running simulations with a range of values of each contact energy, from all the resultant combinations, an appropriate set of contact energies is taken. The contact energies that were established for model simulations included CELL–CELL, CELL–BM, CELL–C1, CELL–medium. Values of these contact energies can be found in **Table 1**. The CELL–medium contact energy can be thought in terms of CELL–CELL adhesion of cancer with proportional correlation. Higher cancer CELL–CELL adhesion will give higher CELL–medium contact energy value. Across simulations, contact energies were established qualitatively motivated by transcriptomic findings including in, but not limited to (Kenny et al., 2007). For example, to mimic the decreased expression of E-cadherin in highly invasive cells, CELL–CELL contact energy was increased and the CELL-medium contact energy reduced.

MMP–tissue inhibitors of metalloproteinase (TIMP) reaction diffusion system

Auxiliary equations in Compucell 3D are used to model chemical fields. These fields store the concentration information of a certain chemical at every location in the simulation grid. Two chemical fields, A and I, are created which are governed by partial differential equations (PDEs) based on R-D dynamics of an activator and its inhibitor. These fields are incorporated with the GGH algorithm to allow interaction between other simulation components and the fields. The governing equations for these two fields are:

$$\frac{\partial [A]}{\partial t} = D_A \nabla^2 [A] + a - \delta_a [a] \quad (1)$$

$$\frac{\partial [I]}{\partial t} = D_I \nabla^2 [I] + b - \delta_I [I] \quad (2)$$

$$b = a = K - (c^* [I] - d^* [A]) \quad (3)$$

Where, $[A]$, $[I]$: concentration values for fields A and I.
D_A, D_I: diffusion constants of A and I
δ_A, δ_I: degradation rates of A and I
a, b: secretion rate of A and I
$t \equiv$ MCS
Default parameterizations, $D_A = 0.01, D_I = 0.8, \delta_A = \delta_I = 0.003$, $K = 2.0$, $c = 4.0$, $d = 2.0$.

Here, A is considered as the activated form of matrix metalloproteinases (MMPs). Its activation (or secretion of the activated form, i.e., "a") is assumed to be dependent on its inhibitors (inversely) and on its own concentration (autocatalysis) in the form of equation 3 (the inhibitor TIMP is known to bind to, and inhibit, the membrane-bound matrix metalloproteinase (MT1-MMP), which in turn activates secretory diffusible metalloproteinases) (Bourboulia and Stetler-Stevenson, 2010; Brew and Nagase, 2010). There are numerous variants of MMPs and TIMPs present in biological tissues (Brew and Nagase, 2010). Their production rates and interdependencies are still not known entirely for cancer cells, so a generalized form of MMP–TIMP interaction (A–I interaction of the model) is assumed in the light of R-D dynamics (Equations 1 and 2). The diffusion rate of MMP is set in the same range that is used by previous literature (Kumar et al., 2016) [that model has $D = 1.0 \times 10^{-9}$ cm^2.s^{-1} $= 0.025$ pixel^2 s^{-1}, as 1 mm $= 500$ pixels]. The difference in diffusion rates between the models (0.01 instead of 0.025) is due to different scaling of MCS with respect to time (s). All other parameters have been set based on previous literature and by optimization of the model. The diffusion rate of I is set higher than A to generate localized "activator" field and delocalized "inhibitor" field of the R-D system.

As all proteins have a lifetime, the degradation rate or decay constant associated with A and I in the model limits the spread of fields. The decay constant is assumed to be similar for A and I due to paucity of rigorous experimental analyses. In the model, the A and I fields are secreted at the boundaries of all "CELL"s, which come in contact with ECM. To initialize the A–I axis, random value of "a" (range is from 0 to 4) is assigned to each *cell*, which is in contact with ECM at MCS < 5. The CC3D package has a forward Euler method-based PDE solver, which was used to solve the PDEs (Swat et al., 2012).

Matrix degradation and regeneration

Investigations into cancer invasion focus primarily on the degradation of matrix by migrating cells that secrete high levels of MMPs. However, cancer cells, while degrading matrices, also secrete their own matrix, which is known as the cancer matrisome. The mechanical properties of the cancer matrisome, the forces it exerts on cells, and its chemical composition are under intense investigation (Naba et al., 2014, 2016; Socovich and Naba, 2018). Degradation of matrix is assumed to be dependent on the ratio of $[A]$ over $[I]$. For each MCS, ECM *cells* access the concentration values of the A and I fields at each of its pixels, and depending on the ratio, those pixels are either degraded or remain unchanged. This degradation is threshold based. Only pixels with the ratio $[A]/[I] > 2.0$ will be converted to the "lysed" form, which is either C_Lysed (C1) or L_Lysed (BM) *cell* type. Motivated by the elegant demonstration by Diambra and colleagues of the regulation of Turing space by cooperativity between the activator–inhibitor reaction dynamics (Diambra et al., 2015), we investigated and observed an appropriate regulatory influence of metalloproteinase degradation dynamics on the relative diffusion between MMP and TIMP (see **Supplementary File 2**). The degraded matrix is assigned different *cell* types by assuming different properties of the degraded form of BM and C1 as the nondegraded BM and C1 also have different properties. As a cost of degradation, $[A]$ and $[I]$ are reduced at a maximum of 1.5 unit/MCS in pixels belonging to the "lysed" *cell* types.

Matrix regeneration is incorporated into the model by conversion of C_Lysed and L_Lysed into C1 (given that cancer cells secrete predominantly fibrillar collagen-rich matrices) after 20 MCS from the degradation event associated with that "lysed" pixel (Socovich and Naba, 2018; Yuzhalin et al., 2018). The regeneration of matrix is essential to eliminate unnecessary free spaces formed as an artifact of matrix degradation, which takes the computational model closer to its experimental counterpart. All the "lysed" *cell* types are subjected to 0.1 volume decrease at each MCS to mimic dissipation of degraded matrix materials *in vivo*.

Cellular growth and proliferation

Growth rate of "CELL" is assumed be a linear combination of nutrient availability at cell boundaries and degradation of matrix. The growth equation is given by,

$$\frac{dV}{dt} = g * p + [GF] * q$$

Where $V =$ volume of "CELL"
$g =$ measure of nutrient availability
$[GF] =$ concentration of GF at center of mass of "CELL"
$p, q =$ constants.

Two quantities, the common surface area of a *cell* with its neighboring *cells* (k) and the total *cell* surface area (s) is accessed to calculate g in this equation as $g = (s\text{-}k)/40$. The denominator in the calculation of g is due to the 2D nature of the simulation as a *cell* can be surrounded by other *cells* only in the *xy* plane and not in the z axis. The scaling of that extra *cell* surface area without any neighboring *cells* in the z axis is provided by the denominator. Another contributor of the growth function is *[GF]*, which mimics the ECM degradation dependence of growth and proliferation (Olivares et al., 2017). The "lysed" *cell* types are programmed to secrete GF at each of its pixel location where the diffusion constant is kept low (0.02) to localize this growth signal to areas of matrix degradation. p (=1/3) and q (=1/21) constant values are set according to the assumed weightage of the two variables in growth equation.

Cell division is incorporated into the cancer cells by a CC3D steppable called "MitosisSteppable" with base function "MitosisSteppableBase." If any "CELL" reaches a threshold volume of 30 units, then those *cells* will be divided in random orientation. The resultant two *cells* will have volumes half of its predecessor with all other properties kept same as the parent *cell*. In this model, growth rate is directly correlated to proliferation as it determines the volume of the *cell* to reach threshold for cell division.

Quantification: invasion of morphology

The quantification for the spread or invasiveness of morphologies has been done in MATLAB using minimal enclosing circle algorithm (**Figure 1D**). Screenshots captured at different MCS from a simulation are used to track invasion of that model. A program was written where for a screenshot, the image is binarized with respect to "CELL," which is represented by red color. From that binarized image, centroids of all cells are accessed by the function "regionprops." In order to find the smallest possible enclosing circle, two bits of information are required, which are position of center and radius of a circle which will encompass all the centroid positions. An arbitrary center for the circle can be selected from which distances are measured to all the centroids. In the smallest possible enclosing circle, center-to-centroid distance will be maximum for the furthest centroid that it needs to cover, and this distance will be the radius of the circle. The function "fminsearch" was used with input of assumed centers and radii (maximum of center-to-centroid distance), which yields a center with minimized radius. Circle formed with this center and radius from "fminsearch" will enclose all the centroids and will be the smallest possible circle to do so. All simulations have "CELL" at the center of the grid as the initial configuration, so the center of smallest enclosing circle can be assumed at the center of that grid to start with, which is also the center of screenshot; this optimizes the programme. Running this program will yield the smallest possible enclosing circle for screenshots at each specified MCS, and the area of this circle is considered as the measure of invasiveness of that phenotype (**Figure 1D**). In our studies, we have explored whether assigning a microenvironment-autonomous motility to *cells* enhances their invasiveness. To address this, we assigned a random motility direction for the "CELL" *cell* type of the model. All of these *cells* are assigned a different direction and a value of the force acting on its center of mass. This assignment of force is randomized but follows a uniform distribution. In Compucell3D, this active motility is incorporated by using "ExternalPotential" plugin and "cellMotility" steppable. The additional contributing term for calculating change in the Hamiltonian (ΔH) is:

$$\Delta H_{AM} = -\vec{F}_{\sigma(i)}.\vec{r}_{ij}$$

where for at any MCS step, for a pixel copy attempt from i to j of *cell* $\sigma(i)$, the force vector is $\vec{F}_{\sigma(i)}$ and the distance vector between those pixels is $\vec{r}_{ij}$. So, their dot product with correct alignment in direction will satisfy the condition to minimize H and the *cell* will move along that direction.

We observe that simulating multiscale invasion with active motility does not significantly change invasion compared to the model, where active motility is not implemented (**Figure S2**).

Statistics

All the biological experiments were repeated three times independently. All the simulations were repeated at least 10 times and the data are represented as mean ± SEM. Parametric Students' t-test was performed with Welch's correction to estimate statistical significance.

RESULTS

Breast Cancer Cells Invade From rBM-Like Matrix to Collagen-Rich Matrix Concurrently Across Multiple Spatial Scales

In order to mimic the invasion of breast cancer cells *in vivo*, we designed a culture model wherein MDA-MB-231 cells were

allowed to form rBM-coated suspended clusters (see Materials and Methods; **Figure S3** showing rBM is spatially limited to the surface of clusters). When such clusters were embedded in type 1 collagen (**Figure 2Ai**) and the cultures were imaged in time lapse, the cancer cells rapidly migrated past the rBM barrier into collagen (**Figure 2Aii**). We observed spatially distinct but temporally concurrent modes of invasion, ranging from bulk motion, where the cells moved centrifugally in an expansive and collective manner (**Figure 2Aiii**), to mesenchymal migration of solitary cells with a slender cytoplasmic front and a nucleus-containing lagging end (**Figure 2Aiv**). We here forward refer to this simultaneous deployment of distinct motility modes as multiscale invasion. Studies concerned with cancer cell migration investigate mechanisms underlying transitions between the modes; however, the studies also note that these modes temporally coexist within histopathological sections of human tumors (Friedl and Alexander, 2011; Friedl et al., 2012; Krakhmal et al., 2015). Our experimental model successfully recapitulates the multimodal and multiscale 3D cancer cell invasion.

We then sought to codify the minimal set of interactive cellular and ECM behaviors that could give rise to such multiscale migratory behaviors of invading cancer cells. Using CC3D, we constructed a computational model wherein for constrained set of values of cell–cell and cell–BM–like ECM adhesion, as well as upon invoking a R-D-based remodeling kinetics of ECM, we observed multiscale invasion of cancer epithelia from a nonfibrillar to a fibrillar *in silico* ECM microenvironment (**Figure 2Bi** represents the *in silico* cluster at MCS = 10; **Figure 2Bii** represents the same cluster at MCS = 440; emergence of expansive collective invasion seen in **Figure 2Biii**, emergence of single cell invasion within the same culture seen in **Figure 2Biv**; see also appropriate sections in Materials and Methods for details of model construction). The use of an R-D-based modulation of ECM steady state was motivated by the morphology of the invasion phenotypes in our experimental assay, wherein invading cell populations exhibited a discernibly iterative spatial pattern in which invading cells were surrounded by lateral zones of inhibition (activator and inhibitor concentration fields in the simulation shown in **Figure S4**). In addition, the use of R-D based microenvironmental regulation has strong precedence in the literature on cancer progression (Chaplain, 1995; Gatenby and Gawlinski, 1996; Roque et al., 2018; Zhang et al., 2018). Time series of both bulk and single cell invasion were tracked and found to increase in a similar fashion in culture and in simulations (See **Supplementary File 2**).

Nature of the "Stromal" ECM May Determine Mode of Cancer Cell Invasion

We sought to know whether the multiscale invasion of cancer cells was a function of the prototypical outwardly radial arrangement of cancer cells inside a thin intervening layer of rBM and an outer presence of type 1 collagen. To verify if the initial rBM coating was required for cluster shape and integrity, MDA-MB-231 cells were clustered in the absence of rBM. The cell clusters that formed had an irregular shape with ill-defined contours and were inherently unstable (**Figures S5A,B** showing irregular and regularly shaped clusters in the absence or presence of rBM coat, respectively). When rBM-coated MDA-MB-231 clusters were cultured entirely in rBM; clusters exhibited collective motility dynamics with most cells still attached to the kernel of the cluster (control multiscale invasion shown in **Figure 3Ai**; rBM-exclusive control shown in **Figure 3Aii**). Solitary invading cells were scarcely seen in the periphery. On the contrary, rBM-uncoated clusters upon embedding in type 1 collagen gels rapidly disintegrated into a small kernel and mostly single cells that exhibited mesenchymal single cell migration (**Figure 3Aiii**).

We used the phenotypic observations to further train our computational model and chose parametric combinations for (i) contact energies of cell–cell, cell–rBM, and cell–type 1 collagen interactions; (ii) R-D-based remodeling of ECM; and (iii) proliferation and death of the cancer cells. We were able to successfully narrow down parametric combinations for which simulations mimicking "only rBM" and "only collagen" controls predicted predominantly collective and single cell migration, respectively (**Figure 3Bi** represents control, **Figure 3Bii** shows emergence of collective invasion in an exclusive rBM-like nonfibrillar ECM environment, and **Figure 3Biii** shows emergence of single cell invasion in an exclusive collagen-like fibrillar ECM environment). Since the parameter combinations were kept identical in the controls, the divergent phenotypes suggest that the identity of the stromal ECM and its spatial arrangement may determine the mode of outward migration of cancer epithelia.

Metalloproteinase Activity and N-Linked Glycosylation Regulate Multiscale Invasion

We next sought to test our assumption that a locally auto-active regulation of ECM remodeling is essential for multiscale invasion. For MMPs with their cognate lateral inhibitors, TIMPs are putative activator–inhibitor couples, given their diffusivity and the nature of interactions. Treatment of cultures with a broad-spectrum MMP inhibitor Batimastat resulted in an abrogation in transition of cells into the stroma, although the leading cytoplasmic head of cancer cells in the periphery of the cluster could still be visually discerned in the surrounding collagen (**Figure 4Ai** represents vehicle control; **Figure 4Aii** represents treatment with 10 μM Batimastat). This suggested that the transition of cancer cell nuclei across the rBM–collagen interface is dependent on protease-dependent remodeling of the stromal ECM. Interestingly, for amoeboid migration (which we have not investigated in our paper, see Discussion), nuclear softening has been proposed to be crucial for protease-independent migration (Das et al., 2019). Decreasing the activator levels within our computational model brought about a decrease in *in silico* migration of cells with sparse transitions into the fibrillar matrix environment (**Figure 4Bi** represents control; **Figure 4Bii** represents simulation that shows inhibition of invasion upon downregulating levels of activator A).

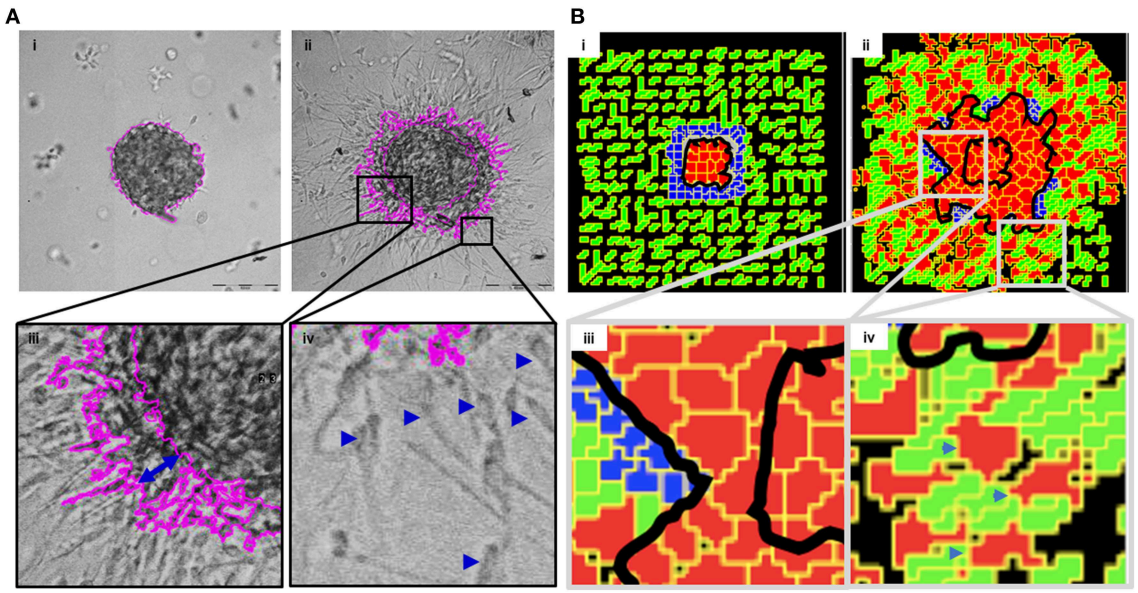

FIGURE 2 | Multiscale multicellular invasion of breast cancer cells in culture. **(A)** Representative phase contrast micrographs from time-lapse imaging of MDA-MB-231 cells showing multiscale invasion into fibrillar matrix. rBM-coated MDA-MB-231 clusters embedded within type 1 collagen **(Ai)** invade into the latter within 24 h **(Aii)**. Cells show expansive migration [**(Aiii)**; double-headed blue arrow shows the extent of collective migration between initial boundary (0 h) and final boundary (24 h) of the cluster (boundaries shown in pink)]. Single mesenchymal cells are also observed in type 1 collagen [**(Aiv)** right inset; blue arrowheads]. Scale bar: 200 μm. **(B)** Multiscale invasion exhibited by computational model. Initial pattern [**(Bi)**; MCS10; cancer cells (red) packed within a BM-like nonfibrillar matrix (blue) and further outward by fibrillar collagen-like matrix (green; inter-fibrillar gap = 3 unit pixels)], and final pattern [**(Bii)**; MCS 440] showing invasion of cells. *In silico* cells show expansive migration [**(Biii)** left inset; bulk movement visible through the spatial gap between two black lines denoting boundary at initial MCS and final MCS]. Single cells are also observed in non-fibrillar ECM [**(Biv)** right inset].

Second, we tested the role of cell–cell and cell–matrix interactions by treating our cultures with an inhibitor of N-linked glycosylation: tunicamycin. Tunicamycin affects the glycosylation and trafficking of cell surface proteins (Elbein, 1991). Molecules involved in cell adhesion such as cadherins and cell adhesion molecules (CAMs) are N-glycosylated. Moreover, while E-cadherin expression is epigenetically silenced in invasive MDA-MB-231 cells, N-cadherin is expressed and promotes their motility (Nieman et al., 1999). Treatment with tunicamycin does not alter the trafficking of N-cadherin but affects its function by interfering with its binding to catenin (Youn et al., 2006). Tunicamycin is also known to abrogate the matrix binding functions of integrins (Chammas et al., 1991). The effect of tunicamycin on metalloproteinases is context-dependent (Kim et al., 2010; Lee et al., 2019). Treatment with tunicamycin may increase the expression of MMPs, but due to associated endoplasmic reticulum (ER) stress and unfolded protein response, their secretion is inhibited (Duellman et al., 2015). Treatment of our complex experimental system with tunicamycin completely abrogated stromal transition of cancer epithelia (**Figure 4Aiii**). The cytoplasmic leading-edge extensions, likely mediated through outside-in integrin signaling, which were observed upon MMP inhibition, were also absent upon tunicamycin exposure.

In our computational model, the phenomenological equivalent of tunicamycin treatment would be to increase contact energies and, hence, downmodulate adhesion between cells and matrices. Additionally, secretion of MMP and TIMP was also downregulated as part of the initial conditions for simulation. Upon doing so, we found impaired invasion of cells into the fibrillar *in silico* environment compared to control conditions (**Figure 4Biii**). We could also observe inhibition of invasion despite retaining the secretion of MMPs and TIMP but only under parametric combinations when the secretion rate of TIMPs exceeded that of MMPs (**Figure S6**). Our experimental and computational results suggest that adhesive interactions and local auto-active ECM remodeling dynamics operative within the invading milieu are necessary for stromal migration of cancer cells, and inhibiting them significantly downregulates the latter (**Figure 4B**).

Collagen Density Alters Multiscale Invasion

We next sought to test whether the arrangement of type 1 collagen fibers surrounding rBM-coated clusters could regulate the nature of cancer cell migration. rBM-coated clusters of MDA-MB-231 cells were embedded within a higher density of type 1 collagen (2.5 mg/ml) scaffolds compared with control (1 mg/ml) (**Figure 5Ai**). The transition of cancer epithelia into high-density collagen was found to be attenuated (**Figure 5Aii**). Dense collagen may impede nonproteolytic migration of cancer cells allowing movement only upon mounting a protease-based degradation of ECM. In keeping

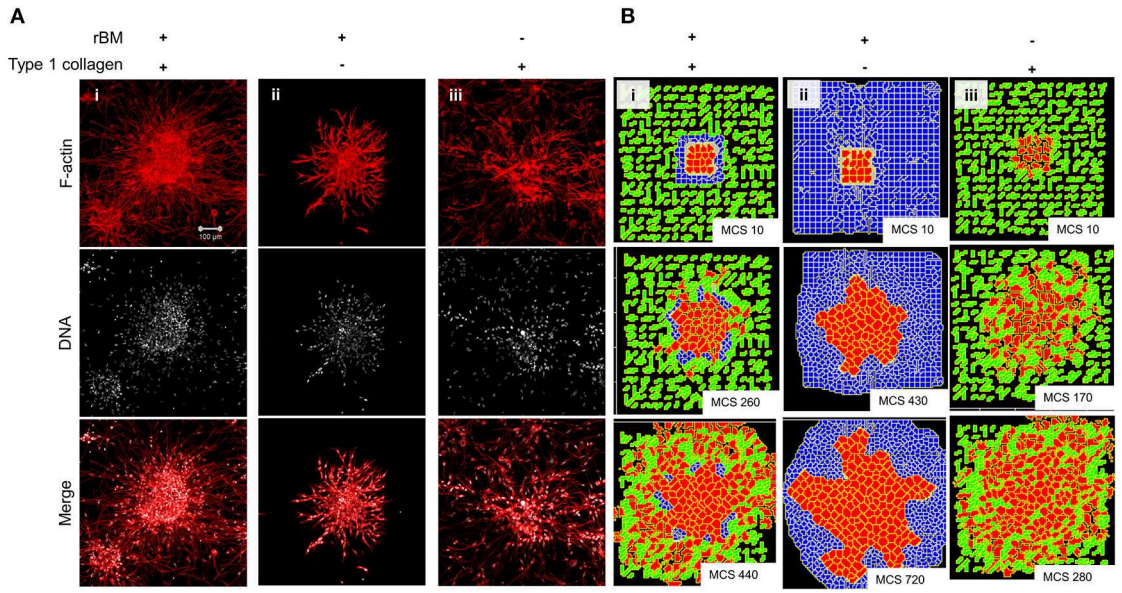

FIGURE 3 | Single-matrix controls of models show simpler modes of invasion. **(A)** Maximum intensity projections of laser confocal micrographs of MDA-MB-231 cell clusters cultured within specific matrix milieu, fixed and stained for F-actin (using phalloidin; red; top row), DNA (using DAPI; white; middle row), and with both signals merged (bottom row). **(i)** rBM-coated clusters embedded in type 1 collagen show multiscale invasion (left column). **(ii)** rBM-coated clusters embedded in rBM show collective or streaming migration of cells, **(iii)** Uncoated MDA-MB-231 clusters in type 1 collagen show predominantly single cell invasion. Scale bar: 100 μm. **(B)** Representative images from simulations of invasion of cancer cells at early (top row), intermediate (middle row), and late MCS steps (bottom row). Simulations mimicking cells encapsulated within nonfibrillar and then fibrillar ECM exhibit multiscale invasion (left column). Simulations of cells cultured exclusively in nonfibrillar and fibrillar ECM show collective and single cell migration (**Bii** and **Biii**). Interfibrillar gap of C1 = 3 unit pixels.

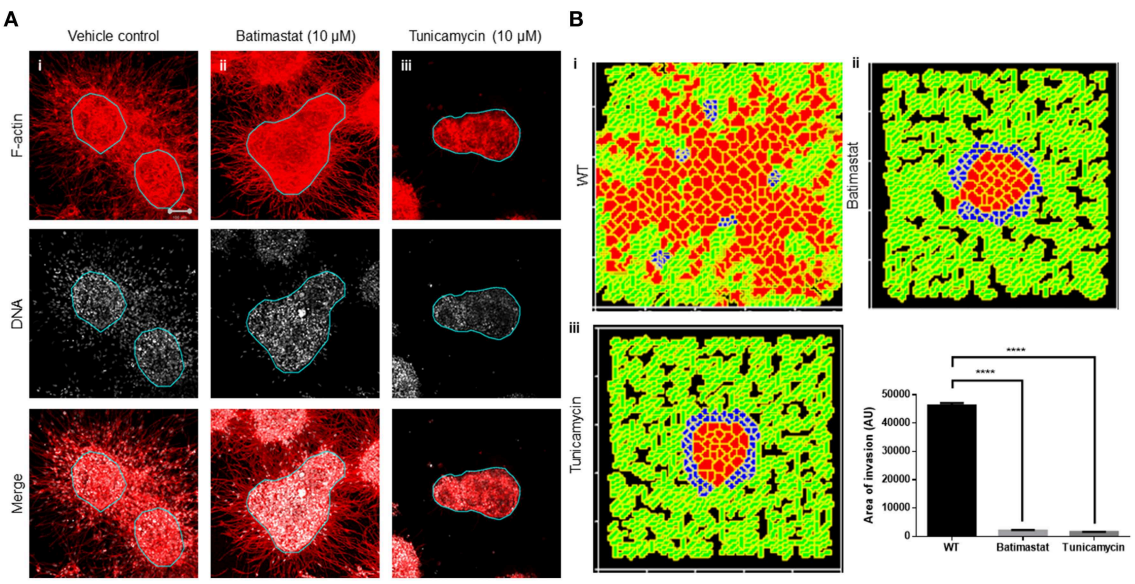

FIGURE 4 | Inhibition of matrix metalloproteinase activity and N-linked glycosylation inhibit multiscale invasion. **(A)** Maximum intensity projections of laser confocal micrographs of MDA-MB-231 cell clusters cultured within specific matrix milieu, fixed and stained for F-actin (using phalloidin; red; top row), DNA (using DAPI; white; middle row), and with both signals merged (bottom row). **(i)** rBM-coated clusters embedded in type 1 collagen treated with vehicle control DMSO show multiscale invasion (left column). **(ii)** Treatment with 10 μM Batimastat leads to inhibition of transition of cells to type 1 collagen although cytoplasmic projections of cells in the periphery of the cluster are visible in the fibrillar matrix. **(iii)** Treatment with 10 μM Tunicamycin results in complete abrogation of multiscale invasion. **(B)** Simulations of control conditions **(i)**, parametric variations analogous with inhibition of R-D **(ii)**, and parametric variations analogous with inhibition of cell–cell, cell–fibrillar ECM, and R-D **(iii)** at MCS590. Graph represents invasiveness of cells in simulations associated with **(Bi–iii)**. Each bar represents mean ± SEM. ****$p < 0.0001$.

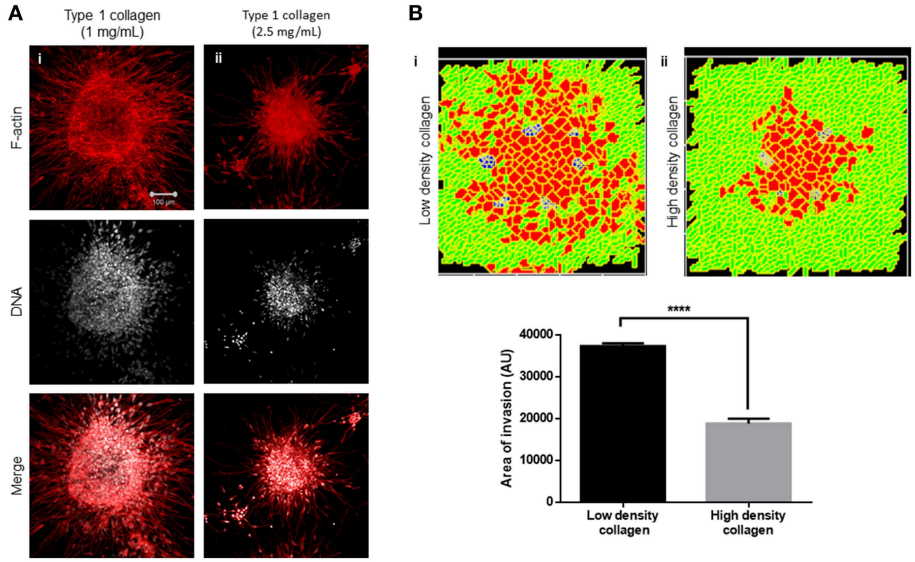

FIGURE 5 | Increased collagen density impairs multiscale invasion. **(A)** Maximum intensity projections of laser confocal micrographs of MDA-MB-231 cell clusters cultured within specific matrix milieu, fixed and stained for F-actin (using phalloidin; red; top row), DNA (using DAPI; white; middle row), and with both signals merged (bottom row). **(i)** rBM-coated clusters embedded in 1 mg/ml type 1 collagen show multiscale invasion. **(ii)** rBM-coated clusters embedded in 2.5 mg/ml type 1 collagen show impaired invasion of cells into surrounding high-density type 1 collagen. **(B)** Simulations of control conditions **(i)** and high-density arrangement of fibrillar ECM showing impaired migration of cells at MCS 540. Graph represents invasiveness of cells in simulations associated with **(Bi,ii)**. Each bar represents mean ± SEM. $^{****}p < 0.0001$.

with our experimental findings, in our computational model, we observe that all other parameters being kept constant, crowding the fibrillar ECM space with a higher density of collagen-like fibers decreased the migration of cells (**Figure 5Bi** represents control multiscale invasion; **Figure 5Bii** represents simulation in high-density fibrillar ECM; **Figure 5B** shows statistically significant impairment of cellular invasion in the computational environment).

Diversity in Morphological Phenotype Can be Explained by Variation in Interplay Between Cell Adhesion and R-D

Our computational model, trained on controls, successfully predicted the consequences on the phenotype of various perturbations. We asked whether it could also accommodate, with suitable changes in its formalism, the possibility of formation of homeostatic nonmalignant phenotypes as well as precancerous and subinvasive phenotypes? If so, what changes in the underlying coarse-grained physical mechanism could be responsible for those?

We obtained a non-invasive homeostatic lumen-containing phenotype (**Figure 6A** represents phenotype at MCS = 10; **Figure 6B** represents emergence of the phenotype at MCS = 580) by assigning the cells within our *in silico* framework, certain properties similar to noncancerous ductal epithelial cells: BM-regulated survival of the cells. By simply implementing the rules that (1) cells that are not anchored to the BM-like nonfibrillar ECM die (Frisch and Francis, 1994) and (2) cells anchored to the fibrillar ECM remain quiescent (Spencer et al., 2011), we were able to achieve growth-restricted lumen-containing acini-like structures that resemble the structures formed by the non-malignant cell line HMLE in 3D (**Figure S7A**). *In silico* phenotypes similar to the pre-cancerous carcinoma *in situ*-like condition, which comprises filled multicellular masses of cells (similar to the mass morphology) (Kenny et al., 2007) (**Figure S7B** shows MCF7 cells forming similar architectures within our 3D assay) could be observed by increasing intercellular and cell–rBM adhesion (**Figure 6C**). A more subinvasive morphology, which resembles the precancerous phenotype, but within which cells have lost their polarity and could give rise to indolently progressive tumors, has been referred to as "grape" (Kenny et al., 2007). We simulated outcomes resembling this phenotype upon further relaxing the intercellular and cell–matrix adhesion (**Figure 6D**). It is crucial to note for simulating both the precancerous and indolent progression phenotypes, the R-D-based ECM remodeling network was not deployed. Invoking the same and decreasing intercellular and cell–rBM adhesion brought about multiscale invasion in simulation (**Figure 6E**). Comparison of invasiveness between the simulations of three cancerous morphologies (**Figure 6F**) reveals that multiscale migration exhibits the highest invasiveness followed by the indolently growing cluster phenotype and, in turn, by the precancerous morphological phenotype.

Finally, we asked whether a decreasing gradient of cell–cell and cell–rBM adhesion was required for increased invasion as predicted by our simulations. Could merely deploying the R-D-based ECM remodeling at higher adhesion regimes bring about greater invasion? Simulating diffusion-driven instability

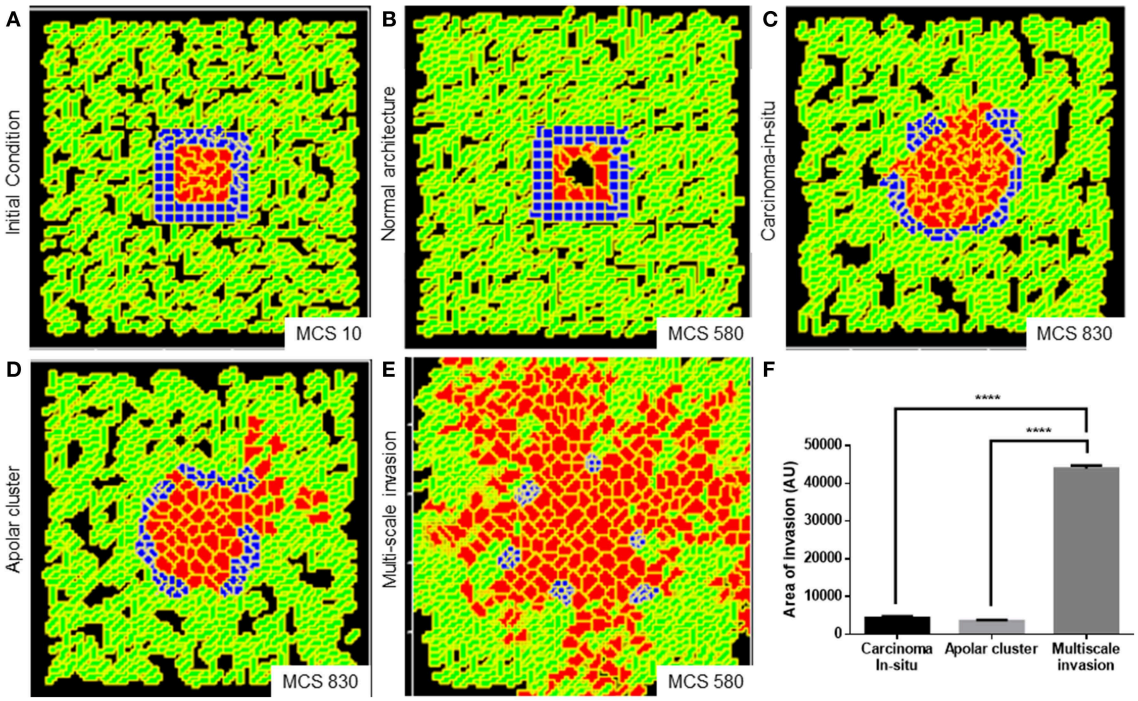

FIGURE 6 | Parameter variation in the computational model can simulate homeostatic, precancerous, and indolently cancerous phenotypes. **(A)** Initial configuration of all components of the computational model at MCS 0. **(B)** Simulation of a homeostatic growth-arrested phenotype with a central lumen obtained upon imposing a nonfibrillar ECM-based rules for regulation of cellular quiescence and death of anchored and detached cells, respectively. **(C)** Simulation of a carcinoma *in situ*-like phenotype obtained by maintaining high values of cell–cell and cell–nonfibrillar ECM adhesion. **(D)** Decreasing cell–cell and cell–ECM adhesion in simulation leads to a phenotype that shows further loss of polarity (as evidenced by a roughness in the outer contour of the clusters) and occasional subinvasive single cell phenotypes. **(E)** Further decreasing cell–cell and cell–matrix adhesion and deployment of an R-D-based kinetics of ECM remodeling leads to multiscale invasion. **(F)** Quantification (bottom right) of the invasiveness of cells from simulations of homeostasis, carcinoma *in situ*, apolar clusters, and multiscale invasion. Scale bar: 100 μm. Each bar represents mean ± SEM. ****$p < 0.0001$.

in ECM degradation in the context of the precancerous adhesion parameter values resulted in increased invasion that was exclusively collective and expansive (**Figure 7A** represents multiscale invasion; **Figure 7B** represents exclusively collective invasion upon simulating R-D in the context of precancerous adhesion parameter values), and phenocopies the only rBM-like *in silico* morphology (see **Figure 3Aii**). On the other hand, simulating the same in the context of the adhesion regimes cognate to subinvasive clustered morphologies did result in multiscale invasion (**Figure 7C**). It is to be noted, however, that the invasion seen in **Figures 7B,C** was significantly lesser than that of **Figure 7A** but more than when in such same phenotypes and R-D-based ECM modulation was off (**Figure 7D**) Our results implicate a threshold that lies between the precancerous and clustered adhesion regimes; the lower the cell and matrix adhesion, the greater the invasion.

DISCUSSION

In this paper, we adopt a coarse-grained systems–theoretical approach toward the exploration of the mechanisms of stromal invasion of breast cancer epithelia. We designed an experimental organo- and pathotypic culture setup wherein not just the 3D behavior of cancer cells could be studied, but also their transition from non-fibrillar (BM-like) to fibrillar (collagenous) ECM environments, as occurs *in vivo* could also be investigated. Using this assay, we observed epithelial transition both as multicellular collectives and as single mesenchymal cells. In contrast, embedding cells in either (but not both) rBM or collagen (as controls) resulted in predominantly discrete collective and single cell migration, respectively. Our observations imply that the complex multimatrix nature of the assay presented here emulates *in vivo* invasive behavior to a better extent than existent single matrix assays.

Our experimental framework led to the construction, in parallel, of a computational model, whose parameters were trained on the phenotypic outcomes of various experimental controls. The design of the computational model takes inspiration from the concept of dynamical patterning modules (DPMs), autonomous heuristic agents that connote discrete physicochemical phenomena, such as adhesion, differential sorting, R-D, polarity, etc. (Newman and Bhat, 2008, 2009). DPMs, when deployed singly or in combination, are useful for understanding the transformation of cellular patterns in distinct ways. DPMs have been used to investigate mechanisms of developmental morphogenesis in plants and animals

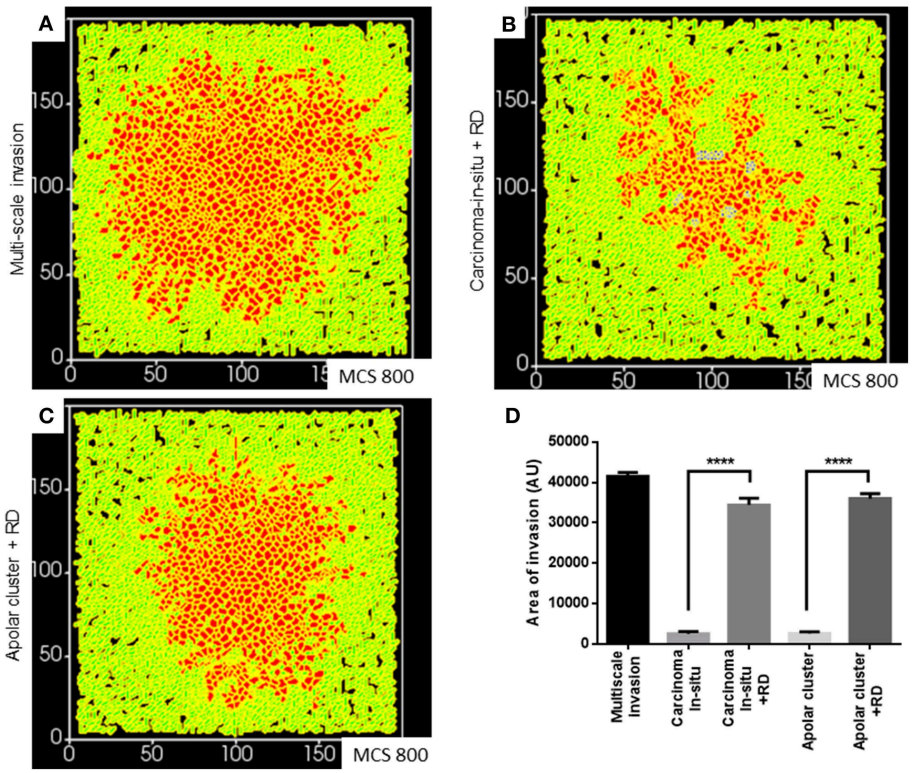

FIGURE 7 | Simulations of the deployment of R-D-based kinetics in carcinoma *in situ* and subinvasive cluster phenotypes. **(A)** Simulation of a control multiscale invasion. **(B)** Simulation, within which regulation of the ECM was modeled using R-D kinetics in parameter regimes of adhesion cognate to carcinoma in situ phenotypes, predicts collective but not single-cell invasion. **(C)** Simulations, within which regulation of the ECM was modeled using R-D kinetics in parameter regimes of adhesion cognate to subinvasive apolar cluster phenotypes, predict multiscale invasion. **(D)** Quantification of the invasiveness of cells from the above simulations in comparison with control multiscale invasion. Each bar represents mean ± SEM. ****$p < 0.0001$.

(Hernández-Hernández et al., 2012; Niklas and Newman, 2013; Benítez et al., 2018). In addition, a DPM-based understanding of the evolution of development provides an explanation of how body plans of animals showed an accelerated period of origination (known as the Cambrian explosion) followed by a relative stasis (Newman et al., 2009).

In the specific context of breast cancer invasion, using DPMs, we have been able to treat much of the intracellular genetic repertoire and its associated dynamics (mutation and epigenetic regulations) as a black box. Instead, we concentrate on phenotypic traits that manifest at the spatial scales of cells and multicellular populations (**Supplementary File 3**). We then asked whether specific combinations of parameters pertaining to these traits are permissive to the diversity of morphologies and cellular patterns seen in breast cancer progression. Given discrete assumptions that are confirmed by experiments, the same framework could give rise to phenotypes exhibited by non-malignant, malignant but non-invasive, subinvasive, and aggressively invasive malignant cells. In the case of noncancerous cells, their quiescent and lumen-containing architecture was dependent on adhesion to BM matrix; inability to do so resulted in anoikis (Frisch and Francis, 1994; Schwartz, 1997; Bissell et al., 2002; Furuta et al., 2018). The model predicts that the transition from homeostatic to a precancerous carcinoma *in situ*-like (DCIS) structures involves anchorage-independent survival and division. The transition from DCIS-like states to subinvasive phenotypes that are characterized by complete loss of cell polarity involves a decrease in adhesion, both intercellular and between cells and BM-like matrices. On the other hand, the transition from subinvasive phenotype to a full-blown invasive multiscale phenotype is predicted to be achieved through specific interplay between decreased cell–cell and -matrix adhesion and R-D-based cross-modulation between regulators of ECM remodeling, with neither physical process being sufficient by itself to bring about the phenotype. The computational model upon being asked to deploy R-D in the presence of high cell–cell and cell–matrix adhesion predicted an exclusively collective invasion phenotype. The latter resembles morphologies obtained when cancer cell clusters are cultured in rBM scaffolds in the absence of type 1 collagen. This suggests that the progression between two given morphologies can be achieved through distinct and dissimilar trajectories in parameter space.

R-D-based mechanisms have been proposed to regulate the spatial patterning of iterative structures in development such as hairs, feathers, and digits (Sick et al., 2006; Glimm et al., 2014; Raspopovic et al., 2014). This occurs through interaction between an autocatalytic mediator of a morphogenetic step and its inhibitor (Gierer and Meinhardt, 1972; Meinhardt and Gierer, 2000). Both the mediator and its inhibitor are, as per the R-D formalism, expected to be diffusible in nature

(Turing, 1952). Their interaction would lead to spatial foci of morphogenesis separated by lateral zones of inhibition. It is reasonable to hypothesize the mediator to be a negative regulator of a morphological trait and its inhibitor to therefore antagonize the mediator's inhibition of morphogenesis. MMPs and TIMPs are exemplars of such processes. They have been shown to play significant roles in mammary gland branch patterning (Wiseman and Werb, 2002). Their interaction dynamics in the context of mammary morphogenesis and elsewhere has been proposed to act through R-D (Grant et al., 2004; Hoshino et al., 2012; Skaalure et al., 2016; Kumar et al., 2018).

A brief survey of expression patterns of genes across multiple cell lines grown on top of rBM matrices provides support for our predictions (Kenny et al., 2007). Cell lines exhibiting subinvasive and invasive morphologies exhibit a progressive decrease in E-cadherin expression for which experimental support is available (Hiraguri et al., 1998). Cell lines with subinvasive morphologies showed decreased levels of β1 integrin, which participates in multiple integrin heterodimers that bind to laminin. Invasive cells specifically expressed an aberrantly glycosylated levels of a β1 integrin (the consequences of glycosylation of β1 integrin have been reviewed in Bellis, 2004). Invasive cancer epithelia are known to express matrix metalloproteinases to a greater extent than untransformed cells: MDA-MB-231, for example, shows high levels of multiple MMPs as well as TIMP, relative to poorly invasive MCF7 cells (Balduyck et al., 2000; Bachmeier et al., 2001).

The modeling approach we have used successfully distinguishes between collective and single-cell growth dynamics. However, it is not able to parse mesenchymal vs. amoeboid motilities. This is because we have modeled cells as bounded units that show little change in shape as they move. We aim to overcome this limitation in the future by constructing multicompartment cells wherein intracellular cytoskeletal dynamics will be incorporated and will also be allowed to respond to inhomogeneities in ECM patterns. Our black-box approach also assumes a direct intracellular linkage between the various extracellular phenomena that mediate invasion. The introduction of interprocess linkages with added feedbacks, delays, and cooperativities as a means of linking adhesion, proliferation, motility, and ECM remodeling, and the (non)linear dynamics associated with the links would further enrich our understanding of the coordination between the diverse cellular phenomena and will be taken up in future efforts. In our computational model, cells proliferate copiously. On the other hand, our culture assays are grown for 24–36 h; cell proliferation can at best be construed to play a mild role in the overall invasion. These two observations are not inconsistent with each other though; proliferation is also observed in cultures grown for longer time periods but does not alter the pattern of invasion that has been initially set by cell migration. The activator–inhibitor couple in our simulations diffuse through and act on matrices: we have therefore not simulated the effect of boundary constraints on the spatial patterns of cellular invasion as explored by others (Diambra and Costa Lda, 2006). In forthcoming papers, we will supplement the collagenous matrix in our experimental assay with cells such as fibroblasts, macrophages, and noncancerous breast epithelia: therein, we intend to computationally explore the effect of spatial constraints on the R-D-based regulation of MMP-TIMP diffusion.

3D pathotypic cultures from patient cells/organoids are increasingly being considered as standards for personalized therapeutic strategies (Hagemann et al., 2017; Pauli et al., 2017). Their ability to prognose radio- and chemoresistance and match the results of patient-derived xenograft models is backed up by a burgeoning body of literature (Hubert et al., 2016; Zeeberg et al., 2016; Gilles et al., 2018). Most of these culture setups lack a stromal compartment. The addition of the latter, as we have done in our assay, may prove to be a useful spatial milieu wherein the effect of immunotherapeutic interventions is tested. Our experimental breast cancer model can also be adapted for other cancers wherein the effect of stromal constituents on multiscale invasion of transformed epithelia may be studied and targeted.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

DPa and RB designed the experiments. Dpa performed the experiments. DPr and RB designed the simulations. DPr performed the simulations. DPa, DPr, and RB analyzed the results of experiments and simulations and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fphys.2019.00790/full#supplementary-material

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Random Matrix Analysis of Ca^{2+} Signals in β-Cell Collectives

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Even within small organs like pancreatic islets, different endocrine cell types and subtypes form a heterogeneous collective to sense the chemical composition of the extracellular solution and compute an adequate hormonal output. Erroneous cellular processing and hormonal output due to challenged heterogeneity result in various disorders with diabetes mellitus as a flagship metabolic disease. Here we attempt to address the aforementioned functional heterogeneity with comparing pairwise cell-cell cross-correlations obtained from simultaneous measurements of cytosolic calcium responses in hundreds of islet cells in an optical plane to statistical properties of correlations predicted by the random matrix theory (RMT). We find that the bulk of the empirical eigenvalue spectrum is almost completely described by RMT prediction, however, the deviating eigenvalues that exist below and above RMT spectral edges suggest that there are local and extended modes driving the correlations. We also show that empirical nearest neighbor spacing of eigenvalues follows universal RMT properties regardless of glucose stimulation, but that number variance displays clear separation from RMT prediction and can differentiate between empirical spectra obtained under non-stimulated and stimulated conditions. We suggest that RMT approach provides a sensitive tool to assess the functional cell heterogeneity and its effects on the spatio-temporal dynamics of a collective of beta cells in pancreatic islets in physiological resting and stimulatory conditions, beyond the current limitations of molecular and cellular biology.

Keywords: collective sensing, pancreatic islets, random matrix theory (RMT), metabolic code, Ca^{2+} imaging, Ca^{2+} signaling, correlations, intercellular communication

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INTRODUCTION

Pancreatic islets are collectives of endocrine cells. Based on the end hormone that these cells exocytose in a Ca^{2+}-dependent manner after being stimulated, several types of cells have been described to compose an islet, with 3 dominant cell types: alpha, beta and delta (Briant et al., 2017). Islets from different parts of pancreas can contain different fractions of each of these cell types, but the bulk cellular mass in a typical islet is in an non-diabetic organism composed of a collectives of insulin secreting beta cells (Dolenšek et al., 2015; Rorsman and Ashcroft, 2017). Early studies assumed these beta cell collectives to be a rather homogeneous population of cells, however, the subsequent functional analyses have revealed a remarkable degree of heterogeneity even in dissociated beta cells in culture. The beta cells were found to differ in a number of physiological parameters, among others in glucose sensitivity and Ca^{2+} oscillation pattern (Zhang et al., 2003),

electrical properties (Misler et al., 1986), redox states (Kiekens et al., 1992), or pattern on cAMP oscillations (Dyachok et al., 2006). These early quests (Pipeleers, 1992) have been mostly searching for morphological, physiological and molecular features that would presumably satisfy at least 3 criteria: (a) entitle special roles for individual cells within the collectives, (b) remain valid even after cell dissociation, and (c) enable to trace embryonic and postnatal development as well as changes during pathogeneses of different forms of diabetes. Recent onset of efficient high-throughput analyses has catapulted these approaches on mostly dissociated cells to a completely new level and enabled identification of a multitude of functional and non-functional subpopulations with their functional characteristics, gene and protein expression, incidences and diabetes-related changes (for a recent review refer to Benninger and Hodson, 2018). A major limitation of these analyses is that the subpopulations described in different studies have relatively little in common and currently their translational relevance is weak. This is, however, not surprising since these approaches primarily deal with sample averages and present merely a small number of discrete snapshots of a very dynamic complex activity. Nevertheless, they turned out to be extremely useful in initial attempts to construct a pseudotime map of the sequence of events in pancreatic endocrine system (Damond et al., 2019) and its interaction with the immune system (Wang et al., 2019) during the progression of type 1 diabetes mellitus (T1D) in humans. Still, better tools are needed to assess the general rules underlying functional heterogeneity in a real-time spatio-temporal dynamics within collectives of beta cells or collectives of any other cell types in an organism. Instead of an ultra-reductionist approach to precisely associate individual molecular markers yielding more or less random correlations in respect to functional heterogeneity, a network of cells is first segmented to their level of functional influence expressed in high deviating eigenvalues to lead a more efficient further discovery of few collective parameters which may also have an identifiable molecular signature. Pancreatic islets have been described as broad scale network (Stožer et al., 2013b) and the search for important principal components is well justified.

The fact is that most of what we know about pancreatic beta cells has been gained by studying dissociated beta cells in cell culture. Therefore, even phenomena that can only be observed in isolated groups of electrically coupled beta cells, like electrical activity (Rorsman and Trube, 1986) or cytosolic Ca^{2+} oscillation, are currently still mostly modeled within the framework of a single cell excitability (Sherman et al., 1988; Bertram et al., 2018). However, each beta cell interacts with several immediate and distal neighboring cells in a pancreatic islet, implicating high-ordered interactions between a large number of elements. Therefore, there is a rich exchange of signals within such a beta cell collective, both through direct cell-cell coupling (Bavamian et al., 2007) as well as through paracrine signaling (Caicedo, 2013; Capozzi et al., 2019). Such an organization necessarily yields a complex response patterns of cell activity after stimulation with physiological or pharmacological stimuli. Until recently, the richness of the aforementioned cell-cell interactions also could not be experimentally documented. However, recent technological advancements made it possible to use various optical tools to address these issues (Frank et al., 2018). For example, the functional multicellular imaging (fMCI) enabled completely new insights into our understanding of a beta cell in an islet as a biological network (Dolenšek et al., 2013; Stožer et al., 2013a,b). The dynamics of a measurable physiological parameter can namely be recorded in hundreds of beta cells within their intact environment (Speier and Rupnik, 2003; Marciniak et al., 2014) simultaneously. The measured oscillatory cytosolic Ca^{2+} concentration changes, which are required to drive insulin release turned out to be a practical tool to trace cellular activity and fundamental to study their interactions in such big collectives (Dolenšek et al., 2013; Stožer et al., 2013a,b). With the use of the tools of statistical physics we and others reconstructed, for example the complex network topologies in beta cell activation, activity and deactivation during transient glucose challenges (Stožer et al., 2013b; Gosak et al., 2015; Markovič et al., 2015; Johnston et al., 2016). As in some other, previously analyzed biological systems, also for the pancreatic islets, the minimal model incorporating pairwise interactions provides accurate predictions about the collective effects (Schneidman et al., 2006; Korošak and Slak Rupnik, 2018).

Along these lines we have recently shown that beta cell collectives work as a broad-scale complex networks (Stožer et al., 2013b; Markovič et al., 2015; Gosak et al., 2017a), sharing similarities in global statistical features and structural design principles with internet and social networks (Milo et al., 2002; Barabási and Márton Pósfai, 2016; Daniels et al., 2016; Perc et al., 2017; Duh et al., 2018). In addition to complex network description when strong cell-cell interaction are primarily taken into account, the analyses of weak pairwise interaction enabled us to use a spin glass model (Korošak and Slak Rupnik, 2018), as well as the assessment of self organized criticality (Bak, 2013; Marković and Gros, 2014; Gosak et al., 2017b; Stožer et al., 2019), also often found in biological samples (Schneidman et al., 2006). The important result from these functional studies is that a faulty pattern of hormone release due to deviating numbers of individual cell types or changes in their function lead to one of the forms of a large family of metabolic diseases called diabetes mellitus (American Diabetes Association, 2014; Pipeleers et al., 2017; Skelin Klemen et al., 2017; Nasteska and Hodson, 2018; Capozzi et al., 2019).

The basic object that we study here is the correlation matrix **C** with elements computed from measured Ca^{2+} signals:

$$\mathrm{Corr}(y_i, y_j) = C_{ij} = \frac{\langle y_i y_j \rangle - \langle y_i \rangle \langle y_j \rangle}{\sigma_i \sigma_j}, \tag{1}$$

where $y_i(t)$ is the i-th time series of Ca^{2+} signal out of N signals measured simultaneously in a collective of pancreatic beta cells.

Random matrix theory (Guhr et al., 1998; Mehta, 2004) (RMT) is concerned with statistical properties of matrices with random elements. Applying RMT to correlation matrices, we study the spectrum of the correlation matrix **C** given by the set of its eigenvalues λ_n:

$$\mathbf{C}\mathbf{u}_n = \lambda_n \mathbf{u}_n, \tag{2}$$

where $\mathbf{u}_n$ are the corresponding eigenvectors.

Statistical properties of the spectra of random correlation matrices for N uncorrelated time series with M random elements where $q = N/M$ is finite in the limit $N, M \to \infty$ are known analytically (Marchenko and Pastur, 1967; Bun et al., 2017). The eigenvalue probability density is:

$$\rho(\lambda) = \frac{1}{2\pi q\lambda}\sqrt{(\lambda_+ - \lambda)(\lambda - \lambda_-)}, \tag{3}$$

where the spectral boundaries are:

$$\lambda_\pm = \left(\sqrt{q} \pm 1\right)^2 \tag{4}$$

When the spectrum of the correlation matrix is unfolded (Guhr et al., 1998) by mapping eigenvalues $\lambda_k \to \xi_k$ so that the probability density of the unfolded eigenvalues is constant $\rho(\xi) = 1$, the RMT predicts that the distribution $P(s)$ of nearest neighbor spacings $s_k = \xi_{k+1} - \xi_k$ is approximately given by the Wigner surmise (Mehta, 2004):

$$P(s) = \frac{\pi}{2} s \exp\left(-\frac{\pi}{4}s^2\right). \tag{5}$$

Possible pair correlations in the eigenvalue spectrum on scales larger than nearest neighbors can be revealed with the use of variance of $n_\xi(L)$, the number of eigenvalues in the interval of length L around eigenvalue ξ. This number variance (Mehta, 2004) is given by:

$$\Sigma^2(L) = \langle\left(n_\xi(L) - L\right)^2\rangle. \tag{6}$$

If the eigenvalue spectrum is poissonian the number variance is $\Sigma^2(L) \sim L$, while real, symmetric random matrices exhibit correlated spectra for which RMT predicts $\Sigma^2(L) \sim \log L$ (Mehta, 2004).

Previous work using RMT in different systems, e.g., on statistics of energy levels of complex quantum systems (Guhr et al., 1998; Mehta, 2004) or correlations in financial markets (Plerou et al., 2002) identified that a bulk of the eigenvalue spectrum agreed with RMT predictions, which suggested a large degree of randomness in the measured cross-correlations in these systems. Only a small fraction of typically a few percent of eigenvalues were found to deviate from universal RMT predictions and were instrumental to identify system specific, non-random properties of the observed systems and yielding key information about the underlying interactions. Biological systems are often complex with large number of interacting parts, high dimensional systems that are basically black boxes "in which a large number of particles are interacting according to unknown laws" (Dyson, 1962). One way to approach such high dimensional systems is to look at the spectrum of the covariance matrix and try to find few principal components that describer most of system variance. This method, principal component analysis (PCA), has been suggested as "'hypothesis generating' tool creating a statistical mechanics frame for biological systems modeling" (Giuliani, 2017). PCA works best for systems where we can find few eigenvalues in the covariance spectrum well separated from the bulk and the system can be described in low dimensional space. Usually, there is no clear separation in the eigenvalue spectrum and other methods such as RMT or methods using renormalization group approach (Bradde and Bialek, 2017) are more suitable. In biological systems, RMT has been used to filter out critical correlations from data-rich samples in genomic, proteomic and metabolomic analyses (Luo et al., 2006; Agrawal et al., 2014), as well as in brain activity measured by EEG (Šeba, 2003) and dynamic brain networks constructed from fMRI data (Wang et al., 2015, 2016). While eigenvalue spacing distributions showed agreement with RMT predictions, the number variance distributions often display deviations pointing to the physiologically relevant reduction in correlated eigenvalues fluctuations with partially decoupled components transiting toward Poisson distribution (Šeba, 2003). Such transitions have also been used as an objective approach for the identification of functional modules within large complex biological networks (Luo et al., 2006). Additionally, as for protein-protein interactions in different species, these latter deviation from RMT predictions has been interpreted as an evidence to support the prevalence of non-random mutations in biological systems (Agrawal et al., 2014).

In this paper we used the RMT approach to test the cross-correlations in the cytosolic Ca^{2+} oscillations under non-stimulated and glucose stimulated conditions. We demonstrate that statistical properties of cross-correlations based on functional multicellular imaging data follows those predicted by RMT, with both high- and low-end deviating eigenvalues, suggesting local as well as global modes driving this correlation in functional islet. In addition, our results show that the long range correlations in eigenvalue spectrum deviate in a stimulus dependent manner.

DATASET DESCRIPTION

We define beta cell collective activity to sense nutrients and produce metabolic code as the relevant constraining context for the physical outcomes of analysis (Ellis and Kopel, 2018; Korošak and Slak Rupnik, 2018). Our data consist of a typical Ca^{2+} activity recorded by multicellular imaging on an islet of Langerhans of fresh mouse pancreatic slice. All data analysis has been performed using custom scripts in Python 3.5 software and customized scripts (RMThreshold) in R software. We used raw data for each calcium signal, but we detrended the signals to remove possible sources of spurious correlations due to systematic slow variations caused by the imaging technique. A common problem in the analysis of fMCI Ca^{2+} signals in living tissue is selection of regions of interest corresponding to a true signal originating from a cytosolic area of an individual cell and not two or more neighboring cells. In practice the reproducibility of the results depends on the level of experience of the operator to subjectively recognize structure from the patterns of activity. While we are primarily interested in the activity of a large population of cells, their interactions/correlations and their collective response, it is crucial that this signals originate from regions of interest that

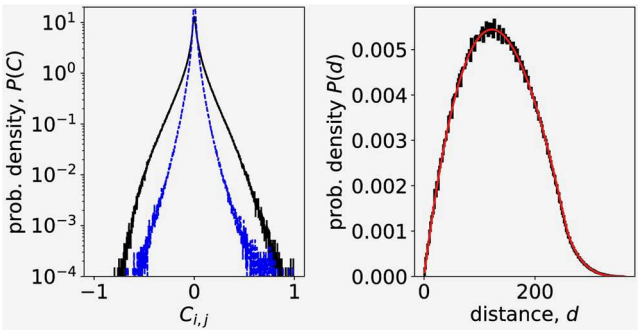

FIGURE 1 | (Left) Correlation coefficient distribution of $N = 4000$ Ca^{2+} signals randomly chosen from experimental dataset (outer, black line). Dashed blue line is the distribution of correlations between residuals after removing the influence of the largest eigenvalue in signals. **(Right)** Distribution of distances between signals randomly chosen from Ca^{2+} imaging data. Black line is the experimental data, thinner red line is the theoretical distribution of distances between random points in a square (Philip, 2007).

correspond to individual cells. Collectives of cells, like beta cells in the islet of Langerhans are densely packed structures, where extracellular space and the cell membrane represent a relatively small to negligible cross-section area on the image of two-dimensional optical section obtained by confocal microscopy. Therefore we decided to avoid the aforementioned subjectivity issue by the random sampling of pixel level signals in the recorded time series. For this analysis we randomly selected $N = 4000$ signals out of the complete dataset of 256 × 256 signals each $M = 23{,}820$ timesteps long (about 40 min recordings at 10 Hz resolution). Glucose concentration was changed during the recording from 6 mM (lasting about first 5,000 timesteps) to 8 mM and back to 6 mM (approx. last 5,000 timesteps) near the end of experiment.

The source of correlations in a cell population where the terminal action is a calcium-dependent process (e.g., exocytosis of insulin in beta cells) are the individual events in a form of plasma membrane ion channel or transporter activity, internal membrane ion channel or transporter activity, as well as calcium leak from activated immediate neighboring beta cell (Berridge et al., 2000). The correlations between the activities of beta cells depend strongly upon the glucose concentration (Dolenšek et al., 2013; Markovič et al., 2015), however in the physiological plasma glucose range (6–9 mM), most correlations are weak (Korošak and Slak Rupnik, 2018), so that the probability of detecting co-activation basically equals the product of the probabilities of activities of individual beta cells. The correlations are statistically significant for almost all pairs of immediate neighbors.

RESULTS

The distribution of correlation coefficients reveals that most of the correlations between the pairs of Ca^{2+} signals are weak, but there is also non-negligible contribution of highly correlated pairs of signals (**Figure 1**, left, black outer line). We also checked the sampling procedure by comparing the computed distribution of distances between pairs of randomly chosen points from 256 × 256 image square to the analytical probability distribution of distances between two random points in a square (Philip, 2007) (**Figure 1**, right). We found a perfect match between the distance distribution computed from data and the theoretical distribution, confirming that our random sampling of data points was non-biased.

Guided by the observed non-gaussian nature of correlation distribution (**Figure 1**, left) we explored a detailed structure of the correlation matrix, since distribution of correlation coefficients only partially hints to the nature of cell to cell coordination. To this end we computed the eigenvalues and eigenvectors of the correlation matrix (Equation 2) and compared the obtained eigenspectrum with the RMT prediction. In **Figure 2** (top left) we show the distribution of eigenvalues that belong to the empirical correlation matrix (black trace) and the RMT prediction (red line) given by Equation (3). While most of the eigenvalues falls within the limits $\lambda_{\pm}$ of the RMT spectrum, there are also significant deviations from RMT prediction. We found the largest empirical eigenvalue λ_{max} two orders of magnitude away from the upper limit of the RMT spectrum, and also a part of the empirical spectrum that extends below the lower RMT limit. To see if the deviations from the RMT are inherent to the measured Ca^{2+} signals, we prepared a surrogate dataset by randomly shuffling each signal's time series. We then computed the correlation matrix and its eigenvalue spectrum from randomized surrogate dataset. As shown in **Figure 2** (top right), the match between the eigenvalue distribution of randomized dataset and RMT is perfect.

Previous RMT analysis of stock correlations in financial markets consistently showed (Laloux et al., 1999; Plerou et al., 1999, 2002) that the distribution of components of the eigenvector $\mathbf{u}_{max}$ corresponding to largest eigenvalue λ_{max} strongly deviates from Gaussian form, suggesting that this mode reflects the collective response of the system to the stimuli. In our case this corresponds to collective response of beta-cells to glucose stimulus. In a linear statistical model for Ca^{2+} signals, we model the response common to all beta-cell with $Y(t)$ and the signals are expressed as:

$$y_i(t) = a_i + b_i Y(t) + \delta y_i(t), \tag{7}$$

where $\delta y_i(t)$ is the residual part of each signal. Coefficients a_i, b_i are obtained by regression. Following Plerou et al. (2002) we approximated the common response $Y(t)$ with the projection of all signals on the largest eigenvector:

$$y_{max}(t) = \sum_{i=1}^{N} u_i(\lambda_{max}) y_i(t), \tag{8}$$

where $u_{max,i}$ is the i-th component of the eigenvector corresponding to largest eigenvalue λ_{max}. To see the influence of the collective response to the distribution of correlation coefficents, we computed using $Y = y_{max}$ the residuals $\delta y_i(t)$ for all N signals and their correlations $C_{res(i,j)} = \mathrm{Corr}(\delta y_i, \delta y_j)$. The dashed blue line (inner trace) on **Figure 1** (left) shown

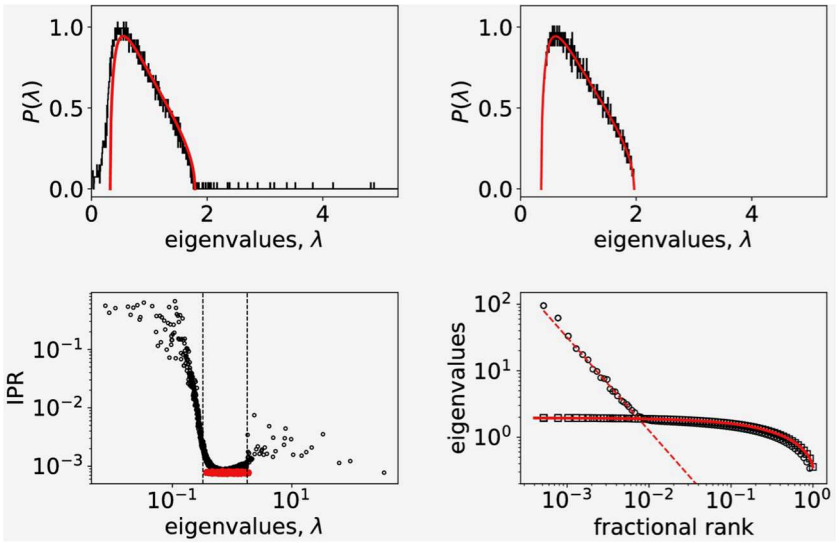

FIGURE 2 | (Top left) Probability distribution of eigenvalues of the empirical correlation matrix for $N = 4{,}000$ randomly picked signals (black solid line) compared to the distribution of eigenvalues of random correlation matrix of the same size (red solid line). **(Top right)** Probability distribution of eigenvalues of the surrogate correlation matrix constructed from shuffled empirical values (black solid line) compared to the random correlation matrix of the same size (red solid line). **(Bottom left)** Inverse participation ratio plot of eigenvalues showing a random band matrix structure of C with large IPR values at both edges of the eigenvalue spectrum. The dashed vertical lines show RMT bounds. The IPR spectrum for randomized correlations is shown in red. **(Bottom right)** The fractional rank plot of the entire spectrum of eigenvalues (open dots). For comparison we added the same plot of eigenvalues of correlation matrix computed from randomized data (open squares). The full line shows the fractional rank plot of eigenvalue spectra obtained from distribution given by Equation (3). The shape of the distribution of large eigenvalues points to a scaling relationship.

the distribution of $\mathbf{C}_{res}$ and reveals that the collective response predominantly contributes to large correlations.

To test further if the largest eigenvalue and the corresponding eigenvector capture the collective calcium response we compared the average signal $\overline{y}(t) = 1/N \sum_j y_j(t)$ with y_{max}. The correlation between signals projected on the largest eigenvalue mode and mean signal was high: $Corr(y_{max}, \overline{y}) \approx 0.8$, confirming the expectation that the largest eigenvalue represents collective effect. Similarly, we checked how similar are the signals corresponding to the bulk RMT eigenvalues:

$$y_{bulk,i}(t) = \sum_{j=1}^{N} u_j(\lambda_i) y_j(t), \tag{9}$$

where λ_i is the eigenvalues from the RMT interval $[\lambda_+, \lambda_-]$. The computed correlation between signals projected on bulk eigenvectors and mean signal, averaged over all signals was $< \mathrm{Corr}(y_{bulk,i}, \overline{y}) >= -0.0044 \pm 0.0047$ suggesting no correlation between the mean signal and signals coming from the bulk RMT regime. To further characterize the eigenvector structure of the empirical Ca^{2+} correlation matrix, we looked at the inverse participation ratio (IPR) of eigenvector $\mathbf{u}(\lambda)$ corresponding to eigenvalue λ defined as (Plerou et al., 1999, 2002):

$$I(\lambda) = \sum_{j}^{N} \left(u_j(\lambda)\right)^4. \tag{10}$$

The value of $1/I(\lambda)$ reflects the number of nonzero eigenvector components: if an eigenvector consist of equal components $u(\lambda)_i = 1/\sqrt{(N)}$ then $1/I(\lambda) = N$, in other extreme case $1/I(\lambda) = 1$ when an eigenvector has one component equal to 1 and all others are zero.

Figure 2 (lower left) shows the computed values of IPR for all eigenvectors as function of corresponding eigenvalue. The red datapoints are the IPR data computed for the surrogate, randomized timeseries data for which we found $1/I \sim N$ as expected. We found similarly values $1/I \sim N$ for the largest eigenvalues of the empirical spectrum (black datapoints) suggesting that to this eigenvectors almost all signals contribute. Deviations from flat RMT prediction at the edges of the RMT spectrum ($[\lambda_+, \lambda_-]$ interval, vertical dashed lines) with large $I(\lambda)$ values suggests that these states are localized with only a few signals contributing. This points to a complex structure of the empirical correlation matrix **C** with coexisting extended and localized eigenvectors similar to one found in correlations in financial markets (Plerou et al., 1999, 2002). In addition, as shown in **Figure 2** (lower right, open dots), we observe a scaling behavior in rank-ordered plot of eigenvalues of empirical correlation matrix that has been connected with a fixed point in renormalization group sense (Bradde and Bialek, 2017; Meshulam et al., 2018). For comparison, we plot also the rank-ordered eigenvalues of randomized data (open squares) and RMT prediction based on eigenvalue density given by Equation (3) (full line) which perfectly describes the randomized dataset. The observed scaling of eigenvalues hints toward the critical behavior that was conjectured for beta-cell collective at the transition from

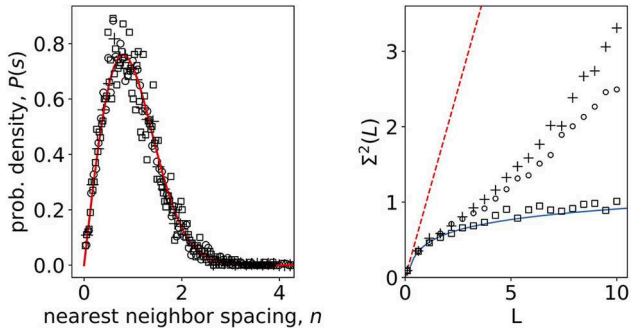

FIGURE 3 | (Left) Nearest-neighbor spacing distribution of empirical correlation matrix eigenvalues of calcium signals in non-stimulatory and stimulatory regime. Open squares: shuffled, randomized data; open dots: 8 mM glucose; crosses 6 mM glucose; full line Wigner surmise (Equation 5). **(Right)** Number variance of eigenvalue spectra of calcium signals. Open squares: shuffled, randomized data; open dots: 8 mM glucose; crosses 6 mM glucose; full line RMT prediction $\Sigma^2(L) = 1/\pi^2(\log(2\pi L) + 1 + \gamma - \pi^2/8)$ (Mehta, 2004); dashed line Poissonian limit $\Sigma^2(L) = L$.

glucose non-stimulated to stimulated phase (from 6 mM to 8 mM) (Gosak et al., 2017b; Stožer et al., 2019).

To explore the statistical differences of signals in non-stimulated and stimulated phase, we separated the original data into two groups of N signals each with $M = 10^4$ timesteps corresponding to response to 6 and 8 mM glucose stimuli. For each group we computed the unfolded eigenvalue spectra and also for randomized data. The results for the nearest-neighbor spacing and number variances are shown in **Figure 3**. For nearest-neighbor spacing distribution we find a good agreement with the RMT prediction both, for non-stimulatory and stimulatory conditions, as well as shuffled stimulated data. All three datasets are well described with the Wigner surmise (Equation 5), so nearest-neighbor spacing does not seem to be sensitive to stimuli changes. On the other hand, however, the number variance is sensitive to stimuli change already during physiological stimulation of the beta-cell collective. The random matrix prediction is in this case valid for shuffled stimulated data only (**Figure 3**, right).

DISCUSSION

The unique spatio-temporal resolution of functional multicellular imaging and sensitivity of advanced statistical approaches for a plethora of different modes of complex network scenarios and levels of criticality, makes these approaches a method of choice to assess the nature of cell-cell interactions under different stimulation conditions. At the same time it enables us to test the validity of experimental designs for study of beta cell function, primarily in the domain of stimulation strength and dynamics. We suggest that without such validation the most critical events in the activation chain within the beta cell collective have been and shall be overlooked or misinterpreted (Stožer et al., 2019). The predominant use of supraphysiological glucose concentration can namely severely deform the relatively slow beta cell recruitment in a collective at physiological glucose concentrations (Stožer et al., 2013a; Gosak et al., 2017a) and miss the typically segregated network clusters of Ca^{2+} events (Benninger and Piston, 2014; Markovič et al., 2015; Westacott et al., 2017), turning critical behavior into disruptive supracritical activity (Gosak et al., 2017b; Stožer et al., 2019). Only under rather narrow physiological conditions it shall be possible to extract the fine structure of cell-cell interactions causing long-term and efficient cell collaboration with the collective. Breaking apart this delicate structure of cell-cell interaction does result in a massive activity, which can be readily described by tools of statistical physics, but this activity does not necessarily serve its physiological or biological purpose (Ellis and Kopel, 2018).

A common denominator of the previous attempts to categorize different beta cell types points to some metabolic and secretory features that can be either reproduced between different classifications or not. Usually there exist a bulk of one subtype and one or more less frequent subtypes (Benninger and Hodson, 2018). These less frequent subtypes can nevertheless have important regulatory roles that may not be immediately apparent. This issue is particularly critical if the frequency of a beta cell subtype represents only a couple of percent of the entire beta cell population in an islet. Along these lines there have been some indications regarding the beta cell subtypes that can serve as pacemakers or hubs within a dynamic islet cell network (Johnston et al., 2016; Lei et al., 2018), however due to the nature of complexity of network features, we may still be short of evidence for definitive conclusions. The full description of heterogeneity of endocrine cells within an islet, ultimately producing a adequate release of hormones is therefore still lacking. In trying to grasp this complexity, it is important to take into account interaction of beta cell collectives with other cell types in and around an islet, like glucagon-secreting alpha cells (Svendsen et al., 2018; Capozzi et al., 2019) or somatostatin secreting delta cells (Rorsman and Huising, 2018) as well as neurons and glial cells (Meneghel-Rozzo et al., 2004), but also endothelial, immune cells (Damond et al., 2019), as well as acinar and ductal cells (Bertelli and Bendayan, 2005).

Random matrix theory is a fitting mathematical framework which provides powerful analytical tools to separate cell-cell interactions happening by chance from those produced by specific coordinated interactions after a changed chemical composition of cell's surrounding. In the financial sector, adequate asset allocation and portfolio risk-estimation can lead to a higher profit and is therefore clear why it makes sense to invest time into cross-correlation analyses (Plerou et al., 2002). But what would be the gain of knowing that randomness of cell-cell correlation matrices is physiologically regulated? Firstly, we suggest that the analysis of the universal properties of empirical cross-correlations is a valuable tool to identify distinct types and further subtypes of endocrine cells within an islet through their non-local and local effects. The largest eigenvalue of **C** namely represents the influence of non-local modes common to all measured Ca^{2+} fluctuations. Other large eigenvalues can be used to address cross-correlations between cells of the same

type, cells with specific functions in the collective or that these cells reside in topologically similar area of the islet. Quantifying correlations between different beta cells in an islet is therefore an exciting scientific effort that can help us understand cell communities as a complex dynamical system, estimate the amount of factors ruling the system or potential presence of a stress situation (Gorban et al., 2010).

The large values of inverse participation ratio (IPR) (**Figure 2**, bottom left) compared to the IPR values in the bulk, indicate that only a few cells contribute to these eigenstates with eigenvalues at the edges of the RMT bulk spectrum. In contrast, all cells contribute to the eigenstates corresponding to the largest eigenvalues. This means that we find delocalized states for the largest eigenvalues and localized states as we move toward the RMT edge of the spectrum. Similar findings were recently reported in RMT analysis of single-cell sequencing data (Aparicio et al., 2018), where the spectrum of covariance matrix of single-cell genomic data followed RMT predictions with deviations at the bulk edge. The localized states at the edge of the bulk spectrum were connected with the true biological signal.

Our results show that the number variance reflecting the correlation between subsequent eigenvalues (a measure for long range correlations in eigenvalue spectrum) follows the RMT predictions up to a certain distance L, however at larger distances it starts to deviate in a stimulus dependent manner, suggesting structural features in the beta cell network. Transitions between Poissonian and GOE statistics in biological systems have been previously described during the process of either integration or segregation of complex biological networks, showing various degrees of long range correlations at various physiological conditions (Luo et al., 2006). This understanding has a vital practical value since it can help decipher different roles that beta cells can play in a collective and to further validate the importance, if any, of previously defined and continuously appearing novel molecular markers of beta cell heterogeneity (Benninger and Hodson, 2018; Damond et al., 2019; Wang et al., 2019). An advanced knowledge about the dynamic properties of the functional cell types will shed a new light into understanding of physiological regulation of insulin release and the assessment of perils of stimulation outside of the physiological range. Furthermore, it can help us elucidate the mechanisms on how this function changes during the pathogenesis of different forms of diabetes mellitus and lead us to novel approaches of therapy planning and prevention. And finally, it can help us understand the general principles ruling the interactions in collectives of other cell types.

DATA AVAILABILITY STATEMENT

The raw data used in this research is available upon request to the corresponding author.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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CORRECTION
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Corrigendum: Random Matrix Analysis of Ca^{2+} Signals in β-Cell Collectives

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In the original article, there was a mistake in **Figure 3** as published. Despite careful examination of the text and references in the proof, we unfortunately failed to notice that the incorrect **Figure 3** was used. The correct **Figure 3** appears below.

The authors apologize for this omission and state that this does not change the scientific conclusions of the article in any way. The original article has been updated.

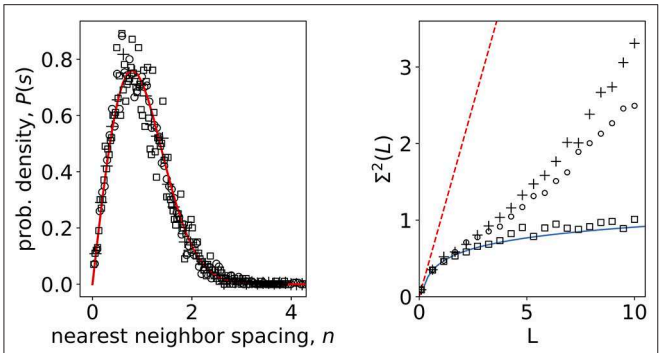

FIGURE 3 | (Left) Nearest-neighbor spacing distribution of empirical correlation matrix eigenvalues of calcium signals in non-stimulatory and stimulatory regime. Open squares: shuffled, randomized data; open dots: 8 mM glucose; crosses 6 mM glucose; full line Wigner surmise (Equation 5). **(Right)** Number variance of eigenvalue spectra of calcium signals. Open squares: shuffled, randomized data; open dots: 8 mM glucose; crosses 6 mM glucose; full line RMT prediction $\Sigma^2(L) = 1/\pi^2(\log(2\pi L) + 1 + \gamma - \pi^2/8)$ (Mehta, 2004); dashed line Poissonian limit $\Sigma^2(L) = L$.

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Understanding Multicellularity: The Functional Organization of the Intercellular Space

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The aim of this paper is to provide a theoretical framework to understand how multicellular systems realize functionally integrated physiological entities by organizing their intercellular space. From a perspective centered on physiology and integration, biological systems are often characterized as organized in such a way that they realize metabolic self-production and self-maintenance. The existence and activity of their components rely on the network they realize and on the continuous management of the exchange of matter and energy with their environment. One of the virtues of the organismic approach focused on organization is that it can provide an understanding of how biological systems are functionally integrated into coherent wholes. Organismic frameworks have been primarily developed by focusing on unicellular life. Multicellularity, however, presents additional challenges to our understanding of biological systems, related to how cells are capable to live together in higher-order entities, in such a way that some of their features and behaviors are constrained and controlled by the system they realize. Whereas most accounts of multicellularity focus on cell differentiation and increase in size as the main elements to understand biological systems at this level of organization, we argue that these factors are insufficient to provide an understanding of how cells are physically and functionally integrated in a coherent system. In this paper, we provide a new theoretical framework to understand multicellularity, capable to overcome these issues. Our thesis is that one of the fundamental theoretical principles to understand multicellularity, which is missing or underdeveloped in current accounts, is the functional organization of the intercellular space. In our view, the capability to be organized in space plays a central role in this context, as it enables (and allows to exploit all the implications of) cell differentiation and increase in size, and even specialized functions such as immunity. We argue that the extracellular matrix plays a crucial active role in this respect, as an evolutionary ancient and specific (non-cellular) control subsystem that contributes as a key actor to the functional specification of the multicellular space and to modulate cell fate and behavior. We also analyze how multicellular systems exert control upon internal movement and communication. Finally, we show how the organization of space is involved in some of the failures of multicellular organization, such as aging and cancer.

Keywords: control, extracellular matrix, mobility, functional integration, physiology, development, immunity

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INTRODUCTION

This paper addresses the theoretical issue of functional differentiation, integration, and coordination in multicellular systems. Our claim is that in order to understand multicellularity and its variety of instances, a crucial dimension of this phenomenon has been neglected and needs to be taken into consideration and analyzed: the intercellular space and its functional organization. By developing a theoretical framework focused on the organization of space in multicellular systems, we argue that: (1) cells are not the only and main actors of multicellularity, as highly organized dynamic structures such as the non-cellular ECMs also play a decisive role in the origin and current realizations of functionally integrated multicellular systems; (2) functional spatial differentiation, the control of motility/fixity of cells and the organization of mobility (e.g., vascularization, immune cells, etc.), are three of the main features that allow multicellular systems to overcome bottlenecks of complexity and realize highly integrated and internally differentiated organisms.

Multicellularity is a widespread phenomenon that cuts across all the domains of life, spanning from bacterial biofilms to plants and metazoans. Its most ancient eukaryotic instances date back to around 1.6 billion years ago in the case of red algae (Bengtson et al., 2017). It has been realized independently several times (Bonner, 2000; Grosberg and Strathmann, 2007; Knoll, 2011) including at least one case, *Volvox*, which transitioned to a multicellular form as recently as 200 million years ago (Kirk, 1998). Multicellularity is neither a unique nor a rare phenomenon in the biological word. When it comes to understand it, therefore, it is not a question of explaining its uniqueness, but rather its generality, with deep theoretical implications for our understanding of life in general. It is not by chance, then, that the multicellular dimension of life is at the center of thriving debates in biology and philosophy regarding development, individuality, integration, etc. (Buss, 1987; Arnone and Davidson, 1997; Santelices, 1999; Wilson, 1999; Okasha, 2006; Grosberg and Strathmann, 2007; Michod, 2007; Queller and Strassmann, 2009; Folse and Roughgarden, 2010; Herron et al., 2013; Niklas, 2014).

As argued by several works, the adaptive advantages of realizing multicellular organizations are usually related to increase of size, accompanied by division of labor and increase in complexity, in such a way that multicellular systems can escape predators and occupy different niches with respect to unicellular organisms (Bonner, 2000; Knoll and Bambach, 2000; Rueffler et al., 2012). While doing so, multicellular systems, from biofilms to metazoa, have faced several problems in order to achieve a viable integration between their cellular components. Among the main ones, are the trade-off between cell differentiation and avoidance of conflict, the control and coordination of cells, the availability of nutrients, the access to signal molecules and the possibility of intercellular communication, modularity, structural cohesiveness, to mention the main ones.

To explain how living systems found solutions to these problems, different theoretical approaches emphasize different aspects as the core of multicellularity: self-organization (Newman and Forgacs, 2005), the capability to interpret positional information (Wolpert, 1969), gene regulation (Arnone and Davidson, 1997), cell-to-cell communication (Bonner, 2000; Niklas, 2014) and its role in cell differentiation (Wolpert and Szathmáry, 2002; Arnellos et al., 2013; Veloso, 2017), division of labor between reproductive and vegetative functions (Michod, 2007), genetic homogeneity (Santelices, 1999), low conflict (Queller and Strassmann, 2009), metabolic integration (Pfeiffer and Bonhoeffer, 2003), increase in the energy available and the development of larger genomes (Lane and Martin, 2010), among others.

Yet, as pointed out by Grosberg and Strathmann, multicellularity is not a problem of principle, insofar as many of the requirements that have been suggested for multicellularity – such as cell-adhesion, communication, differentiation, coordination, etc. – have already evolved in unicellular organisms (Grosberg and Strathmann, 2007, see also Brunet and King, 2017). That should not be a surprise, given the importance of the social/communitarian dimension of life (Shapiro, 1988), which has inspired even possible scenarios on origins of life as emerging from colonies of protocells (Woese, 2002; Carrara et al., 2012; Mansy, 2017). The question, thus, is not as much to explain how and why multicellularity originated, as it is of understanding what is specific of multicellular organizations, and how to account for the different forms they can achieve.

The question, then, is what is so special about the interactions that take place in a multicellular system that is not already realized by unicellular organisms. In order to find an answer, it is necessary to understand how cells are *controlled* or *constrained* in their living together in multicellular systems in such a way that they realize and maintain viable organized entities. When these forms of control fail, or their properties change in certain ways, this change may give rise to different (transient or stable) forms of multicellular organization or regressions, more often incompatible with the original one, such as in cancer (Sonnenschein and Soto, 1999; Bissell and Radisky, 2001; Soto and Sonnenschein, 2011) and aging (Moreau et al., 2017).

Our thesis is that in order to understand how cells are constrained and integrated in higher order systems and how several structural and organizational bottlenecks are overcome, looking at cells and their interactions is not enough. We argue that the debate on multicellularity has actually been driven by an implicit cellular bias, so that some fundamental features of multicellular organization have been overlooked by a perspective that identifies in cells the main and only actors of multicellularity. We show that multicellular forms of life cannot be explained exclusively in terms of cellular interactions and their biochemical mechanisms. Rather, we argue that in order to provide a theoretical framework to understand multicellularity, it is necessary to also take into account a dimension that is missing or underdeveloped in current accounts, that is, the intercellular space. By that we mean not only considering the space in which cells operate, and how they specify it, but also how the organization of space, in turn, has a direct influence on cell fate and behavior. It is our contention that the increase in size which characterizes multicellular organisms, and which enables cell differentiation and division of labor, goes hand in hand with and directly depends for its viability on the capability to organize the intercellular space.

Multicellular systems, in fact, are not just made of cells, but of highly dynamical and active structures such as extracellular matrixes (ECMs), which do not just provide structural support for cells, but give rise to a variety of inherently organized intercellular spaces. The importance of space, form, and physical constraints in general has been stressed in the past, but in this paper we will develop a different and more specific point, i.e.: that the organization of space plays a functional role, and the non-cellular structures involved are to be considered as actors of multicellularity together with cells. We will show that the functional properties related to space contribute to many of the features that are considered as fundamental in the debate on multicellularity and that the dynamic nature of space organization has relevance for development and robustness. How the intercellular space is organized is crucial in the control of the fate and activity of groups of cells, in the differentiation of functionally distinct areas, in providing nutrients and enabling communication, ensuring protection, etc. In addition to that, the increase in overall size, accompanied by the loss of the capability of motility in the majority of the cells of a multicellular system, requires the reorganization of mobility and realization of distinct communication subsystems (i.e., the vascular system, the immune system, and the nervous system).

Despite its crucial role at the multicellular level, the organization of the intercellular space has not been the object of a sufficient attention in the literature. Therefore it requires a comprehensive theoretical account capable to bring together and to make sense of the different contributions from biology and medicine, and to inspire further research. This paper aims to do so by developing a theoretical framework that takes into account at its core the question of the organization of space and can contribute to a better understanding of multicellular phenomena. Moreover, we argue that in order to understand the organization of space, the roles of ECM and of mobility and communication systems need to be analyzed. In particular, ECM structures act as control subsystems through mechanical as well as molecular interactions. They play a crucial functional role in maintaining systems of cells viably together, and are instrumental in the transition from unicellular to multicellular systems, as it is shown in the case of *Volvox carteri* (Kirk, 2005). Moreover, not only the organization of space is crucial to understand the realization and viability of multicellular systems in all domains of life, but also different ways of organizing space can account for the distinctive features of different multicellular forms, such as biofilms and eukaryotic multicellular systems, from *Volvox carteri* to metazoa.

The paper will proceed as follows. In Section "Why Multicellular Systems Are Not Just Balls of Cells: The Limits of Current Accounts of Multicellularity," we analyze the main conceptual issues related to multicellularity and how different theoretical approaches address them. We show that these accounts exhibit some deep conceptual problems, and we argue that they derive from the fact that they do not directly tackle, at their foundations, questions regarding the organization of space. In Section "The Functional Organization of Space," we introduce and develop the idea of functional organization of the intercellular space by analyzing the role of extracellular structures as control subsystems, and by specifying their functional contribution to the integration of multicellular systems. In Section "Motility, Mobility, and Communication Within Multicellular Systems," we analyze how multicellular systems organize mobility and communication, by focusing on the role of the vascular system, of mobile immune cells, and the nervous system. In Section "Concluding Remarks," we conclude with a recapitulation and a discussion of the implications of this theoretical framework for our understanding of distinctively multicellular phenomena such as cancer and aging.

WHY MULTICELLULAR SYSTEMS ARE NOT JUST BALLS OF CELLS: THE LIMITS OF CURRENT ACCOUNTS OF MULTICELLULARITY

Multicellularity is a highly diversified phenomenon. Having emerged independently over 25 times in the history of life on earth (Grosberg and Strathmann, 2007), it is realized, in different ways and with different degrees of integration, by bacterial biofilms (Shapiro, 1988; Ereshefsky and Pedroso, 2012), and by eukaryotic systems giving rise to social organisms (Strassmann and Queller, 2010), colonies, chimeras, clonal, and aggregative entities (Herron et al., 2013), plants, fungi, and metazoa. The literature on multicellularity is characterized by a proliferation of accounts that aim to capture the distinctive features, and internal differences, of this class of biological organizations in general[1].

Most accounts of multicellularity are characterized by a specific attention to reproduction and evolution, and aim to provide an understanding of multicellular systems as evolutionary individuals (Buss, 1987; Santelices, 1999; Michod et al., 2006; Michod, 2007; Pepper and Herron, 2008). In doing so, they highlight features such as reproductive bottlenecks, differentiation between reproductive and non-reproductive tasks, high-cooperation and low conflict, as central to account for the capability of multicellular systems to work as "bundles of adaptation", where all elements work toward a common evolutionary goal (Queller and Strassmann, 2009; Strassmann and Queller, 2010; Herron et al., 2013).

Yet this is not the only way to look at the problem. While not denying the importance and role of evolutionary considerations for the study of the origins and the histories of the lineages of multicellular systems, another possible research avenue is to investigate the distinctive features of their physiologies. This alternative approach implies looking at how these systems are organized and how their organization is necessary for their persistence. In this paper, we pursue this latter strategy and we focus on the capability of multicellular

[1]A special interest has been placed upon the subclass of metazoa, because they exhibit the highest structural and behavioral complexity, and the degree of cohesiveness that we usually associate to organismality. See, for example, Arnellos and Moreno (2016) for a detailed review of this literature. We will not focus here specifically on the issue of multicellular organismality but, rather, on the distinctive features of multicellular organizations in general.

systems to realize viable dynamic physiological networks capable of self-production and self-maintenance[2].

In the past, this type of approach has been carried out mostly by taking the living cell as the paradigmatic case (see Moreno and Mossio, 2015). Consequently, organismic and organizational frameworks have been primarily developed by focusing on unicellular life. Multicellularity, however, presents additional challenges to our understanding of biological systems, related to how cells are capable to live together in higher order entities, in such a way that some of their features and behaviors are constrained and controlled by the system they realize. Whereas in the case of cellular systems, this goal has been pursued by focusing on molecular and macromolecular mechanisms and structures, in the case of multicellularity, it needs to be pursued by focusing on *cells and extracellular structures*, by showing how these components are physically and functionally integrated into cohesive systems.

Functional integration is a central concept in order to understand how different types of multicellular entities give rise to viable systems in which the activity of individual cells is recruited and coordinated. In a minimal sense, functional integration consists in the degree in which in a biological dynamic regime of self-maintenance the different components that collectively realize the system as a viable unit depend on one another for their production, maintenance, and activity (see, for example, Bich, 2016).

To achieve functional integration, a biological system requires some internal differentiation – i.e. the presence of components that contribute in different ways to the realization of the system (Mossio et al., 2009) – which constitutes the basic requirements for division of labor. Most evolutionary accounts of multicellularity put special emphasis on the differentiation between germ and soma cells and on their mutual dependence on a reproductive and evolutionary scale (Buss, 1987; Michod et al., 2006). A minimal case of this type of cell differentiation in multicellular systems can be found in one species of the Volvox genus, called *Volvox carteri* (Kirk, 1998, 2005; Herron, 2016; Matt and Umen, 2016). This collective entity is realized by green algae of the order Volvocales, which give rise to a spherical system of thousands of cells immersed in a highly differentiated, modular ECM. As a result the system is capable to move as a unit in space toward sources of light to perform photosynthesis (**Figure 1**).

Volvox carteri is characterized by the differentiation into only two types of cells: somatic and germ cells. Both types of cells are capable of photosynthesis. Somatic cells are equipped with flagella and located at the periphery of the system. They are immersed, with a fixed orientation, in a complex ECM structure differentiated in zones which constitutes most of the mass of the whole system. Due to their specific positioning, these cells provide a coherent propulsion for the whole system. Germ cells are bigger in size and are located inside the system.

The integration between the two types of cells accounts for the maintenance of this class of systems on the inter-generational and phylogenetic scales. Yet, in a system such as *Volvox carteri*, the functioning and maintenance of the system as a whole at the *physiological scale* is not achieved through cell differentiation, insofar as the germ cells do not play a specific role in it. Focusing on differentiation into somatic and germ cells does not say much about how the current system is maintained physically and functionally cohesive and, ultimately, alive during its ontogenetic time scale.

To understand how the current system, rather than the lineage, is maintained, one has to focus on its physiology and, in particular, on the fundamental metabolic functions and mechanisms of intercellular control and communication. When considering the problem from this perspective, several types of features have been proposed as necessary for multicellularity, including genetic homogeneity (Santelices, 1999) and unicellular

[2]This approach is in agreement with the line of investigation in physiology traditionally inaugurated by Claude Bernard, centered on the idea that the activity of the components of a biological system contributes to the *realization* and *stability* of their internal milieu which, in turn, is the condition for their own existence (Bernard, 1865, 1878; see also Bechtel, 2007; Mossio and Bich, 2017).

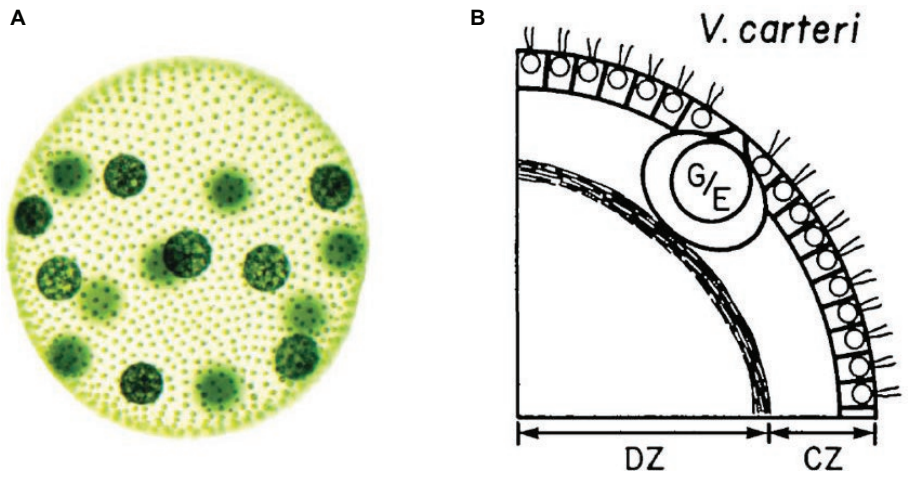

FIGURE 1 | (A) Image of an adult *Volvox carteri* (Kirk, 2005, p. 300. *Reproduced with permission from John Wiley & Sons, Inc.*). Gonidia are the bigger spheres; somatic cells are the smaller dots. **(B)** Position of gonidia (G/E) and somatic cells (small circles with flagella) within the *Volvox carteri*'s ECM (Kirk et al., 1986, p. 226. *Reproduced with permission from Company of Biologists Ltd.*).

bottlenecks (Grosberg and Strathmann, 2007), low conflict (Queller and Strassmann, 2009), metabolic integration (Pfeiffer and Bonhoeffer, 2003), genetic control (Arnone and Davidson, 1997; Sebé-Pedrós et al., 2017), patterns of self-organization (Newman and Forgacs, 2005), etc. In particular, two closely interdependent characteristics have been suggested as distinctive of multicellularity and crucial for the functioning, maintenance, and viability of multicellular systems: cellular differentiation (Bonner, 2000; Wolpert and Szathmáry, 2002; Arnellos et al., 2013; Veloso, 2017) and increase in size with respect to unicellular systems (Bonner, 2000; Knoll and Bambach, 2000; Rueffler et al., 2012).

Cellular differentiation is a distinctively multicellular feature. It might seem trivial to say, but unicellular systems can only produce different phenotypes and play distinct functions *in time*. Multicellular systems, from biofilms to metazoa, can instead exhibit several differentiated phenotypes at the same time. Such a capability is an essential requirement for functional integration. Through cell differentiation, multicellular systems become in principle capable to harbor components playing different functional tasks, and hence to realize division of labor under certain conditions. A cohesive functional integration between these different tasks is achieved when the differentiation of functions is coordinated at the system level, and the differentiated components contribute through their activity to the maintenance of the system. By showing the fundamental role it plays in the developmental processes of metazoans (**Figure 2**), Arnellos et al. (2013) and Veloso (2017) have argued that cell differentiation, through intercellular signaling and the formation of self-organized gradients, is the crucial element to account for multicellularity.

Increase in size has also been invoked by many as a crucial factor in the origin and evolution of multicellular systems (see Grosberg and Strathmann, 2007): a mean to avoid unicellular predators and, in turn, to expand feeding opportunities by predating upon unicellular organisms. From a physiological standpoint, the increase in size achieved through multicellularity allows increased storage reserves, the generation of an internal environment and new metabolic capabilities. Importantly, the increase in size allows to reach the critical mass necessary for differentiated somatic cells to realize division of labor. Following this line of argument, Bonner (Bonner, 1998, 2000) argues that the increase in size comes first (for example in *Volvox carteri*) both logically and historically in the origins of multicellularity, followed by the emergence of intercellular mechanisms of cell differentiation and communication.

From a physiological standpoint, accounts of multicellularity built upon either of these two factors, or both together, exhibit several conceptual limits. Let us start with (intercellularly induced) cell differentiation. This property is not sufficient, or maybe not even necessary, in order to account for functionally integrated multicellularity. Differentiation might not be *necessary* for minimal multicellularity, at least in principle, because basic functional integration would not require different types of cells, but just different types of components, to play different physiological functions that contribute to the activity and maintenance of the system. As we will argue in the next sections, these components can be cells but also non-cellular structures like ECM. Thus, in principle, there can be a functionally integrated system – like *Volvox carteri* – with only one type of somatic cells interacting with ECM structures.

Cell differentiation is not *sufficient* for multicellularity either. Differentiation between somatic and germ cells for example, does not entail physiological functional integration. What would be needed to achieve it by means of cell differentiation, instead, is different types of somatic cells. Moreover, even when somatic differentiation is achieved, it needs to be employed to realize division of labor and integration. And the expression of the functional potential of differentiated types of cells in the maintenance of the system requires that the system has reached a critical size, thus enabling the coordinated activity of a critical number of cells of different cell types.

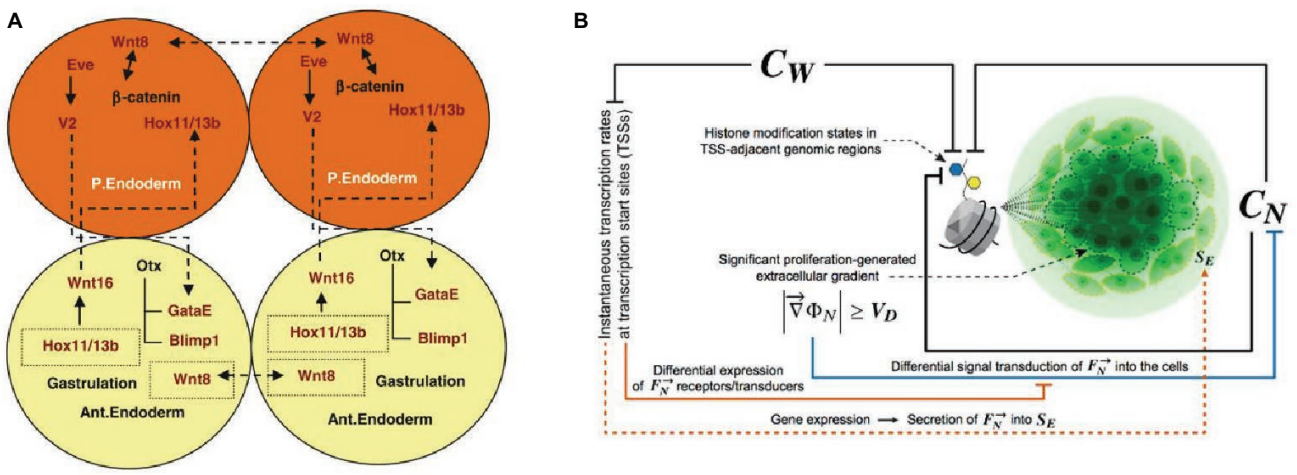

FIGURE 2 | Accounts of multicellularity centered on cell differentiation achieved through intercellular signaling in the development of metazoa. **(A)** The intercellular cross-effects of *Wn8* and *Delta* activate *Hox1/13b* and trigger cells differentiation (Arnellos et al., 2013, p. 869. *Reproduced with permission from Springer Nature*). **(B)** The role of intercellular constraints in histone modification and consequent cell differentiation and proliferation, with generation of intercellular gradients [Veloso, 2017, p. 90. *Reproduced under the terms of the Creative Commons Attribution License (CC BY)*].

Finally, cell differentiation alone cannot make sense of the differences between distinct types of multicellular systems. While it might allow distinguishing *Volvox carteri* from other systems, it might fail in other cases. Single species biofilms exhibit at least nine types of cells that are the result of differentiation induced by intercellular signals, like in the case of *Bacillus subtilis* (Lopez et al., 2009; Mielich-Süss and Lopez, 2015), let alone multispecies biofilms. This is comparable to cell differentiation in hydra, one of the simplest metazoan, which has 20 cell types.

Increase in size, while it constitutes an enabling condition for division of labor in systems with cell differentiation, incurs into several problems and unpassable bottlenecks as well. Even in the minimal case of biofilm, the required increase in size cannot be achieved without solving some problems related to the circulation of nutrients in all areas of the system and the elimination of waste and toxic compounds. It also requires the implementation of medium- and long-range mechanisms of coordination of cell fate and behavior and of communication (transmission of signals), that go beyond direct cell-to-cell interaction and self-organized cellular gradients. Diffusion, as the mean to provide the molecules necessary for the functioning of an organized system, puts strict limits to the size of a system. As it has been argued (Knoll, 2011), in order to increase in size beyond a thin layer of cells, all multicellular systems require solving the problem of diffusion by realizing, among other things, differentiated structures for the transport of oxygen, nutrients, and molecular signals.

In sum, focusing either on the increase in size or on signal-induced cell differentiation, or even on both factors together, cannot explain why multicellular systems are not limited to just small balls or thin layers of cells, but instead give rise to complex, differentiated and integrated structures. In our view, something more fundamental is missing to understand the reason why the size and number of cells can increase in such a way to take advantage of cell differentiation and allow cells to coordinate and actually carry out activities with different functional roles. Filling this gap, by developing a framework capable to overcome the limits put into evidence in the current accounts of multicellularity, will be the aim of the rest of the paper.

THE FUNCTIONAL ORGANIZATION OF SPACE

The missing piece of the puzzle discussed in the previous section, we argue, is constituted by the consideration of the intercellular space: a highly dynamic and differentiated context whose internal organization is of paramount importance for the realization and maintenance of viable multicellular systems. As we argue in this section, the intercellular space is more than just a medium for the activity of cells and for passive diffusion and the storage of molecules. The way it is organized plays a fundamental role in making growth in size and functional differentiation possible (**Figure 3**).

Increase in size cannot happen without organizing the intercellular space, as it would encounter impassable bottlenecks such as the one constituted by the case of vascularization vs. diffusion. Moreover, dynamic structures of the intercellular space, such as those made of ECM[3], play a direct role in determining

[3]The eukaryotic ECM is a complex three-dimensional structure that characterizes the intercellular space. It is a network of several types of proteins and carbohydrates (collagen, enzymes, glycoproteins, proteoglycans, etc.) which can include several types of other molecules, such as growth factors (see Hynes, 2009; Mecham, 2011 for an overview). Bacterial biofilms are also characterized by a specific extracellular matrix, which has different composition and structure compared to those found in eukaryotic systems, notably including DNA (see, for example, Steinberg and Kolodkin-Gal, 2015).

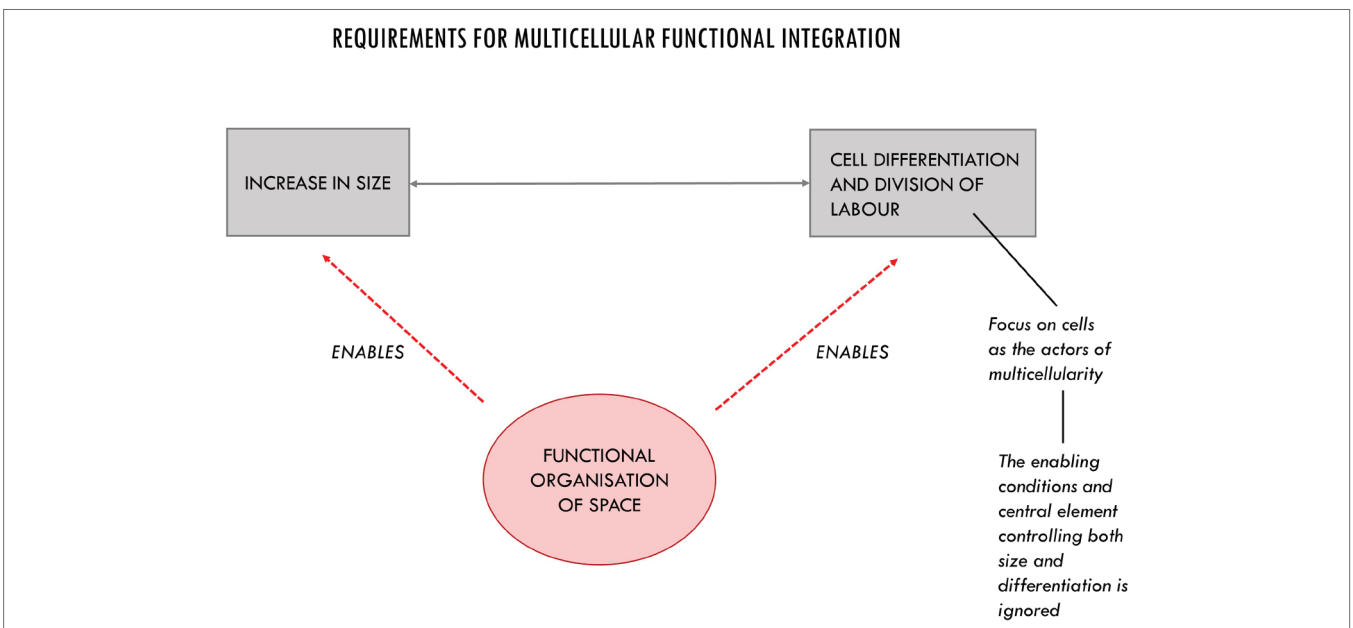

FIGURE 3 | The three main requirements for multicellular functional integration. The organization of space is an enabling condition for the increase in size and cell differentiation. Its functional role in multicellular systems is often underestimated due to an implicit cellular bias.

cell fate and behavior and in achieving functional differentiation. They do so by means of mechanical and molecular mechanisms resulting from the 3D anchorage of cells. A fundamental role in this context is played, among others, by mechanotransduction mechanisms responsible for spatial control and proliferation (Alenghat and Ingber, 2002; Wang et al., 2009; Dupont et al., 2011; Piccolo et al., 2014). Variations in mechanical constraints, such as changes of ECM stiffness or changes in cell shape controlled by the ECM, have a profound impact on cell behavior. As showed by Piccolo et al. (2014), for example, transcriptional regulators YAP and TAZ are intracellular mechanisms by which the mechanical properties of the ECM and the cell geometry within the intercellular space instruct cell behavior. YAP and TAZ have been linked to a universal system that controls proliferation, space localization, and organ size. Changes in ECM stiffness and, consequently, in cell shape, constrain the structure of the cytoskeleton and modulate the activity of the YAP/TAZ regulatory transcriptional mechanism: they keep this protein complex in a transcriptionally active state in the nucleus (stiff ECM), or located in the cytoplasm where it is subject to degradation (softer ECM). In other cases, the ECM modulates the activity of cells by acting upon membrane proteins, such as integrins which, in turn, trigger regulatory changes within cells themselves (Adams and Watt, 1993; Hynes, 2009; Rozario and DeSimone, 2010; Tsang et al., 2010; Faulk et al., 2014).

These dynamic structures functionally specify the intercellular space. Despite the increasing amount of data on the involvement of ECM structures in several phenomena of relevance for biology and medicine, their role as active players in the *control*, *coordination*, and *integration* of the components of multicellular systems, together with cells, has been underestimated in the main theoretical accounts of multicellularity and requires further conceptual scrutiny. Supported by the data available, in this section, we proceed in this direction by developing a theoretical framework that takes into account the organization of the intercellular space to the core, starting by addressing the problem of control in multicellular systems. We show that cells are not the only actors of multicellularity. And we argue that the intercellular space, rather than a mere background for cell-to-cell interactions, should be understood as an organized *milieu* populated by extracellular components that play a crucial role together with cells in controlling the dynamics of the multicellular systems.

The Problem of Control in Multicellular Systems

Control is generally understood in biology as the capability to actively modify the dynamics of a system toward certain states (Rosen, 1970). In living systems, such capability to steer or harness a process, or to modulate the activity of the constituents of the system, can be understood in terms of *constraints*: structures that act as local boundary conditions that enable specific processes and activities. A general definition of constraint can be given in the following terms: given a particular process P, a structure C acts as a constraint upon P if: (1) at a time-scale characteristic of P, C is locally unaffected by P; (2) at this time-scale C exerts a causal role on P, i.e., there is some observable difference between free P, and P under the influence of C (Mossio et al., 2013, p. 164)[4].

In a nutshell, a constraint reduces the degrees of freedom of processes or collections of elements, in such a way that they exhibit specific behaviors and they can be used to perform some coherent activity in the context of the system (Pattee, 1972, see also Kauffman, 2000; Umerez and Mossio, 2013; Winning and Bechtel, 2018). A biological example of a constraint is an enzyme, which by lowering the activation energy necessary for a reaction, catalyzes it toward an otherwise improbable product. At a different scale, another paradigmatic case is the vascular system, which canalizes the stream of blood toward different parts of the organisms, while in unconstrained conditions the process would take place in a very different way and rate by diffusion, and with different outcomes. The peculiarity of living systems with respect to other natural and artificial systems is that they produce some of the constraints – such as enzymes, membranes, or the vascular system – that are necessary for their own internal functioning (Montévil and Mossio, 2015; Moreno and Mossio, 2015).

Distinct types of constraints play different functional roles. A crucial distinction can be made between those *structural constraints* which statically and passively reduce the degrees of freedom of the processes they canalize, and those dynamic *control constraints* that actively select between the degrees of freedom available (Pattee, 1972). Structural constraints can be realized by static structures, or by purely physical interactions like in the case of the restriction of spatial freedom by neighboring cells. Control, instead, as the capability to actively modify the dynamics of a system toward certain states, requires the presence of dynamic constraints that are characterized by both sensory and effector capabilities, and that exhibit differential activity (e.g., activation or inhibition) in presence of specific boundary conditions or interactions, for example, with signal molecules (Bich, 2018). Control constraints do not reduce degrees of freedom once and for all. Instead, they modulate the controlled processes depending on their activation status[5].

Control is crucial for an organized system, such as a living one, in which processes are stochastic, and constantly require modulation in relation to changes in external and internal conditions. Moreover, different subsystems might present different ways of operating, and their activities and rates need to be coordinated to avoid conflict and to ensure their joint functional contribution to the maintenance of the system. To do so, the activity of basic control constraints is directly modulated by other specialized regulatory constraints in the system. The result is the realization of control architectures capable to implement those of differential and specific responses necessary for the integration and coordination of the activities of several subsystems and, ultimately, the maintenance of a complex organization (Bich et al., 2016).

[4]A more detailed characterization can be found in Montévil and Mossio (2015).
[5]David Fell, for example, in a classic textbook on metabolism in cellular and multicellular systems, stresses these features of control: "we can regard metabolic control as the power to change the state of metabolism in response to an external signal" (Fell, 1997, p. 3).

Addressing the question of functional integration in multicellularity requires taking into consideration how control is realized at the intercellular scale. At this level, what is controlled and coordinated is, among other things, the activity of cells. Cells are themselves living autonomous entities that exhibit agential capabilities, and which need to be organized into integrated and cohesive systems, by avoiding conflict and making their activities compatible and mutually sustaining (Soto and Sonnenschein, 2018).

Focusing on the specificity of control at the intercellular level requires considering how a system is capable to constrain and coordinate the activity of cells together with that of other extracellular components at short, medium, and long ranges, in such a way that they achieve a viable way of living together. For example, if we think of proliferation and motility as the properties that are characteristic of unicellular organisms – their "default state," as defined by Soto and Sonnenschein (2011) – a challenge multicellular systems face is how to exert a differential and dynamic control upon these properties in a way that is functional for the whole. Not all the cells can proliferate and not at any time. Therefore, the system activates the division of certain cells in specific moments in time and inhibits it in others. Moreover, depending on the state of the system, the capability of *motility* is also inhibited in most cells. When those constraints that act on proliferation, motility, mobility, etc. fail, or their properties are modified, these changes may give rise to different forms of multicellular organization, more often incompatible with the original one, such as in cancer, and contribute to the development of several human diseases, such as osteoarthritis, fibrosis, etc. (Bonnans et al., 2014).

Spatial Control and the Organization of the Intercellular Space: Overcoming the Cell Bias

In order to understand multicellular control, it is important to put into evidence three closely interconnected theoretical aspects. The first is that control is not exhaustively accounted for by cell-to-cell interactions alone: extracellular components produced by the system also play an active role in controlling the dynamics of the system and contribute to its realization and viability. Second, these additional control subsystems populate and functionally specify the intercellular space, which is not an inert background for cells, but a dynamic *milieu* which constitutes the boundary conditions for cells activity and whose properties directly influence the behavior of cells. Third, as a result, this space is functionally organized in such a way that it enables increase in size and division of labor at the system's level.

One of the reasons why the full role of the organization of the intercellular space and of extracellular control structures has been overlooked as a theoretical principle in understanding multicellularity – for example, in favor of cell differentiation based on cell-to-cell interactions – lies in an implicit cellular bias, which identifies in cells the *only* active players in this class of biological systems. Placing the focus on cells is not surprising, and it is also correct, albeit partial. Cells are the most fundamental biological units. As correctly stated by Pier Luigi Luisi, a biochemist and synthetic biologist involved in origins of life research "It is well known that life is cellular and only cellular: all tissues and organs of all animals and plants are organized assemblies of cells – so that we can consider the cell as the elemental constituent of life on this planet" (Luisi, 2017, p. 353). Whereas this statement may be coherently and successfully applied in research on origins of life – i.e. how the first living cells originated – it would be problematic if, when addressing multicellularity, the statement "all life is cellular" was interpreted as the fact that cells are all that is important, or the only building blocks necessary to understand multicellular systems.

Even accounts of multicellularity which take space into account, such as Wolpert's concept of "positional information" (Wolpert, 1969), do not address the properties and organization of the intercellular space *as such*. Instead, they assume a perspective centered on cells, and focus on: (1) how cells detect their surroundings, interpret their position in space, and change their activity accordingly and (2) how spatial differentiation results from a "process by which the individual cells within a population are specified to undergo a particular molecular differentiation, which results in a characteristic spatial pattern" (Wolpert, 1969, p. 2). More recent accounts also address space from a cellular perspective, by putting into evidence the importance of the transition from a temporal differentiation to a spatial segregation of cell types in the origin of multicellularity (Brunet and King, 2017)[6].

Yet "all life is cellular" does not necessarily mean that cells (or groups of cells) are the only active functional components in multicellular systems. In fact, there are other dynamic components produced within a multicellular system, such as ECM structures, that play an active role in it and provide a decisive functional contribution to its maintenance and integration. They do so by acting through spatial relations. It is specifically ECM structures that exert a fundamental constraining function upon the cellular default state of proliferation and motility in multicellular systems (see, for example, Montévil et al., 2016). The ECM gives rise to structures that exhibit a trade-off between stability and dynamicity which allows them to play a control function within multicellular systems. These extracellular components cannot be understood only as *structural constraints* which statically reduce the degrees of freedom of the constrained cells once and for all. They are dynamical components that actively select between the degrees of freedom available, thus exhibiting – together with cells – the basic sensory-effector capability characteristic of *control constraints* at the intercellular level.

While providing stable anchorage, and exhibiting specific features in different tissues, ECM structures also carry out differential constraining activity that functionally modulates the state of cells. They are dynamical constraints because, at different physiological time-scales, they can change their physical state, density, composition, 3D shape, or the state of activation of their proteins, in relation to the state of the system or of a specific tissue. For example, mechanical forces and molecular interactions can alter the functional domains of proteins

[6]Accounts focused on intercellular self-organization and the formation of patterns of cell, from Weiss' (1968) to Newman's (Newman and Forgacs, 2005), are characterized by a similar conceptual attitude according to which the distribution in space is understood as resulting from cell-to-cell interactions more than a causal actor in itself.

embedded in the matrix; building and dissolving the matrix also selectively modifies its control capabilities in time. In addition, enzymes can act as regulatory switches that modulate the control capabilities of the ECM by creating and modifying collagen cross-links (Chen et al., 2015).

In turn, depending on their (activation) state, ECM structures can constrain in different ways the behavior of cells, by acting upon specific membrane receptors, by inducing changes in cells shapes, or by modulating the activity of signaling molecules and morphogens. These activities are functional insofar as they contribute to the overall maintenance of the system (**Figure 4**). The ECM, for example, modulates cell fate and activity by interacting with mechanosensitive proteins such as integrins in cell membranes and by activating gene transcription (Rozario and DeSimone, 2010; Dupont et al., 2011; Piccolo et al., 2014). As dynamic repositories of signal molecules, morphogenetic and growth factors, ECM structures are capable of modulating their availability to cells on the basis of specific interactions in the intercellular space, thus controlling the behavior of cells both at short and medium ranges (Hynes, 2009; Rozario and DeSimone, 2010; Tsang et al., 2010). Changes in the stiffness of the matrix also control cell differentiation (Lv et al., 2015) as well as migration, apoptosis, and proliferation (Wells, 2008). These features are not limited to eukaryotic multicellular systems; the matrix plays similar roles in bacterial biofilms as well (Sutherland, 2001; Branda et al., 2005; Steinberg and Kolodkin-Gal, 2015).

In metazoa, ECM structures interact with cells in assembling some of the supra-cellular structures that give rise to space differentiations and play a functional role as boundaries and interfaces: i.e. the endothelium with respect to the vascular space, and the epithelia in the case of tissues. Epithelial tissues play a fundamental role in animal development and in the organization of complex animal bodies (Tyler, 2003; Arnellos and Moreno, 2016). They also support animal movement and sensory-motor capabilities (Keijzer and Arnellos, 2017). The formation of the epithelium is not determined by the intrinsic properties of cells only. The ECM generally plays an active role in controlling the features and positions of cell-cell junctions and, consequently, cell assembly (Tseng et al., 2012). In the specific case of the epithelium, the ECM structure involved in the assembly and

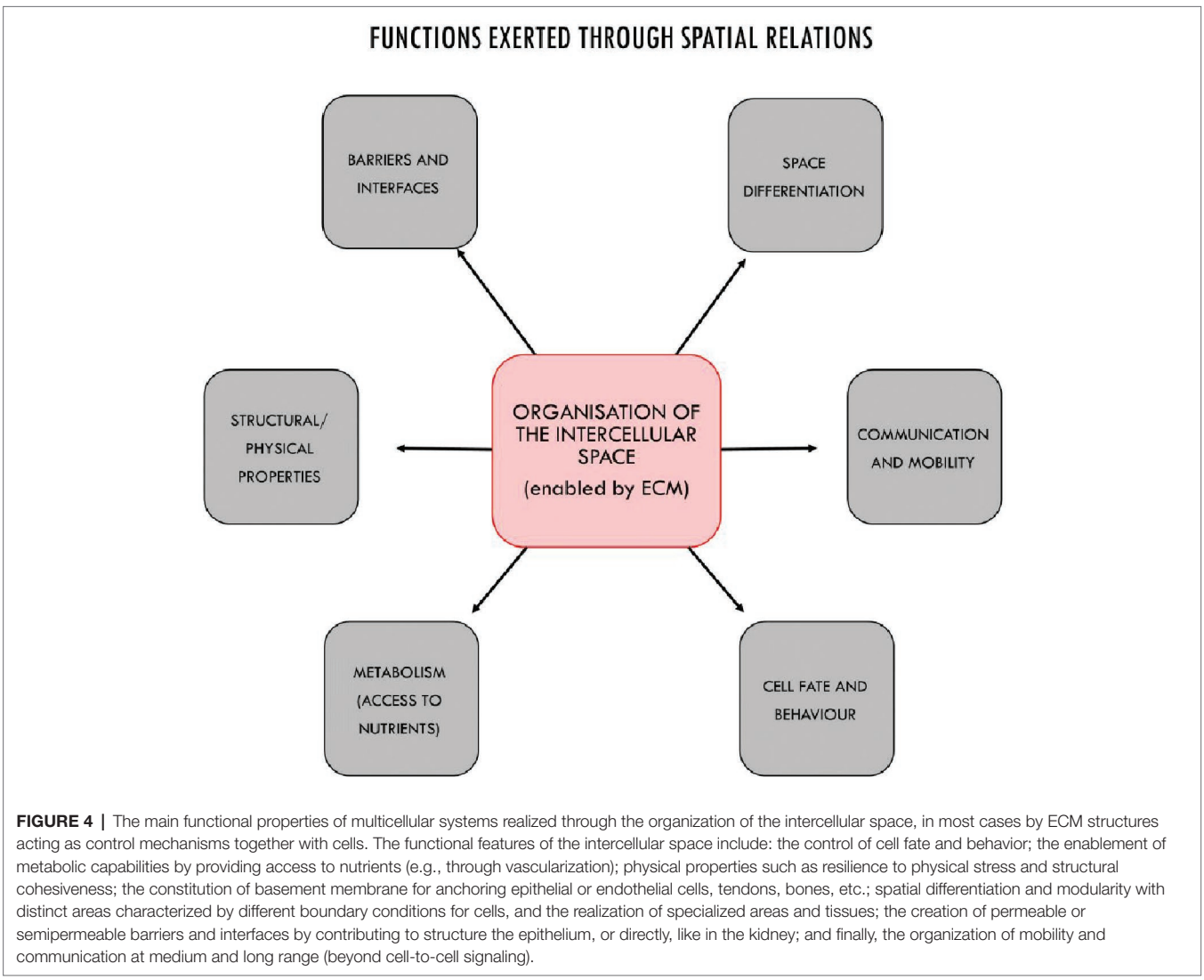

FIGURE 4 | The main functional properties of multicellular systems realized through the organization of the intercellular space, in most cases by ECM structures acting as control mechanisms together with cells. The functional features of the intercellular space include: the control of cell fate and behavior; the enablement of metabolic capabilities by providing access to nutrients (e.g., through vascularization); physical properties such as resilience to physical stress and structural cohesiveness; the constitution of basement membrane for anchoring epithelial or endothelial cells, tendons, bones, etc.; spatial differentiation and modularity with distinct areas characterized by different boundary conditions for cells, and the realization of specialized areas and tissues; the creation of permeable or semipermeable barriers and interfaces by contributing to structure the epithelium, or directly, like in the kidney; and finally, the organization of mobility and communication at medium and long range (beyond cell-to-cell signaling).

stabilization of this organized intercellular structure is the basement membrane (BM). Alteration in the properties of the BM in relation to the epithelial cells – through release of cell-BM contacts or through degradation of the BM by proteolytic enzymes – is associated with and can trigger the epithelial-mesenchymal transition (EMT), with strictly controlled or chaotic disaggregation of the epithelial organization. These processes are involved in the formation of tissues and organs during development and in cancer, respectively (Nisticò et al., 2012).

All together, the functions exerted within an organized intercellular space make fundamental contributions to the realization of the two features that are usually considered as the basis of multicellularity: increase in size on one side and cell differentiation with division of labor on the other. The former is enabled, among other things, by the control exerted by the ECM upon cell proliferation, and by the fact of providing structural support and cohesiveness to the system, anchoring for cells, and acting as a scaffold for shape transitions during development (Sherwood, 2015)[7]. Moreover, increase in size is also achieved by means of spatial organization that makes it possible the distribution of nutrients to cells, for example through vascularization.

The ECM also controls cell differentiation and contributes to coordinate the activity of different cell types in such a way that the system achieves division of labor and functional integration. Matrix structures modulate cell differentiation by sensing and transducing mechanical signals that have precedence over short range cell-to-cell control mechanisms (Guilak et al., 2009; Piccolo et al., 2014; Lv et al., 2015, see also Streuli et al., 1991). By controlling increase in size, they allow the system to achieve the critical mass that is necessary to take advantage of the presence of different types of cells. By exerting control at medium ranges, they can coordinate the activities of groups of cells and specify tissue phenotype (Lelièvre et al., 1998). In particular, matrix structures exhibit properties specifically related to their localization in the system. ECM's exhibits specific features vary from tissue to tissue, thus exerting a distinctive type of control upon cells in each one. In such a way, it provides a decisive contribution to the spatial localization, differentiation, and stabilization of specific cell types in distinct tissues, and it contributes to functionally employ the potentiality provided by the spatially concentrated, coordinated activity of different cell types to realize organ differentiation and division of labor[8].

In sum, the intercellular space cannot be considered a mere background for cells. Its functional organization – especially, due to the control capabilities of ECM structures – on the one hand accounts for the possibility of increasing the numbers of cells living together. On the other hand, it puts together, integrates, and coordinates the activity of several types of cells toward physiological goals. In such a way, it enables division of labor by allowing types of cells to realize different functions and give rise to tissues and organs: the latter considered as integrated ensembles of cells capable to perform functions, in the physiology of an organism, that are necessary to maintain this organism alive.

MOTILITY, MOBILITY, AND COMMUNICATION WITHIN MULTICELLULAR SYSTEMS

When a multicellular biological system grows in size and in the number and types of cells, it becomes more and more necessary to coordinate the activity of different components at medium and long ranges and to transport nutrients everywhere in the system. Multicellularity has solved this problem of spatial organization in different ways and degrees, by developing internal control and communication mechanisms that coordinate the parts of the systems at distance.

The solution to the issue of integration at longer spatial scales is achieved by organizing movement: i.e., by controlling what moves in space, how it does so, and when. Movement, which is a widely shared property in unicellular organisms, needs to be under control to realize multicellularity. Unlike in unicellular organisms, motility is inhibited in most cells of multicellular systems. This is true for all multicellular systems, including biofilms. Inhibiting motility prevents the disaggregation

[7]A minimal example this latter function can be found in *Volvox carteri* where the ECM structures that connect cells play a crucial role in in the development of the system. After cleavage, the embryo cells are in an inside-out configuration that does not allow directional swimming toward light sources: the gonad cells are on the outside and the flagellar ends of somatic cells on the inside. At this stage, the embryo inverts by means of changes in cells shapes and using cytoplasmatic bridges between cells as pivots. It brings the flagella on the exterior of the system and sequesters the gonad cells on the interior (Kirk, 1998, 2005). The role of ECM structures in this process of development is shown by mutants. The extra-embryonic vesicle needs to expand to give enough space to allow inversion, and this does not happen in Inv C and InvB mutants, where the secretion of extra-embryonic ECM is affected, modifying the properties of the vesicle (Matt and Umen, 2016). Other mutations affecting the properties of the ECM cause the system to disaggregate just at the end of inversion, as cells are not kept together and disperse (Kirk, 1998). Moreover, at the end of inversion, the production and organization of the ECM plays also a central role in establishing the orientation of the flagellated cells (Hallmann and Kirk, 2000).

[8]As previously argued (Section "Why Multicellular Systems Are Not Just Balls of Cells: The Limits of Current Accounts of Multicellularity"), cell differentiation is not always found associated with division of labor in a functionally integrated system. In fact, a minimal example of functionally integrated multicellular system is for example *Volvox carteri*, which is characterized by two types of physiologically active components – i.e., the flagellated somatic cells and ECM structures – despite exhibiting only one somatic cell type. The integration between these two types of components allows the system to behave as a unit, capable to swim toward light sources during the day and toward the ground during the night. The ECM constitutes around the 99% of the volume of the system (Adams, 2013). In addition to contributing to development, to giving structural cohesion, and to enabling coordinated movement by organizing the position of the flagellated cells, the ECM plays other physiological roles (Kirk, 1998). ECM performs enzymatic activity by providing the system with an extracellular phosphatase with a broad substrate specificity which is used when reaching the phosphate-rich ground during nights (Hallmann, 1999). The liberation of daughter spheroids occurs through local enzymatic process of lysis of the ECM (Sumper and Hallmann, 1998). ECM promotes gonadal growth by storing nutrients, and gonadal cells grow twice as fast then when ECM is ruptured (Hallmann and Kirk, 2000). ECM is also involved in sexual induction through the phosphorylation of ECM glycloproteins in response to pheromones with cAMP as second messenger (Kirk, 1998; Hallmann, 2003).

of the system, and it allows to spatially concentrate groups of cells that collaborate to perform certain functions[9].

The functional properties involved in the organization of the intercellular space play a primary role in the control of motility. In bacterial biofilms, motility is directly modulated by the matrix, which mechanically inhibits the rotation of the flagella and triggers intracellular signal cascades[10]. As a result, the immobile bacterial cells differentiate into *persisters*, which increase the production and deposition of matrix molecules and, in turn, inhibit the motility of other cells, thus further amplifying matrix production with a cascade effect that contributes to the overall growth of the biofilm (Cairns et al., 2014; Steinberg and Kolodkin-Gal, 2015; Waters et al., 2016). Moreover, the inhibition of motility contributes to the realization of a regime of spatial and functional differentiation, where groups of cells or mixed-species microconsortia locally share a similar extracellular environment and work together, thus contributing to division of labor (Flemming and Wingender, 2010).

In *Volvox carteri*, the ECM controls the motility of flagellated somatic cells. By immobilizing them in place on the exterior of the system with a fixed outward orientation, the ECM prevents them from swimming independently, dispersing and, thus, disaggregating the system. At the same time, it enables them to perform their function, which consists in collectively realizing a coherent movement for the whole system (Kirk, 1998).

In metazoa, the ECM also contributes to modulate motility. During specific stages in development, entire groups of cells migrate, and their movements are highly canalized by the dynamic properties of ECM structures. Interaction between ECM and integrins controls cell adhesion and deadhesion. Contractions of the cytoskeleton generate traction on the ECM and allow locomotion in combination with ECM-associated growth factors, signals, cytokines, mechanotransduction, etc. (Rozario and DeSimone, 2010). Moreover, different degrees of stiffness of the ECM allow or inhibit cell motility. Stiffness is influenced by crosslinking of matrix components such as collagen, or by the modification of proteoglycans, for example, by the amount of hyaluronans which connect proteoglycans to collagen. In different tissues and at different times, cells are controlled by the degree of stiffness of the ECM. Experiments show that normal mammary epithelial cells, when growing in a soft 3D matrix similar to the basement membrane found in *in vivo* tissues, adopt an acinar structure organized in spherical monolayers of cells with a central lumen. Increase in ECM stiffness first causes loss in the spherical monolayer organization to give rise to a tight ball of the same cells with no internal lumen and, if further increased, it triggers migratory behavior within the 3D matrix (Halder et al., 2012).

Given that motility is highly controlled, and most cells are immobile in multicellular systems, the latter employ different ways of delivering signals, of exerting coordinated and localized control when and where needed. As already discussed, ECM structures can exert medium-range control at the tissue level beyond the range of cell-to-cell interactions. In addition to that, multicellular systems achieve integration by organizing space at longer ranges, by controlling the movement of some cells and of those nutrients, signals, control molecules, etc. that are necessary for the coordinated activity of the components in different areas of the system. Long-range control upon movement and communication within the system is achieved in at least three different ways (**Figure 5**): (1) by making components mobile in a fluid through vascularization; (2) by means of cells, such as the immune ones, that retain the capability of motility and move in the blood or through the ECM in tissues; and (3) through signal transmission architectures realized by networks of neurons.

When the size of a multicellular system increases, and the cells in the interior do not have direct access to the external medium, directly or through diffusion, mechanisms that distribute nutrients to all the cells are employed. Vascularization plays a fundamental role in this respect, by providing all cells with an efficient access to nutrients, oxygen, and other essential molecules. It constitutes the fundamental long-range space-organizing subsystem in multicellularity, specialized in the control of movement of nutrients, hormones, signal molecules, and cells, by making them mobile within the flow of a liquid medium. Vascularization is common to all multicellular systems which exert control upon their internal mobility. This is also true for biofilms, where the organization of the hydrophobic molecules of the matrix gives rise to channels that harness the flow of fluid and allow nutrients from the periphery to reach the bacteria residing in the inner region (Sutherland, 2001; Cairns et al., 2014). It is absent from few multicellular systems that, like *Volvox carteri*, do not have integrated metabolism and have their somatic cells localized on the outside.

The ECM contributes to the development of vascularization and to the organization of the vascular tree. It constitutes one of the control mechanisms for the differentiation and for the modulation of the behavior of endothelial cells (Newman et al., 2011). More specifically, the ECM basement membrane of vessels (VBM) provides growth factors, signals and the mechanical support for the construction of blood, lymphatic, and kidney tubule vessel structures. Growth factors such as VEGF-A (vascular endothelial growth factor A) are stored in the VBM and can be released to stimulate the differentiation and proliferation of cells in vasculogenesis and angiogenesis (van Genderen et al., 2018).

The strict relationship between vascularization and growth of the system can be observed in tumors. In humans, tumor may have limited growth in 3D (1–2 mm^3) before their demands of oxygen and nutrients cannot be met by diffusion alone. When cells become hypoxic, to enable further growth a tumor promotes several distinct mechanisms of vascularization to properly bring oxygen: e.g., sprouting and intussusceptive angiogenesis, mobilization of endothelial progenitor cells from the blood, and vasculogenic mimicry by tumor cells that differentiate to an endothelial phenotype and realize tube-like structures. These processes of neovascularization are carried out through the activation of endothelial cells by means of growth factors from the FGF (fibroblast growth factors) and VEGF

[9]Cases in which the multicellular system fails to functionally control motility include for example metastasis.

[10]Changes in the properties of the matrix can reverse the process in distinct phases of biofilm development (O'Toole and Kolter, 1998).

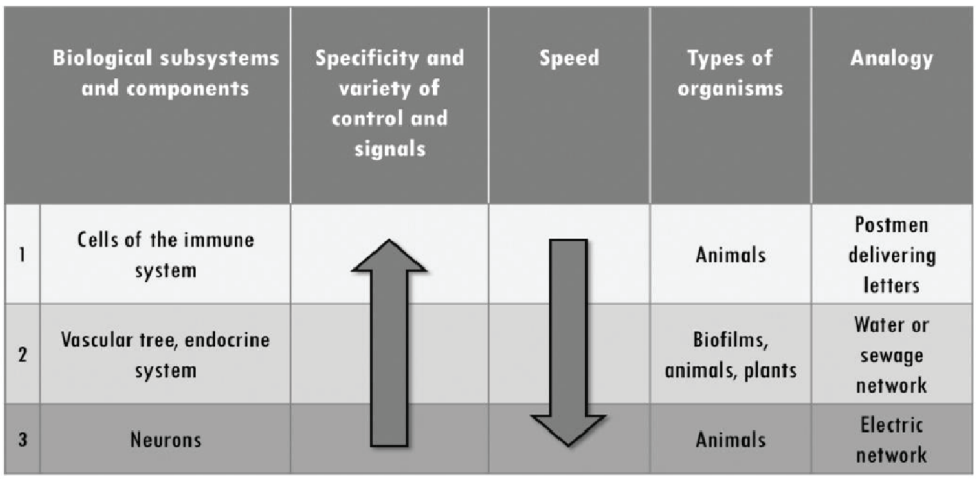

FIGURE 5 | Subsystems for the control of mobility and communication in multicellular systems. They differ with respect to the degree of specificity and the variety of control mechanisms they implement, and for their speed. While vascularization is common to almost all multicellularity, the other subsystems are specific of metazoa. We associated to each of them an analogy from city organization to exemplify their distinctive functioning in multicellular systems. Immune cells' high specificity and low speed of movement is analogous to a postman delivering letters. The transport of water, nutrients and waste through vascularization can be associated to a hydraulic network which, at least in very basic form, can be found in most human settlements over a certain size. Finally, neuronal architectures in the body can be associated with electric networks in terms of high speed of movement and lower specificity of individual signals.

families stored in the matrix, and through the remodeling or the co-opting of ECM structures (Hillen and Griffioen, 2007).

While vascularization is a common feature in multicellularity, other mechanisms are more specific of animal life. In metazoa, not all cells lose motility or rely only on vascularization for their transport within the system. The immune system is a way to organize movement and communication by controlling, in a way that is functional for the whole, those cells that have retained intrinsic motility.

Immune cells can move through blood, but in most cases they reside in tissues, where they are highly mobile within the ECM network that fills the space between tissues cells (Purwar et al., 2011)[11]. There, these primed and memory cells, called "T resident memory cells", provide for a primary system of immune surveillance at the level of tissues and organism's barriers. Through their mobility among the cells that constitute the tissue, they can exert a localized and specific control. By delivering highly specific signals to cells within tissues, they play important fine-grained coordinating functions, such as, among others, tissue repair, the regulation of fat cell metabolism to adapt to prolonged exposure to environmental cold (Lee et al., 2015), and communication with the nervous system in the guts (Gabanyi et al., 2016). In the case of zebra fish, they even connect xanthoblasts to melanophore, which then can be loaded with melanin and give rise to the stripes that characterize this fish (Eom and Parichy, 2017). The movement of immune cells in tissues is afforded by the porosity of the molecular network that makes up different types of ECM, depending on the orientation and density of the fibers. It is made possible also by the ability of immune cells to modify their shape, which in turn is limited by the nuclear size and shape and by its intrinsic ability to deform as well. Changes in the properties of the ECM, such as its porosity, can modulate this mobility of immune cells in the extracellular environments.

The third subsystem that contributes to the long-range spatial and functional integration of multicellular systems is the nervous one. Specific of metazoa, it establishes quick long-range communication networks that enable the control of the activities of a large number of cells at short time scales, thus allowing fast coordinated behavior. This is consistent with the thesis on the origin of neurons advanced by Keijzer et al. (2013) as control mechanisms for the activity of muscles: the function for which neurons first evolved in metazoan was to enable muscles to coordinate their contractions by transmitting signals between muscles and realize a synchronized propulsion movement, like it happens in the jellyfish[12].

CONCLUDING REMARKS

In this paper, we addressed the problem of multicellularity from a physiological point of view, focused on how the components of a multicellular system are functionally integrated into a viable organization. We argued that the limitations of the main accounts of multicellularity, based on increase in

[11]For example, there are roughly 20 billion T cells just in the skin of an adult human, twice the total number of T cells in blood (Purwar et al., 2011).

[12]As argued by Keijzer and Arnellos (2017), a basic nervous system allows a faster and more extensively coordinated control upon a contractile body than, for example, the mechanisms employed by sponges, which are based on contractile cells and chemical signaling among them.

size and cellular differentiation to explain this phenomenon, depend on an implicit cell bias – that sees only in the cells the active players within multicellular system – and are missing an important conceptual point, that is, the organization of the intercellular space and the role of extracellular structures in it. Realizing a multicellular system, instead, requires solving the problem of controlling, integrating, and coordinating the activity of the components at different spatial scales and providing the nutrients and oxygen necessary for their maintenance.

We provided a theoretical framework to understand the role of spatial organization in multicellular systems, based on the role (1) of ECM structures as control mechanisms that organize the system at short (together with cell-to-cell interactions) and medium ranges and (2) of vascularization, immune cells, and neural cells, which control movement and communication at longer ranges. The central idea is that the intercellular space is internally differentiated and functionally organized by these dynamic extracellular (ECM) or supracellular (endothelium, epithelium with their BMs) structures that play an active role as control mechanisms. The intercellular space can be understood as functionally organized because it brings together this set of mutually dependent, yet functionally differentiated, extracellular control components that contribute to the viable integration of the system. These components are sensitive to changes in spatial properties, and by acting as selective constraints upon dynamic spatial relations, they control the system's processes (e.g., transport of metabolites through vascularization), the activity of other cellular or non-cellular components, and the boundary conditions that allow cells to survive and carry out their activity. They are functional insofar as their activities contribute to the overall maintenance of the system.

This approach can provide a theoretical perspective to integrate contributions from the growing field of studies on the ECM and its functions, and to support further research hypotheses. A theoretical framework centered on the organization of space can contribute to provide an understanding of why ECM is so important and why it is crucially involved in all these phenomena. It can provide insights into the main causal factors underlying the distinctive features of different types of multicellular organizations (from biofilms, to plants and metazoa), understood as different ways to organize space and, possibly, of their dependence on differences between types of ECM. It allows also investigating possible parallelisms between the evolution of metazoa and the evolution of ECM molecules such as collagen. As it has been argued in the case of animal multicellularity, many of the capabilities required for it had already evolved in unicellular systems (Grosberg and Strathmann, 2007; Brunet and King, 2017): there does not seem to be massive appearance of radical genetic novelties between metazoa and their closest unicellular relatives. As shown by Sebé-Pedrós et al. (2016), cell differentiation is already present in unicellular organisms such as *Capsaspora*, which has three temporal life stages, one of which is an aggregative multicellular stage with production of ECM. What characterized the origins of metazoan multicellularity might have been the emergence of regulatory novelties related to genetic and spatial control: dynamic regulatory gene networks that allow fine tuning of activities of cells (Sebé-Pedrós et al., 2017), together with the emergence of new ECM molecules and structures such as Collagen IV and basement membrane (Fidler et al., 2017). The genetic requirements for the fine-tuned capability of modulation of cell fate and activity might have proceeded hand in hand with the emergence of new extracellular structures.

Thinking in terms of the organization of the intercellular space has also important implications for our understanding of multicellular phenomena of both biological and medical relevance, such as immunity, cancer, and aging. Until recently, immune cells present in the blood of vertebrates captured most of the attention of immunologists, especially in humans, where blood remains the obvious way to look at the functioning of the immune system. However, it was recently discovered that the amount of lymphocytes in the blood was drastically outnumbered by a factor of 50 by those residing within the tissues and organs (Purwar et al., 2011). These tissue resident memory T lymphocytes are seeded into the tissues from the blood in the course of an immune reaction. Yet they do not recirculate back to the blood, but stay *in situ* within tissues for the entire life of the organism. There, they are very active patrolling these tissues, where they constitute the first line of defense. They can do so because they have the capacity to move between the cells that constitute the tissue, through a space filled with the ECM, as discussed in Section "Motility, Mobility, and Communication Within Multicellular Systems" (see also Ariotti et al., 2012).

The link between immune protection and mobility became obvious when studying certain hereditary immune deficiencies which are uniquely induced by impeding solely the capacity of immune cells to move, such as by knocking down actin remodeling, for example, by deadly mutation of DOCK8 or Coronin 1, two gene products necessary for actin remodeling. Crippling down the motility of these cells creates by its own a deep immune deficiency. Importantly, when observed *in vitro* in a liquid medium, these mutated lymphocytes do not show any defect, whereas, when put in a 3D matrix, they prove to be incapable of movements and die by plasma membrane ripping (Zhang et al., 2014).

Numerous intimate immune functions rely on the ability of the cells mediating them to be mobile. This is the case for T regulatory lymphocytes which need to be mobile to move toward the sites where they carry out their regulatory activity, even though some of them could be generated *in situ*. Another demonstrative example is provided by the way cytotoxic T lymphocytes can get rid of the influenza virus. It has been known for a while from experiments with mice that the survival of the mouse relies on a vigorous response by CD8+ T lymphocytes, which can kill parenchymal infected cells, thus exhausting virus replication. Obviously, as these effector cells are generated outside the infected tissues, they need to move to the place where infected cells are. This condition can only be realized if they could follow cues left within the ECM by neutrophils under the form of tiny fraction of their cytoplasm loaded with chemokines attached to the scaffold of ECM (Lim et al., 2015). Here, again the importance of mobility and its 3D organization within the ECM finds its necessity and proves to be a major explanatory factor.

The organization of the intercellular space plays also an important role in cancer. Cancer could be regarded as an organoid including tumor cells and normal cells such as endothelial cells or fibroblasts, also set around the organization of a heterogeneous scaffold of ECM (Marie and Merlino, 2019). The high stiffness and cross-linking of the ECM that can be found in tumors, provide cancer cells with the necessary signals of proliferation and/or invasion, transmitted through the mechanotransduction pathway of signaling (Halder et al., 2012; Acerbi et al., 2015). Pioneering work in cancer biology has advanced the thesis that alteration of ECM may anticipate cell transformation (Bissell and Radisky, 2001; Soto and Sonnenschein, 2011)[13]. At the same time, and for the same reasons, tumor ECM shields the tumor from the possible invasion of immune cells. This aspect of cancer biology is under scrutiny and may even change the therapeutic way with which we consider acting on tumors, or even on the immune system, in the case of cancer. For example, if the physical properties of ECM are structuring the relationships between itself and the immune system, one can envision ways to externally (physical agents) or internally (enzymes regulating the ECM scaffold) act on it in such a way as to have a deep effect on the tumor itself.

Many other fields in biology and medicine could benefit from thinking in terms of the active organization of space by the ECM (Lampi and Reinhart-King, 2018). The case of aging is of some interest in this regard. Changes in the properties of the ECM are involved in the aging of vascular systems (Kohn et al., 2016). Moreover, as mentioned previously in this section, the intrinsic alteration of lymphocytes motility can lead to a profound state of immune deficiency. During aging, the alteration of the physical properties of the medium where lymphocytes are moving, namely ECM, could similarly lead to severe and progressive alterations of the immune system and its responses. So far, a unifying picture of the mechanisms of aging is not available. Yet, for what concerns the aging of the immune systems (immunosenescence), it is possible to imagine or even find in the literature clues leading to think that the alterations of ECM known to occur during aging and to be an hallmark of it, may severely impinge on cell differentiation and stem cells numbers, thymus aging, T-cell repertoire shrinkage, to cite few, or even on the individual inflammatory status. Their effect is to create a state of immune deficiency similar to the one found in the hereditary diseases mentioned previously, as discussed in Moreau et al. (2017). Increase in ECM stiffness due to progressive crosslinking of matrix proteins associated with a low turnover, profoundly affects the possibility of lymphocytes to travel through it. Changes in ECM porosity affect another important parameter in cell biology, which is cell deformation itself, linked to nuclear size, shape, and deformability, as the nucleus is the biggest organelle of a given cell. Nuclear mechanical stress is a new part of biology which begins to produce new interesting data, shedding light on new mechanisms of DNA mutations, rupture of nuclear membrane, changes of epigenetic traits, to cite a few.

In sum, the importance of a theoretical framework that focuses on multicellular systems in terms of organization of the intercellular space and the relative functional properties, does not only contribute to an understanding of how multicellular systems are organized and to the formulation of research hypotheses on their origins and evolution. It also provides a unified perspective that puts together different work on ECM and on spatial functional features of multicellular systems, to understand failures of multicellular organizations, with important implications for medicine.

[13]See Bertolaso (2016) for a discussion of different approaches to cancer, including those focused on tissue properties as opposed to cell-centered ones. As an alternative to both positions, Bertolaso proposes a dynamical and relational view of biological organization and cancer, focused on the dialectic between different types of processes and on the role played by context. See Green (2019), instead, for a discussion of tumor progression in hierarchical terms as a case of downward causation.

AUTHOR CONTRIBUTIONS

J-FM provided the original idea. LB and J-FM developed the theoretical framework and wrote Section "Concluding Remarks." LB wrote Sections "Introduction," "Why Multicellular Systems Are Not Just Balls of Cells: The Limits of Current Accounts of Multicellularity," "The Functional Organization of Space," and "Motility, Mobility, and Communication Within Multicellular Systems" of the manuscript and drew the diagrams. All authors discussed the general outline of the article and contributed to comments and revisions.

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CONCEPTUAL ANALYSIS
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The Origin of a Trans-Generational Organization in the Phenomenon of Biogenesis

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One of the central issues of the whole process of biogenesis is how to understand the progressive constitution of a large (in spatial and temporal terms) system that transcends the individual sphere of proto-metabolic organizations and includes collective networks, both synchronous (i.e., proto-ecosystem webs) and asynchronous (i.e., trans-generational protocell populations). This paper analyzes the appearance of a minimal form of reproduction in the process of biogenesis from an organizational perspective. This perspective highlights the problem of how a process transcending the actual organization of the reproducing entities (i.e., protocells) could have a causal power. It is proposed that this problem may be explained if we consider that reproduction generates a kind of feedback between the actual concatenation of the processes of each reproducing cycle and the type continuity that a reliable iteration of these cycles creates. Thus, reproduction generates a new form of self-maintaining system linking "organismal" and "evolutionary" domains, since the consequence of the iteration of self-reproducing cycles is the long-term continuity of a specific type of SM compartmentalized organization, and the functional role of a particular self-reproducing organization (token) lies in its capacity to trigger a diachronic succession of similar self-reproducing organizations, i.e., a lineage.

Keywords: biogenesis, organization, (self)reproduction, individual-collective duality, trans-generational causal entailments

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INTRODUCTION

Physiology is usually understood as the study of the (current) organization of a living system, or in other words, the study of its mechanisms and functions. It is normally implicitly understood that the term "living system" refers to a cohesive and individuated entity, namely an *organism*. As stated by Widmaier et al. (2016), physiology deals with fundamental biophysical and biochemical phenomena, or in simpler terms, the control mechanisms that constitute organisms' actual organization. The evolutionary perspective, on the other hand, deals with changes in the hereditary features of certain collections of organisms (termed *populations*) over successive generations.

However, as we shall argue, looking at this issue from the perspective of its origins makes it easier to understand the connection between the "physiological" and the "evolutionary" dimensions of the phenomenon of life. This is because one of the first things to consider when we address the question of the origin of biological organization is the fact that the appearance

of full-fledged living systems was necessarily the result of a historical process. Only through a historical process could those rare changes and variations (particularly functional innovations) which occurred in the actual (and ephemeral) organization of earlier individuated systems have been preserved and accumulated, thus enabling a gradual and ongoing increase in complexity.

Life itself is, of course, an example of an actual organization, but because this organization is so complex, it cannot have appeared spontaneously: high complexity cannot emerge "from scratch". Consequently, the process by which physico-chemical systems have managed to generate increasingly complex systems, capable somehow of retaining their acquired complexity, should be viewed as an entailed process of accumulative inventions. Hence, the appearance of life is, also, a historical process: each prebiotic entity, characterized by an actual organization, is only possible due to a set of inherited factors, which include everything ranging from internally inherited components ("genes") to collectively built environmental conditions ("ecological niches"). The study of the origins of life is therefore the study not only of how sets of chemicals have created localized and cohesive forms of self-maintaining organizations ("proto-organisms"), but also the study of how they have reproduced to enable the emergence of lineages and historical changes. It is the study of how sets of these proto-organisms have progressively changed their local environments, and how in turn these changes have facilitated the appearance of more complex proto-organisms. In sum, the question of how the connection between current organization and historicity has appeared and developed seems of paramount importance. In other words, to explain the origin of life, we must explain how a form of organization that was both sufficiently simple so as not to require any form of historical inheritance, but complex enough to generate an entailed process of accumulative inventions, namely, a historical process, could have appeared. Hence, this process (called "prebiotic evolution") requires as its starting point the appearance of a relatively complex organizational threshold, given that several non-trivial conditions would have to have been met.

There are two aspects to prebiotic evolution. One is the emergence of spatially bounded forms of organization, capable (at least in a minimal sense) of self-maintenance. The other is the development of a mechanism ensuring the accumulative retention of the organizational and structural innovations generated within these localized systems, beyond their presumably ephemeral lifespan. This mechanism was reproduction. This is why reproduction is probably one of the most salient milestones in biogenesis. Yet, the two aspects are really two sides of the same coin, since reproduction is a form of organization that copies itself and, in consequence, is merely a very specific way of self-maintaining an organization, through propagation. Seen from the most generic perspective, reproduction is a consequence of the way in which a system (or two systems, if reproduction is sexual) is organized; and since (reliable) reproduction leads to lineages and historical changes, the question of how the connection between the current organization and reproduction emerged and developed in early proto-organisms seems of paramount importance.

For these reasons, in this paper, we will analyze how the appearance and evolution of reproduction provides insight into the progressive constitution of a large (in spatial and temporal terms) system that transcends the individual sphere of proto-metabolic organizations and includes asynchronous (i.e., trans-generational protocell populations) and collective networks which are both synchronous (i.e., proto-ecosystem webs) in nature. As we shall argue, this approach will enable us to develop an organizational account that may help bridge the gap between the physiological and evolutionary research traditions.

The paper is organized as follows. First, we will analyze which kind of system could be a candidate for initiating a process of chemical complexification leading to life, in the form of self-maintaining compartmentalized organizations. Next, we will discuss how this initial organizational complexity could have been retained, along with the role played by an early form of reproduction in the creation of an entailed process of complexification beyond the "lifespan" of the aforementioned compartmentalized forms of organization. The emergence of reproduction raises many conceptual paradoxes, which will be discussed in the Section "The Paradoxical Nature of Reproduction." Finally, in the Section "The Consequences of Reproduction: The Appearance of a Multidimensional Organization," we will show how, as a consequence of earlier forms of reproduction, prebiotic evolution unfolded the progressive constitution of a multi-scale system, consisting of one domain with individuated proto-metabolic organizations, and another with a large (in spatial and temporal terms) system of ecological networks.

THE ORIGIN PROBLEM: WHAT IS THE STARTING POINT OF PREBIOTIC EVOLUTION?

The origins of prebiotic evolution are probably plural. One of the initial paths for the origin of life is, of course, the complexification of chemical processes, which occurred in concrete places under very specific boundary conditions. At first, relatively simple systems could have appeared spontaneously, and their persistence was ensured because, given the favorable environmental conditions, they could easily have arisen spontaneously time and time again. Hence, one obvious requirement for prebiotic evolution is that we must start with systems which, in principle, were sufficiently simple so as to have emerged in a spontaneous manner from a series of material aggregates under the conditions which existed at the time of the primitive Earth. Consequently, a series of highly specific boundary conditions must be envisaged (see, for example, Nisbert and Sleep, 2001) that could lead, on the one hand, to the production of new and more complex compounds, endowed with catalytic or template properties; and on the other, to the generation of far-from equilibrium self-maintaining (SM) reaction networks. However, such systems most likely came after many other systems, the maintenance of which basically depended on

boundary conditions that, in terms of complexity, were much more advanced than they themselves[1].

In certain places and under very specific boundary conditions, different sets of reactions may give rise to increasingly complex cyclic processes, leading to the appearance of increasingly complex *self-maintaining* networks (Martin and Russell, 2003). The core of a SM network is an autocatalytic reaction loop. An autocatalytic reaction loop arises as a result of the exploration of the structure of a chemical environment and its starting components' reactivity space, connecting intermediate products (substrates rather than catalysts) in a cyclic pathway. Yet, in an autocatalytic cycle, the reactions are controlled kinetically, i.e., are prompted by catalysts to produce and maintain a set of thermodynamically unstable compounds. This means that the system is maintained away from equilibrium (Pascal et al., 2013). Catalytic networks, or the more abstract concept known as "catalytic task space" (Kauffman, 2000, pp. 13–14), refers to a framework of synchronic systems comprising different reactions, which together form an integrated organization. In other words, the term denotes a group of molecules which affect each other catalytically in order to coordinate the specific places, times, and speeds of their chemical transformations[2].

As the name indicates, a SM network is a means of preserving the existing organizational structure. Indeed, because they generate a mutual entailment of processes, SM networks tend to persist: they are maintained because of the cyclic nature of the processes that define them. Since, in a SM network, some components make a specific causal contribution to the maintenance of the whole network, variations that do not destroy the organization could in fact be recruited when they contribute to its maintenance. Hence, a SM network tends to incorporate/recruit any contingent organizational or structural modification that may coherently enter it, providing it contributes to its maintenance. Thus, many of the innovations generated among the myriad of reactions occurring in the local environments would have been retained.

However, what is still required is a means of managing the exchange of energy and matter between the organization and its environment. In a prebiotic scenario, the only alternative for achieving this would have been a selectively permeable compartment[3], which would have helped ensure correct concentrations and would have generated an internal environment which was both differentiated and adjustable in terms of pH, volume and chemical composition, etc. This environment would, in turn, have fostered the development of a metabolic process (and vice versa). The establishment of a division between the internal and external parts of the system would have resulted in concentration gradients (Mitchell, 1961; Harold, 1986), as well as pH and oxidation-reduction differences (Morowitz, 1981, 1992; Chen and Szostak, 2004). These differences may have served as mechanisms for storing energy, perhaps later on forming the basis for endothermic transformations and against gradient active solute transport, which are present in all existing cells (Skulachev, 1992). For these reasons, encapsulation can be seen as a prerequisite for the evolution of a primitive SM organization able to manage the energy flows required to maintain the system (Ruiz Mirazo and Moreno, 2004; Shirt-Ediss, 2016). For its part, the encapsulated organization would have been an instrument for the evolution of a compartment, offering catalysts and other compounds to enable selective permeability to emerge.

Moreover, the formation of self-assembling vesicles and their early association with autocatalytic networks (namely, the formation of "protocells"[4]) may harbor a minimal form of functional variety[5]. For instance, various components of the basic self-maintaining cycle may participate in the generation or composition of the physical border, which in turn would ensure better conditions for recursive self-maintenance. It is also conceivable that the basic self-maintaining cycle would have generated certain components (i.e., catalysts) that would have promoted the processes to follow pathways that were chemically unlikely; or they may have coordinated their inter-conversion processes in space and time in such a way as to ensure a greater number and variety of reactions and components involved in the organization. Thus, to be capable of prebiotic evolution, the organization of a chemical system must have harbored a potential variety of internal constraints which somehow contributed to the system's self-maintenance (Moreno and Ruiz-Mirazo, 2009; Mossio et al., 2009). This (at least minimal) internal functional differentiation is what is implicit in the concept of a metabolic organization. And this is also the root of a physiological system.

[1]This is a key question in relation to the Origins of Life because a chemically stable and diverse environment would, in turn, enable individual systems with a lower level of metabolic complexity. As argued by both Morowitz (1992) and Morange (2008), metabolic simplicity is dependent on how chemically demanding the environment is. A changeable and chemically restricted environment would be more demanding than a very stable and chemically rich one. Nevertheless, no one has, to date, clarified the exact nature of the relationship between environmental and metabolic complexity in early evolutionary stages. Another issue linked to the stability of far-from-equilibrium chemical organizations is the interaction between individuated systems and collective networks, defined as sets of different types of localized systems in constant interplay with environmental compounds, which together constitute a stable kind of collective self-maintaining system. We will address this question in Section "The Consequences of Reproduction: The Appearance of a Multidimensional Organization."

[2]More technically, this idea of SM network is similar to that of a "reflexively catalytic network," a more abstract concept that denotes a series of cyclic reactions in which each reaction is driven by one (or more) module from one of the other reactions. Furthermore, all reactants within the system are generated from a small central group of components (Hordijk and Steel, 2004). Although such a system has yet to be reproduced *in vitro*, various theories postulate that they may have emerged prior to the RNA stage (Hordjik et al., 2012; Vasas et al., 2012). See also next section.

[3]Prebiotic compartments would likely have emerged due to the spontaneous assembling of amphiphilic molecules in water (Deamer, 1985; Deamer and Dworkin, 2005).

[4]The term "protocell" is used to denote any experimental or theoretical model involving a self-assembling compartment connected to chemical processes taking place around or within it (Rasmussen et al., 2008; Ruiz-Mirazo, 2011). Here, the term is used slightly more specifically, referring to a compartmentalized system that is both far-from-equilibrium and self-maintaining, and which has some prebiotic properties, including growth, autocatalytic activities, and reproduction.

[5]As argued by Bickhard (2000), Mossio et al. (2009), and Mossio and Moreno (2010), a system shows functional diversity if it is a dissipative SM organization made up of a series of structures (catalysts, membrane, etc.) which constrain energy flows in such a way that they maintain the system where they exist and thus reproduce themselves.

Interestingly, a selectively permeable compartment acts as a global constraint affecting all the internal functions of the system. Indeed, a selectively permeable compartment is able to selectively control transport processes, thereby modifying its constitutive processes to adapt to the environment and ensure the maintenance of its identity. Thus, compartmentalization defines a specific organization in terms of cohesion, physical boundedness and organizational asymmetry with its environment. In sum, since they constitute a specific and physically bounded set of processes, giving rise to a cohesive and functionally integrated organization, self-maintaining protocells realize a minimal form of biological individuality (Moreno, 2016). The capacity to remain in far-from-equilibrium conditions and realize functional differentiation follows on from this cohesive organization.

THE APPEARANCE OF REPRODUCTION

In a scenario such as the one described in the previous section, we can conceive of the existence of populations of diverse protocells undergoing processes of fusion and fission, and therefore, of a mechanism of protocell "multiplication." Of course, this "multiplication" would not have guaranteed organizational similarity; however, it would have permitted a certain degree of diversification and even a minimal form of complexification. Yet, a sustainable process of increase in complexity requires methods of preservation, the reason being that noise will accumulate errors, and since the mechanism ensuring the persistence of the identity of this type of system lies in their organizational circularity, even slight variations would have been deleterious.

The simplest way to overcome this problem is by means of some mechanism allowing organizational redundancy. In other words, it is a mechanism that controls the process of fission so as to guarantee at least a minimal form of organizational repetition. How? If, instead of producing a chaotic form of growth leading to duplication and fission, the internal organization of a type of protocell was able to produce at least a small percentage of new protocells that maintain a sufficient level of organizational similarity with it, the production of an indefinite number of similar organizations would be ensured. This process is reproduction.

The appearance of protocells capable of reproduction (i.e., self-reproducing protocells) would not only have ensured their persistence but would also have rendered them dominant. In a noisy environment that tended to eliminate the less robust forms of protocells (and those slightly more complex ones would most likely have been less robust), only reproduction could have enabled the long-term persistence of the different forms of protocell organization that may have appeared. Despite requiring a more complex organization, due to its inherent capacity to create an indefinite number of spatially separated copies, as well as the fact that it allows some degree of variability, a self-reproducing protocell offers a powerful and robust way of maintaining both past inventions and new innovations.

But how could a minimal form of reproduction have appeared? It is worth to clarify that when we speak here about self-reproducing protocells we do not refer to simple vesicles or other forms of very simple protocells, but of functionally differentiated compartmentalized organizations (as described in the last part of previous section). Whereas the reproduction of the formers is relatively simple (and even, is the usual way they occur in nature), this capacity is instead unlikely in the second case. Not a precise model of a reproduction of such type of a protocell exists yet, but there are different studies showing that a SM protocell can become a self-reproducing organization (and not merely a self-replicative structure) when the ongoing processes of a self-maintenance/production occur in certain specific conditions (Zepik et al., 2001; Solé et al., 2007; Mavelli and Ruiz-Mirazo, 2013; Murtas, 2013; Stano and Luisi, 2016). For example, the deployment of a protocell's continuous process of self-production may generate growth, and upon reaching a certain size, the compartment (and its contents) may end up in fission, producing two (or more) new similar protocells. And given that the new system and the original one are similar, then, environmental conditions permitting, the process can be reiterated time and time again. Accordingly, reproduction occurs when the ongoing processes of a self-maintaining entity occur in certain specific conditions[6]. In other words, reproduction could only have occurred when a self-maintaining system was organized in a very special way. Let us see now how.

Now, since what we call "growth" implies a process of duplicating certain structures of the system, coordinated with changes in the compartment, a minimal control of the temporal and spatial allocation of the components is also required. This is especially important when the system becomes more complex. For example, when a bacterium reproduces, it triggers fission at just the right moment, ensuring that a set of specific components (not only the genetic ones) are in place to endow each new bacterium with a complete copy of its essential material. However, before fission is achieved, the bacterium must copy many of its components, especially its genetic material, which is segregated and allocated to opposite ends of the system. Next, the different proteins involved in the reproduction process come together at the site where the division is to take place. One vital component of this process is the FtsZ protein, whose monomers arrange themselves into a ring in the middle of the bacterial body, with other components involved in the process then assembling around it. All these elements are positioned in such a way as to ensure that the division divides the cytoplasm without damaging DNA. When division takes place, the cytoplasm splits in two and (in many bacteria) a new cell wall is synthesized. The order and timing of these processes (component replication and segregation, division site

[6]Actually, rather than a form of self-maintenance/production, reproduction constitutes *a duplication*: while SM is a temporally indefinite repetition of a localized unique form of organization, (self)reproduction is an indefinite spatial propagation of a similar organization. As a consequence, whereas in a continuous process of SM, the eventual variations lead to the forgetting-erasing of the initial organizational identity, in a process of reproduction, variations may occur in some reproductive sequences, while others maintain the previous identity. Moreover, SM does not propagate, while reproduction does, thanks to an organizational discontinuity between the reproducer and the reproduced, which occurs in the form of a spatial discontinuity resulting from the achievement of a growth process ending in (at least) two similar organizations.

selection, invagination of the cell compartment, and synthesis of the new one) are tightly controlled (Weiss, 2004).

Obviously, reproduction would have been much simpler in protocells. It is likely that, when certain parameters were met, simple protocells would have spontaneously settled into a stationary reproducing regime, characterized by regular growth and a division cycle. This would also have involved maintaining a standard size and chemical composition down the generations. As Mavelli and Ruiz-Mirazo (2013) showed, under certain specific conditions, anosmotic synchronization is generated between membrane and core volume growth, and this in turn gives rise to a stationary reproduction regime[7]. In other words, reproduction is not possible without synchronized coordination between the changes taking place in the compartment and the encapsulated SM network.

Of course, reproduction implies also a mechanism that ensures at least a certain degree of similarity between the reproducer and the reproduced. The most primitive form of inheritance would have been probably statistical, namely, only a percentage of the "offspring" would be similar to their ancestors. And only when the earlier genetic components appeared, a mechanism of reliable reproduction could be in place. One possible form of pre-genetic inheritance may be what Segré and co-workers (Segre and Lancet, 2000; Segré et al., 2001) have called "compositional genomes," which consists in systems that, after duplication, are capable of (statistically) transferring their compositional specificity. More recently, Vasas et al. (2012) and Hordijk and Steel (2014) have presented a model of protocell that could ensure a form of statistical inheritance. Accordingly, even primitive self-maintaining systems lacking template components could be capable of reproduction with a certain degree of identity transmission (although the reliability of the copies would be very low).

All things considered, the appearance of protocells capable of self-reproduction, even in its minimal form, clearly required that these systems be capable of achieving a certain degree of functional integration between all the aforementioned processes, especially between changes in the compartment and changes in the internal network. For all these reasons, reproduction could not have appeared very early on in the process of biogenesis.

THE PARADOXICAL NATURE OF REPRODUCTION

As seen above, the appearance of reproduction should be considered the consequence of a very special form of compartmentalized SM organization. However, at the same time, the set of processes that generate reproduction are fundamentally different from those that generate self-maintenance: whereas self-maintenance implies a clear organizational continuity of the same system, reproduction implies a physical discontinuity at the end of the process that connects the reproducing system with the reproduced one. Thus, to what extent can the set of processes that constitute an entire reproductive event be considered an uninterrupted succession of states?

On the one hand, the reproductive process is an actual organization in the following sense: it is a continuous set of processes that progressively build a duplicate organization, with all processes occurring within the same compartment. Let us call this set of processes the *reproducing cycle* (we use the term cycle because the end stage is similar to the initial one). A reproducing cycle is a specific set of processes generated within the temporally larger SM organization of an individuated system, which eventually results in two separate (yet similar) individuated systems. As an actual organization, the reproducing cycle is a far-from-equilibrium concatenation of constraints, each of which functionally contributes to the SM of said organization (in which they operate). Take, for example, the components involved in growth and duplication, or those involved in ensuring adequate temporal control; all these components act as functional constraints, ensuring the correct fulfillment of the reproducing cycle. They act as functional components to the extent that they locally constrain the system's flows of matter and energy in such a way as to contribute to the maintenance of the global reproducing cycle and, insofar as they themselves are produced within the system, they indirectly exist because of their own action (for more details, see Mossio et al., 2009).

Yet, as mentioned earlier, although the reproducing cycle implies a duplication of the specific SM organization in which it exists, its working is fundamentally supported by this very SM organization: the reproducing cycle itself exists only insofar as it is embodied in the SM (metabolic) organization, which constitutes the individual system[8]. Focusing on the concept of "reproducing cycle" highlights the relevance of an organizational and material connection between parental and filial individual organizations as a necessary mechanism for ensuring sufficiently reliable reproduction. Thus, although in the end the reproducing cycle implies the production of a new, spatially distinguishable system, for reproduction to occur, the underlying organizational continuity between the producing and the produced system(s) cannot be disrupted. This continuity is a necessary condition, because otherwise, as Mossio and Pontarotti (2019) have recently argued, an adequate degree of functional similarity between the producing and the produced systems would not be achieved. And as Griesemer (2002) points out, reproduction is not only the transmission of a "form"; *it* also implies a material connection between the system that reproduces and that which is reproduced. In other words, the similarity between reproducer and reproduced is supported by a material and organizational connection between the metabolic organization of the parental system and that of the filial system.

[7]Thus, even the simplest forms of self-maintaining organizations, which have no template components, can transmit a certain amount of identity during reproduction (although the fidelity of the copies would, of course, be very low).

[8]Moreover, reproduction implies a cost for the standard functioning of the basic metabolic organization (because it makes no contribution to the actual system in which it operates). In operational terms, a reproducing SM system can survive/persist indefinitely only because reproduction is a cycle that (sooner or later) ends, and the system recovers its standard metabolic regime. However, from a broader perspective, this "dysfunctional" regime (at the level of the individuated system) is the way by which intergenerational causes could operate.

However, on the other hand, reproduction also implies an organizational discontinuity, because it generates a spatial separation: at the end of the reproducing cycle, part of the set of causal connections occurring in an individuated organization is interrupted and a new, spatially distinct form of individuated organization appears. Gradually, the organization is duplicated, and once the process of duplication and allocation is complete, the border is also duplicated and an organizational discontinuity occurs. Following this disruption, the individual fates of the reproduced and the reproducer, considered as tokens, are different (although, of course, both will be functionally similar).

All these considerations lead us to the following conclusion: in a prebiotic, pre-genetic context, a self-reproducing organization is a specific form of a compartmentalized SM organization, characterized (among other features, like the presence of certain constraints able to control the temporal and spatial distribution of molecules) by the fact that it triggers an indefinite production of similar[9] (yet spatially separate) organizations. Because of this, each separate entity (*token*) resulting from a reproductive cycle (generation) inherits a specific organizational identity, and the sequence of generations (lineage) therefore constitutes a unique *type*. In a reproductive process, there is type continuity, since there is a mechanism that ensures a similarity between the generator and the generated, and this creates an uninterrupted temporal succession of similar organizational tokens. This is the basis of the *type continuity* between two systems (the reproducer and the reproduced) and, by extension, between all the members of an entire lineage[10].

The paradoxical fact is that, due to both the maintenance of the organizational continuity of the reproducing cycles and, at the same time, the disruption of this continuity, reproduction generates *another form of continuity*: an organizational *similarity* between an indefinite set of spatially (and temporally) separated protocells. In other words, a self-reproducing organization triggers an indefinite production of similar, yet spatially and temporally separate, organizational tokens. As a result, each separate entity resulting from a reproducing cycle (generation) inherits a specific organizational identity and the sequence of generations (lineage) therefore constitutes a *type*.

The consequence is that, indirectly, reproduction yields an evolutionary history, namely, a causal process that is not apparently based on a continuous entailment of current states. This is the domain of what Mayr (1961) termed "historical causes". However, as Bickhard (2001) has argued, "history can have causal consequences only insofar as history factors through current state. And appeal to distal causes, such as evolutionary history, is legitimate only insofar as those distal causes factor through current state without loss. Simply put, the past cannot cause anything in the future without the full mediation by the present (state)" (p. 462). Hence, according to Bickhard, we have a problem, since to be causally effective, trans-generation entailments must operate as an entailed set of causal interactions, namely, as an actual organization.

A solution to this problem may be to consider that historical (i.e., trans-generational) causal continuity does not (only) rely on the continuity of the actual organization of the reproducing cycles (which, as we have seen, are interrupted every time a new individual is born). Rather, it exists, fundamentally, because the reproducing cycles ensure a sufficient degree of similarity between the reproduced and the reproducer, such that an indefinite set of iterations of the cycle can be ensured, thus maintaining the *specific form* of organization that reproduces itself and is reproduced. In other words, historical continuity is based on a kind of feedback loop between the actual concatenation of the processes of each reproducing cycle and the type continuity that a reliable iteration of these cycles creates. Thus, reproduction generates a kind of feedback between the "organismal/physiological" and "evolutionary" domains, since the consequence of the iteration of self-reproducing cycles is the long-term continuity of a specific type of SM compartmentalized organization; and the functional role of a particular self-reproducing organization (token) lies in its capacity to trigger a diachronic succession of similar self-reproducing organizations, i.e., a lineage. A lineage, in turn, contributes to the maintenance of a specific type of self-reproducing organization.

The specific functional components involved in the accomplishment of a self-reproducing cycle do not contribute directly to the token organization in which they operate, but are retained because, indirectly (i.e., through the iteration of reproductive cycles), they contribute to creating and maintaining intergenerational similarity, which in turn, (and again indirectly) contributes to the maintenance of the very specific type of self-reproducing organization in which they are generated[11]. In other words, to be functional, the structures involved in the reproductive cycle of an individuated organization require the establishment of an intergenerational lineage, namely, the participation of successive different (yet organizationally similar) tokens.

This entangled relationship between reproducing cycles and their consequences is the basis for the unfolding of evolutionary historicity. This, in turn, generates a completely new set of conceptual categories: populations, lineages, selection[12], fitness landscape, etc., which together define what is termed "the evolutionary domain". Next, we will analyze the consequences of the origin of this entangled relationship.

[9]Similarity is ensured by organizational continuity (as we have explained) and by the transmission of genetic materials. Although the appearance of each new individual (generation) implies a complete renewal of the material elements, this renewal occurs without interrupting the individual's "basic" (lineage) identity, thanks to both organizational continuity and the stability of the hereditary constraints.

[10]A lineage is a series of generations derived from an ancestral genetic type. In other words, it is a temporal series of organisms connected by a continuous line of descent from ancestor to descendent. In a pre-biotic pre-genetic context, a lineage is more difficult to define; but we can say that a temporal series of self-reproducing protocells, which are connected by a continuous line of descent from ancestor to descendent, and which maintain a certain degree of functional identity, could be considered a primitive form of lineage.

[11]Actually, the chain of causes and effects forming this causal loop is more complex. The dynamic effects between each protocell and its environment (which is of course also constituted by other protocells and their side products) affect its offspring and therefore slowly shape the type of inherited components received by each new generation of protocells (and vice versa).

[12]Selection here means the process whereby self-reproducing protocells that generate more offspring will prevail at the expense of other types of protocells. Notice that this concept of selection is trans-generational (and therefore diachronic). It is therefore different from that of strictly synchronic competition which depends on the more or less successful management of environmental conditions by different types of protocells, since selection in this case will occur regardless of reproduction. Nevertheless, a synchronic disadvantage will usually lead to a diachronic disadvantage also.

THE CONSEQUENCES OF REPRODUCTION: THE APPEARANCE OF A MULTIDIMENSIONAL ORGANIZATION

As we have seen, even before the appearance of genes, self-reproduction with some form of inheritance led to intergenerational entailments between protocells. These entailments are the basis of a very primitive form of evolution, understood as the intergenerational change of populations.

According to this view, the two dimensions (the physiological and the evolutionary) are not symmetrical. Whereas the physiological dimension appears as an actual SM organization, the evolutionary domain appears as an unfolding of a mechanism, full of contingent events. Although, strictly speaking, an evolutionary perspective is not only a diachronic view, because it implies also a continued process of selection between synchronically competing phenotypes, it is usually understood as a historical phenomenon: Thus, evolutionary Biology is essentially focused on the study of changes in the heritable characteristics of reproducing sets of organisms (and even proto-organisms) over successive generations (Hall and Hallgrimason, 2008). And, given that to a large extent these changes are contingent, evolution is also, largely, a historical field of study.

However, the appearance of populations and lineages also created a new synchronic domain, this time operating at a different temporal (and spatial) scale than that of individuated protocells. This domain appeared as a consequence of the long-term action of different communities of metabolically similar protocells modifying their collective environment. Thus, these different sets of protocells may have generated mutually self-sustaining and complementary interactions.

Of course, this would have required several conditions to be met: first, the existence of stable communities at a long-term temporal scale (compared with the short-term scale of the protocell's lifespan), and therefore of reproduction with a minimal form of inheritance; and second, a transition from a domain where the number of different types of individualities was very small to another where the variety of types increased considerably (which in turn is linked to an increase in the reproductive reliability of the individualities). In other words, at any given time, the combination of individual action and evolutionary processes generated communities of different types of protocells which mutually and synchronously affected their environmental conditions.

Because of their similar metabolic identity, each of these different types of communities would have collectively modified their environment, together constraining the flows of matter and energy in a specific way; and when these different flows of matter and energy found a complementary relationship, they became stabilized and the communities created the conditions required to ensure their global long-term maintenance. Thus, although evolution (based on heritable reproduction) laid the groundwork for the appearance of increasingly complex individuated prebiotic organizations, the emergence of these collective self-maintaining webs is another equally important prerequisite for the long-term sustainability of the process of biogenesis.

These collective SM webs can be seen as constituting the earlier forms of an ecological domain. Odenbaugh (2010) defines the essence of an ecosystem in terms of energy flows and biogeochemical cycles involving components of both a biotic and abiotic nature, stating that: "an ecosystem exists just in case biotic and abiotic members of a set are closed under these ecosystemic causal relations" (p. 245). What the ecological perspective introduces is an intrinsically cooperative and systemic approach, where the actors of the relevant interactions are collections of (reproductive) physiological individuals, which together construct an environment (niche construction). Moreover, whereas selection has often been seen as an action carried out by the environment on almost passive organisms, niche construction is an active process performed by specific collections of organisms[13]. The systemic dimension of ecological interactions lies in the complementary functionality of the different communities in a given ecosystem, which together constitute a global self-maintaining network (Nunes-Neto et al., 2014). What defines ecological interactions is the fact that an action carried out by a particular kind of organism (or proto-organism) on a particular environment has an effect on the inflow of energy and material pertaining to another type of organism, and this, in its turn, carries out an action which affects another group, and so on, until the folding up of the entire set of interactions. This closed network of interactions constitutes a far-from-equilibrium SM organization.

The importance of the appearance of ecological networks lies in the fact that they enabled the long-term sustainability of prebiotic systems in both energy and material terms. The action carried out by the different types of (proto)organism guaranteed the constraint of the energy and matter flow required for both their own maintenance and that of other types of (proto)organisms, thereby ensuring their indefinite maintenance (provided that certain geological and astronomical conditions were met: for example, ultimately, the network had to be driven by a stable external energy source).

Ultimately, of course, each of these different constrained flows of energy would have been based on the "microscopic" environmental action of long-term stable communities of similar individuated protocells. Globally, the creation of stable ecological systems would in turn have helped to ensure the long-term maintenance of differentiated protocells, since it enabled them to sustain not only evolution but also the environmental conditions necessary to develop more complex metabolic organizations. This is why an ecological system became, as Dagg (2003) points out, a kind of "biologically constructed environment".

It is certainly difficult to determine in which stage of prebiotic evolution a primitive form of ecosystem may have appeared. Even at the level of very simple artificial protocells, some experiments have observed the development of "colonies" which might have facilitated vesicle fusion and solute capture, generating a positive feedback loop between "individual" systems and the

[13]For the purpose of the argument, a self-reproducing protocell can be considered equivalent to a very primitive organism (see below). Hence, in this case, communities of protocell lineages can be considered "biotic" entities which may, under certain circumstances, achieve the ecological relationships defined here.

"colony" (Carrara et al., 2012; Stano et al., 2014). Since this interdependency between the individual and the colony may be observed even in the simplest of protocells, during later phases, this interaction would probably have undergone a process of reinforcement and complexification. It is therefore sensible to hypothesize that, as self-reproducing[14] protocells became more metabolically complex[15] and capable of producing more complex compounds within themselves, a fairly dense group of said compounds would have begun to accumulate in their vicinity, with some being harnessed and used by other protocells, providing, of course, they were functional for them. This would have led to one of two outcomes: a dead end, resulting from the ongoing rise in the quantity of protocells and scarcity of resources in the surrounding area; or the generation of a network of metabolic dependencies among the individuated systems. Furthermore, as Guerrero (1995), Guerrero et al., 2002), and Ruiz-Mirazo et al. (2004) have argued, this proto-metabolic complementarity among different kinds of protocells would have eliminated the need to "clean" up the growing quantity organic waste that could not be digested. Similarly, and more recently, Briones et al. (2015) stated that:

> "Thanks to the fundamental connection between the membrane and the metabolism, a continuous flow of energy and matter between each system and its environment began to occur. This would have prompted a movement of substances between different protocells living in close proximity, which is essential because each compartmentalized system would have been slightly different from the others, and none would have been able to produce all the molecules needed. Moreover, lacking mechanisms for the reuse of certain basic chemical compounds, sooner or later a global crisis would have occurred due to depletion of available resources. Therefore, moving on from their earlier steps, groups of protocells began to establish ecological relationships with each other: the beginning of life not only marked the beginning of evolution, but also the beginning of ecology." (p. 266, our translation).

Thus, the origin of ecosystems can be traced back to a scenario in which a certain *community* of protocells would have affected the inflow of energy and material pertaining to another community of protocells, whose own metabolic impact on its environment would have in turn affected another group of protocells, with the cycle repeating itself until the loop was closed. As a result, a web of metabolic dependencies would have been established among them. Hence, over time, different types of populations would have established functional interactions and relatively stable collective networks.

This self-maintaining network of (proto)ecological interactions is important because, while said interactions are the result of long-term metabolic interactions among a diversified community of protocells, the (proto)ecosystem also enables the sustainability of this community of protocells in the long term, in terms of both energy and materials. Seen from this global perspective, the ultimate consequence of the appearance of reproduction in biogenesis was the creation of a new *self-maintaining organizational* domain, operating at a much broader temporal and spatial scale than that of the physiological domain of proto-organisms. Long-term stable groups of individuated self-reproducing systems, characterized by their different forms of metabolism, created the mechanisms and interactions that allowed the emergence of a long-term (yet synchronic) macroscopic self-maintaining network (an ecosystem). From this perspective, evolution is the means by which individual entities became ecological communities, and the mechanism that allowed the progressive plasticity and diversity of such communities.

CONCLUDING REMARKS

In this paper, we have tried to show that understanding the origin of life requires the explanation of the early appearance of a new form of circular causality in the process of biogenesis, articulated at different spatial and temporal scales. This multilevel form of causality, in turn, is based on the appearance of reproduction, which, being itself the expression of a special form of SM organization endowed with a form of cohesive individuality, permitted at the same time a cumulative historical process and the generation of synchronic collective SM ecological networks.

In this context, we should reconsider the concept of "actual organization." As we have argued, reproduction creates a temporal multi-scale organization, thereby implying (at least) two different classes of current states: those happening at the temporal scale of individuated systems and those happening at the temporal scale of the ecosystem. And in a multiscale organization such as this, short-term processes are in turn affected by slower and spatially larger processes. Also, in this context, a type is not a purely abstract entity; rather it occupies a certain space (although not continuous) and endures over a period of time.

At the lowest level, this form of organization is represented by cells (or protocells), which are spatio-temporally bounded organizations, and could be considered as individuated identities. This form of organization is also the most basic and the first to appear. Their organizational identity is a *token* identity. But when they reproduce, they generate an organizationally discontinuous set of similar entities, which in turn will generate a spatio-temporally broader form of organization: an evolving ecosystem. So, if one changes the time-space scale, a former type could be seen as a token, and thus, the type-token distinction appears as scale dependent. In a similar vein, therefore, the concept of "current organization" is also scale dependent, and what actually matters is the connection created between different scales.

Of course, processes occurring within protocells are far from the hyper complex physiological processes that constitute present-day organisms; and, similarly, the pre-Darwinian (and

[14]Because trans-generational continuity (i.e., lineages) is a requirement for the emergence of an ecological domain.

[15]Especially after the appearance of the earlier genetically based proto-organisms.

proto-ecological) processes here sketched are much simpler than those happening in the biological world. But what I am arguing is that the proto-metabolic organization of these types of protocells *points to* what in a full-blown biological organism is a physiological organization; and that their reproductive dynamics, similarly, *foreshadows* the evolutionary and population dimension of life.

If this interpretation is right, the physiological domain has its roots in the constitutive self-production of the earlier localized entities that will progressively become organisms. But this path will require that at the same time a class of these primitive entities develop a reproductive capacity and as a consequence, they will generate a fist form of a multiscale phenomenon, that expressed itself in the form of individuated, cohesive systems (proto-organisms), as well as in collective, physically unbounded networks (proto-ecosystems; the whole biosphere); in actual organizations (self-maintaining metabolisms, food-webs), and in causally correlated sets of diachronic/historical processes (represented by phylogenetic trees). Thus, even before the appearance of a full-fledged biological domain, biogenesis would have enacted an entangled multidimensional and multiscale self-maintaining system that was both individual and collective; synchronic and diachronic. And it is due to the generation of this multiscale organization that prebiotic evolution will progress and become a robust process.

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The author confirms being the sole contributor of this work and has approved it for publication.

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Bodily Complexity: Integrated Multicellular Organizations for Contraction-Based Motility

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Compared to other forms of multicellularity, the animal case is unique. Animals—barring some exceptions—consist of collections of cells that are connected and integrated to such an extent that these collectives act as unitary, large free-moving entities capable of sensing macroscopic properties and events. This animal configuration is so well-known that it is often taken as a natural one that 'must' have evolved, given environmental conditions that make large free-moving units 'obviously' adaptive. Here we question the seemingly evolutionary inevitableness of animals and introduce a *thesis of bodily complexity*: The multicellular organization characteristic for typical animals requires the integration of a multitude of intrinsic bodily features between its sensorimotor, physiological, and developmental aspects, and the related contraction-based tissue- and cellular-level events and processes. The evolutionary road toward this bodily complexity involves, we argue, various intermediate organizational steps that accompany and support the wider transition from cilia-based to contraction/muscle-based motility, and which remain insufficiently acknowledged. Here, we stress the crucial and specific role played by muscle-based and myoepithelial tissue contraction—acting as a physical platform for organizing both the multicellular transmission of mechanical forces and multicellular signaling—as key foundation of animal motility, sensing and maintenance, and development. We illustrate and discuss these bodily features in the context of the four basal animal phyla—Porifera, Ctenophores, Placozoans, and Cnidarians—that split off before the bilaterians, a supergroup that incorporates all complex animals.

Keywords: muscle, tissue contraction, animal sensorimotor organization, physiology, development, epithelia, animal evolution, Cnidaria

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INTRODUCTION

Multicellular systems evolved from unicellular ancestors on at least 25 independent occasions, both in prokaryotes and eukaryotes (Bonner, 2000). Of these 25 occasions, six eukaryote lineages gave rise to complex multicellularity. Complex multicellularity is characterized by intercellular adhesion and communication, and by (tissue) differentiation beyond the one between somatic and

reproductive cells that is common among simple multicellular systems (Knoll, 2011). Within a (current) total of 119 eukaryotic clades, complex multicellularity originated three times in 'plant-style' lineages—leading to green algae and plants, red algae, and brown algae—as well as twice within the branch leading to modern fungi—leading to basidiomycetes and ascomycetes. Finally, complex multicellularity arose once within the animal (metazoan) lineage (e.g., Bonner, 1988; Medina et al., 2003; Knoll, 2011). Thus, while the evolution of complex multicellularity itself is not a unique evolutionary event, the evolution of animal-style multicellularity did happen only in one lineage. Accordingly, and based on previous work (Arnellos and Moreno, 2016; Keijzer and Arnellos, 2017), our goal in this paper is to further discuss and argue on the special organizational characteristics and the related bodily complexity necessary for the evolution of animal multicellularity.

Typical animals exhibit a free-moving motile lifestyle that allows them to actively change position with respect to the substrate they are situated on (or in), to actively manipulate their environment, ingest large chunks of foodstuff and so on.[1] A similar free-moving lifestyle is widespread among unicellulars – whether they are bacteria or protists like ciliates or amoebae—and it seems an obviously adaptive lifestyle under a wide variety of conditions. Letting aside very complex behavior that involves increasingly complex nervous systems, the fundamental difference in comparison to these unicellular free-moving organisms is that many animals have extended such a motile lifestyle to much larger organizations, expanding from a submillimeter size to species measured in tens of meters, a difference of five orders of magnitude. It is for this reason that, superficially, typical animals may just seem to consist of larger, more complex versions of free-moving unicellulars, suggesting a gradual and piecemeal transition, similar to the later evolution of eyes (e.g., Nilsson and Pelger, 1994). Also, like plants, animal life is such a major component of modern macroscopic life that its presence may look like an unavoidable outcome of evolutionary pressures leading to a multicellular equivalent of the unicellular free-moving lifestyle (Conway Morris, 2003).

However, given (a) the obvious adaptive advantages of a free-moving lifestyle at small as well as at larger scales, as currently seen in typical animals, and (b) the presence of only one evolutionary lineage that actually led to complex free-moving multicellulars, an interesting option comes forward: The rise of typical animals did not consist of a straightforward gradual transition from small-scale to large-scale motility. The initial transition to the typical animal lifestyle was a very difficult one, which involved major transformations in organization, life-style and development (Arnellos and Moreno, 2016).

In particular, the organizational requirements for animals involved new emergent forms of complex integration implemented in bodies. As a matter of fact, animal multicellularity is unique in the sense that it exhibits a level of complexity that brings about typical bodies: differentiated, potentially large and relatively stereotypical multicellular organizations that are capable of self-initiated reversible motility—either as a whole or partially—and sensing as a single unit. This goes far beyond the capacities for self-initiated motility that are present in other multicellular organizations, especially with respect to the increase in force and speed enabled by contractions. It has been previously argued that the key difference that ultimately enabled the rise of typical animals was the development of integrated multicellular organizations implemented in complex body plans (Arnellos and Moreno, 2016) with motility based on tissues capable of reversible contractions, most notably muscle (Keijzer et al., 2013). In this paper, we posit and explain why and how contraction-based motility is one of the most characteristic and fundamental properties of large motile bodies, and we also show and argue for its indispensably mutual role in the development and maintenance of such specifically integrated multicellular organizations.

The use of muscle-based contraction was an essential condition for typical modern animals and the famous Cambrian Explosion, starting 542 million year ago, would not have been possible without it. During the Cambrian Explosion, animals with "complex adaptive bodies" – so called CABs by Trestman (2013) and centralized brains—'typical animals'—became the dominant fauna (Fedonkin et al., 2007). Complex prey-predator relations sprung up, possibly driven by the evolution of eyes (Parker, 2003) and reinforcement learning (Ginsburg and Jablonka, 2010). From here on the prominent story of animal evolution took off. The central animal lineage in these subsequent events were the *bilaterians*. This is a very large evolutionary group that is often defined in terms of having bilateral symmetry, a head, a tail, a back and a belly. They have a nervous system, muscles and a gut and include most commonly known animals, such as the vertebrates, arthropods, mollusks and many other groups.

The bilaterians thus represent a stage where the animal bodily organization exhibits the key features—muscle-based contractility, neural control, a gut, complex development and physiology—that made them so successful as motile multicellular organizations. In this paper, and considering, as mentioned above, previous work on the particularity of the form of integration and of the pivotal role of contractility in multicellular organizations, we further investigate *what set the basis for such complexly organized (animal) bodies.* Thus, here we focus on the space of more basic forms of animal organizations from which the bilaterians evolved. There are four known basal phyla that split of before the bilaterians: Porifera (sponges), Ctenophora (comb jellies), Placozoa, and Cnidaria (jellyfish). These basal phyla constitute a major source of information concerning the various forms of basal animal organization. We will sketch a range of configurations for bodily complexity to illustrate the intrinsic difficulties involved in functionally integrating many different organizational factors and keeping them coordinated. The typical animal body and its multicellular reinvention of the free-moving 'unicellular' lifestyle is only one of these options.

More specifically, in this paper, we focus on the various conditions that must have been fulfilled for typical animal bodies to become a reality. For this reason, we formulate a *thesis of*

[1] Here we discuss what is *typical* for animals and set aside various exceptions. With 'typical' we refer to free-moving animals like vertebrates, arthropods, jellyfish, etc. These typical features are not present in all animals, in particular the most basic animal phyla. Nor do we deny that many animals are sessile, live in colonies, such as coral reefs attest, or are part of multi-multicellular entities such as the Portuguese man o' war.

bodily complexity: The multicellular organization characteristic for typical animals requires the integration of a multitude of intrinsic bodily features between its sensorimotor, physiological, and developmental aspects, and the related contraction-based tissue- and cellular-level events and processes. As we argue, at least at such levels of organizational complexity, many of these conditions are best seen as being constrained by the requirements imposed by the specific form of integration of the bodily organization itself, and much less, if not at all, by environmental features acting through selective pressures. Only by first fulfilling a wide array of such organizational requirements did the animal multicellular configuration eventually—and only in some lineages—become capable of regaining the free-moving lifestyle of many unicellulars in a highly successful way. The initial hurdle here was not to gain more complex behavioral capacities, but to reacquire behavioral capacities already present in organisms using cilia, but now based on (muscle) contraction.

The *thesis of bodily complexity* highlights the complex routes traversed through that space of organizational forms of animal multicellularity, and the various ways of integrating contraction-mediated sensorimotor, physiological, and developmental aspects into functional organisms. We have three objectives. *First*, we aim to clarify the various features that are central to bodily complexity. These features are often taken as background structures in selection-oriented accounts. Here, we stress their role as basic requirements for the kind of functionality that selection might work on. *Second*, we will develop and discuss these features by turning to the various ways in which they come together and are integrated in the four basal phyla that split off before the bilaterian supergroup: Porifera, Placozoa, Ctenophora, and Cnidaria. Animals of these groups show interesting mixes of the various features and help to illustrate why the animal organization that is often taken for granted as a self-evident form of good design results from a complex integration of bodily features in ways that are not self-evident at all. As a *third* and additional aim, we hope to show how this mixing and integrating of features paved the way to the bodily complexity that eventually lead to the Cambrian Explosion.

The paper is structured as follows: In section two, we introduce the several features that play crucial roles in the origins of animal multicellularity and the forms of complexity present in animal bodies. The guiding thread here will be the role played by contractile tissues and cells (of muscular nature, in general) and its impact on bodily complexity as this is realized based on the integration between physiological, developmental, and sensorimotor processes in a multicellular system. The next four sections treat in detail the four different ways in which these features have been integrated in the four different basal phyla that are still extant today: Porifera, Placozoa, Ctenophora, and Cnidaria, again with an idea to the role played by contractile cells and tissues and the related form of integration achieved between development, maintenance and behavior. We compare the four phyla to the typical animal organization based on their organizational forms. Finally, in the concluding section we draw our conclusions concerning the major questions addressed, and develop our claim that establishing bodily complexity itself was a major evolutionary hurdle that cannot be seen as a reaction to evolutionary pressures. We argue that the key to understand the emergence and evolution of animals is to be found also at the level of the respective *bodily complexity*, i.e., at the properties and the characteristics of the early multicellular organizations exhibiting contraction (muscle)-based motility and at the related organizational requirements for the development and physiological maintenance of their integrated bodies.

SETTING THE STAGE FOR BODILY COMPLEXITY: CONTRACTION-BASED MOTILITY REQUIRES INTEGRATED BODIES AND VICE-VERSA

Epithelial Contractility and the Animal Sensorimotor Organization (ASMO)

Animals are not simply scaled-up unicellular agents but exhibit new and complex multicellular organizations that could have taken many different forms (Arnellos et al., 2014). Typical animal organizations require the fulfillment of a broad range of preconditions in order to develop and function. The switch to contraction-based motility is a crucial and fundamental precondition for several reasons. First, this switch enabled a much more forceful and fast form of motility that allowed the rise of (typical) animals. Second, coordinating contractions imposes an evolutionary need for tissues that control these contractions, for example excitable epithelia (Mackie, 1970) and, most notably, nervous systems (Pantin, 1956; Keijzer et al., 2013). Third, coordinating contractile tissue by means of a basic nervous system arguably provides a route toward new multicellular forms of sensing and multicellular sensors that work as single units (Arnellos and Moreno, 2015; Keijzer, 2015).

This latter capacity for sensory feedback derived from self-initiated contraction-based activity across a body surface forms the kernel of what we call the *animal sensorimotor organization* or ASMO. Keijzer and Arnellos (2017) specify the following conditions: (1) a multicellular body, constituting an 'inner space' or domain, which is differentiated from the body's 'outer space' or environment; (2) the presence of contractile epithelia; (3) complex, standardized body architectures; (4) sensitivity to tension and stress at the level of (intra) cellular processes; and (5) reversible, contraction-based changes in body-shape. Here, we will use the ASMO as the key condition for typical animals.

The ASMO is an evolutionary key transition because it ties (literally) large collections of cells into a single motile unit, turning it into a large-scale structure of tensile and compression resistant elements that can act as a motile multicellular agent of macroscopic size. This amounts to a soft-bodied precursor of a tensegrity structure of hard skeletal struts and opposing muscles and tendons (Turvey and Fonseca, 2014). Such an agent can access its environment by multicellular senses that are sensitive to patterns of stimulation across surfaces and guide its own motility on the basis of such information (Keijzer, 2015). This organization is so familiar to us that it becomes almost invisible

and hard to appreciate as a separate achievement of life. Still, it amounts to a major and specific evolutionary transition.

From here on, many evolutionary innovations can be made, as we can witness in the multitude of forms of typical animals. The important message that we want to bring across here is that a 'typical animal' configuration depends for its existence on the initial fulfillment of a broad variety of bodily constraints integrated in a specific organizational form in order to function as a free-moving agent (Arnellos and Moreno, 2015). These include controlled (muscle-based) contractions that actually achieve reliable motion and enable macroscopic feeding guided by sensory devices. Larger and differentiated bodies require various internal transport systems and an ongoing control of physiological processes, as well as a developmental system that guides cell differentiation and produces these complexly integrated multicellular organizations. So, while typical animal bodies are a very successful evolutionary invention, their initial evolution seems to depend a lot on the initial evolution and reciprocal calibration of these bodily features. The major asset of typical animals—free multicellular motility—only became established when these bodies were already in place.

The ASMO notion introduced above provides a suitable point within the space of possible animal configurations, and in particular, it provides a set of minimal conditions that form the basis for the behavioral characteristics of typical animals—muscle-based motility, complex sensory devices, and a centralized nervous system. Beginning by the empirical[2] background of ASMO (Keijzer and Arnellos, 2017)—namely epithelial cell and tissues—we will focus on the evolutionary route toward an ASMO and the various ways in which basal phyla show different forms in which the various ingredients were combined and integrated in functioning organisms.

Epithelia provide the central constructive material and developmental scaffolding for much of the animal body (Tyler, 2003). They are two-dimensional sheet-like tissues, made up from epithelial cells connected to one another by a variety of specialized molecules (Tyler, 2003; Magie and Martindale, 2008). The cytoskeletons of epithelial cells are linked by these connections and the sheet can impose (contract) and resist mechanical tensions in various ways. Epithelial cells have an outward-facing apical side with a primary cilium, and a basal side anchored in an underlying extra-cellular basal membrane, which is part of the underlying extracellular matrix (ECM). The latter is a continuum of collagen fibers and fibrils that integrates and reinforces the contractile epithelium, making it more resistant to tension and provides a stable setting for the cells (Timpl, 1996). As epithelia provide the basic animal building block, they provide a plausible starting point for tracking the changes leading to complex animal bodies. Epithelial tissues are central to multicellular integration and when widely interpreted may constitute a very primitive multicellular configuration on its own. As a matter of fact, even the most minimally complex epithelial unit can be considered a body that exhibits integration between sensorimotor coordination, physiology, and development (Arnellos and Moreno, 2016).

In this context of contractile epithelial cells and tissues, we will develop two leading ideas. First, while contractility is a basic feature of eukaryotic cells that is directly linked to the presence of a cytoskeleton and widely used, turning contraction into a multicellular motility device is not an easy transition. It involves traversing what Kauffman (1993) calls a rugged fitness landscape with many local optima. Second, when it occurred, the switch from motility by cilia to contraction (muscle)-based motion was a central driving factor for the evolution of the ASMO: Using cellular contractions for motility requires a large number of organizational features in order to function. Both ideas will be developed by a systematic discussion of the main organizational characteristics of the four known basal phyla. The first one deals with the ways in which contractility is present in these phyla and is involved in the related constitutive and interactive functions. The second idea targets the ways in which the relevant organizational aspects like development, physiology and sensorimotor coordination guided by the presence of a nervous system—together with their integration to various animal body plans—correlate with and are potentially driven and mediated by contraction (muscle)-based motility. We provide here a short and general introduction of the two ideas and of the related features, while in the next four sections we will specifically explain how these features are exhibited and implemented in the four basal phyla.

Contraction-Based Motility

Active motility plays a key feature in animal interaction because it allows the repositioning of the organism and environmental features with respect to one another. While cilia are extremely important and handy features as a motility device they are limited to small-sized organisms of a few millimeters, barring some specially adapted organisms like Ctenophora (Tamm, 2014). For large-scale motility as exhibited by typical animals, a different mechanism is required that comes in the form of tissue contraction, most notably muscle. Tissue contractions provide a much larger source of force that can be scaled to large organisms in the centimeter to meter range. The use of tissue contraction for motility does also require a much more complex coordination mechanism compared to cilia and this need for coordination will be our main focus in the following sections. A central idea here is that contraction control was a major driver toward the evolution of sensorimotor coordination as specifically realized through nervous systems.

Contractility and Modes of Feeding

An implication directly related with motility in animals is feeding. As heterotrophs, organisms within the animal lineage acquire their energy and constitutive substances from the intake of food particles. A motile body has some interesting ways to be fed. Sperling and Vinther (2010) differentiate three general modes of feeding that, barring some derived exceptions, cover the whole animal lineage. Contraction-based motility characterizes all modes as it mediates between the animal and its food source in the environment in all three

[2]For the theoretical background of ASMO see Keijzer (2015).

feeding types (though in different forms and degrees as discussed below). Microphagy – the direct uptake of bacteria and dissolved organic carbon from the water column – is found in Porifera. Placozoa feed with a ventral sole where the area between the animal's ventral side and the underlying substrate acts as an 'external stomach' where food is externally (pre)digested before it is absorbed by the ventral surface. All other phyla – Ctenophora, Cnidaria, and the Bilateria – exhibit macrophagy. They take in large food particles, which are then internally digested in a gut. Interestingly, the presence of a gut goes hand in hand with the presence of both muscle and a nervous system, which suggests a strong connection between these features.

Another central idea here is that contraction-based motility provides behavioral implications such as the modes of feeding discussed before, which in turn entail organizational requirements related with sensorimotor, physiological, and developmental aspects of the animal body.

Contractility and Sensorimotor Coordination (Coordinating Sensors and Effectors)

In an epithelial body exhibiting reversible contraction-based changes in body-shape together with sensitivity of environmental stimuli and stress at the level of each individual contractile epithelial cell, sensorimotor coordination happens primarily through the epithelial structure itself. This is characteristic for animals like the Porifera and the Placozoa, whose bodies are in constant and total contact with the water, and whose modes of feeding require an external or a cell-dependent micro-digestion. In these cases, epithelia (either at tissue level or as individual cells) function as sensor and effector structures. As the multicellular body becomes more robust with respect to its shape, and capable to implement more complex movements—especially with respect to finding and catching food—there is a need for more efficient and elaborated sensorimotor coordination. In such cases (e.g., Cnidaria, Ctenophora) where preys are hunted, caught, directed into a mouth and then pushed down into a gut, specialized cells stimulated by small 'organs' such as statoliths or explosive cells such as cnidocytes, and myoepithelia and/or true muscle cells function as effector structures, while coordination between sensors and effectors is implemented through a nervous system—i.e., a cellular tissue that enables the plastic and selectively combinatorial coordination of distant contractions in a multicellular body. Again, it is noted that macrophagy—having a gut—is always empirically combined with muscle-based motility and the presence of a nervous system (with various degrees of centralization).

Physiology

The coordination of sensors with contractile effectors enables movements with various degrees of freedom for the animal, which in turn provides a context where physiological housekeeping processes related to the material, energetic, and metabolic requirements of such movements become relevant. As mentioned before, a fundamental and characteristic feature of animal bodies is their epithelialization due to which their cells are held together, oriented and attached to an ECM. This creates a three-dimensional tissue that separate an inner (systemic) and an outer (environmental) space with various degrees of sealing in between these two domains and with intercellular means of molecular, electrical, and signal communication between the cells of the tissue. In these three-dimensional bodies some cells will be in contact with the environment while other cells will be secluded in the inner space. This implies certain novel requirements for the transport of oxygen and nutrients required by all cells. Considering the low solubility of oxygen in the water and that that cell size should be restricted to ∼1 mm when oxygen availability is limited by diffusion, there is a challenge for 3-dimensional multicellular bodies to maintain the appropriate oxygen supplies (Knoll, 2011). Sperling et al. (2015) suggest this can be achieved either by maintaining body shapes that minimize diffusion distance or by evolving oxygen-transport systems. All basal metazoa have implemented the first strategy.

Contractility and Development

Body motility in combination with a certain physiology requires a division of labor that cannot be achieved but through different cell types.[3] In the transition from microphagy to macrophagy, multifunctionality and its subsequent segregation to sister cell types seem to have played an important role (Arendt, 2008). In this context, apart from the primary division between germ and somatic cells, epithelial, muscle, sensory, and neuronal cell types as well as some other very specialized ones (such as the cnidocytes and the nematocysts in Cnidaria or the collocytes in Ctenophora) were evolved from a cell type that most likely resembled a choanoflagellate.

The diversification of cell types as well as their differential constitution and spatial arrangement in a three-dimensional body requires certain forms of developmental regulation. In the evolutionary scenario from a colonial multicellular choanoflagellate to a metazoan, where the cellular functionalities of the latter are more or less encoded within the choanoflagellate genome (King et al., 2008), there is no need for evolution of different cell types but for the constant appearance of cell types that in their unicellular phase were alternating in time as well as for their spatial differentiation and arrangement within the three-dimensional body. In simple multicellularity, gradients of available light, oxygen, and nutrients of the environment together with signals transduced from exterior cells may induce differentiation of interior cells along the gradient (Schlichting, 2003). In more complex multicellularity, where, in general, development begins with a fertilized egg (Newman, 2014), it is epithelia[4] that —provide a reliable scaffold

[3]Our intention is not to discuss each cell type analytically (after all, there is no such complete record) but to mention the cell types required for the realization of body motility and the related physiology in the transition from microphagy to macrophagy. Several cell types will be mentioned in Sections "Porifera," "Placozoa," "Ctenophora," and "Cnidaria."

[4]Epithelia are generally considered as cells clonally derived from a fertilized egg that remain attached to one another by cadherin-based membrane proteins, and

to guide diffusing morphogens that initiate developmental processes, which in turn combine various (contraction-mediated) morphogenetic movements with mechanisms of inter- and intra-cellular signaling that mobilize and drive morphogenesis (Newman, 2012).[5] To this, a diverse developmental signaling and transcription factors repertoire that seems to be present in all early metazoa plays a great role (e.g., Srivastava et al., 2008, 2010). This repertoire seems to have preceded the genomic developmental toolkit of bilaterians (Degnan et al., 2009; Riesgo et al., 2014), let alone the fact that this toolkit is not only present in the early metazoan genome but it is also expressed during their development.[6]

Contraction-Based Motility and Integrated Bodies

We argue that enabling contraction-based whole-body motility—which in turn makes large and fast-moving animals possible—is not a self-evident evolutionary development but intrinsically tied up with an array of interdependent and crucial conditions for it to function. Together these conditions constitute the requirements for an integrated multicellular organization that involves the need to coordinate these contractions and to use the environmental sensory feedback they create (ASMO), the need to build complex and standardized multicellular bodies by a set of complex developmental processes, the need to maintain some chemical interaction space with the environment (such as a gut) for the organism as a whole, and the need for a broad array of physiological and homeostatic maintenance processes.

All these features are organized in (sometimes) fundamentally different ways in the four early diverging metazoan bodies. We argue that the ways in which *bodily complexity* can be said to increase (or at least differs) over these four phyla supports the idea that organizing contraction (muscle)-based free motility requires a highly complex integrated multicellular organization. These four phyla show how contractile capacities are present in all cases, while their use and characteristics differ in important ways. Enabling contraction as the primary source of motility, switching away from the use of cilia, occurs only in one of these four phyla. The discussion on these various uses of contraction and the specific ways in which this use is deeply connected to feeding-style (and general behavior), development and physiology show that the evolutionary route toward the replacement of ciliary by contraction-based motility is not a self-evident evolutionary improvement at this stage of multicellular organization.

In the following four sections we will describe in detail the different bodily complexities as they are materialized in each one of the form of integration of these four phyla with a particular focus on the presence and use of contraction for organizing body plan and motility. In all four animal cases, such use is intrinsically related to a specific organizational context, which sets its own restrictions on evolutionary changes.

PORIFERA

Multicellular suspension feeders like the *Porifera* (sponges) have a water canal system (a network of chambers containing flagellated cells—the choanocytes) whose beating pumps water through the body. The choanocytes are then filtering bacteria (even less than 1 μm in size), which are digested intracellularly, making this a form of microphagy.[7] But even in this form of feeding contractility plays an important role as sponges are able to contract their canals (slow contractile events that propagate through cellular sponges) so as to arrest the feeding current by closing the intake system in order to prevent damage to the feeding chambers and/or to expel inedible material that has entered the filtration system (Leys, 2015). Such behavior is enabled by the bodily organization and its tissue- and cell-related components.

Cell Types

In Porifera there are around 11–16 cell types (Simpson, 1984; Bell and Mooers, 1997; Carroll, 2001). Few of them are found in the two spatially differentiated epithelia (the pinacoderm and the choanoderm) with different physiological roles in filtering, maintenance, and structural integrity of the animal (Leys et al., 2009). The pinacoderm consists of pinacocytes that make up the external surface (exo-pinacoderm) and the water channels (endo-pinacoderm), and porocytes that are miniature sphincter cells.[8] The choanoderm is the feeding epithelium composed by choanocytes that have a single flagellum surrounded by a ring of microvilli.[9] The two epithelial layers surround the mesohyl, which is a collagenous non-epithelial layer that provides the gross morphology of the sponge. It is primarily composed of an ECM secreted by motile amoeboid cells, and of archeocytes that can differentiate into all other sponge cell-types. Among these cell types are the lophocytes that secrete collagen fibers supporting the mesohyl as they move through it, the spongocytes and the sclerocytes that polymerize into thick skeletal fibers and secrete the mineralized skeletal spicules in many sponges, respectively. There are also the oocytes and the spermatocytes, which are reproductive cells undergoing gametogenesis to form sperm and eggs.

which are at the same time being independent while also being attached to each other thus forming embryonic tissues that behave like liquids (Newman, 2016b).

[5]For the moment, we are just stating the common aspects of what we refer to as 'epithelial development' that characterizes all metazoa. However, as we have previously explained, a genuinely epithelial developmental organization is to be met only in the Eumetazoa (Arnellos and Moreno, 2016). As we explain in Sections "Porifera," "Placozoa," "Ctenophora," and "Cnidaria," the developmental process is coordinated in different ways in the four early metazoa, resulting thus in body plans with crucial differences in their complexities.

[6]Again, striking absences in the genomes of each of the four early metazoa lineage and their implications in development of the body plans will be discussed in Sections "Porifera," "Placozoa," "Ctenophora," and "Cnidaria."

[7]Food particles are taken up by phagocytosis or pinocytosis followed by intracellular lysosomal degradation (Leys and Eerkes-Medrano, 2006).

[8]The pinacoderm functions as a stable sheet of cells with properties for skeletal support and protection by the outer and for transport of nutrients by the inner epithelia, respectively.

[9]The choanocytes form a round chamber and beat their flagella to draw water from the incurrent endopinacoderm-lined canals into the chamber where bacterial food are filtered by the microvilli. The water is then expelled through the osculum (see Ruppert and Barnes, 1994 for details).

Sensorimotor Coordination

In *sponges*, epithelia function as sensory and contractile tissues, and they are the primary conduits of behavior coordination (Nickel et al., 2011; Leys and Hill, 2012). The osculum (via the short, non-motile, primary cilia that line its inside) seems to be the main sensory organ for sensing environmental stimuli that trigger responses by the sponge (Ludeman et al., 2014). The effectors are either the flagella of the choanocytes (in the case of glass sponges)[10] or contractile epithelial cells (i.e., actinocytes)[11] that reduce the water flow into the sponge by reducing the size of the canals and chambers, or 'sphincters' formed by other specialized pinacocytes arising from the inner epithelium enabling thus the narrowing of the canal, and consequently, the prevention of the uptaking of unwanted particles that might damage their microvilli-based filter.

Sponge larvae first swim and then settle on a solid surface before their metamorphosis into a juvenile sponge. Each cell of the larval epithelium possesses a single, motile cilium and its apical end facilitates larval dispersal through swimming or crawling. Phototactic swimming seems to be the combination of the rotation of the larval body around its AP axis (due to metachronal beat of the cilia) and the shading by pigment of a region of cilia (Leys and Degnan, 2001). In some cases, phototactic responses of sponge larvae are the result of independent responses (either photonegative or photopositive) of individual cells (operating both as sensors and effectors) in the ciliated posterior tuft of the larva (Maldonado et al., 2003; Collin et al., 2010).[12]

Adult sponges coordinate the contractions of their canals (or the arrest of their flagella in the case of glass sponges) in order to prevent the damaging of their filter system by the uptake of large particles. In the absence of a nervous system there must be some other type of spatio-temporal coordination of the adult sponges' cellular activities in order to detect and respond to the changes in water quality. Sponges are basally epithelial animals. The sponge larva consists of polarized epithelial cells, and adult sponges exhibit spatially differentiated epithelia with different physiological roles in filtering, maintenance, and structural integrity of the animal (Leys et al., 2009; Leys and Hill, 2012). The inner epithelium *(the endopinacoderm)* has a sensory function. Specifically, the osculum is the part of the inner epithelium that (via the short, non-motile, 'primary' cilia that line its inside) seems to be the main sensory organ for sensing environmental stimuli that trigger responses by the sponge (Ludeman et al., 2014). The sensory signals received by the cilia propagate to the rest of the sponge body mainly through cells of the epithelium. Sponges lack nerve cells in the sense that they cannot transmit signals along several cells to communicate to other cells via a chemical synapse. However, sponge cells send chemical signals in neighbor cells but in very low speeds compared to the conventional neuron-based transmission.[13] Only glass sponges (Hexactinellida) have electrical signals conducted through the syncytium that trigger the halt of beating of the flagella of choanocytes in order to stop the feeding current.[14] This has an 'all or none' effect (Leys and Mackie, 1997). Sponges of all other groups have no electrical signals. In these cases, the effectors are contractile epithelial cells (actinocytes) that reduce the water flow into the sponge by reducing the size of the canals and chambers, or 'sphincters' formed by other specialized pinacocytes arising from the inner epithelium enabling thus the narrowing of the canal. All these cases of coordination between environmental stimuli and effector contraction are realized through juxtacrine signaling (namely, a type of cell signaling where a signal released by a cell triggers a response in a neighboring cell (see Leys, 2015 for details). In all, epithelia in sponges function as sensory and contractile tissue, and they are the primary conduits of behavior coordination.

Physiology

Porifera are in constant contact with the water through their highly porous bodies, while the flagellar movements of cells facilitate the circulation of water through the internal canals. Practically, the whole animal is within its environment. Nutrients are transported by the water that flows through the system of canals, whereas the exchange of nutrients takes place directly at the cellular level (microphagy).

Signal propagation through juxtacrine signaling is made possible due to sponge epithelia being able to control the ionic milieu of their extracellular space. Particularly, the outer epithelium (the pinacocytes that make up the external surface called exo-pinacoderm) functions as a stable sheet of cells with properties for skeletal support and protection by the outer and for transport of nutrients by the inner epithelia, respectively. More specifically, adherent junctions are formed in the epithelia of all sponge groups but their ultrastructure is nowhere the same as the one in eumetazoa. And although all sponge groups show fine septae in their epithelial junctions, distinct septae junctions are only known in calcarea, while in demosponge and homoscleromorph tissues the ladder-like structure of the septate junctions is particularly faint (Leys and Riesgo, 2012). However, it has been shown that demosponge outer epithelia do seal and control the ionic composition of their ECM (Adams et al., 2010). Also, homoscleromorphs epithelia are the only ones that possess a distinct basement membrane (Ereskovsky et al., 2015).

[10]Glass sponges (Hexactinellida) are different from all other sponges because they have a syncytial organization. They cannot contract their "epithelia" because their choanocytes are actually a syncytium.

[11]The pinacoderm does not qualify as a muscle tissue. However, it contains contractile filaments traversed by extended actin networks. The elementary contractility of these filaments has been experimentally verified (Nickel et al., 2011).

[12]In general, sponge larvae move by independent ciliary beating. However, they rarely perform horizontal displacement by ciliary beating during their dispersal in open waters. Instead, the cilia are only used to keep larvae rotating along their longitudinal axis, which apparently allows for the re-adjusting of the depth range while drifting (see Maldonado, 2006 for details).

[13]Propagation along the whole sponge can take from 30 min to a full hour. In general, signaling in cellular sponges is three orders of magnitude slower than neuronal signaling, while the fastest rate of contraction in sponges is still 10 times slower than action potential in plants (Leys, 2015).

[14]Choanocytes have a single flagellum surrounded by a ring of microvilli and constitute the choanoderm (the feeding epithelium).

Overall, all sponge epithelia seem to demonstrate a degree of structural integrity and a barrier function in the sense that they are able to control the transepithelial passage of ions (see Leys et al., 2009).

Development

Porifera have also a simple body plan compared to the eumetazoa, and similarly to the Placozoa, the adults also lack anterior-posterior (AP) polarity (however, the larvae do swim directionally), typical neurons and muscle cells, and a central gut. Sponges possess a rich developmental gene toolkit with many transcription factors, however, their body plan lacks symmetry and it is quite indeterminate (i.e., without fixed number of morphological characteristics in a standard arrangement). Several of their transcription factors are even involved in determining and differentiating muscles and nerves (Srivastava et al., 2010). However, similarly to Placozoa, sponges do not develop a neuromuscular system (Mah and Leys, 2017). Sponges are undergoing an intermediate 'epithelial development.' All sponges but the homoscleromorphs lack basal lamina, while no sponge contains the planar-cell-polarity-inducing *Wnt* non-canonical pathway, which is characteristic of diploblasty (Newman, 2016b). Consequently, in Porifera, blastulation, gastrulation, and histogenesis are not quite distinct (Ereskovsky, 2010). In addition, it is still undecided whether sponge cell layers are homologous to eumetazoan germ layers, as they do not undergo progressive fate determination (Degnan et al., 2015). This means that it is highly likely that the existence of a regulatory network guiding progressive germ layer determination (through gastrulation) and the related capacity for diverse cellular differentiation and integration are characteristics of eumetazoan organizations (Nakanishi et al., 2014). Moreover, most sponge cells reside within the mesohyl to which they bind via integrins and within which they can move freely past one another. Such mainly mesenchymal organization (absent in diploblastic Cnidaria and Ctenophora) makes the sponge cells subjects of morphogenetic capacities and dynamics different than those of genuine epithelial tissues (Newman et al., 2009; Newman, 2016a for details).[15] Therefore, even if some sponges can undergo morphogenetic processes very similar to those met in 'epithelial development,' it is surely a development with such a regulatory capacity (by the interplay of epithelial morphogenetic movements and intercellular signaling) that cannot provide standardized body plans. However, contrary to the Placozoa that lack molecules involved in the transduction of the Notch signal (Srivastava et al., 2008), the appearance of the complete *Notch* signaling pathway appears to operate as an intercellular regulator of alternative cellular fates within a common tissue layer (Ehebauer et al., 2006), resulting thus in the high number of cell types exhibited by the Porifera (as opposed to the Placozoa).

[15]The continuous movement of individual cells allows the actively remodeling of the branched skeletal structures in Porifera, thereby yielding a non–precisely determined anatomy in a body form that is shaped asymmetrically and plastically by its environment (Meroz-Fine et al., 2005).

PLACOZOA

Trichoplax[16] (small, flat marine animals) exhibits a motile feeding mode based on external digestion by a direct endocytosis at the surfaces of ventral epithelial cells (Smith and Reese, 2016). Although placozoa use their cilia to glide over patches of algae that are digested extracellularly, once the nutrients have been ingested the animal starts to change its shape and to make churning movements by contracting its fiber cells in order to help digestion and circulation of the nutrients (Smith et al., 2015). How is this behavior supported organizationally?

Cell Types

There are six cell types in *Placozoa* with five of them located in the ventral epithelium. The small ventral epithelial cells are the most widespread having apical junctions joining them into an epithelium, but they lack tight junctions and a basal lamina (Smith et al., 2014). Lipophil cells are newly recognized cells that are interspersed regularly within the ventral epithelium and are a good candidate for digestive cells as they are likely to secrete digestive enzymes. Gland cells is another type of newly recognized cells that, contrary to the lipophils, are widespread around the rim of the ventral epithelium but sparse in its central regions. Gland cells seem to be neurosecretory chemosensory cells playing a role in feeding as their secretion modulates the activity of the ventral ciliated and lipophil cells in the presence of food (Smith et al., 2015). Fiber cells are found in a single layer in the interior of *Trichoplax*, and they are also in contact with the dorsal epithelium. These cells are usually considered to be forming a connective tissue through syncytial junctions (a fiber syncytium). This syncytium resembles the excitable epithelial cells in glass sponges though its conduction efficiency and its contractility are uncertain (Ruppert and Barnes, 1994). The upper (dorsal) epitheloid layer contains epithelial cells with junctions as the ventral cells but with fewer microvilli. In most strains of Placozoa each one of these dorsal cells contains a large spherical lipid droplet thus resembling some of the chemical properties of the large lipophil cells. Crystal (birefringent) cells have been lately reported to be found around the entire rim of *Trichoplax* lying underneath the dorsal epithelium near the fiber and lipophil cells and in contact with the fiber cells. It is hypothesized they could be either stem cells or they might have some sensory function as there are reports that Trichoplax could be able to respond to light (Smith et al., 2014).

Sensorimotor Coordination

In *Placozoa*, the ciliated cells of the ventral epithelium are clearly the effector cells that are responsible for gliding motility. *Trichoplax* will pause much more frequently and for longer time when food is present in the environment. Accordingly, there

[16]This is the originally discovered and most widely described placozoan species. *Hoilungia hongkongenesis* (the second placozoan species) and all yet undescribed species are morphologically indistinguishable. *Polyplacotoma mediterranea* is a newly discovered species that can adopt ramified body shapes. No structural differences to other placozoans have been detected for *P. mediterranea* so far (Osigus et al., 2019).

appears to be a way of sensing the underlying algae as well as triggering of the secretion of granules from the lipophil cells for the algae's lysis. The gland cells (those interspersed in the inside of the animal together with other ciliated ventral epithelial cells) are supposed to be chemosensory cells that are modulating the activity of the ciliated ventral and of the digestive cells (Smith et al., 2014).

The gliding movements (either for migration or rotation in place) of *Trichoplax adhaerens* are propelled by the asynchronous beating of the cilia of the ventral epithelial cells. The direction of these movements can be changed by the reorientation of the strokes of the animal's cilia (Smith et al., 2014). The participation of all cells in these movements is not due to any active coordination in between those cells. Probably, each individual ciliated cell senses and responds independently to a chemoattractant, while collective movement toward the food target is ensured by the system of adherens junctions that holds neighboring cells in the two epithelia that enclose the animal and by elastic forces arising from other cells with which they are linked directly and via intervening cells they thus holding the whole animal together during crawling[17] (Smith and Reese, 2016; Smith et al., 2019). When the animal finds itself over algae the gliding stops as it pauses to feed. Its cilia at the entire ventral epithelium stop beating and specialized cells (lipophils) that are widely separated with each other simultaneously start secreting large ventral granules that lyse the algae. In the absence of a nervous system there must be some other type of spatio-temporal coordination of cellular activities during pausing of locomotion and feeding. The gland cells located at the circumference of the ventral epithelium are chemosensory neurosecretory cells that coordinate the uniform arrest of cilia beating by secreting endomorphin-like peptides upon contact with the algae. Specifically, the peptides are secreted as a gradient in the epitheloid environment and they spread by diffusion. The signals are amplified through a type of positive feedback loop relayed among the secretory cells. It is in this way that ciliary arrest spreads across all over the animal (Senatore et al., 2017).

Physiology

Similarly to Porifera, Placozoan cells are in constant contact with the water. Placozoa have two layers of cells with the in-between substance being (practically) seawater. In Placozoa the food is ingested through the churning movements of the ventral epithelium that forms a primitive 'digestive bag.'

The coordination of external digestion through the secretion of granules by the lipophil cells is done through a highly localized secretion that happens by paracrine signaling pathways either based on peptides by the gland cells found in the periphery or by other cells (Smith et al., 2015). Once the group of lipophils have simultaneously secreted their digestive granules, groups of (epithelial ventral and fiber) cells located in the center of the Trichoplax will begin to make elliptical churning movements in order the lysed algae to be endocytosed by the ciliated ventral epithelial cells. The digestive movements resemble those of myoepithelial cells, and their coordination could be realized based on the (so far) suggested contractility of the fiber cells that, to some extent, seem to be interconnected by electrically syncytial conductive junctions (like the ones found in glass sponges) and are connected to all other cell types (Smith et al., 2014). Again, the adherens junctions of the epithelium hold the cells together during digestive movements.

Development

Placozoa have a very simple body plan compared to the other metazoa. They lack anterior-posterior (AP) polarity, they have no neurons neither muscle cells, and they also lack a proper gut. Contrary to the low morphological complexity of *Trichoplax*, and while its genome is relatively small, the gene content is complex containing genes that specify structures and processes such as ECM production, germline sequestration and neural signaling that are characteristic of higher animals but are absent in *T. adhaerens* (Schierwater et al., 2009). This low morphological complexity in the presence of complex gene content is possibly due to the absence of 'epithelial development' and with developmental regulation taking place (mostly) at the cellular level and far less at the whole animal. Specifically, although the cells of the epithelium in Placozoa exhibit apico-basal (AB) polarity, thereby being able to laterally attach to their neighboring cells while also being basally attached to acellular surfaces, they lack a basal lamina—a specialized ECM necessary for a complete epithelium and for the formation of cell sheets in metazoa as it regulates cell behavior and function in tissue genesis and homeostasis (Fidler et al., 2017). This prohibits the epithelia in Placozoa to exhibit interesting morphogenetic movements that will provide germ layers and consequently the capacity for distinct and diverse morphological outcomes in a body plan (Newman, 2016b). Moreover, the epithelium in Placozoa is leaky, lacking any septate and gap junctions (Smith and Reese, 2016), thus not enabling efficient and rich intercellular signaling that could hypothetically mobilize various morphogenetic movements. Consequently, in spite the rich and complex developmental gene toolkit, development in *T. adhaerens* provides a non-standardized and indeterminate body plan (i.e., without fixed number of morphological characteristics in a standard arrangement), with no A/P polarity, and a few cellular types.[18]

CTENOPHORA

Ctenophora are fed through a combination of swimming and retracting of their two branching tentacles. Swimming is realized by the coordinated beating of the cilia of the comb plates (ctenes). Preys that touch the tentacles that are usually trailing behind the animal (or are extended like a net) are entangled and stuck by the colloblast cells (the major component of the tentacle epidermis)[19]. The tentacle will

[17] This is an epithelium that lacks any tight or gap junctions.

[18] However, little is known about the development of *T. adhaerens* and of Placozoa in general, yet.

[19] All ctenophores except the beroids have tentacles and thus possess colloblasts. Another exception is *Euchlora* that does not possess colloblasts

then retract and the prey will be transferred to the mouth for consumption. In ctenophores with reduced tentacles of reduced size (such as lobate adult animals) or with no external tentacles (such as the Thalassocalycida) food is captured through the oral lobes or through a rapid bell contraction of the peripheral sphincter muscle of its medusa-like body, respectively (Swift, 2009).

Apart from the feeding process described above, ctenophores have an interesting repertoire of motility-based interactions with the environment such as forward swimming, escape swimming, and geotaxis. Let's describe the complex bodily organization behind such behaviors.

Sensorimotor Coordination

Ctenophores have an apical sensory organ that is supposed to be the main sensory organ region due to the high concentration of nervous elements surrounding it. It includes a gravity-sensing statolith, mineralized lithocytes that together with balancer cilia serve as mechanoreceptors (sensitive in changes in pressure), and the lamellate bodies that are supposed to be sensitive to changes of light. The main effectors related to the locomotion (swimming) of the animal are the cilia of their comb plates, while true smooth muscle cells[20] are used for maintenance of the body structure as well as for catching and digesting preys. In all motile interactions the muscles are used to maintain body shape while the animal is moving forward by the beating of cilia as well as for the contraction of the tentacles, the ingestion of food and the egestion of waste in and out of the pharynx, respectively. The coordination of the effectors in all these behaviors is realized through hydrodynamic (beating of the cilia in comb plates) conduction or/and through electrical (in the case of mechanoresponses of the balancing system, of ciliary inhibition for changing of direction of swimming and escaping or for feeding) conduction via the nerve nets. In general, there is a more confined and local (and only indirectly global) and less directly global through-conducting modulation compared to the Cnidaria (Tamm, 2014; Simmons and Martindale, 2015).

Physiology

Ctenophora, whose body is, similarly to Cnidaria, composed of two epithelial layers (the outer epidermis and the inner gastrodermis) and the jelly like mesoglea in between, have a similar but more elaborated digestive system than Cnidaria. Indeed, the digestive tract of Ctenophora is complete. They have a through-gut with mouth and separate anus. There are two anal pores at the anterior end of the organism for excretion of (mainly) soluble wastes. The pharynx connects the oral with the anal openings via the center of the gastrovascular cavity. Its morphology enables a much more efficient food processing and distribution compared to Cnidaria. The food is processed in three phases as it passes through the acidic environment of the pharynx and then it moves through one alkaline phase along the folds of the pharynx and through another one near the so-called stomach, where food particles are broken down by cilia. The small particles are distributed to the body by the endodermal system of meridional canals that run subjacent to the comb rows, while the remaining larger (mainly exoskeletal) particles will be egested back out of the pharynx (Bumann and Puls, 1997; Tamm, 2014, 2019).

Food digestion and nutrients distribution is realized in a complete digestive tract and it mainly involves cilia beating[21], and secondarily, epithelial and muscle cells. The esophagus is lined up by cilia pointing in opposite directions on its two sides. The continuous beating of the cilia makes the esophagus a gastric mill that cuts pre-digested prey into small particles, which are then directed to the stomach again through cilia. In some species (beroids) the ingested prey is cut in pieces through the peristaltic waves of muscle contractions. Food particles are distributed via the branched gastrovascular canal during brakes between bouts of defecation. Indigestible fragments are egested through the mouth and small waste particles are egested through one of the two anal pores (defecation). Ingestion and egestion cannot happen simultaneously as bouts of defecation are shutting down food handling and distribution in the digestive system. Localized tracts of opposite beating cilia line up all canals. Defecation is preceded by the combination of the constriction of the walls of esophagus together with the slowing down of cilia beating. Although these systems haven't been thoroughly studied, there is experimental evidence that the adjustment of the ciliary beating in opposing directions and the various muscle contractions for food ingestion/digestion and fluid circulation, as well as the modulation of cilia beating prior to defecation is likely to be regulated by local electrical conduction realized through epithelial gap junctions or by neural nets (see Tamm, 2014, 2019 for details; Simmons and Martindale, 2015 for a relevant review).

There is an apparent absence of classic neurotransmitters (i.e., acetylcholine, serotonin, histamine, dopamine, adrenalin, noradrenalin, and octopamine) and of neurotransmitter receptors in Ctenophores compared to the Cnidaria (Moroz et al., 2014). However, there is glutamate and GABA, acetylcholinesterase is present in *Mnemiopsis leidyi* (Ryan et al., 2013), while acetylcholine (together with adrenaline) have been shown to play a role in *M. leidyi* bioluminescence (Marlow and Arendt, 2014). Ctenophores also contain many G-protein-coupled receptors and several neuropeptide precursors (Moroz et al., 2014). Moreover, ctenophores express a diversity of innexins (also used to form gap junctions in Cnidaria), and opsins necessary for a sensory neuron for photo-reception (Jekely et al., 2015). All these findings suggest the existence of a complex peptidergic armory in the Ctenophores nervous system that, similarly (though likely not that widely and globally) to the Cnidaria regulate the animal's physiology.

but instead has nematocysts that it obtains from the medusae it consumes (Pang and Martindale, 2008).

[20]Contrary to the Cnidaria there is no epitheliomuscular component (Hernandez-Nicaise, 1991).

[21]In a recent work Tamm (2019) reports that the widening and narrowing of the endodermal canals that play a major role in food distribution and waste elimination are more possibly due to fluctuations in hydrostatic pressure within the canal lumens caused by fluid flow driven by the cilia that are lining the canal walls.

Development

Ctenophores have several unique morphological features that distinguish them from all other animals. They are diploblastic epithelial animals with a gelatinous mesoglea penetrated by true muscle cells, nerves, and mesenchymal cells. They have a branching ciliated gastrovascular system that runs the body along the oral/aboral axis. They consist of four quadrants separated by the tentacular and the esophageal plane. There are two comb rows, a half of a tentacle and a quarter of the apical organ in each one of the quadrants. Since adjacent quadrants are not morphologically identical to each other (anal canals are found only in two diagonally opposed quadrants) ctenophores have a rotational symmetry (this is neither a radial nor a bilateral one) (Martindale and Henry, 2015). Ctenophora don't have as rich developmental genetic toolkit as Cnidaria. Indeed, the gene content of ctenophores is much more similar to the one of sponges than it is to any other metazoan (Martindale and Henry, 2015).[22] Ctenophora—most likely because they are holopelagic and thus they should get to a free-living feeding stage very quickly—are (contrary to Cnidaria) direct developers. The polarity of the primary axis is provided by cytoplasmic maternal products, while the cWnt signaling pathway appears to be important in the establishment of polarity later in their development of ctenophores (Pang et al., 2010; Jager et al., 2013). Also, although both their epithelia form basal laminas—contrary to Cnidaria, where the basement membrane plays numerous important roles in directing cell differentiation, cell migration and tissue regeneration through intercellular signaling—the basal lamina of Ctenophores seem to play only a structural role rather than a role in cell signaling (Babonis and Martindale, 2016). Therefore, although Ctenophora exhibit epithelial development and a standardized and determinate body plan as Cnidaria do, it seems that their development is regulated differently than in Cnidaria, demonstrating a more egg-based stereotyped regulation than a developmentally rich regulation that endogenously emerges as development unfolds.

CNIDARIA

The rest of the animals (belonging to the Eumetazoa) are macrophagous, i.e., they can be fed on large items through a gut. In this case food is inserted inside the body but it is digested outside of the cells. It is in macrophagy that motility becomes extremely important because for a gut to work the body must be positioned in relation to food items (locating, catching and directing them in the mouth/gut), which involves a sensorimotor process that actively and precisely manipulates the environment at the bodily scale.

In general, Cnidaria have the capacity for diverse motility-based interactions with the environment that are mainly manifested as highly directional movements of various parts of their body that in several species (jellyfish) is combined with swimming with increased maneuverability. Cnidaria catch their preys through a combination of the internally generated fast rhythmic movements used for swimming with the sharp and directed movements of their tentacles[23] and the timely activation of nematocysts—which are discharged upon a prey touches the tentacles, thereby capturing or paralyzing it.[24] The swimming is realized through the contraction of their bells in several different rhythms, thus reducing the space inside the rim of the bell, while forcing water out through the opening, and the springiness of the mesoglea (the jelly like part of the Cnidarian body) powers the recovery stroke. What is the bodily organization behind all this interesting behavior?

Cell Types

Cnidaria (as well as ctenophores) have almost a similar number of cell types as porifera (Bell and Mooers, 1997; Carroll, 2001), however, their cellular diversification is different, and this is strongly related to the difference in the mode of feeding. Passing from distributed microphagy to a more centralized external digestion without a gut to an external digestion within a gut cavity (macrophagy) there is a need for distinct cells that circulate nutrients (through contractility), uptake, and excrete nutrients in the internal cells of a three-dimensional body. Apart from the feeding-related cells in Cnidaria and Ctenophora we also find distinct cells related to the sensorimotor behavior of the animal such as myoepithelial cells or pure muscle cells (in the case of Ctenophora), nerve cells (mostly arranged in nerve nets) and several sensory cells (see text below for cnidaria and in section 5 for Ctenophora, but also Arendt et al., 2015).

Sensorimotor Coordination

Jellyfish have primitive 'sense organs' that form part of the epidermis. They consist of neurosecretory cells and of small arrays of sensory neurons, which function as sensory and motor-neurons that establish bi-directional synapses (mainly) with myoepithelial cells and nematocysts, thus providing particular sensory modalities—i.e., light reception through simple eyes, chemoreception through epithelial sensory cells, sensation of gravity through statocysts, and mechanoreception (touch and vibration) through cilia and cnidocytes or nematocytes (see Jacobs et al., 2007 for details).

The activity of all moving parts (bell and tentacles) is realized through the cnidarian musculature that is formed by myoepithelia[25] that together with the epithelial tissue are the effectors that provide coordinated (synchronized) local

[22] Ctenophora lack most of the secreted antagonists to the TGF-B pathway, appear to lack Hox and ParaHox genes (Moroz et al., 2014), the orthologs of several HedgeHog pathway components, and the JAK/STAT pathway. Nuclear receptors (NR) are expressed only after the onset of gastrulation, and the same goes (most importantly) for the canonical WNT pathway, while they have fewer ligands and receptors for the canonical WNT pathway than do cnidarians (Mullikin et al., 2010; Pang et al., 2010).

[23] In the case of Anthozoa there is no swimming but predation happens through the use of moving tentacles and their nematocysts.

[24] A nematocyst is a stinging organelle contained in the specialized stinging cells (cnidocytes) that are located primarily at the tips of the tentacles, and secondarily in the two epithelial layers (the outer layer called epidermis, and the inner one called gastrodermis).

[25] All muscular structures described so far in Cnidaria are epitheliomuscular. Epithelial smooth muscles are generally regarded as primitive features and typical for Cnidaria. Anthozoa (corals and anemones) possess only smooth muscles and striated muscles have only be reported in one very derived clade of swimming

contractions. However, this comes with important limitations on the variety of patterning possibilities (i.e., the degree of plasticity and flexibility achieved by excitable myoepithelia)[26]. The cnidarian body overcomes this limitation with the differentiation of a nervous system (NS), which is composed of distributed nerve nets associated with simple sensory receptors that are distributed radially around the body of the animal.[27] The effect of this arrangement of nerve nets permits the generation of different action potentials by the rapid communication of a stimulus (through the release of neurotransmitters) from a part of the animal to any other part, over relatively long distances and with a significant degree of modulation, which is not possible in animals lacking neurons. This allowed for both quicker responses and the enhancement of functional diversification of the myoepithelial patterning capabilities.

As described above, Cnidaria have primitive 'sense organs' that form part of the epidermis, and which consist of neurosecretory cells and of small arrays of and sensory neurons operating in certain different modalities. However, none of these sensorial modalities could be put into work without the existence of a nerve net operating as an intermediate layer integrating the sensory stimuli with several motor outputs in a timely and diverse manner. Nerve nets are used to support and integrate multiple distinctly different actions generated by different patterns of neural activity (Satterlie, 2008). In the hydrozoan jellyfish *A. digitale*, for instance, some parts of the nerve net are fused forming longitudinal or circular tracts innervating its swimming muscles thus allowing very fast signal conduction, which in turn can support fast attack or escape reactions or slow rhythmic swimming for feeding. The centralization of the nervous circuitry in *A. digitale* and in other hydrozoans has resulted in the formation of two nerve rings along the bell margin (inner and outer), both of which play the role of extra distinct neural components in the modulation of its motility. The outer nerve ring is connected to the sense organs and has a sensory function, whereas the inner nerve ring has a motor-sensory function, regulating the contractions of the umbrella as a pacemaker (Satterlie, 2008). This instance of implicit functional subdivision in *A. digitale* results in an underlying neural circuitry, in which the fast escape part can override the part responsible for the regular swimming but the former part can also be co-opted by the latter for its own uses (Satterlie, 2002, 2008).

Physiology

Cnidaria (and Ctenophora) have two epithelial layers of metabolically active cells separated by the mesoglea (a metabolically inert substance) thus achieving diffusional contact with seawater for each one of their cells. Contrary to sponge and placozoa, in cnidaria (as well as in ctenophora) there are primitive organs related to the transportation and absorption of nutrients. In cnidaria the endoderm (or gastrodermis – one of the two epithelial layers) forms an internal body cavity called the gastrovascular cavity that lines the gut forming thus a proto-coelenteron. Intake and excretion of materials in and out this gastrovascular cavity happens through a single opening that serves as both mouth and anus. This allows for the consumption of greater food size compared to placozoa and porifera. The coelenteron is almost completely closed and separate from the environment and digestive enzymes can be released and concentrated within this cavity. Food is thus extracellularly digested within the gastrovascular cavity and then it is intracellularly digested within each gastrodermal cell. The transport of digested (soluble) nutrients happens intercellularly via the epithelial cells.

The NS in Cnidaria (together with other epithelial cells) regulates various parts and aspects of their physiology via a neuroendocrine-like activity (Tarrant, 2005). Cnidaria achieve physiological regulation thanks to neuroendocrine[28] cells synthesizing peptide-signaling molecules (acting as neurotransmitters or/and neurohormones)[29] whose circulation occurs primarily through epithelial diffusion, and which regulate a variety of physiological processes such as ingestion of prey, digestion, and excretion of nutrients, oocyte maturation, the pumping activity of the body column, and spawning.

Development

Cnidaria have a more complex body plan compared to the Placozoa and to the Porifera. They are diploblastic, radially[30] symmetrical animals with an oral/aboral axis, a subumbrellar cavity (that is not a coelom), and primitive organs (gut, pharynx, tentacles, and sense organs), and of course, an epithelio-neuromuscular structure. Notwithstanding the richness of the developmental genetic toolkit of placozoa and especially of sponges, the developmental toolkit of Cnidaria is richer than the one of all other basal metazoa in all important aspects characterizing the metazoan body plans such as morphogenesis, regional tissue patterning, axis formation, and cell-type specification.[31]

But equally importantly and complementary to the rich developmental gene toolkit is the fact that Cnidaria possess two layers of complete epithelia that enable their embryonic

anemones. All three classes of Medusozoa are described as possessing both striated and smooth contractile fibers. See Seipel and Schmid (2005) and Burton (2008) for details.

[26]The main problem of epithelial conduction in coordinating and synchronizing muscle sheets of effector cells is the lack of directional and selectively targeted propagation of impulses (i.e., an epithelial conduction cannot circumvent an intermediate tissue without activating it, neither can modulate nor regenerate a signal, see Anderson, 1980 for details).

[27]Actually, as argued by Mackie (2004), the superficial name of 'nerve nets' undermines the neuronal organization of Cnidaria, since some of these nerve nets are considered to reflect a considerable degree of centralization (Satterlie, 2011 for details).

[28]The role of neuroendocrine cells in Cnidaria is played by both sensory cells integrated into the epidermis and by sub-epidermal ganglion cells, where endocrine cells are epidermal epithelial cells and gastrodermal neurons.

[29]Almost all neurotransmitters, neurohormones and non-neuronal hormones are present in Cnidaria. Most signaling molecules in Cnidaria are peptides, but there are also small non-peptide regulators (Kass-Simon and Pierobon, 2007).

[30]Some adult anthozoa even present bilateral symmetry and thus axes differentiation (Leclère and Rentzsch, 2014).

[31]Cnidaria have a complete TGF-b signaling pathway, a complete canonical WNT signaling as well as a surprising diversity of WNT ligands, nuclear receptors (NRs), the Notch/Delta signaling cascade, a complete set of HedgeHog signaling components, Hox and ParaHox genes, the RTK/FGF signaling, and components of JAK/STAT signaling (Babonis and Martindale, 2016).

multicellular masses to clearly exhibit gastrulation and PCP-based body elongation (see Newman, 2016b for details and references therein) in a fashion that in turn allows an efficient and rich intercellular signaling, which propels the further mobilization of various morphogenetic movements providing thus a standardized and determinate body plan with a fixed number of morphological characteristics in a standard arrangement.

The role of the NS is also of great importance in a variety of developmental processes. Specifically, neuropeptides are regulating a variety of developmental and physiological processes, such as the induction or inhibition of neural differentiation, neurogenesis, and oocyte maturation, and morphogenesis (Tarrant, 2005; Takahashi and Takeda, 2015).

DISCUSSION: A THESIS OF BODILY COMPLEXITY

Typical 'complex adaptive bodies' (CABs) with their immense behavioral potential are a very successful evolutionary invention. However, the preceding evolution of the basic configuration of such typical animal bodies involved a set of evolutionary changes that could not have been driven by the eventual fitness options that, after all, only became available when these basic ingredients were in place. In Section "Setting the Stage for Bodily Complexity: Contraction-Based Motility Requires Integrated Bodies and Vice-Versa," we presented and we argued why the animal sensorimotor organization (ASMO) is such an evolutionary key transition. This organization is so familiar to us that it becomes almost invisible and hard to appreciate as a separate achievement of life. Still, it amounts to a major and specific evolutionary transition. The road traveled to this evolutionary point (ASMO) is not straightforward but dependent on bringing the central ingredients together in a specific form of organization.

What is characteristic of this evolutionary road is the transition from cilia-based to contraction/muscle-based motility. The latter enabled large and fast organisms that could move about as large motile agents, which resulted in animals being the dominant macroscopic life-form together with plants. So, organizing bodies capable of motility mediated by muscle-based (and myoepithelial) contractility is a key feature that must have been in place for large and fast animals to become possible. But muscle-based motility itself depends on the combination of many other factors being integrated into a multicellular organization as a single unit. More specifically, as we show, the transition from widely available (and used) cilia to muscle-based motility is a major one that involves: the presence of a body that relies on contraction for reversible motility; developmental processes that generate complex and standardized body forms; a nervous system that coordinates the whole organism in a way that is fast and unifying; complex physiology to keep all cells involved alive and thriving by keeping the whole body 'energized' and oxygenated and that weighs these needs depending on the requirements of the moment (active or resting, etc.); a molecular interface (a gut or what is present for this purpose).

However, as we show (in Sections Porifera, Placozoa, Ctenophora, and Cnidaria) in our discussion of the bodily organization of the four known basal animal phyla and the eventual space of possible multicellular configurations toward ASMO, contraction-based motility is not a self-evident switch from cilia but something that is just one of the many options available for the use of tissue contraction that was present, and which in all these cases is used in some way to change their body configuration and to control their feeding, though in very different ways. Porifera are mainly sessile, and use contraction to control their water canal system, not to move about or manipulate their environment. Placozoa move around by using cilia while exhibiting contractions to change their body shape, and presumably to help digest their food. Neither have standardized bodies in contrast to both Cnidaria and Ctenophora.

Ctenophora have a standardized diploblastic epithelial body with muscles and a NS. However, they appear to have a major organizational difference with respect to how they use their contractions compared to the Cnidaria; Ctenophores rely on cilia as a prime mover of the body, while contraction is mostly used to maintain body shape and (only) secondarily to coordinate the through gut. This is something very different from cnidarians (and bilaterians), which are fully depending on muscle control to interact with their environment. In addition, Ctenophora appear to exhibit less intercellularly regulated development than the Cnidaria since epithelial intercellular signaling doesn't seem to be very operative during their (at least) early developmental stages and their genetic developmental toolkit is significantly decreased (compared to the Cnidaria) from the typical bilaterian genome (Martindale and Henry, 2015, see also footnote 22). Also, the genomic content of nervous system genes is the most reduced of any animal (Simmons and Martindale, 2015).

From a merely biological point of view, these aspects do not necessarily have positive or negative implications. After all, nature has been always exploring several different multicellular organizational forms. However, from the perspective of the evolution of biological organizations (especially when one considers the road toward ASMO), there is a notable implication of the different integration exhibited by the Ctenophora (compared to the Cnidaria). In particular, when one considers the fact that Ctenophora have the most advanced (and complete, though transient) digestive system of all four basal phyla, this suggests that their bodily organization uses tissue contractility much more for the shaping of the animal and the manipulation of digestion than it does for interaction. As a matter of fact, as Tamm (2014) notes, in contrast to the global functionality of nerve nets and myoepithelial conduction that can trigger behavioral responses anywhere on the animal and spread them to all related effectors, the neuromuscular organization of ctenophores is spatially confined and interacts only indirectly with other biomechanical and neural conducting pathways. Consequently, as ctenophores move by cilia, which is characteristic of unicellular organisms, and exhibit a rather minimal presence of reversible, contraction-based changes in body-shape, leave alone in body appendages, we conclude that they exhibit an ASMO only in a basic and atypical way.

So, while contractile free-movement is key and the capacities for contraction are present from the epithelial start, their use for motility is hard. And while the use of cilia can be seen as generating a local optimum, there is, as we show, no straightforward pathway from the presence of contractile epithelia to contraction-based motility. This would require an evolutionary benefit over cilia that are much easier to use and control. But getting to this evolutionary point, is difficult because, as we argue, it first requires the fulfillment of various organizational conditions.[32] This is exactly what happens in the Cnidarian organization.

In Cnidaria, the whole body is hold together due to its genuinely epithelial nature. Nervous systems operate as a regulatory sub-system that spatiotemporally coordinate the combinatorial execution of local contractions of distant groups of epitheliomuscular cells throughout the whole animal body, thereby maintaining sensorimotor loops. Cnidaria show a diverse repertoire of movements in which either the whole body (in jellyfish), its parts or several combinations of them (in all species) can perform fast, reversible, and well-coordinated movements. This coordination results in a functionally rich behavior only because it is integrated in a body plan with a set of primitive and differentiated organs that provide the animal with the proper physiology (metabolic and biomechanical requirements) for its behavior. As discussed in detail (see Section Cnidaria), the common basis of integration between sensorimotor and physiological coordination is the diploblastic epithelial organization and the NS that, together with an abundance of neuro-transmitters, neurohormones and non-neuronal hormones, modulate physiological processes and ensure global homeostasis and growth. The emergence of such a coordinated and standardized body is only possible through specific developmental regulation exhibited only in a genuinely epithelial development (Arnellos and Moreno, 2016), where the combination of epithelial morphogenetic movements with the rich intercellular signaling provide a continuously unfolding system coordinating the development of such complex bodies (Arnellos et al., 2014). Again, the NS (together with various neuropeptides) is actively modulating the development of several (even late) developmental stages of the adult Cnidaria. This is exactly how the neuro-epitheliomuscular Cnidarian body achieves such as strong integration between its behavioral, physiological and developmental processes.

The bodily complexity (and the related form of integration) exhibited by Cnidarian organizations has several implications. A most prominent one is that Cnidaria show motility-based interactions that are completely new (and thus different) compared to the ones demonstrated by their unicellular parts. This is not the case neither for the Porifera, where most of their contraction-based actions are similar to the ones of choanoflagellates nor for the Placozoa where motility is done by cilia and digestion is external just as the case of unicellular eukaryotes, nor quite exactly for the Ctenophores which, after all, they still move via cilia. In this way, the Cnidarian organization makes *a qualitative jump—a major transition*—regarding the capacity of a multicellular body for contraction-based interactions compared to the other early metazoa. In the account we sketched, the Cnidarian body exhibits the special organizational conditions necessary for muscle-based contractions to be reused as a motile force. This is why Cnidaria show an unequivocal ASMO. Consequently, of the four phyla discussed, the cnidarians come closest to the features that are central to the bilaterian 'complex adaptive bodies' (Trestman, 2013). They exhibit a specific form of integrated organization, where multicellular contraction and its sensory potential are fully established, even when the various senses remain rather limited, barring a few exceptions.

The events constituting the evolutionary transition that resulted in modern animals, including the bilaterians, happened a very long time ago and we do not yet have a sufficiently clear grasp of these events (e.g., Erwin, 2015). In order to assess some of the possibilities for basic animal configurations, we looked at the characteristics of the few early diverging phyla that still have living representatives. As we are all highly familiar with typical bilaterian animals, it may seem that this configuration is actually the most natural and normal one. By surveying the four basal animal phyla, we hope to have shown that this is not the case and that the basic features that are, to some extent, present in all animal phyla, can come together as functioning organisms in very different ways. In this way an organizational account helps to set aside bilaterian-influenced descriptions of non-bilaterian animals. Moreover, it seems that the evolutionary road toward a muscle-based system of motility is not a straightforward one. The ability to use contractions for efficient movement and sensing at a multicellular scale—which must be considered the key to the major success of bilaterians as typical animals—can only be achieved on the condition of a complex, highly differentiated and specifically integrated bodily organization. Getting such a complex bodily organization in place sets up a major evolutionary bottleneck when it comes to the evolution of typical animals.

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AA and FK contributed equally to the conception, design, preparation, and writing of the manuscript.

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[32] This is a characteristic and generic requirement for all biological organizations, namely, their differentiated functionality should first be in place in order for natural selection to be able to operate on them (see Arnellos and Moreno, 2012; Moreno and Mossio, 2015 for extensive discussions).

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CONCEPTUAL ANALYSIS
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Glycemia Regulation: From Feedback Loops to Organizational Closure

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Endocrinologists apply the idea of feedback loops to explain how hormones regulate certain bodily functions such as glucose metabolism. In particular, feedback loops focus on the maintenance of the plasma concentrations of glucose within a narrow range. Here, we put forward a different, organicist perspective on the endocrine regulation of glycaemia, by relying on the pivotal concept of closure of constraints. From this perspective, biological systems are understood as organized ones, which means that they are constituted of a set of mutually dependent functional structures acting as constraints, whose maintenance depends on their reciprocal interactions. Closure refers specifically to the mutual dependence among functional constraints in an organism. We show that, when compared to feedback loops, organizational closure can generate much richer descriptions of the processes and constraints at play in the metabolism and regulation of glycaemia, by making explicit the different hierarchical orders involved. We expect that the proposed theoretical framework will open the way to the construction of original mathematical models, which would provide a better understanding of endocrine regulation from an organicist perspective.

Keywords: organicism, feedback loop, organizational closure, glycemia regulation, proof of concept (POC), functional constraints

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INTRODUCTION

In recent years, an increasing number of contributions in theoretical biology and philosophy have been advocating an organicist perspective (Gilbert and Sarkar, 2000; Etxeberria and Umerez, 2006; Soto and Sonnenschein, 2006; Moreno and Mossio, 2015; Soto and Sonnenschein, 2018). According to organicism, theoretical and experimental biology – and notably physiology – should address aspects of living systems in light of their integration into a coherent unit, understood as a natural system endowed with a distinctive complexity[1].

One of the fundamental notions of organicism is 'organization,' a concept more specific than a mere synonym of 'configuration' or 'arrangement,' which relies on a rich theoretical tradition

[1]By 'distinctive complexity' we mean those aspects that are *specific* to living systems, notably linked to their functionality (Longo et al., 2015). It refers to their proper "way of being." Understanding biological complexity is therefore a central task of biological research. Complexity should not be confused with notions as 'complication,' which has a rather negative connotation and refers to aspects that might be difficult to access theoretically or experimentally, or both.

inspired by the work of Kant (1790) and Bernard (1865) and further developed in the 1960's and 1970's (Piaget, 1967; Rosen, 1972; Pattee, 1972; Varela et al., 1974; Gánti, 1975). We use the term 'organization' to refer to a certain mode of interaction between the parts of a system, distinctively realized by biological organisms when compared to other kinds of natural systems or to artifacts. In a first approximation (see Section "Organizational Principles" for more details), organization refers to a regime in which (1) a set of parts are related to each other so as to constitute a system that displays both functional differentiation and integration; (2) the activity of the whole system plays a role in producing and maintaining its parts over time: organized systems maintain themselves.

Although organicism is gaining momentum in the theoretical literature, a wider reception in biology would be achieved if its applications were shown to improve experimental and modeling practices. In the context of the issue topic "Multilevel Organization and Functional Integration in Organisms," the general objective of this investigation is to propose a "proof of concept," an illustration of how organicist – and more precisely, organizational – principles can advantageously modify biological modeling. We do so by focusing on a particular case study: the regulation of plasma glucose concentrations (glycemia) in mammals.

Our aim is to show that the representation of this phenomenon substantially changes when shifting from a standard characterization in terms of feedback loops to an original one grounded in organizational principles. Feedback loops are control devices that, although not specific to biology, have become since the 1930's an important tool in different areas of biology (e.g., neurophysiology, see for instance Lorente de Nó, 1934), often in association with the idea of homeostasis (Cannon, 1929). Since then, they have been used to model and understand dynamically stable situations in which the value of a variable appears to be actively maintained within a given range (Wiener, 1948).

Feedback loops also provide a useful description of certain complex physiological control phenomena. In endocrinology, the concept of negative feedback plays a central role in the understanding of the maintenance of calcium and glucose plasma concentrations within a narrow range (Widmaier, 1992; Wilkin, 1997; Mundy and Guise, 1999; Carmeliet et al., 2003). Positive feedback also plays an important role in endocrinology although, in contrast to the negative feedback, it is used to explain how an effect is amplified by creating instability, such as the positive oxytocin loop of parturition and the estrogen-triggered positive feedback of ovulation (Higuchi and Okere, 2002; Russel et al., 2003; Christian and Moenter, 2010).

In spite of their widespread and practical use in developing dynamical models of how physiological variables are maintained as stable, we claim that feedback loops might bring about a weakening of biological explanation. In particular, the description of glucose regulation – the phenomenon on which we focus here – in terms of feedback has three main problematic implications: first, it tends to neglect the nature of the relations between the parts and their place within the whole organism; second, it flattens the description of the system by overlooking the various categories of objects in play as well as their hierarchical relations; third, it is built on the relationship between concentrations of glucose, insulin and glucagon, and does not foster the inclusion of further factors involved in the regulation of glucose metabolism, such as the nervous system and the gut. In contrast, we posit that the organicist perspective promotes a more specific understanding of how biological organisms realize homeostatic behavior in relation to certain variables. Biological homeostasis is derived from more fundamental organizational principles and explained as a result of the regulatory capacity of a functionally differentiated hierarchical biological organism.

It is worth underscoring that our goal here is not to provide a full-fledged model of the regulation of blood glucose concentration, although we do provide some preliminary guidelines. Rather, we aim to show how organizational principles allow us to take into account the characteristic complexity of biological systems when focusing on a specific phenomenon and, therefore, open the way to the elaboration of richer and more appropriate models.

THE STANDARD REPRESENTATION OF GLYCEMIA REGULATION: THE FEEDBACK LOOP

The model of homeostasis through negative feedback has a long history. It dates back to the introduction of the notion of "conservation of the internal milieu" by Bernard (1865) in the 19th century and Cannon's (1929) subsequent introduction of the concept of homeostasis in the early 20th century. Negative feedbacks were explicitly formalized by cybernetics (Wiener, 1948) and traditionally used in engineering in the 1960's (see Lewis, 1992, chapter 1). Standard accounts of feedback can also be found in the classical literature of systems theory (see for example Rosen, 1968, p. 37; Jones, 1973, p. 80).

A negative feedback describes phenomena in which the value of the output of a system is used to modify the activity of the system (the output "feeds back") in such a way as to create a loop that reduces fluctuations in the output itself, and to make its value homeostatic within a specific range. According to Rosen (1968, p. 37), a feedback loop is characterized by the presence of a controller and a controlled system: "The relation of the controller and the controlled system is the following: the inputs to the controller are the original inputs to the system, together with the outputs of the controlled system; and the inputs to the controlled system are the outputs of the controller. Thus, in effect the controlled system is supplied with a new input set, determined partly by its own past activity; i.e., we have a system with a feedback." As mentioned, feedback loops are widely used to model homeostasis in numerous biological fields. Although they share the core idea, different models vary regarding the number and kind of elements constituting the feedback system. In what follows, we rely on a recent characterization provided by Modell et al. (2015), which gives a general and complete description of the feedback loop, and is considered as a standard for education purposes in physiology. While most feedback models usually focus on the relationship between a reduced set of variables, this

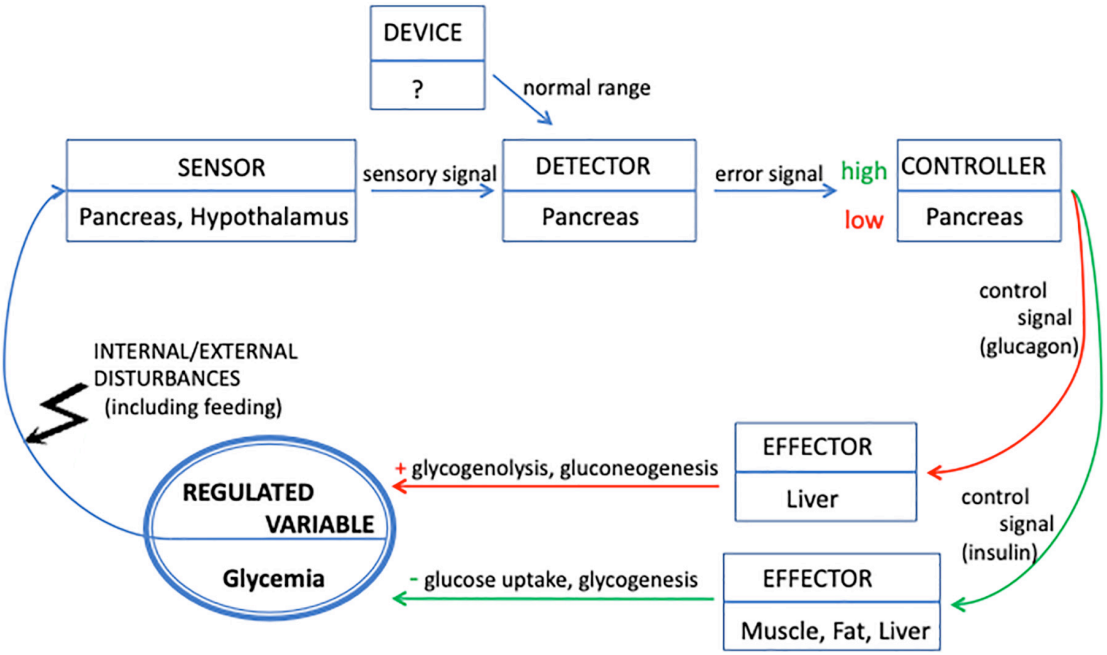

FIGURE 1 | Representation of glycemia regulation in terms of a feedback loop. The regulated variable (glycemia) is represented by the bold circle. The functional components involved in the feedback loop are represented by rectangular boxes indicating their localization in the organism. Blue arrows indicate interactions between components. Red arrows indicate interactions (signals and metabolic processes) that, triggered by low glycemia, produce an increase in glucose concentration. Green arrows decrease the glucose concentration as a response to high glycemia. The system feeds back into itself by sensing the value of glycemia, which is the target of its regulatory activity. The feedback loop maintains the value of glycemia as homeostatic by modulating the release of insulin and glucagon on the basis of the difference between the actual value and the normal range.

description has the advantage of making explicit *all* the elements that are often left implicit.

There are five components that a system must contain in order to realize a feedback loop and to maintain a target variable – the "regulated" one – as homeostatic, i.e., within a specific interval (see **Figure 1**). Paraphrasing Modell et al. (2015), these are:

(1) A *device/mechanism* that establishes the *normal range* ("set point") of values for the regulated variable;
(2) A *sensor* that measures the actual value of the regulated variable and emits a *sensory signal*;
(3) A *detector* that compares the actual value (through the sensory signal) with the normal range. The result of this comparison is an *error signal* that is sent to the controller;
(4) A *controller* that interprets the error signal and determines – through a *control signal* – the value of the outputs of the effector;
(5) An *effector* that responds to the *control signal* and modifies the value of the regulated variable.

As Modell et al. (2015, p. 261) explain: "Such a system operates in a way that causes any change to the regulated variable, a disturbance, to be countered by a change in the effector output to restore the regulated variable toward its set point value. Systems that behave in this way are said to be negative feedback systems."

Let us apply this description to the case of glycemia regulation and locate the components of the feedback loop within the organism. The description of glycemia homeostasis should show how the organism manages to maintain a steady supply of glucose, regardless of whether it is feeding or fasting. Glucose, the *regulated variable*, is the principal source of energy for the organism in general, and particularly for the brain.

During fasting, the liver (the main *effector*) breaks down stored glycogen, and glucose is secreted into the bloodstream. The activity of the liver is modulated by hormones that, in the terminology of feedback control, are said to carry signals. Glucagon, a hormone produced by the pancreas, increases glycogenolysis and the synthesis of glucose from amino acids and lipids (gluconeogenesis) in the liver. In turn, the increase of glycemia triggers in the pancreas the release of insulin, the hormone that facilitates the entry of glucose into the muscle compartment (the other *effector*) where it is needed for physical activity and inhibits glucagon secretion. Muscular uptake decreases glycemia, which induces the pancreas to secrete more glucagon, and so forth (see **Figure 1**). In this picture, that focuses on the role of hormones in modulating the effectors' activity, the role of *sensor* is supposed to be played by chemosensors located in the pancreas and hypothalamus, structures that also play the role of *controllers*. In contrast, the *detector* is not clearly distinguished and located; however, it is inferred to be located in the pancreas. A very important point here is that the rate of secretion of hormones, as well as their effect in controlling glycemia, is not an "all or none" phenomenon.

That is, glucose, insulin and glucagon are always present in the bloodstream.

Feeding leads to the storage of glucose as glycogen. More precisely, food intake, digestion and absorption increase glycemia in the systemic circulation that reaches all organs; in particular, the increase is massive in the portal blood that supplies the liver. High glycemia is sensed by the pancreas that, by also playing the role of *detector* and *controller*, triggers a response mediated by its beta-cells. These cells release insulin into the bloodstream. Insulin promotes the transport of glucose into cells, predominantly those in skeletal muscle and adipose tissue (both tissues are *effectors*), and its conversion into glycogen in these compartments as well as in the liver. As a result, glycemia decreases.

In both situations, a negative feedback is said to occur between low glycemia and insulin concentration, because pancreatic beta-cells slow down the rate of insulin secretion when glycemia is low, which results into the stabilization of glycemia at some low concentration. Additionally, a second and simultaneous chain of events takes place. Low glycemia, stabilized by the decreased rate of insulin release, suppresses the inhibition of alpha-cells, which increases the rate of glucagon release. Glucagon promotes the hydrolysis of glycogen in the liver and release of glucose in the blood, which increases glycemia. This set of coupled processes generates a homeostatic situation, in which glycemia is maintained within a viable range by compensating for both decreases and increases of glycemia. During both fasting and the intestinal absorption of sugars, the circularity of glucose homeostasis is given by the fact that, starting from normal glycemia, variation in glucose concentration results in the restoration of normal glycemia. Modell's description of the feedback system now applied to the control of glycemia is shown in **Figure 1**.

The diagrammatic representation of glycemia regulation in terms of feedback loops serves the purpose of focusing on and making explicit the circular relations between coupled variables (the concentrations of glucose, insulin, and glucagon) that are relevant to explain the homeostatic behavior of the organism, in relation to one of these variables, namely, glycemia. The negative feedback diagram provides a useful tool for global evaluation of the ability of the organism to blunt the increase of blood glucose concentrations after administering a glucose overload. Indeed, the glucose tolerance test provides a measure of the ability of the organism to regulate glycemia and thus to evaluate normalcy, pre-diabetes and diabetes.

Yet, in spite of the descriptive role and clinical usefulness of the feedback loop, we submit that such a representation does not foster an adequate understanding of biological organization because most functional, topological and hierarchical features of the organism remain hidden. What is put forward is the relation between variables, without revealing much about the complexity of the organism that should be spelled out to better understand how it controls its own glycemia.

We think that the inadequacy of feedback loops takes four forms. First, they tend to favor the idea of a neat localization of functional components. While it usually works for manmade machines because their parts have been designed separately and assembled, a neat localization applies much less clearly for organisms, in which a given function can be jointly performed by several components and a given component or structure can perform different functions. In addition, some functions can be distributed over the entire system and thus are non-localizable. Second, feedback loops tend to represent the system as a *flat* chain of interacting components. As the above diagram illustrates, the system is described as a set of functional components realizing a chain of steps, with no hierarchies or distinction of levels. Although they perform different functions, each component interacts in the same way with the following one in the chain, by either activating or inhibiting its activity (be it through a signal or not), following the kind of perturbation affecting the system. In this respect, there seems to be – to use a philosophical expression – only one kind of "causal relation" at work in a feedback loop, which makes us claim that the resulting representation flattens the characteristic complexity of the system. Third, feedback loops do not foster the search of additional components and variables that might play a role in the homeostatic behavior. Of course, feedback diagrams can be enriched by new empirical knowledge; yet, they focus exclusively on the relation between several variables (in our example, the concentration of glucose, glucagon and insulin) so as to understand the stability of the variable of interest (glucose). Accordingly, they ignore – and do not encourage exploring – the relationship between these variables and other physiological components which converge in diverse ways to control the concentration of glucose. Fourth, a description in terms of feedback assumes the existence of a value (or, more precisely, an interval of values) to be kept stable – a set point – without providing an explanation of how it is established or how it can be modified.

The epistemological stance that lies behind these weaknesses, we submit, is the classical cybernetic analogy between organisms and machines, which are supposed to realize the same kind of control capacities. Although some aspects of biological organisms can certainly be described in a way commonly used to describe machines, the analogy can be misleading in several ways as it risks concealing crucial differences between the two classes of systems. In particular, this holds for homeostasis, whose description in terms of feedback emphasizes the common capacity of organisms and machines to maintain a given variable as stable while overlooking biological specificities. While in machines homeostasis is a goal determined by an external designer, in organisms it constitutes a means to achieve the more fundamental goal to maintain oneself as alive. Contrary to machines, organisms maintain some variables as stable only insofar as this promotes their self-maintenance, which means not only that homeostasis is achieved in a different way, but also that alternative behaviors and variations can be observed in some circumstances. In this respect, a fundamental difference between organisms and machines points to the device that establishes the normal range of the regulated variable. In machines the normal range, set by the human designer, is recorded in some component of the system, while in organisms, as mentioned above, it is unclear what process sets the normal range (many biologists would presumably appeal to evolution). Furthermore, it is highly debatable whether the normal range is recorded in some specific

component of the system (Fitzgerald and Bean, 2018). While feedback loops are blind to these differences, we submit that the organizational framework fosters a more adequate understanding of organismal homeostasis (and specifically glucose homeostasis) as a manifestation of distinctive biological capacities.

ORGANIZATIONAL PRINCIPLES

The theoretical framework built on the notion of organization characterizes biological systems as autonomous, i.e., endowed with the distinctive capability to constantly produce, transform, repair and replace their own components, and maintain themselves through exchanges of matter and energy with the environment (Piaget, 1967; Rosen, 1972; Varela et al., 1974; Kauffman, 2000; Moreno and Mossio, 2015). Unicellular and multicellular autonomous systems locally oppose the increase of entropy and the thermodynamic tendency toward equilibrium. They maintain themselves in far-from-equilibrium conditions – i.e., in highly improbable dynamic distributions of energy and matter – by *controlling* the thermodynamic flow.

Biological control can be defined as the capacity to modify the dynamics of a system toward a certain state (e.g., an enzyme acting upon concentrations of metabolites, see Rosen, 1970; Fell, 1997). It implies an asymmetry between the controller and what is controlled[2]. In biological systems control is exerted by molecular and supra-molecular structures (such as enzymes or membranes), by cells, extracellular structures, tissues and organs that are produced and maintained by the system itself. These structures act as *constraints* on the thermodynamic flow. A constraint is a structure that has a causal effect on a process (or transformation) while being locally unaffected by the process at the time-scale in which it takes place (Montévil and Mossio, 2015). Constraints play the role of local boundary conditions that enable specific processes to take place by reducing their degrees of freedom[3] (Pattee, 1972; Kauffman, 2000). By doing so they locally channel the flux of energy and matter, chemical reactions, etc. toward outcomes that can contribute to the functioning of the system, and that would be extremely improbable (or practically impossible) in the absence of such constraints. A paradigmatic example of a biological constraint is an enzyme that, by lowering the activation energy necessary for a reaction, catalyzes it toward an otherwise improbable product, which in turn can be employed to perform some functional activity for the system. Another example is given by the structures constituting the vascular system, which constrain the circulation of oxygen to the neighborhood of cells, where it participates in respiration. As a matter of fact, it has been argued that any biological structure or part to which biologists ascribe a *function* can be conceptualized as exerting a constraint on a process or transformation (Mossio et al., 2009).

The specificity of living systems is that they are *organized*, by which we mean that their constitutive constraints collectively produce and maintain each other and, ultimately, the whole system itself. The resulting organization realizes a distinctive regime, called *organizational closure* or *closure of constraints*, in which the very existence and activity of a set of constraints depends on their mutual relations and interactions (Moreno and Mossio, 2015; Montévil and Mossio, 2015). Unlike other natural self-maintaining systems such as dissipative structures – which are spontaneous, are mostly or fully determined by external boundary conditions and emerge anew each time under specific environmental conditions – living systems are historical and vastly contribute to determine their own conditions of existence (Mossio and Bich, 2017).

At the intracellular level, the coordinated activity of organized constraints such as proteins, membranes and nucleic acids, contributes to the realization and maintenance of the organized system that contains them, by channeling the flow of matter and energy necessary to build these components and to run the internal processes of the system (Ruiz-Mirazo and Moreno, 2004). Multicellular systems, while constraining thermodynamic processes to sustain their own metabolism, also exert control upon the activity of the cells that constitute their tissues and organs (Arnellos et al., 2014; Longo et al., 2015; Montévil et al., 2016; Soto et al., 2016; Veloso, 2017; Bich et al., 2019). At both organizational levels, the set of mutually dependent control constraints is responsible for the realization of what we label the *first-order regime of closure*[4] (**Figure 2A**).

Yet the first-order regime is only one dimension of the living organization. In fact, one of the distinctive features of biological autonomous systems is their adaptivity, and in particular the capacity to modify their own first-order regime in relation to changes in internal needs and external conditions. Such a capacity can take two main forms. On the one hand, the first-order regime, while performing metabolic activity, exhibits some dynamic stability that makes it capable of compensating *as a network* for small perturbations, mostly stoichiometrically[5] by means of changes in concentrations (for example, by relying on the balance between supply and demand of metabolites). In such cases the network is maintained by the same attractor or shifts toward a new one among those available (in case of multi-stable networks).

On the other hand, living systems possess a specialized class of organized constraints (which means that they are also maintained by the organism), that we label *regulatory*, that act as higher-order controllers upon first-order constraints. Regulatory constraints modulate the first-order regime of closure in response to specific variations induced by external perturbations, but also by internal dynamics of the organism (Bich et al., 2016). They do so by selectively inducing shifts between distinct available

[2]By 'asymmetry' we mean here that control is not a mutual interaction between entities, but a relation in which it is possible to distinguish between what acts and what is acted upon.

[3]By 'degree of freedom' we mean the number of variables of a process that are free to vary independently.

[4]The organizational framework considers that the hierarchies of *levels* and *orders* are distinct. A 'level' designates a closed regime of constraints that is either made of constituents, or included in an encompassing system, realizing themselves a distinct closure. An 'order,' in turn, designates a kind of functional object that operates over (modulates) other functional objects, within the same regime of closure. See Moreno and Mossio (2015, p. 143) for details.

[5]That is, in terms of quantitative relationships between different compounds involved in chemical reactions.

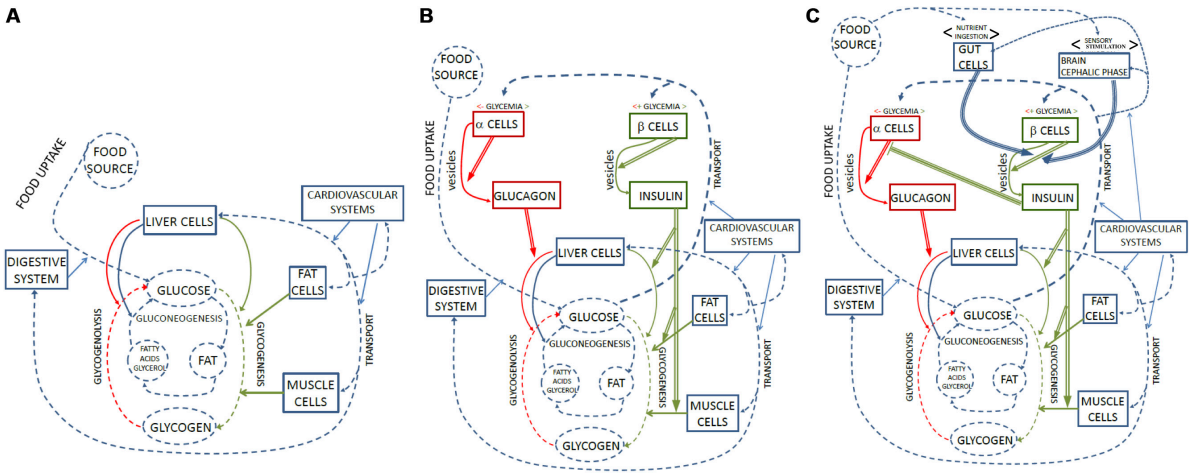

FIGURE 2 | Representation of glycemia regulation in terms of organizational closure. **(A)** Represents the first-order functional regime, in which a set of functional parts (rectangular boxes) constrain (blue arrows) processes and transformations (dotted blue arrows) involving glucose as a product or metabolite. Metabolic substrates and products are in dotted circles. Constraints and processes in red and green are those that affect glucose and glucagon concentrations. Organizational closure is realized by the fact that each part acting as a constraint is also the product of a metabolic process within the system. As a result, the system self-maintains. **(B)** Includes second-order regulatory functions to organizational closure. The regulatory subsystem comprises parts which play the role of constraints that are sensitive to glycemia (sensitivity to *x* is marked by <*x*>above the pertinent box) and second-order constraints (double arrows), which modulate the activity of first-order ones. Regulatory constraints marked in red are sensitive to low glycemia and accelerate glycogenolysis; regulatory constraints marked in green are sensitive to high glycemia and accelerate glycogenesis. The overall result of regulation is the homeostasis of glycemia, which is not represented as a separate 'component' in this figure, but constitutes an effect of the biological dynamics controlled and regulated by different orders of organized constraints. **(C)** Adds third-order regulatory functions. The regulatory subsystem includes parts that play the role of constraints sensitive to food (either by ingestion or perception) and exert third-order constraints (triple arrows) on second-order constraints. The inhibitory action of insulin on alpha-cells is also interpreted as a third-order constraint. Second- and third-order regulatory constraints are also the target of metabolic processes, which contribute to their maintenance: therefore, the whole hierarchical system realizes organizational closure.

physiological[6] or agential regimes[7]. A typical example is the regulation of the direction of movement in bacteria performing chemotaxis, which depends on the sensing of sugar gradients and is achieved by shifting the motion of the flagellum between tumbling and rotating[8]. Another example of dynamics requiring regulation is protein synthesis. Not all possible proteins can be available simultaneously in the system, due to spatial and energetic limitations; therefore, the various components should be synthesized just when they are needed. Also, some subsystems may work differently and with different requirements, not always compatible, and their operations need to be modulated in such a way that they can jointly contribute to the maintenance of the system while avoiding potential conflicts (Bich, 2018)[9].

Regulation provides the organism with the possibility of acting upon its own dynamics. Conceptually, a regulatory subsystem includes a set of dynamic constraints operating in a way that is distinct from first-order constraints, and *collectively* satisfying two main requirements:

(1) The presence of constraints that act as second-order controllers, which means they modulate the activity of other constraints in the system, instead of directly channeling metabolic processes (which is what first-order constraints do)[10];
(2) The presence of constraints that, by being specifically sensitive to variations in internal or external conditions, begin to perform a qualitatively different function and, thereby, bring about the activity of the regulatory subsystem.

The first requirement implies that there is no regulation when the change of first-order dynamics is not due to the action of a constraint but, instead, to a variation in the concentration of a metabolite, which changes the rates of the metabolic pathways because of the effects of the law of mass action. The second requirement, in turn, means that regulation is a context-sensitive phenomenon, which is triggered by specific conditions, as for example when an enzyme reacts to an allosteric interaction. In particular, there must be constraints that perform a function only when a given internal or external

[6]For example, diauxic shifts in the metabolism of bacteria between different carbon sources, regulated by switching the genes involved in the synthesis of the relative enzymes on and off.

[7]Autonomous agency can be defined as the set of activities that modify the environment of the system that generates them and are "performed according to a certain goal or norm" (Moreno, 2018, p. 290), which is related to the maintenance of the system itself.

[8]See Bich and Moreno (2016) for an analysis of bacterial chemotaxis in terms of regulatory constraints.

[9]For a discussion of the importance of regulation to understand life and its origins, see also Cornish-Bowden and Cárdenas (2019).

[10]An example of a second-order constraint is a kinase enzyme catalyzing the phosphorylation of another enzyme in the system, leading to the activation or inhibition of the latter, while being locally unaffected by such interaction (for example, the kinase is not consumed in the reaction).

variable (to which they are "sensitive") undergoes a specific change[11] (**Figure 2B**).

For regulation to be functional in the context of the organism, there must be a connection between what stimulates the regulatory subsystem and what this subsystem acts on. It means that the regulatory action should make the first-order dynamics viable in the context that activated regulation in the first place. For instance, there is a relation between blood glycemia and glycogenesis, so that a modulation of the latter constitutes an adaptive response to the viability challenge raised by a significant variation of the former. Yet, this relation does not take the form of a direct dependence, which means that variations of the conditions that activate a sensitive constraint (e.g., through an allosteric interaction) affect the subsystem differently than variations of the processes on which second-order constraints exert their control[12]. This asymmetrical relation is what we have referred to elsewhere as "dynamical decoupling," between regulatory and regulated constraints (Bich et al., 2016), by which the regulatory subsystem exhibits degrees of freedom that are not specified by the dynamics of the regulated one. Such a local independence allows regulatory subsystems to modulate first-order constraints in a relatively autonomous way.

In brief, regulatory subsystems exhibit context-sensitive activity[13] that adaptively modulates metabolic processes by acting upon first-order constraints. They act when specific conditions are met, so as to maintain the overall viability of the system. Let us apply these ideas to the case of glucose regulation.

REINTERPRETING GLUCOSE REGULATION FROM ORGANIZATIONAL PRINCIPLES

How do organizational principles guide the elaboration of a biological model? To study a phenomenon, the organizational framework requires the identification of the relevant processes, the time scales at which they can be described, and the first-order constraints acting upon them at those times scales. Once these objects are identified, the next step consists of determining the dependencies between the constraints so as to obtain a closed graph, in which at least a subset of constraints are maintained by processes under the control of other constraints, so that the entire network can be said to realize collective self-maintenance. Moreover, if the aim is to understand how this network of constraints is modulated in response to external perturbations or changes in the internal state of the system, second-order dynamically decoupled constraints need to be identified and integrated into the closed graph of dependencies[14].

Let us illustrate how this general procedure can be applied by considering the case of glycemia regulation in multicellular systems, specifically mammals. Glucose *as a metabolite* constitutes a primary energy source for these organisms. The plasma concentration of glucose depends on food uptake and on those internal metabolic processes (as well as organismal activities, such as exercise) that consume, transform, store and release this sugar. As shown in the feedback loop diagram, glycemia is kept within a specific interval in the blood despite variations affecting the supply and consumption of glucose. From an organizational perspective, such a homeostatic behavior is achieved through the contributions of both first-order compensatory capacities and higher-order regulatory functions, the latter ones involving different organs and resulting in the modulation of the whole first-order metabolic regime.

To describe the regulation of glucose metabolism that results in homeostasis, it is first necessary to identify the key processes involving glucose as a metabolite and the first-order constraints that control it. This first-order regime is a theoretical extrapolation, because there is no real organism devoid of regulatory constraints. However, experiments removing the constraining action of insulin and glucagon in mice give us a "real-life" approximation of how the first-order regime would work in the absence of the main regulatory constraints (Unger and Cherrington, 2012). As we will see, some of these first-order constraints are responsible for the presence of glucose in the blood, which makes it available to all cells, others for its removal from the bloodstream for utilization or storage. Glucose metabolism consists of different processes, which are:

(1) Glucose *uptake* by the cells of different tissues (brain, intestine, liver, etc.), constrained by glucose transporters in the cell membrane;
(2) Food *intake*, which includes the ingestion and digestion of carbohydrates and drastically changes the amount of glucose in the system. It is constrained by the digestive system (its dynamic structural constraints, the digestive enzymes and the absorption by the epithelial cells of the intestine). Food intake is the main source of variation for the first-order regime of utilization and production of glucose;
(3) Intracellular *glycolysis*, the breaking down of glucose molecules into pyruvate as part of the process of production of ATP. This process is constrained by enzymes in the cell metabolism;
(4) *Glycogenesis*, which consists of the transformation of glucose into glycogen for storage: a process constrained mainly by liver cells, striated muscles cells and cells of the white adipose tissue in which it is then stored;
(5) *Glycogenolysis*, the transformation of stored glycogen into glucose, constrained by all cells that store glycogen.

[11] In this framework, biologically relevant entities can have a twofold role. A molecule, for instance, can be characterized as a *stimulus* when, by interacting with a sensitive constraint, induces the activation of the latter. The same molecule, instead, can play the role of a *metabolite* when consumed as a substrate in a metabolic process.

[12] This is exemplified by the kinases. Their activation is triggered by an interaction on a different site than the active one that catalyzes the phosphorylation of other enzymes. In this case, the regulatory subsystem is realized by a single enzyme. In other cases, as we will discuss, the subsystem is constituted by a set of different constraints playing different roles, and collectively realizing the regulation.

[13] On the biological importance of control constraints that exhibit context-sensitive activity, see also Pattee (1972); Abel (2010), and Winning and Bechtel (2018).

[14] Veloso (2017) provides a mathematical approach to detect regulatory decoupling in the case of epigenetic control during the development of multicellular systems.

Glycogenolysis by hepatic cells is the main source of blood glucose during fasting;

(6) *Gluconeogenesis*, also constrained by liver cells, which produce glucose anew starting from amino acids, lipids, pyruvate and lactate;

(7) Glucose *transport*, responsible for the distribution of glucose in the system: once this sugar reaches the bloodstream (after its absorption in the intestine, or after its secretion by the liver during fasting) its distribution in the body is constrained by the vascular system (e.g., the portal vein for transport to the liver, the hepatic veins for release into the general circulation).

All the constraints acting on the previous processes (namely: the digestive system, the vascular system and different organs and cell types) are in turn maintained by the glucose supply they constrain. By controlling processes involving glucose, they therefore contribute to their own conditions of existence and to the overall maintenance of the biological system that harbors them. In sum, they contribute to the realization of a regime of first-order closure (see **Figure 2A**).

How does the system manage changes in glucose concentration? In basal conditions, in which there is neither an abrupt intake nor a high need for glucose, the organism is constantly subject to small variations in glycemia due to stochastic interactions and the dynamic balance among the first five coupled processes. This first-order regime exhibits dynamic stability with respect to such variations mainly through a balance of processes 4 (glycogenesis) and 5 (glycogenolysis), plus 3 (glycolysis)[15]. Supply and demand effects such as a small increase in glucose concentrations in the blood, or of glycogen content in the cells, can speed up, dampen or inhibit the processes that rely on or produce these metabolites. For example, the glycogenesis pathway in muscle cells is characterized by two steps: the phosphorylation of glucose into G6P, constrained by the transport/hexokinase (GT/HK) subsystem; and the production of glycogen from G6P, constrained by the enzyme glycogen synthase (GSase). Due to both allosteric interactions and the effect of mass action, an increase in the supply of glucose can favor these reactions, while the accumulation of glycogen in turn inhibits the pathway. Because of these network properties the first-order regime achieves – with no regulation in the organizational sense – a balance that allows it to compensate for small variations in glycemia and at the same time exert a tight control upon this and other individual pathways (**Figure 2A**).

Such a delicate balance of first-order control mechanisms is however insufficient to maintain glucose homeostasis in the organism, both in basal conditions and – a fortiori – in response to greater alterations of glycemia caused by food intake or sudden high energy needs[16]. This is why regulatory constraints intervene to selectively modulate the first-order constraints in a coordinated way. By doing so, they allow the metabolism to speed up glycogenesis and inhibit glycogenolysis in the presence of high glucose concentrations, and vice versa in the case of low concentrations. A central role in glucose regulation is played, as discussed in Section "The Standard Representation of Glycemia Regulation: The Feedback Loop," by the hormones insulin and glucagon released by the pancreas. From an organizational perspective, they can be treated as *second-order* constraints that modulate the functioning of other first-order constraints. Insulin does so in three ways: it facilitates glucose uptake in muscle and adipose tissue; it increases glycogenesis in liver, striate muscle and white fat cells; and it inhibits glucagon secretion by pancreatic alpha-cells. Glucagon, in turn, increases glycogenolysis and gluconeogenesis in the liver, and thus the release of glucose in the bloodstream.

Let us follow the conceptual scheme introduced at the end of Section "Organizational Principles" and focus first on the activation of the regulatory subsystem. Pancreatic beta-cells continuously produce and store insulin in vesicles and secrete it into the bloodstream. The secretion of insulin occurs when the insulin containing vesicles already present in the cells fuse with the cell membrane and release the hormone. In basal conditions, the release of insulin is low, but when the glycemia rises, beta-cells secrete it abundantly, thus modulating the metabolic activity of the first-order regime. Note that the activation of regulation is independent of insulin synthesis and the subsequent glycolysis, which is consistent the idea of dynamical decoupling discussed above. As a matter of fact, as we discuss just below, the activation does not even depend directly on glucose, but rather on the capacity of the regulatory subsystems within these cells to sense the energetic state of the intracellular metabolism.

As discussed in the previous Section, the regulatory subsystem includes two fundamental functional parts: the second-order constraints and the sensitive constraints (**Figure 2B**). From the organizational perspective, the elaboration of the model should therefore identify the parts that perform these functions. What are the sensitive constraints in this case? When glucose circulating in the blood is transported into beta-cells, it is metabolized and transformed into ATP. The increase in the ratio between ATP and ADP triggers a sequence of changes in some of the constraints in the cells (ion channels and membrane polarity), starting from the closing of ATP-sensitive K^+-channels and the consequent depolarization of the membrane. The depolarization of the membrane is followed by the opening of voltage-dependent calcium ions channels. The channels opening activates the proteins of the SNARE[17] complex of the membrane, which in turn controls the fusion of the membrane and the vesicles containing insulin, with the consequent release of this hormone in the bloodstream (see for example Röder et al., 2016). We submit that the channels are the best candidate to be understood as the sensitive constraints of the glycemia regulatory subsystem: they convert a quantitative change in the cellular metabolism (increased production of ATP) – induced by an increase of glycemia – into a qualitative change in the regulatory subsystem (the closing of the K + -channels and

[15]Gluconeogenesis (process number 6) contributes to this balance during fasting, exercise and when other sources are mostly depleted.

[16]Variations in glucose concentrations can only change glycogen production linearly in the absence of a significant release of insulin (Schafer et al., 2005).

[17]SNARE (SNAP receptor) is an acronym designating a group of proteins that mediate membrane fusion and exocytosis. SNAP is an acronym for Soluble NSF (*N*-ethylmaleimide-sensitive factor) attachment proteins.

the depolarization of the membrane)[18]. From this point on, the activation state of the regulatory subsystem results into the release of the second-order constraint (insulin) through a series of intermediate steps involving changes in potential. Glucagon secretion by pancreatic alpha-cells is activated in a similar way through the calcium-mediated fusion of glucagon-containing vesicles with the cell membrane, controlled by the SNARE complex. Yet, activation in alpha- and beta-cells is triggered by opposite glucose concentrations because of the specificity of their sensitive constraints (the ion channels). In alpha-cells, activation is triggered by cAMP[19] when glucose concentration, and consequently that of ATP, is low (Gaisano et al., 2012).

With regard to the second-order action of the regulatory constraints, insulin plays three functional roles: (1) it facilitates glucose uptake into cells of muscle and adipose tissues, (2) it stimulates glycogenesis and (3) it inhibits the release of glucagon by pancreatic alpha-cells and, consequently, glycogenolysis. The first function is exerted by facilitating the migration of the glucose transporter GLUT4 to the plasma membrane by stimulating the glucose transport/hexokinase (GT/HK) first-order constraints. The second function is exerted by directly controlling those first-order constraints responsible for the process of production of glycogen from glucose through the phosphorylation of the enzyme glycogen synthase (GSase). The third function of insulin is to inhibit the production of glucose from glycogen (glycogenolysis) by liver cells, by activating phosphoprotein phosphatases 1 (PP1), which dephosphorylate glycogen phosphorylase, thus inhibiting its activity (Hye-Sook et al., 2016), and by inhibiting the release of glucagon by alpha-cells. The later effect on alpha-cells occurs by modifying one of the first-order constraints involved, the membrane potential of alpha-cells (see for example Quesada et al., 2008).

The secretion by alpha-cells of glucagon, the other second-order regulatory constraint, is markedly increased when glycemia is low. In this circumstance insulin release is strongly decreased, and the intracellular ATP concentration in alpha-cells is low, while cAMP concentration is increased. Glucagon triggers a cascade of phosphorylation in liver cells, activating the enzymes (first-order constraints) responsible for glycogenolysis, which is the transformation of glycogen back to glucose. In addition to the increase of glucagon secretion triggered directly by hypoglycemia, there is a counterintuitive increase of glucagon triggered by hyperglycemia. This effect manifests spontaneously when there is a severe loss of beta-cells in diabetes type-I, because insulin secreted in paracrine fashion normally inhibits, as mentioned, the release of glucagon by the alpha-cells adjacent to beta-cells. However, it has been recently postulated that prior to the destruction of beta-cells in diabetes type I an increased glucagon concentration is observed and is attributed to hyperglycemia. Such an effect of hyperglycemia on glucagon release could be obtained experimentally in normal animals infused with glucose to obtain a chronic hyperglycemia (Jamison et al., 2011). Hence, the paradoxical induction of glucagon secretion is due to direct effects of hyperglycemia in the alpha-cell (Knudsen et al., 2019) and it is exacerbated indirectly by loss of beta-cells, when the inhibitory paracrine effect of insulin is lost.

A simple feedback model cannot adequately capture the two effects of hyperglycemia underlying the paradoxical increase of glucagon secretion (Wang et al., 2015), because it is centered on negative control loops between glycemia and two pancreatic hormones, insulin and glucagon. As a consequence, the feedback model makes organismal hierarchies collapse. This concerns all constraints from the second-order (as insulin and glucagon), to the higher-order constraints– such as the intra-islet interactions that regulate glucagon secretion, including beta-cells constraining glucagon secretion by the alpha-cells. The more orders of constraints are identified, the more the model *flattens* the functional organization of the organism (see for instance Röder et al., 2016, p. 5) the more the model loses explanatory adequacy. In fact, the feedback loop is useful to depict the situation in diabetes as long as the disease is seen as a problem due to lack of insulin (type 1 diabetes, T1D) or to insulin resistance (type 2 diabetes, T2D). However, since the discovery of the role of beta-cells on suppressing glucagon secretion by alpha-cells, the centrality of insulin deficiency on the genesis and maintenance of diabetes was re-evaluated, if not contested. As a result, the role of excess glucagon is given equal importance if not supremacy over the classical insulin centered view (bi-hormonal hypothesis). Intra-islet paracrine regulation of glucagon by the beta-cells, as well as by paracrine secretion of additional pancreatic hormones and by the nervous system are higher-order order constraints that are not represented *as such* in the feedback model[20].

As discussed in the previous Section, regulation consists of the capacity to selectively modulate the first-order self-maintaining regime in response to specific variations of the internal and external environment, due to the action of a dynamically decoupled dedicated control subsystem that is sensitive to these variations. Regulation allows the new first-order regime to cope with the changed environmental conditions and internal requirements. Constraints exerted by alpha- and beta-cells from the pancreas comply with this characterization of regulation, by constituting a subsystem that is sensitive to specific conditions and exerts second-order control upon first-order constraints in a way that is dynamically decoupled from them. More specifically, we have identified seven sets of processes, seven sets of associated first-order constraints, and a regulatory subsystem endowed with two sets of second-order constraints. Depending on the capacity

[18]It is important to point out that glucose is part of the metabolic process constrained by first-order constraints, in this specific case the enzyme glucokinase, which catalyzes the same process in a multitude of different cell types. The sensitive constraint belonging to the regulatory subsystem should not be part of the process or the first-order constraint, but should bring forth a qualitative change. In our proposal this corresponds to the ion channel. Regulation is activated in beta-cells with the polarization of the membrane, a qualitative change that is not metabolic anymore and which for example does not take place in other types of cells.

[19]Cyclic adenosine monophosphate (cAMP), obtained from ATP, is a second messenger involved in various physiological processes. The activation of glucagon secretion in alpha-cells is triggered by cAMP when ATP concentration is low.

[20]Another interesting example of therapeutic effects that are not easily explained by the feedback loop is the remission of T2D by bariatric surgery. Although this procedure was originally used to reduce weight in obese patients, it was found that it also induced remission of T2D. In particular, this procedure revealed the importance of the small intestine in glucose homeostasis. Several hypotheses were advanced on the importance of each of the components underlying remission, from glucose and nutrient sensing (sensitive constraint) to incretin secretion (third order constraint) (Laferrère, 2016). While these functions could be integrated into a hierarchical organizational description, they would suffer from the same problem of flattening in a feedback loop.

of some of the constraints of the regulatory subsystem to sense variations in the metabolic state of pancreatic cells, which in turn is affected by the supply of glucose, the system can selectively modulate the regime of first-order constraints: when perturbed by food intake, it does so through insulin, resulting in an increased production of glycogen from glucose; in the case of high energy demands, through glucagon, resulting in an increased production of glucose from glycogen. The overall result is the self-maintenance of the organism in varying conditions, achieved in particular through the homeostasis of glucose concentration in the plasma. While models relying on feedback loops only describe the relations between the variables involved in homeostasis, models relying on organizational closure can also *derive* these relations from the underlying functional organization of the organism. It is for this reason that, we hold, the organizational framework has a potentially higher explanatory power.

The above schema, however, focuses exclusively on metabolism and, accordingly, is still the first step in a more elaborate description. Many other, non-metabolic factors, including the nervous system, the intestine, and adipose and muscle tissues also participate in the modulation of insulin and glucagon secretion in relation to other environmental or internal conditions (Röder et al., 2016)[21]. This means that second-order regulatory constraints acting on the first-order regime are not at the top of the hierarchy but can themselves be modulated by higher-order constraints belonging to regulatory subsystems sensitive to different variables (**Figure 2C**). Unlike the feedback model, the organizational framework can naturally handle these additional regulatory subsystems by including in the closed graph the pertinent processes and constraints, and by making explicit the different orders involved. The procedure applied so far to identify and integrate first- and second-order functional constraints can be further iterated to obtain a richer and more adequate representation of the biological organization involved in the phenomenon under scrutiny.

DISCUSSION AND CONCLUSION

Let us compare the two different descriptions of glucose regulation as represented in **Figures 1**, **2**: feedback loops vs. organizational closure.

The representation in terms of feedback loops – and more generally in terms of single level networks – explains glucose homeostasis by focusing on the values of three variables, i.e., the concentrations of glucose (glycemia), insulin and glucagon. The relationships between these variables are determined by a set of functional components, which are included in the system as *independent* objects, i.e., as objects whose conditions of existence do not depend on the dynamics they control. As a result, feedback loops only include those relations among components – usually described as signals – that are relevant to explain the values of the target variables.

The main motivation behind the adoption of the organizational framework is radically different from that underlying feedback loops. Rather than focusing only on a specific relationship between variables, it also aims at understanding glucose homeostasis as a manifestation, as a consequence of the distinctive biological capacity of self-maintenance and, in particular, of organisms' capacity to manage energetic resources. Accordingly, functional components contribute to the maintenance of a biological organization that, in turn, contributes to maintain their own conditions of existence. In the organizational terminology, functional components are subject to closure. Understanding glucose homeostasis in light of biological self-maintenance leads to a significant enrichment of the quantity and kinds of functional objects and processes involved. In particular, we have emphasized the crucial distinction between different *orders* of functionality, each of them implying different kinds of constraints and processes. The regulation of glucose concentrations and metabolism is achieved through the coordinated activity of a *hierarchy* of functional regimes, which seem to be overlooked by descriptions appealing to feedback loops.

While feedback loops rely on the machine analogy, organizational closure emphasizes the *dis*analogy. Most of the differences between the two frameworks, we submit, derive from this central epistemological divergence. But what is at stake is not just a matter of difference. It is our contention that the organizational framework possesses a higher explanatory power than descriptions in terms of feedback loops, insofar as the former can replace the latter, *but not vice-versa*. As we suggested with the example of glucose regulation, the organizational framework can explain the homeostasis of a variable as the result of the functional and hierarchical complexity of an organism. In contrast, a description in terms of feedback loops cannot capture the functional complexity of the organization underlying biological homeostasis. There are many ways to realize the same feedback loop, and the organizational framework aims at understanding how biological organisms achieve that goal in each specific circumstance (e.g., through coupled processes, loops involving one order of constraints, regulatory loops involving second-order constraints, higher level regulatory loops including third order constraints and so on and so forth) and explaining its functional significance. In a word, we argue that organizational models can explain all what feedback models explain, and *more*.

The higher explanatory power of the organizational perspective has further implications, that allow going beyond the weaknesses of feedback loops mentioned at the end of Section "The Standard Representation of Glycemia Regulation: The Feedback Loop." A description of regulation in terms of organizational closure, by placing it in the context of the organism, enables the ascription of several functions to the same component, or the ascription of a given function to a set of distributed structures or even – to refer again to the "device" that sets the normal range of a target variable – to the organism as a whole. The different posture with respect to

[21] The fact that alpha- and beta-cells are sensitive to their internal state rather than directly to glucose, makes it possible for distinct processes (not directly dependent on glucose) to coordinate different steps of the activation process of insulin and glucagon secretion. For example "insulin release is stimulated by the so-called cephalic phase, which represents the conditioned reflex of increased hormone secretion, referred to as cephalic phase insulin response, even in the absence of nutrients/glucose as a trigger, such as when anticipating a meal, to prepare the organism to adequately respond to incoming nutrients"(Röder et al., 2016, p. 5).

an engineering conception that describes a phenomenon by appealing to a system of fixed components and localized and univocal functional roles appears clearly here. Also, unlike the feedback description, the organizational perspective fosters the progressive integration of additional processes and functional constraints into the description, insofar as its main explanandum is not the homeostasis of a variable *per se*, but the capacity of self-maintenance of the organism as a whole. In particular, it encourages improving the description of a system by specifying several hierarchical orders of regulatory constraints.

Another important implication, already evoked at the end of Section "The Standard Representation of Glycemia Regulation: The Feedback Loop," is that the organizational framework leaves room for variations in the regulation of glucose (as well as other variables) and departures from a given range of homeostatic values. The reason, again, is that glucose homeostasis is not a goal in itself, but a means to achieve self-maintenance; accordingly, an organism can vary its behavior as soon as the specific internal and external conditions require an adaptive response, be it a temporary or irreversible shift. The difference with regulation in machines, which are designed *for* maintaining a variable within a given range (deviations from the latter being therefore conceived as an error), is blatant here. Lastly, and symmetrically with respect to the previous point, the understanding of homeostasis from an organicist perspective opens the possibility of connecting homeostasis with biological norms, and thus with judgments about health and disease, while feedback loops cannot. Just as deviations from the normal range are not necessarily bad, the maintenance of homeostasis is not necessarily good: their biological significance depends on the general state of the organisms and its adaptive needs. Fever in response to infection is one of the best-known adaptive responses, as is the stress response. The recent availability of instrumentation to perform continuous glucose monitoring also revealed that normoglycemic individuals (according to standard clinical measurements) exhibit high interstitial glucose concentration variability; their glucose concentrations may reach prediabetic and diabetic ranges during a significant portion of the monitored time. This interindividual variability also appears as a response to standardized meals, thus suggesting that glucose homeostasis within a specific range is not as "normal" as previously thought (Hall et al., 2018). In this regard, it is useful to think on the adaptive potential of interindividual variability. For example, insulin resistance is considered by some as an adaptive response that protects the cardiovascular tissues from nutrient-induced injury, rather than the main culprit of the T2D syndrome (Nolan et al., 2015).

Because of these implications, we argue that the organizational framework is worth exploring. The representation of glycemia regulation offered here constitutes what is sometimes called a "proof of concept," i.e., an illustration of how the framework could apply to a specific biological phenomenon and how it can be represented through diagrams[22]. Its epistemological role consists in fostering the elaboration of more precise organizational *models*, which would make explicit many other aspects, starting with the times scales and the topology of the constraints involved in the control and regulation of the relevant processes. One recent example of a model relying on organizational principles is provided by the work of Montévil and coworkers on mammary organogenesis (Montévil et al., 2016).

To be sure, the scientific fruitfulness of the organizational framework can be adequately assessed (as is the case for any theoretical proposal) only by looking at the quantity and quality of the models that it could generate. Yet, the illustration provided here has the merit of making explicit some guidelines for elaborating models, which should focus – as discussed in Section "Reinterpreting Glucose Regulation from Organizational Principles" – on those objects that play a central role in the organizational framework: processes, time scales, constraints, constraint dependencies, closure and the hierarchy of functional orders. It also shows that the organizational framework tends to promote the integration into a single model of experimental data that are usually obtained and exploited separately by different experimental groups and scientific communities (see also Veloso, 2017). Accordingly, the framework can make an important contribution in overcoming the compartmentalization that sometimes characterizes experimental research. Conversely, the inherent tendency to integrate data into organism-centered models may induce the search of new experimental data required to "bridge the gap" between aspects and phenomena that have never been treated and interpreted jointly.

The proof of concept provided in this study also fulfills another epistemological function, which consists of putting some of the challenges that an organization-centered modeling strategy has to face into the foreground.

One challenge consists of integrating a variety of processes, interactions and associated functional constraints realizing organizational closure, by making their topological and quantitative relations explicit. Insofar as they would explore new ways of looking at biological organisms, organizational models may require developing original formal and mathematical tools. Moreover, the various levels and orders that constitute the hierarchical organization of the organism should be discriminated and integrated. However, the task of drawing the functional boundaries of a given regulatory subsystem, by identifying both its sensitive and higher-order constraints, may prove to be difficult to achieve in some cases. What is at stake here is the specific nature of biological organisms, whose functional organization is the result of an ontogenetic process (instead of an assemblage of pre-existing parts, as in machines) and an evolutionary history.

The organizational framework also faces the reciprocal challenge vis-à-vis complexity. A satisfactory model does not need to include all the details about the processes and constraints at play in the organism: a trade-off between precision and comprehensiveness must be found. The proof of concept

[22] As argued by Bechtel (2008), diagrams are an important component of scientific reasoning, useful in order to represent how the phenomenon under study is generated through the 'well-orchestrated' organization of the components that participate in it. Diagrams are needed precisely to develop a reasoning that captures both the operations performed by the parts and the relationship between these operations.

presented here shows that an organizational framework can provide theoretical guidelines for locating a target phenomenon in the overall organization, and for removing dispensable aspects. The organizational diagram given above, for instance, focuses on various constraints that play a direct role in the regulation of glycemia and, in turn, neglect other constraints involved in the metabolism. The possibility of simultaneously detailing some functional aspects while bracketing others allows the framework to make the model more relevant with regards to the specific phenomenon under scrutiny, while maintaining the general characterization of closure. More precisely, the fact of detailing or neglecting specific aspects is achieved by "zooming" in or out when describing the constraints and the processes on which they act. In the above diagram, for instance, constraints such as insulin, glucagon, alpha- and beta-cells and their target processes are described in a more detailed way when compared to the digestive system or the vascular system. Also, the framework allows neglecting details at some level of description, when it focuses on processes and functions located at other levels. For example, when considering a tissue as a constraint performing a function (and not as a collection of cells), one may concentrate on collective aspects like cell junctions, cell adhesion to the substrate and cell–matrix interactions, and ignore – at least to some extent –intracellular aspects and components.

As a result, even though the organizational diagram includes more objects than the feedback loop, it succeeds in providing a tractable and useful description of biological complexity. Most of the time, the decision about which functional aspects should be detailed and which one should be bracketed depends on the phenomenon being considered and the explanatory aim of the model. Yet, the organizational framework might also be able to elaborate some general guidelines in this respect: exploring this question could elicit a fundamental epistemological discussion on modeling practices from an organicist perspective.

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All authors listed have made an equal contribution to the work, and approved it for publication.

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REVIEW
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Fluid Shear Stress Sensing by the Endothelial Layer

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Blood flow produces mechanical frictional forces, parallel to the blood flow exerted on the endothelial wall of the vessel, the so-called wall shear stress (WSS). WSS sensing is associated with several vascular pathologies, but it is first a physiological phenomenon. Endothelial cell sensitivity to WSS is involved in several developmental and physiological vascular processes such as angiogenesis and vascular morphogenesis, vascular remodeling, and vascular tone. Local conditions of blood flow determine the characteristics of WSS, i.e., intensity, direction, pulsatility, sensed by the endothelial cells that, through their effect of the vascular network, impact WSS. All these processes generate a local-global retroactive loop that determines the ability of the vascular system to ensure the perfusion of the tissues. In order to account for the physiological role of WSS, the so-called shear stress set point theory has been proposed, according to which WSS sensing acts locally on vessel remodeling so that WSS is maintained close to a set point value, with local and distant effects of vascular blood flow. The aim of this article is (1) to review the existing literature on WSS sensing involvement on the behavior of endothelial cells and its short-term (vasoreactivity) and long-term (vascular morphogenesis and remodeling) effects on vascular functioning in physiological condition; (2) to present the various hypotheses about WSS sensors and analyze the conceptual background of these representations, in particular the concept of tensional prestress or biotensegrity; and (3) to analyze the relevance, explanatory value, and limitations of the WSS set point theory, that should be viewed as dynamical, and not algorithmic, processes, acting in a self-organized way. We conclude that this dynamic set point theory and the biotensegrity concept provide a relevant explanatory framework to analyze the physiological mechanisms of WSS sensing and their possible shift toward pathological situations.

Keywords: endothelial cell, shear stress, vasoreactivity, vascular remodeling, angiogenesis, regulation – physiological, tensegrity

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INTRODUCTION

Blood flow through the vascular network produces mechanical forces exerted within the blood vessels, mainly the blood pressure, and the wall shear stress (WSS) exerted on the endothelial layer that lines the lumen of the vessel. Both forces are sensed by the cardiovascular system, which in turn modifies its activity and hence the mechanical characteristics of the blood flow. As a consequence,

a dynamic causal loop exists between the morphofunctional state of the cardiovascular system, which mechanically determines the blood flow and the mechanical constraints it exerts on this system, and the blood flow which, through the mechanical sensing pathways stimulated by these constraints, modifies this morphofunctional state. Since the cardiovascular system develops early during embryonic development, being functional approximately around 10 weeks of gestation, mechanosensitivity may play an important role during prenatal and postnatal vascular morphogenesis as well as during adulthood. Recent studies have shown that the endothelial cell (EC) sensitivity to WSS is involved in several developmental and physiological vascular processes such as angiogenesis and vascular morphogenesis, vascular remodeling, and vascular tone (Chen and Tzima, 2009; Franco et al., 2015; Carter et al., 2016; Poduri et al., 2017; John et al., 2018; Iring et al., 2019). In contrast with barosensitivity, which involves central control by the brainstem of the heart activity and the peripheral arterial resistance and endocrine blood volume control, shear stress sensitivity seems to be determined and act locally. Despite the absence of central control, FSS sensitivity contributes to ensure an adequate behavior of the cardiovascular system as a whole, i.e., able to ensure adequate oxygen and nutrient delivery to the tissues. Regarding WSS sensitivity, the vascular system can hence be viewed as a self-organized homeostatic system. In order to account for the WSS-sensitive regulation of the vascular system, some authors have applied the general set point theory (SPT) of homeostatic processes to WSS-dependent vessel modeling (Baeyens et al., 2015; Baeyens and Schwartz, 2016). However, on the one hand, WSS sensitivity is involved in a variety of short-term and long-term modulations of the vascular activity, and, on the other hand, the classical SPT is initially a theory of central control. The question then arises whether this theory is applicable to the variety of behavioral processes determined by WSS. This is an important question since WSS sensing is a physiological phenomenon but is associated with several vascular diseases (Ong et al., 2020). If valid, the SPT would be a useful tool to understand how WSS sensing can shift from normal, beneficial processes toward pathological ones. The aim of this article is (1) to summarize the physical characteristics of the WSS applied to the vascular wall in physiological conditions, (2) to review the existing literature on the effects of WSS sensing on the behavior of ECs and its short-term (vasoreactivity) and long-term (vascular morphogenesis and remodeling) effects on vascular functioning in physiological condition, and (3) to analyze the relevance, explanatory value, and limitations of the SPT applied to WSS.

PHYSICAL CHARACTERISTICS OF FLUID SHEAR STRESS IN BLOOD VESSELS

Theoretical Principles

The shear stress exerted on the vessel wall is a physical phenomenon that is the consequence of the frictional forces generated by the blood flow on the luminal surface of the wall. Unlike the pressure pulse, which creates a radial and circumferal strain that generates the distension of the artery wall, the frictional forces of the blood create a WSS tangential to the direction of the blood flow and the vessel axis. The WSS corresponds to the viscous drag that the fluid exerts, due to its viscosity, on the wall of the vessel, i.e., the lumen side of the endothelium. For an ideal fluid, for which the viscosity is null, there is no shear stress. The shear stress is schematically represented in **Figure 1A**.

The shear stress τ is then defined as the ratio of the tangential force F to the surface area A to which it is applied, and is hence a pressure.

$$\tau = \frac{F}{A} \tag{1}$$

The shear stress depends on the flow and the rheological properties of the fluid, and the geometrical characteristics of the pipe through which it flows. Assuming several simplifications, it is possible to calculate the WSS exerted by the blood flow in a vessel segment, under the application of fluid mechanics principles (Marchandise et al., 2007; Fung, 2010; Caro, 2012; Koeppen et al., 2018). For a Newtonian fluid, for which the viscosity is constant, the shear stress depends on the viscosity of the fluid and the shear rate. This can be illustrated by a plate moving at constant velocity on a homogenous fluid, as shown in **Figure 1B**.

For a Newtonian fluid, the shear rate, defined as the ratio of the displacement τ of the fluid at a given distance y, du/dy, is constant, and the viscosity η is the ratio of the shear stress to the shear rate du/dy.

$$\eta = \frac{\tau}{du/dy} \tag{2}$$

Considering a vessel segment as a linear tube of constant diameter, and assuming the linear flow of a Newtonian fluid, application of the Poiseuille's law allows formulation of the quantitative relationship between the shear rate as a function of the geometrical characteristic of the vessel segment and of the flow (**Figure 1C**). From the definition of the Newtonian viscosity, the shear stress is given by the product of the viscosity and the shear rate (Eq. 2). For a cylindrical segment, the shear rate α can be expressed as a function of the diameter D of the segment and the mean velocity V_m.

$$\alpha = \frac{8 \times V_m}{D} \tag{3}$$

The shear stress can then be expressed as a function of the viscosity of the fluid, the mean flow velocity, and the diameter of the segment:

$$\tau = \eta \times \frac{8 \times V_m}{D} \tag{4}$$

For a cylindrical tube, the flow rate Q is the product of the mean velocity of the fluid and the cross sectional area of the tube. Hence, the mean velocity can be expressed as follows:

$$V_m = \frac{4 \times Q}{\pi D^2} \tag{5}$$

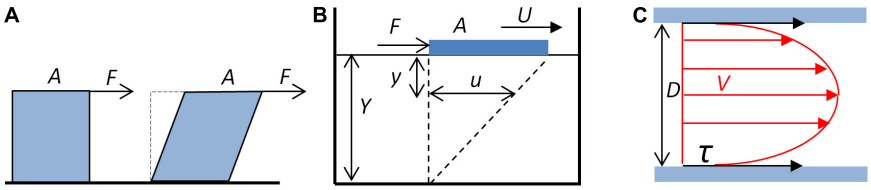

FIGURE 1 | Fluid shear stress. **(A)** Schematic representation of the shear stress. The fluid exerts a tangential force *F* on the surface *A* of a solid (blue cube), which tends to deform the solid. **(B)** Shear stress, shear rate, and viscosity. A plate (blue block) of area *A* submitted to a tangential force *F* moves on the surface of a liquid of depth *Y*. The displacement at the surface of the liquid (*U*) generates, at the *y* distance to the surface, a movement of the fluid *u*. The shear rate is the ratio *du*/*dy*. For a Newtonian fluid, *du*/*dy* is constant. **(C)** Schematic representation of the shear stress exerted by the blood flow on the wall of a cylindrical vessel. Red arrows represent the velocity (*V*) of laminar blood flow. The shear stress (τ, black arrows) exerted by the blood on the wall of the tube depends on the blood flow mean velocity, the blood viscosity, and the diameter (D) of the tube (Eqs. 3 and 4).

From Eqs 4 and 5, the shear stress exerted on a wall is proportional to the viscosity of the fluid and the flow rate and inversely proportional to the diameter D (or the radius r) of the vessel segment at the power 3:

$$\tau = \eta \times \frac{32Q}{\pi D^3} = \eta \times \frac{4Q}{\pi r^3} \quad (6)$$

The shear stress can also be expressed as a function of the pressure differential in the segment, taking into account the Poiseuille's equation:

$$Q = \frac{\pi r^4}{8\eta L} \times \Delta P \quad (7)$$

L is the length of the segment and ΔP the pressure differential between the extremities of the segment. Replacing Q in Eq. 6 by Eq. 7 gives the following equation:

$$\tau = \frac{r}{2} \times \frac{\Delta P}{L} \quad (8)$$

Under the simplifications already mentioned, the shear stress exerted on the vessel wall (WSS) can hence be calculated from the geometry of the vessel segment and the characteristics of the flow, either the mean flow velocity (Eq. 4) or the flow rate (Eq. 6), or, alternatively, the pressure differential in the segment (Eq. 8) (Reneman et al., 2006). The equations used to calculate experimental WSS values depend on the nature of the data obtained from the experiments to characterize the blood flow. Since measurement of the pressure differential is technically difficult, Eq. 8 is rarely used. Instead, Eqs 4 and 6 are usually applied to calculate the WSS in vessels. In experimental studies in which the flow rate is known, in particular in *in vitro* experiments, Eq. 6 is used, whereas in clinical studies in which the flow velocity is measured (allowing the calculation of the shear rate), Eq. 4 is used. When maximal velocity V_{max} instead of mean velocity is measured, Eq. 6 should be corrected in order to take into account the difference between V_m and V_{max}. Due to the frictional force, the velocity is higher at the center of the segment, and, for a Newtonian fluid, the velocity profile is parabolic, and the ratio of V_{max} to V_m is 2. Hence,

$$\tau = \eta \times \frac{4 \times V_{max}}{D} \quad (9)$$

However, in *in vivo* studies, precise calculation of shear stress values should take into account the fact that the blood in a non-Newtonian fluid, namely, its viscosity, is not constant. An apparent viscosity can be attributed, i.e., the viscosity of a Newtonian fluid showing the same relationship between Q and ΔP, according to the Poiseuille's law. Additionally, the velocity profile is flattened, which changes the ratio of V_{max} to V_m (V_{max}/V_m). In arterioles, this ratio is usually between 1.39 to 1.54, i.e., less than the theoretical value 2 (Reneman et al., 2006). Also, for small vessels with diameters less than 300 μm, blood viscosity is lower than in larger vessels, so that shear stress may differ, due to change in apparent viscosity, depending of the location throughout the vascular tree.

Shear Stress Patterns and Physiological Values

Additionally, these physical characterizations of FSS are done under simplifications, namely, considering a uniform and laminar blood flow through a straight cylindrical vessel segment, conditions that are not always realized in the vasculature. First, in arteries, blood flow is not constant but pulsatile, a consequence of the pulsatile activity of the heart. Second, the vessel segments are not always rectilinear, so that blood velocity and, hence, FSS is not similar at the inner and outer parts of the curved segment. Third, blood flow is not always linear. It can be turbulent, or disturbed, in particular at arterial bifurcations and curvatures. Numerous models based on computational fluid dynamics have been developed to predict these local patterns of blood flow and WSS, in particular for human aorta. Indeed, several pathologies, like plaque formation and atherosclerosis, are associated with zones of disturbed blood flow and low WSS, and such models are helpful tools to predict the progression of the disease and to choose the most appropriate treatment and its timing (Ong et al., 2020).

Despite these local complex patterns of WSS, average values have been calculated by several authors and are available in the literature, either in the international (SI) or the centimeter-gram-second (CGS) system of units (Lipowsky et al., 1978; Kamiya and Togawa, 1980; Koller and Kaley, 1991; Joannides et al., 1995; Cheng et al., 2003; Carter et al., 2016; Saw et al., 2017). In order to help comparing the values published in the literature and applying the equations in a coherent unit system, **Table 1** gives

TABLE 1 | Comparative units.

Physical quantity	SI unit (symbol)	CGS unit
Force	Newton (N)	dyne = 1.10^{-5} N
Pressure	Pascal (Pa)	barye = dyne cm^{-2} = 1.10^{-1} Pa
Viscosity	pascal second (Pa s)	poise = barye second = 1.10^{-1} Pa s
Length	meter (m)	centimeter = 1.10^{-2} m
Velocity	meter/second (m s^{-1})	centimeter/second = 1.10^{-2} m s^{-1}
Volume	$meter^3$ (m^3)	$centimeter^3$ = 1.10^{-6} m^3
Flow rate	$meter^3$/second (m^3 s^{-1})	$centimeter^3$/second = 1.10^{-6} m^3 s^{-1}
Shear rate	$second^{-1}$ (s^{-1})	$second^{-1}$ (s^{-1})

Units of basic physical quantities in the international system (SI) of units and the centimeter-gram-second (CGS) system of units.

TABLE 2 | *In vivo* WSS values in different vascular segments.

Vessel	hAA	hCCA	hBA	hCFA	hIVC	cMA	cMV
D (mm)	16.3	5.5–7.7	3.1–4.4	7.0	18.9	0.029	0.031
mWSS (Pa)	0.35	1.1–1.4	0.3–0.5	0.3–0.4	0.34	4.71	2.9
Viscosity (mPa S^{-1})	4	2.9–4.6	4.8–5.0		4	3.59	5.15

hAA, human abdominal aorta; hIVC, human inferior vena cava (Cheng et al., 2003). hCC, human carotid artery; hBA, human brachial artery; hCFA, human common femoral artery (Reneman et al., 2006). cMA, cat mesenteric arteriole; cMV, cat mesenteric venule (Lipowsky et al., 1978). D, vessel diameter; mWSS, mean wall shear stress.

TABLE 3 | Change in blood flow rate and WSS-induced vasoreactivity.

	hCCA		hBA		rCrA	
	Initial	Final	Initial	Final	Initial	Final
flow rate (mL s^{-1})	*4.82*	*8.3*	0.4	1.22	*2.67 × 10^{-6}*	*2.78 × 10^{-6}*
diameter (cm)	0.639	0.675	0.267	0.277	2.17 × 10^{-3}	9.78 × 10^{-3}
WSR (s^{-1})	188	*275*	*214*	*583*	2,658	3,879
WSS (dyn cm^{-2})	*7*	*10*	*7*	*20*	*53*	*78*
Viscosity (Poise)	*0.035*	*0.035*	*0.035*	*0.035*	*0.02*	*0.02*
S_{WSS} (dyn cm^{-1})	5.91 × 10^{-3}		3.85 × 10^{-4}		1.60 × 10^{-5}	

Experimental data obtained in human common coronary artery (hCCA) (Carter et al., 2016), human brachial artery (hBA) (Joannides et al., 1995), and rat cremaster muscle arterioles (rCrA) (Koller and Kaley, 1991). WSR, wall shear rate; WSS, wall shear stress. Data in italics are calculated from the experimental data given by the authors using Eq. 7. The sensitivity coefficient S_{WSS} is defined as the ratio of change in diameter on change in WSS between initial and final conditions.

a summary of units in both systems and their conversion ratio. **Table 2** presents several *in vivo* measurements of WSS in large and medium arteries, arterioles, veins, and venules (Lipowsky et al., 1978; Cheng et al., 2003; Reneman et al., 2006). Other data are also given in **Table 3**.

It is classically considered that WSS in arteries is around 1–2 Pa (10–20 dynes cm^{-2}), and around 0.1–0.6 Pa in veins (1–6 dynes cm^{-2}), around 10-fold less (Ballermann et al., 1998; Baeyens et al., 2016). Compared to other mechanical forces to which the vessels are submitted, shear stress is very low. For example, mean arterial blood pressure is around 13 kPa, endogenous stress in tissue, as well as focal adhesion (FA) stress, is around 3–5 kPa (Galbraith and Sheetz, 1997; Balaban et al., 2001). Additionally, these average values should be taken as a range of order, since the values depend on where they have been measured and the mode of calculation. In humans, the mean WSS in the common carotid artery (CCA) is around 1.2 to 1.4 Pa, and the peak WSS around 2.5 to 3.6 Pa, whereas in common femoral, superficial femoral, and brachial arteries, the mean WSS ranges from 0.3 to 0.5 Pa (Reneman et al., 2006). The physiological average value of WSS is hence not uniform throughout the arterial network, but may vary locally.

Hence, WSS should be viewed as a physical constraint exerted on the endothelium of the vessels, characterized by a complex spatial and temporal pattern including its intensity, direction, pulsatility, and regularity. As already seen, WSS is about 1,000-fold less than other mechanical stresses, which means that WSS sensitivity is in a range quite different than other kinds of mechanical sensitivity. ECs are sensitive to small variations in magnitude, but also in the direction and regularity of blood flow-induced WSS (Givens and Tzima, 2016). These WSS characteristics are sensed and interpreted locally by the ECs, and this local WSS sensitivity has global physiological consequences. It is indeed an important determinant of the morphology of the vasculature, controlling both its development during embryogenesis and its remodeling during postnatal and adult life. It also modulates the vascular reactivity in response to short-term blood flow change. WSS sensitivity is hence an important physiological property that optimizes blood flow to the tissues and ensures the mechanical integrity of the vessel walls.

SHEAR STRESS AND VESSEL PHYSIOLOGY

Among the large variety of processes involving the endothelial sensitivity to WSS, the present analysis will focus on three main physiological responses to WSS, short-term vasoreactivity, long-term vessel remodeling, and vessel architecture building during vascular morphogenesis.

Shear Stress and Vasoreactivity

Several studies have in past decades evidenced that shear-stress induces vasodilatation in an epithelium-dependent manner (Holtz et al., 1983; Vanhoutte, 1986). Inversely, reduction in blood flow has been shown to induce vasoconstriction, which mechanically increases WSS (Langille et al., 1989). *In vivo* experiments have shown that increase in WSS, either by increase in blood flow and wall shear rate (WSR) (Koller and Kaley, 1991; Carter et al., 2016), or fluid viscosity (Melkumyants and Balashov, 1990), triggered an arterial vasodilatation that occurred within a few seconds. This rapid increase in vessel diameter is due to the relaxation of the arterial musculature induced by WSS-induced endothelial release of vasorelaxant agonists, such as prostacyclin and, more importantly, nitric oxide (NO) (Frangos et al., 1985; Joannides et al., 1995). WSS-induced NO production is due to the phosphorylation of the NO synthase (NOS) at various serine sites, but the molecular interactions that couple WSS sensing and NOS phosphorylations are complex. Recent studies have shown that the mechanically gated ion

channel PIEZO-1, located at the plasma membrane, and the mechanocomplex PECAM-1, vascular endothelial cadherin (VE-cadherin), and VEGFR2 complex, located at cell–cell junctions, are key molecular interactors in WSS sensitivity, but the ways they interplay are controversial. In the model proposed by Iring et al. (2019) PIEZO-1 activation by WSS induced autocrine or paracrine secretion of adrenomedulin and ATP. Each mediator induces NOS phosphorylation by specific intracellular pathways. Adrenomedulin binds to its membrane receptor CALCRL, which is coupled to G_s heterotrimeric G protein, triggering cAMP production, PKA activation and NOS phosphorylation. ATP binds to P2Y receptors coupled to $G_{q/11}$ protein, inducing PLCβ activation, phosphorylation of the mechanocomplex, activation of PI3K, than of AKT, leading to NOS phosphorylation (Iring et al., 2019). However, it has been shown that PIEZO-1 is activated downstream, and not upstream, $G_{q/11}$ protein (dela Paz and Frangos, 2019). Previous studies based on endothelial microtubules disruption have shown that the integrity of the cytoskeleton is necessary for WSS-induced vasodilatation (Sun et al., 2001), and suggested the existence of complex interplays between PIEZO 1 and EC cytoskeleton (Nourse and Pathak, 2017). Whatever the mechanisms responsible for WSS-induced vasoreactivity, its physiological consequence is a retroactive limitation of WSS variation. Indeed, for a given viscosity and flow rate, WSS is inversely proportional to the diameter of the vessel at the power 3 (see Eq. 6), so that physiological WSS-induced vasodilatation or constriction causes mechanically a variation in WSS opposite to its initial change.

Shear Stress and Vascular Remodeling

In addition to short-term vasoactive response to WSS, sustained increase in WSS induces long-term remodeling of vessel walls. It has been shown, using arterovenous fistula techniques in dogs and monkeys to modulate the blood flow rate, which sustained increase in WSS-induced outward remodeling and subsequent growth in vessel caliber (Kamiya and Togawa, 1980; Zarins et al., 1987). As a mechanical consequence of the remodeling and increase in vessel diameter (see Eq. 7), the WSS that had initially increased returned to its initial value around six months post operatively. Inversely, decrease in blood flow induced by ligation has been shown to induce endothelium-dependent inward remodeling and constitutive reduction in artery diameter (Langille and O'Donnell, 1986; Langille et al., 1989). WSS-induced remodeling involves several processes affecting vessel homeostasis such as EC morphology, endothelial permeability, inflammation, etc. In adults, ECs lining the arterial vessel adopt differential morphologies according to the direction and the magnitude of the SS, and their alignment reflects the direction of the flow. Physiological laminar WSS promotes cell elongation and orientation in the direction of flow, suppresses proliferation, stimulates anti-inflammatory gene expression, and suppresses expression of inflammatory pathways. WSS below or above its physiological value induces changes in EC alignment, polarization, and gene expression and activates inflammatory response and remodeling processes (Zhou et al., 2014; Baeyens et al., 2016). The molecular interactors of these processes are multiple and related by complex and not fully understood interplays, but a key element of this WSS sensitivity is the mechanocomplex PECAM-1, VE-cadherin, and VEGFR2 (and possibly VEGFR3). Different reports have demonstrated that PECAM-1 acts in concert with VE-cadherin and VEGFR2 to mediate a large number of shear stress responses. These responses include EC alignment, NF-κB activation, and Akt phosphorylation following application of shear stress (Fleming et al., 2005; Tzima et al., 2005). Importantly, PECAM-1 is required for both anti-inflammatory and inflammatory signaling in ECs (Tzima et al., 2005). *PECAM-1* KO mice subjected to partial carotid artery ligation display defects in flow-mediated vascular remodeling and intima-media thickening, due to defects in the NF-κB pathway (Chen and Tzima, 2009). In the current model proposed by Baeyens et al. (2016), the PECAM-1, VE-cadherin, VEGFR complex, associated with the cytoskeleton, is the mechanotransducer that converts the mechanical stimulus into a biochemical signal responsible for the EC behavioral responses and the shift from the quiescent and steady state of the vessel to its inflammatory and remodeling one. Inward and outward remodeling in response to reduction and increase in WSS, respectively, tend to bring WSS back to its original value, at which the vessel returns to a quiescent state. In this process, VEGF receptors seem to determine the WSS value for this quiescent state, since changing expression of VEGFRs shifts the quiescent state WSS value (Baeyens et al., 2015).

Shear Stress and Vascular Morphogenesis

Wall shear stress sensitivity is not only involved in vessel diameter adjustment in adults, but also contributes to the pattern of the vascular architecture during developmental vessel formation. A primary vascular plexus initially expands by sprouting (Isogai et al., 2003; Potente et al., 2011) followed by remodeling of vessel organization, shape, and size. Superfluous and inefficient connections are pruned away by active regression (Franco et al., 2013). Blood flow triggers several effects such as vessel constriction, EC survival, alignment, and migration that can all contribute to vessel regression (Meeson et al., 1996; Chen et al., 2012; Kochhan et al., 2013; Udan et al., 2013; Lenard et al., 2015; Franco et al., 2016). Interestingly, EC ability to respond to flow seems to be not fixed but dynamically adjusted according to the environment (Kwon et al., 2016). The literature shows many results on the ability of cells not only to respond to a change in flow magnitude but also to sense the direction of flow to control vessel regression. Vascular regression is driven by EC polarization and migration away from low to high flow (Chen et al., 2012). Above a critical value, flow induces axial polarization and migration of ECs against the flow direction. ECs actively migrate from regressing vessel segments to integrate into neighboring vessels (Udan et al., 2013; Franco et al., 2015). Non-canonical Wnt signaling was shown to control vascular remodeling by blocking excessive vessel regression in a flow-dependent manner (Franco et al., 2015, 2016). EC migration against the flow and vessel diameter remodeling emerge as an important mechanism that determines embryonic arterial formation. In this process, the SMAD signaling pathway, including DACH1 and endoglin

(ENG), seems to play an important role (Rochon et al., 2016; Poduri et al., 2017). Smad4 deficient coronary ECs are unable to migrate against the direction of the flow and lead to an abnormal accumulation of cells within the arteries. Dach1 supports coronary artery growth through its regulation by blood flow-guided EC behavior (Chang et al., 2017). *Dach1* deletion in ECs decreases artery size with impairment of EC polarization, which could be due to inefficient directed migration against the flow. Two recent studies using mice and zebrafish mutant and *in vitro* experiments show that ENG controls flow-directed migration and EC shape required for correct vascular morphogenesis (Jin et al., 2017; Sugden et al., 2017). ENG appears to be required for proper EC sensing of both amplitude and direction of shear stress, although some aspects of EC flow-sensing were not impaired in *Eng* mutant (Bautch, 2017).

Taken together, these data show that WSS sensing, both in magnitude and direction, induces spatialized EC responses, in particular EC migration against blood flow. These responses are critical determinants of the architecture of the vascular network during development by vessel regression/stabilization and vessel diameter remodeling.

SHEAR STRESS SENSING: SENSORS OR TENSEGRITY?

The previous section has summarized the major consequences of WSS on the physiology of the vasculature and the experimental evidence of the influence of WSS on vascular cell behavior, what is called WSS sensing. The present section will discuss the models of WSS sensing. Authors working on WSS sensing usually produce models of it, and some of them have been presented in the previous section. Typically, the "model" of PIEZO-1 activation presented by Iring et al. (2019) is an example of a model of WSS sensing. A model of WSS sensing can be basically defined as a representation of biological processes that provide explanations of how WSS induces cellular behavioral changes, and how these changes determine the physiology of the vasculature. It goes beyond the experimental results and articulates them in a network of cause-consequence processes. In this meaning, a model provides causal explanations, namely, it explains a phenomenon by a concatenation of cause-consequence processes. A model of WSS sensing is hence an explanatory framework that gives sense to the experimental results. We propose to classify these models in two main general categories that differ in the kind of explanations they provide, the "sensor-pathway" model and the "tensegrity" one.

The "Sensor-Pathway" Model

In WSS sensitivity of ECs, WSS can be viewed as acting like a pharmacological agent (Fung, 2010). This is obviously a metaphor, since WSS is not a molecule, but the analogy may be relevant in the meaning that WSS is sensed by the ECs and results in change in EC behavior. In the pharmacological stimulus-response coupling, the detection of the presence of the agonist is ensured by the receptor of the agonist. This receptor is hence the primary sensor of the stimulus. The ligand–receptor molecular interaction guarantees the sensitivity and the specificity of the signal detection, and the downstream pathways are responsible for the behavioral changes induced by the pharmacological stimulus. Such a model can be called the "sensor-pathway" model. However, in the case of WSS sensing, where the stimulus is not pharmacological, primary sensing cannot be ligand–receptor interaction. If the "sensor-pathway" model is relevant, the question than arises about the nature of the primary sensor of the WSS, and abundant literature exists about the possible sensors and mechanotransducers of WSS. This terminology requires some conceptual clarification. A primary sensor is a molecule or a molecular complex that is directly submitted to the WSS and is the initial trigger of the downstream pathway. A mechanotransducer is able to transduce a mechanical stimulus into a biochemical process (e.g., phosphorylation). A primary sensor can be a mechanotransducer, but a mechanotransducer is not necessarily a primary sensor.

The most frequently cited candidates as WSS sensors or mechanotransducers are primary cilia, the apical glycocalyx, ion channels such as PIEZO-1, G protein-coupled receptors, protein kinases, and caveolae. The best-studied is the EC–EC junctional complex, composed of PECAM-1, VE-cadherin, and the VEGF receptors 2 and 3. In this model, WSS induces an increase in tension across PECAM-1, mediated by an association with vimentin (Conway and Schwartz, 2015). This leads to the activation of a Src family kinase, which in response phosphorylates and activates VEGFR2, which in turn stimulates PI3K-AKT signaling pathway (Tzima et al., 2005). More recently, a study identified VEGFR3 as a novel component of this complex (Coon et al., 2015). In this model, VE-cadherin acts as an adaptor for the transmission of mechanical signal to VEGFR2 and 3. The components of this complex seem to be particularly important in the sensing of shear stress intensity and in the establishment of remodeling. Indeed, *PECAM-1*$^{-/-}$ mice show defects in inward and outward remodeling in a partial carotid ligation model (Chen and Tzima, 2009). Recent studies have shown that VE-cadherin Y658 phosphorylation was modulated by WSS intensity (Orsenigo et al., 2012) and that this Y658 phosphorylation was crucial for flow sensing through the junctional complex (Conway et al., 2017). VEGFR3 expression was also modulated by WSS intensity, and the level of VEGFR3 was also found to participate in WSS sensing. This study showed that high expression of VEGFR3 was correlated to higher sensitivity to WSS while low expression of VEGFR3 decreased WSS sensitivity. Thus, VEGFR3 expression seemed to determine the standard value for vascular remodeling (Baeyens and Schwartz, 2016).

In addition to the requirement of the junctional complex for shear stress sensing, FAs that ensure the anchorage of ECs to the underlying basement membrane seem to be important in this process (Ando and Yamamoto, 2013). Recently, it has been shown that laminin 511, a key component of endothelial basement membrane, was essential for mouse resistance artery WSS response and inward remodeling (Di Russo et al., 2017). Cells are anchored on the basal membrane by FA via integrins, which have been showed to be a sensor of shear stress direction in ECs. For example, β1 integrins are directly sensitive to mechanical forces and are essential for EC response to unidirectional flux, via activation of Ca^{2+} signaling (Xanthis et al., 2019).

Endothelial cell primary cilia can also act as a mechanosensor, triggering calcium signaling and NO production *in vitro* (Nauli et al., 2008). Studies have shown that the presence of endothelial cilia was regulated by WSS intensity *in vivo*, and contributes to WSS-dependent vessel development. In the embryonic heart, endothelial cilia were found in low shear stress vascular regions whereas in arteries ECs were unciliated (Hierck et al., 2008). In the developing zebrafish embryo (Goetz et al., 2014) and during vascular remodeling in the mouse retina (Vion et al., 2018), primary cilia was most frequently observed in ECs exposed to low and moderate shear stress. Inducible genetic deletion of primary cilia in ECs during postnatal retina development causes premature and widespread vessel regression (Vion et al., 2018). However, since the presence of primary cilia depends on the nature of WSS, they are hence a consequence of WSS, not a primary sensor. The question remains also of how these cilia transduce into intracellular pathways the mechanical forces to which they are sensitive.

As already mentioned about WSS-induced vasoreactivity, the ion channel PIEZO-1 is a mechanotransducer and is considered in some models as the primary sensor of WSS (Iring et al., 2019). However, this model does not account for the fact that PIEZO-1 does not seem to be primarily activated by WSS but activated downstream G protein activation (dela Paz and Frangos, 2019), and that its activation requires the integrity of the cytoskeleton (Sun et al., 2001). Actually, there are two ways to conceive WSS sensing by PIEZO-1. The first one refers to PIEZO-1 as a stretch-activated channel, in which PIEZO-1 is supposed to be sensitive to the stretching of the lipid bilayer produced by the shear stress of the plasma membrane, and can be called the "force-through lipid" sensing. In this view, according to the "sensor-pathway" model, PIEZO-1 is the primary sensor. The second one refers to PIEZO-1 as sensitive to the forces exerted by the cytoskeleton, a "force through filament" sensing. Actually, there is experimental evidence for both types of force sensing but, regarding WSS, whether PIEZO-1 acts as primary sensor remains controversial (Nourse and Pathak, 2017).

From this abundant literature, some key notions emerge about mechanotransducers, in particular the importance of the junctional complex of the adherens junctions (AJs), with PECAM-1, VE-cadherin and VEGFRs, and of FAs and integrins, and the role of PIEZO-1 in WSS vasoreactivity. However, two main questions remain open. As far as the "sensor-pathway" model is relevant, the identification and the nature of the primary sensors remain problematic. Due to their locations, AJs and FAs, though sensitive to mechanical stimulation, are not directly submitted to WSS and can be hardly considered as primary sensors. Second, and most importantly, a relevant model of WSS sensing should be able to link the initial cause (WSS) to its final consequence (cell behavior) by a series of processes that retain all the spatiotemporal informative content of the stimulus (intensity, directionality, pulsatility, and linearity of WSS). In the sensor-pathway model, the cascade of cause-consequence processes from the primary sensor activation and downstream is described in terms of levels of protein expression, phosphorylation/dephosphorylation, and molecular interactions. However, these molecular processes, by themselves, do not ensure the conservation of the spatiotemporal characteristics of WSS. As stated by Baeyens, "a coherent model of flow sensing is lacking" (Baeyens et al., 2016). This questions the relevance of the classical "primary sensor-downstream pathways" model, inherited from the pharmacological conception of ligand-receptor sensor, for the investigation and the understanding of WSS sensing.

The "Tensegrity" Model

In the usual conception on which the "sensor-pathway" model is grounded, a cell is most of all viewed as a viscous protoplasm limited and contained by an elastic membrane. An alternative view is the "tensegrity" model of mechanosensing (Paszkowiak and Dardik, 2003). The concept of tensegrity, or tensional integrity, coined by Richard Buckminster Fuller, is initially an architectural principle (Fuller and Applewhite, 1982). The mechanical stability of the structure built according to this principle is ensured by a net of continuous tension exerted on the components of the structure that are either in tension or in compression. A camping tent is an example of tensegrity. The shape of the tent is the consequence of the equilibrium of the tensile forces to which the different components of the tent, e.g., tent canvass, poles, ropes and pegs, are submitted, each one being balanced by an equal one opposite in direction. Any local change in tension alters this equilibrium and hence has global consequences on the shape of the structure that rearranges until the tensile forces reach a new equilibrium. But, as far as the tensional net is maintained, the structural integrity is retained despite the change in its shape. A structure is a tensegrity when it is in a state of baseline isometric tension, or tensional prestress, which avoids any slack in the tensional structure. This makes it both resilient and immediately responsive to internal and external mechanical stresses.

The concept of tensegrity has been applied to several biological processes at different levels of organization, including cellular mechanosensing (Ingber, 1997, 2008). In the model developed by Ingber (2018), the cell is viewed as shaped by the cytoskeleton whose architecture is ensured by tensional prestress. The three main components of the cytoskeleton are (i) the microfilaments, containing actin and myosin), (ii) the intermediate filaments, basically composed of vimentin, keratin, and desmin, and (iii) the microtubules, hollow polymers of tubulin. All of them contribute to this tensional prestress. The contractile microfilaments generate the active tension to which intermediate filaments and microtubules are submitted. The cytoskeleton is linked to FAs, which are transmembrane macromolecular structures containing talin, vinculin, α-actinin, paxillin, and integrin. Integrins bind with the extracellular matrix, so that FAs are molecular bridges between the cytoskeleton and the extracellular matrix to which it is anchored via integrins. By experimental tuning of the mechanical forces exerted on the cell, it has been evidenced that the cytoskeleton mechanically associated with the extracellular matrix behaves as a tensegrity structure (Kumar et al., 2006; Ingber, 2018). When the cell is submitted to mechanical deformation, the forces exerted on the tensional network are modified, resulting in changes in the tractional forces on FAs and integrins receptors. These changes in the balance of forces activate several biochemical

processes, in particular the activation of small GTPases Rho that modulate F-actin and hence actomyosin-dependent tension generation by contractile microfilaments (Ohashi et al., 2017). A recent minimal theoretical model showed that feedback between mechanical tension and Rho GTPase activity can account for different kinds of spatially organized cell behaviors, such as individual cell relaxation/contraction state, and the propagation of contraction waves in a 2D sheet of interconnected cells (Zmurchok et al., 2018).

While several studies have used tensegrity (whether they use the name or not) to provide an explanatory framework of mechanosensitivity, few studies using tensegrity have been specifically dedicated to WSS mechanosensing in ECs (Lim et al., 2015). Among them, a multicomponent, multicell model of the mechanical effects of FSS on ECs has been recently published (Dabagh et al., 2014). Using the finite-element method, the authors have built a computational model that predicts the deformation of ECs and subcellular organelles, and the stress to which the cytoskeletal stress fibers, the cell–cell AJs, and the cell-matrix FA are submitted when exposed to 1–2 Pa WSS, a value in the physiological range. The model predicts deformation of the cell and the nucleus, change in junctional angles, and an increase in AJ and FA stress. Indeed, the stress at FA increases up to 480 Pa and that at AJ up to 700–1,200 Pa. This corresponds to a 250–600 fold amplification of the intensity of the WSS at FAs, and a 600-fold amplification at AJs. The stress values predicted by the model are also in the range of order (kPa) of the stress values experimentally measured at FAs (Balaban et al., 2001).

According to the model, the architecture of the cell is hence responsible for the transmission and the amplification to the AJ and the FA of the FSS to which the luminal surface of the EC is submitted. In epithelial cells, some experimental results also suggest that changes in cytoskeleton tension are the initial events required for the response to FSS. In cultured MDCK cells, using optical force sensor for α-actinin, and fluorescent E-cadherin, it has been shown that shear stress-induced remodeling of AJs is driven by cytoskeletal forces (Verma et al., 2017). In ECS, as we have seen previously, several experiments have evidenced the key role of the cytoskeleton integrity for WSS sensing.

There are hence several theoretical and experimental results that strongly suggest that tensegrity is responsible for WSS sensing in ECs. In this view, "sensing" is primarily a change in the tensional equilibrium of forces operating in the cytoskeleton, cell-substrate, and cell–cell adhesions, due to the deformation induced by FSS at the lumen surface of the EC. The "primary" sensor, able to transmit and amplify the mechanical stimulus, is not an individualized molecular component of the cell directly submitted to FSS, but the cell architecture as a whole. However, WSS mechanosensing is not limited to mechanical processes. Cellular active processes such as phosphorylation, gene expression, etc., are involved in the cell response to FSS. These processes are physiological ones that are not energetically spontaneous and are distinct from the passive ones, occurring without energy consumption. This makes a critical difference between a passive tensegrity structure, in which the initial external force exerted locally induces a global change in shape, and a biotensegrity, in which active processes are involved. In the biotensegrity approach, these physiological processes are generated by the structural changes in the whole-cell shape induced by FSS, and are the consequence of mechanochemical transducers. They trigger several biochemical processes (e.g., cadherin expression, Rho activation, and microtubule polymerization) in response to the WSS-induced architectural changes. Second, if these biochemical processes modify the tensegrity equilibrium of the cell, which is spatially oriented, this may explain the conservation of the spatiotemporal characteristics in the WSS stimulus-response coupling.

The main difference between the "sensor-pathway" and the "biotensegrity" models does not reside in the molecular components involved in FSS mechanosensing, but in the nature of the explanations of WSS-response coupling they provide. In the "sensor-pathway" concept, the investigation of the mechanisms responsible for WSS-sensing focuses on the identification of molecular interactions and biochemical processes, which indeed occur during mechanosensing, but pay little attention to how these processes are responsible for the spatially oriented behavioral responses of the cells. The biotensegrity concept focuses on the causal continuity of the stimulus-response coupling, explained by the tensional equilibrium of the cell architecture. An important point is that, in the biotensegrity concept, this tensional equilibrium is not just passive adjustment to external mechanical constraints but also includes active internal adjustment due to the activation of biochemical processes. Hence, the biochemical "pathways" are embedded in a network of tensional forces, in the meaning that they are triggered by mechanical changes (via mechanochemical transducers), and modify the tensional equilibrium of the cellular architecture. They hence contribute to the cell response to WSS because they interplay with the structural organization of the cells. The biotensegrity model, which integrates the WSS-dependent biochemical pathways as active internal components of the tensegrity, is not just a model of mechanosensing (how the cell senses WSS) but also a model of mechanosensitivity (how the cell responds to WSS).

UNIFORM SHEAR STRESS AND SET POINT THEORY

Theoretical Formulation

As we have seen, ECs are able to sense the spatiotemporal characteristics of WSS and therefore contribute to determine the morphofunctional properties of the vascular network by at least three kinds of mechanisms, namely vessel regression and stabilization, long-term modulation of vessel diameter by inward and outward remodeling, and short-term vasoreactivity. Moreover, the consequence of these WSS-dependent processes is a retroactive limitation of WSS variation. These observations have lead Baeyens and coworkers to apply the "set point" theory of regulation to endothelial WSS sensitivity (Baeyens et al., 2015, 2016; Baeyens and Schwartz, 2016). Their model was proposed for WSS-induced vessel diameter remodeling, but the concept can also be applied to others vascular properties.

The "set point" theory, or "target point" theory, is a model of regulation of a biological process in which a biological variable remains in a determined range of values despite the environmental changes that modify the initial value of the variable. Applied to WSS sensing, it means that there exist mechanisms that ensure that the WSS characteristics remain in a small range of values despite changes in the conditions that determine WSS in the vessel (i.e., changes in flow rate or velocity, changes in blood viscosity). Expressed in bioengineering terms, such a phenomenon of regulation can be formulated as follows (Fung, 2010):

(a) A variable x, or a relationship among a set of variables (x, y, ...) that describes the phenomenon has been identified.
(b) There exists a standard value of x or a standard relationship among (x, y, ...) that is associated with a stable or optimal living condition.
(c) There exists a sensor that can detect any deviation or error of the variable x from the standard value, or of the relationship among (x, y, ...) from the standard one. The error is monitored all the time.
(d) A mechanism to minimize error exists.
(e) The dynamics of error minimization is biologically satisfactory.

According to this definition, once the variable (WSS) and its standard value have been identified, some key conditions are required to characterize a regulation process. The first one is how can be objectivized the "optimal" living conditions and the adequacy of the biological response (the fact that it is "satisfactory"), notions to which refer to items (a) and (b) of the definition. The second one is the identification of the mechanisms responsible for deviation minimization, which refers to item (c) of the definition. A possible way to define the biological optimization of a vascular network is to consider the minimal energy cost of blood flow. This notion has been formulated since the first half of the 20th century. In a branching network through which flows a fluid, minimum energy expenditure is achieved if the resistance to blood flow remains constant from proximal to distal generations (Fung, 2010). In this minimum cost model, for each vascular bifurcation, the relationship between the radius of the parent segment of generation n (r_n) and the radius of the two child segments ($r_{(n+1)a}$) and ($r_{(n+1)b}$) is:

$$r_n^3 = r_{(n+1)a}^3 + r_{(n+1)b}^3 \quad (10)$$

When applied to WSS, the minimum cost model shows that this condition is achieved when the WSS is equal in all of the segments (Kamiya et al., 1984), due to the fact that WSS is inversely proportional to r^3 (Eq. 7). So, if the WSS value is similar in all of the segments of the vascular tree, this ensures the energetic optimality of the vascular architecture. This has led to the formulation of the "uniform shear stress" principle, responsible for "optimal design," as formulated by Kamiya et al. (1984). According to this principle, maintenance of WSS value in each part of the vascular tree by local adaptive response to WSS change ensures the energetic optimality of the entire arterial tree. In principle, this can be applied to the three above-mentioned mechanisms, namely, vascular morphogenesis, long-term vascular remodeling, and short-term vasoreactivity. In the case of vessel regression and stabilization, an exuberant process of vessel sprouting and random connection, followed by vessel regression below a threshold WSS value, associated with vessel inward or outward remodeling of the remaining vessels until the set point value is obtained, will produce an organized network optimally designed. Uniform shear stress in vascular morphogenesis can hence be viewed as a constructal process that tends to shape vascular networks following thermodynamic constructal principles (Roux and Marhl, 2017). Adult vascular remodeling can also be analyzed in term of uniform shear stress. Indeed, as argued by Bayens et al., inward and outward remodeling are feedback processes ensuring the maintenance of constant WSS. WSS-induced vasoreactivity can be also analyzed as a negative control of WSS. Since this negative feedback maintains the energetic optimality of the vasculature, it can be said to be adaptive.

This adaptive response requires a mechanism responsible for deviation minimization. Actually, since WSS sensing is involved in different processes (vasoreactivity, morphogenesis, and remodeling), acting on different time scale, different mechanisms operate in this feedback. However, these different mechanisms can be analyzed with the common concept of homeostasis. Formulated in accordance with the control theory applied to physiological homeostasis (Carpenter, 2004), the principle of such mechanisms can be represented as an algorithmic process monitoring the WSS value by comparison with a set point value (**Figure 2**).

Uniform Shear Stress and Physiological WSS Sensitivity

In order to examine the relevance of the uniform shear stress principle, and the adequacy of the SPT as a suitable concept for analyzing the WSS sensitivity mechanisms, quantitative experimental data need to be compared with the theoretical requirements of the theory. The uniform shear stress principle has been primarily formulated for WSS-induced vessel remodeling, and several studies have shown that, after remodeling, the WSS remains unchanged (Kamiya and Togawa, 1980; Zarins et al., 1987). For example, in monkey iliac artery, after outward remodeling following arterovenous fistula, WSS, initially 16 dyn cm^{-2}, was 15 dyn cm^{-2} (Zarins et al., 1987). Long-term remodeling adaptation seems hence to follow the uniform shear stress principle. As stated by Baeyens and Schwartz (2016), this can be explained by the balance between quiescent (no remodeling) and active (inward or outward remodeling) states. Quiescence can hence be associated to different diameters.

For short-term vasoactive response to WSS, such a process is not possible. It is certainly possible to define in theory a quiescent state, corresponding to the absence of vasoactive stimulus from ECs to the smooth muscle layer, and a corresponding "set point" value. But the value close to which WSS sensitivity tends to maintain WSS cannot correspond to the quiescent state. Indeed, for the system to be able to react both to WSS

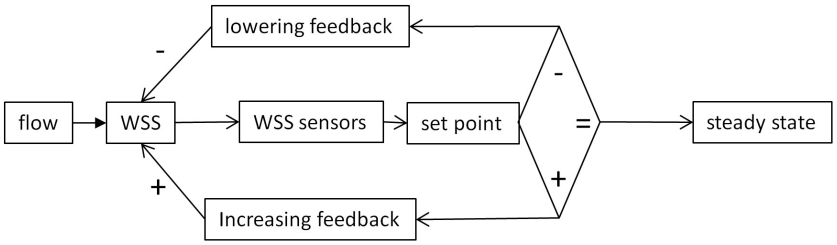

FIGURE 2 | Schematic representation classical set point theory according to the concepts of control theory. The WSS value is sensed by mechanosensors and compared with a reference value. If the two values are similar, the system remains in a steady state. If not, positive or negative variations from the set point activate feedback mechanisms that, respectively, lower or increase WSS value. The diamond represents the decisional step of the regulatory loop.

increase or decrease, the "set point" should be in the range of WSS sensitivity, and hence corresponds to a vasoactive state. Moreover, WSS-induced vasodilatation requires change in WSS. If the response was able to maintain uniform WSS, the consequence would be the annihilation of the WSS change, and subsequent ending of the vasoactive stimulus, and return to the initial vessel diameter. In contrast with remodeling, in WSS-induced vasoreactivity, stable WSS value is not quiescence, but a dynamic equilibrium between two phenomena, the physical relationship between vessel radius and WSS described by Eq. 6 and the physiological WSS sensitivity that links WSS and the vessel radius. Acting together, these two phenomena constitute a feedback loop. We have analyzed several quantitative data of WSS-induced vasoreactivity *in vivo* already published in the literature, in which the authors have experimentally manipulated the blood flow rate and measured the consequence of flow rate change on vessel diameter and/or WSS. Data, taken from several vascular beds, human CCA, human brachial artery (BA), and rat cremaster arterioles (CrA), are summarized in **Table 3**. Since the authors had not always calculated the flow rate, the vessel diameter, and the WSS or WSR, we have applied Eq. 6 to calculate the missing data. For the calculation of WSS from WSR, blood viscosity was set at 0.035 Poise for large arteries, and 0.02 for arterioles, in order to take into account the decrease in apparent viscosity in small vessels, in accordance with experimental measurements (Kamiya et al., 1984). In large arteries, WSR ranges from 188 to 275 s^{-1}, and WSS from 7 to 20 dyn cm^{-2}, values that are in the physiological range. In arterioles, WSR is around 10-fold more, and WSS around fivefold more. These experimental data are in accordance with the proportionality between WSS and pressure differential described in Eq. 8. Indeed, terminal arterial segments are the site of the largest decrease in blood pressure, and hence of highest WSS. This confirms the fact that WSS is not uniform all along the arterial tree.

For a given kind of segment, change both in WSS and diameter values induced by increase in blood flow rate confirms that there exists WSS-induced vasoreactivity (since diameter changes) but not uniform WSS (since WSS increases). We can hence define a WSS sensitivity coefficient, S_{WSS}, as the ratio of vessel radius difference on WSS difference between initial and final blood flow rate. In the absence of WSS sensitivity, $S_{WSS} = 0$, while, for uniform shear stress, $S_{WSS} \rightarrow \infty$.

From the experimental data given in **Table 3** and Eq. 6, it is possible to build a graphical representation of the function $r = f(WSS)$, r being the radius of the vessel. Indeed, from Eq. 6:

$$\mathrm{r} = \sqrt[3]{4\eta \times \frac{Q}{\pi WSS^3}} \tag{11}$$

For a given blood flow rate Q and a given blood viscosity η, the vessel radius in inversely proportional to the cubic square of WSS. Since Q and η are known, the function $r = f(WSS)$ can be built, adjusted to the couple of experimental values for r and WSS. For each arterial segment (CCA, BA, and CrA), $r = f(WSS)$ is given in **Figure 3**, for both initial and final conditions. Vascular adjustment in response to increased blood flow rate corresponds to the shift from the initial curve to the final one. In the absence of vasoactive response, the vessel diameter remains constant, and this shift is horizontal. Hence, the horizontal intercept from the initial WSS and radius with the final curve gives the final WSS in the absence of WSS sensitivity. Under the hypothesis of uniform WSS, WSS sensitivity ensures the conservation of WSS. In this case, the shift from the initial to the final curve is vertical. Hence, the vertical intercept from the initial WSS and radius with the final curve gives the final radius in the case of maximal WSS sensitivity (strict maintenance of WSS). Actually, the real situation is between these two opposite hypotheses. The straight line between the initial and the final WSS and radius values represents the real WSS sensitivity, and the slope of this line is S_{WSS}. As can be seen in **Figure 3** and **Table 3**, S_{WSS} is low, so the effect of WSS sensitivity of vessel radius is modest. This does not mean that it is physiologically unimportant in terms of vascular resistance, because the resistance is inversely proportional to the radius at the power 4. In the absence of WSS sensitivity, the resistance would be greater.

About vessel regression during vascular morphogenesis, the situation appears more complex. Indeed, the first stages of vascular development occur in the absence of blood flow. The initial vascular network should be hence flow-compatible, but is not flow-directed. When flow occurs, WSS sensitivity can take place and contribute to vascular remodeling.

In the model proposed by Franco et al. (2016), with no or reduced flow, ECs do not rearrange according to flow, whereas, above a threshold value, WSS sensitivity takes place. The axial polarity vector (the nucleus to Golgi apparatus vector) of ECs

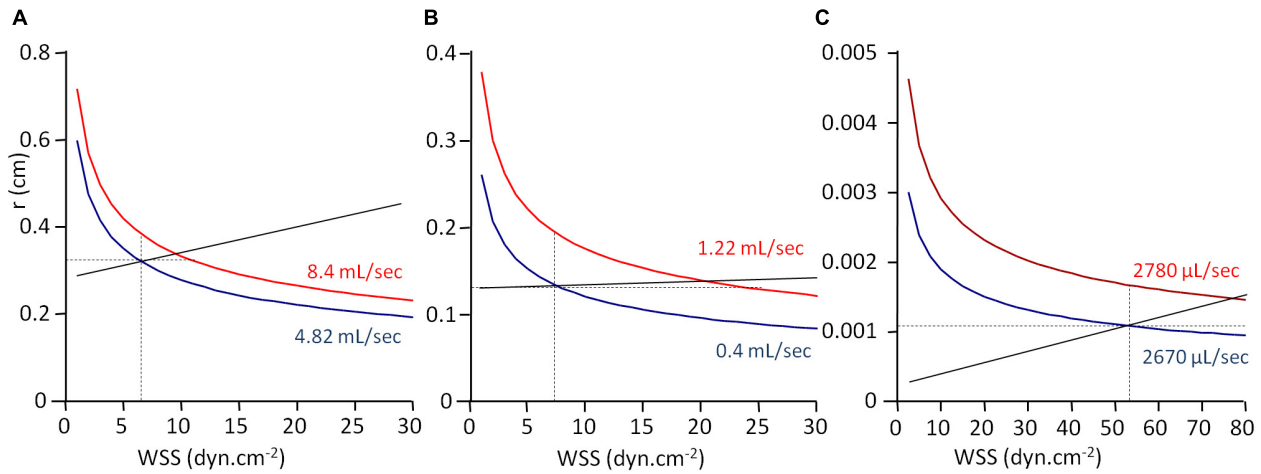

FIGURE 3 | WSS-induced vasoreactivity. Graphical representation of WSS and vessel radius from **Table 3** data. *r*, vessel radius; WSS, wall shear stress. The curves are the graphical representation of the function $r = f(\text{WSS})$ calculated according to Eq. 11. Values for blood viscosity (η), blood flow (*Q*), *r*, and WSS are the experimental ones given in **Table 3**, and the curve is built by varying WSS value and calculating *r* from the equation. Panels **(A)**, **(B)**, and **(C)** are the curves built from the data obtained in human common coronary artery **(A)**, human brachial artery **(B)**, and rat cremaster muscle arterioles **(C)**. Full black lines represent WSS sensitivity, the slope of the line being the sensitivity coefficient S_{WSS}. Intercepts of the line with the curves are the observed radius and WSS values. Horizontal and vertical dot lines correspond, respectively, to the absence of WSS sensitivity, and uniform WSS.

orients against the flow, and ECs migrate from low to high flow zones (Franco et al., 2016). Computational modeling of blood flow and WSS in newborn mouse retinal vascular plexus showed very high WSS values, up to 10 Pa (100 dyn cm^{-2}) close to the optical nerve, at the origin of the plexus, with a more or less linear drop to a WSS value below 2 Pa (20 dyn cm^{-2}) at 1 mm distance from the origin of the plexus (Franco et al., 2016). Cell density decreases with WSS, but not linearly. There seems to be a plateau in vascular density close to 3 Pa (30 dyn cm^{-2}), while EC polarization against the flow (axial polarity vector anti-parallel to flow) is linearly correlated with WSS value. This is compatible with the existence of a WSS value in reference to which vessels stabilize, though further studies are needed to experimentally support the existence of a set point value below which vessels regress and above which they stabilize.

Set Point Theory and Dynamic Equilibrium

Taken together, these experimental data support the existence of regulatory WSS-sensitive mechanisms that tend to stabilize WSS by short-term and long-term vessel diameter adjustments, and suggest the existence of a critical WSS value for vessel regression/stabilization during vascular morphogenesis. The uniform shear stress theory, as a physical principle, and the SPT, as a physiological one, constitute a relevant conceptual framework to analyze the different kinds of WSS sensitivity. However, the uniform shear stress principle should be viewed as an idealization of the vascular network that is not strictly verified experimentally. WSS value is not uniform all along the vascular bed. Moreover, in some cases, as we have seen for WSS-induced vasoreactivity, the mechanisms for WSS regulation are incompatible with strict maintenance of WSS. This does not invalidate the idea that local sensing of WSS contributes to the overall architecture and efficiency of the vascular network, but raises the question of how the reference WSS value is adjusted locally.

Another important issue is how we conceive and represent the mechanisms that minimize deviation from the set point value. As exposed above, in the classical representation of regulatory mechanisms, grounded on the theory of information, regulatory processes are viewed as a flowchart of instructional steps. As highlighted by Baeyens and Schwartz (2016), such an algorithmic view of set point maintenance is unlikely to account for the real processes occurring during vascular remodeling. Instead, they have proposed a model including three pathways, denoted A, B, and C, of different WSS sensitivities and effects on vessel remodeling (Baeyens and Schwartz, 2016). The model presented by the authors is given in **Figure 4A**. A reaches its maximum for low WSS, C begins to increase for high WSS, and B increases linearly with WSS. Additionally, A, B, and C interfere as follows: B inhibits A and C inhibits B. Integration of these interactions produces the following patterns for A, B, and C outputs, denoted A′, B′, and C′, given in **Figure 4B**. A′ is maximal for low WSS, and then decreases while B′ increases. B′ is maximal for middle WSS value and C′ increases with high WSS values. Considering that high B′ induces quiescence, high A′ inward remodeling, and high A′C′ outward remodeling, the quiescent state occurs for a specific WSS value determined by a dynamic equilibrium between these three pathways. This value can be said the "set point" value (Baeyens and Schwartz, 2016).

In their article, the set point model published by Baeyens and Schwartz (2016) was presented graphically, but it can be described mathematically. This allows more precise prediction of the pathway outputs A′, B′, and C′, and the existence of a set point value for WSS. We hence propose the following mathematical

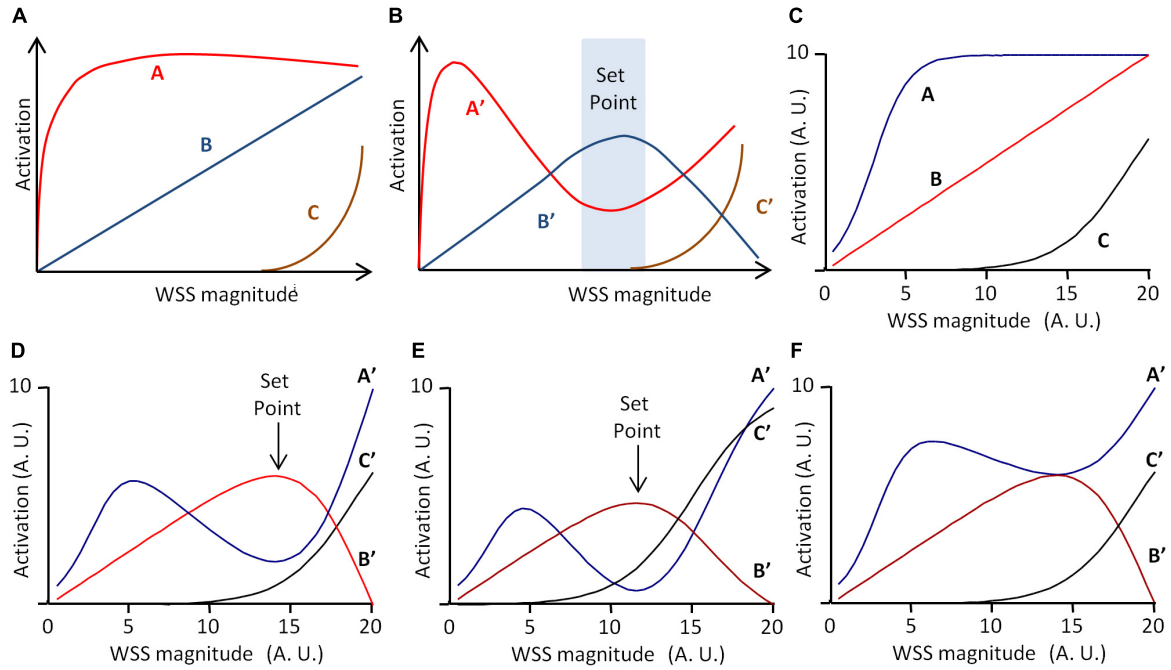

FIGURE 4 | Scheme of the set point model for vascular remodeling. **(A,B)** Scheme of the dynamic model proposed by Baeyens and Schwartz (2016), redrawn from the original publication. A, B, and C are three WSS-dependent intercellular pathways determining vessel remodeling and quiescence. **(A)** Independent activation profile of A, B, and C. **(B)** Activation profile of A, B, and C outputs (A′, B′, and C′, respectively), when C inhibits B, and B inhibits C. If A′ determines inward remodeling, B′ quiescence, and A′C′ outward remodeling, the model predicts a "set point" zone of WSS for which B′ is greater than A′ and C′, corresponding to the quiescent state. **(C)** Graphical representation of the mathematical formulation of the model (Eqs 12–17) with A_m = 10, C_m = 10, α = 3, β = 0.5, γ = 19, *ha* = 0.4, and *hc* = 0.2. **(D)** Model prediction for A′, B′, and C′ for I_c = 100% and I_b = 80%. **(E)** Model prediction for A′, B′, and C′ for γ = 15 instead of 19. Values are expressed in arbitrary units (A.U.). **(F)** Model prediction for A′, B′, and C′ for I_b = 40% instead of 80%. Vertical arrows indicate the set point value, defined as maximal B′ value above A′ and C′ values. Values are expressed in arbitrary units (A.U.).

description, in which A and B are described by two sigmoidal equations and B by a linear one.

$$A = \frac{A_m}{1 + 10^{(\alpha + WSS)^{ha}}} \tag{12}$$

$$B = \beta \times WSS \tag{13}$$

$$C = \frac{C_m}{1 + 10^{(\gamma + WSS)^{hc}}} \tag{14}$$

These equations are purely descriptive, and the parameters A_m, C_m, α, β, γ, *ha*, and *hc* have been chosen so that, for WSS varying from 0 to 20 (arbitrary units), the profile of the equations corresponds to the profile of the pathways in the original publication (**Figure 4C**). The inhibitions of B by C and of A by B are described by two inhibitory coefficients I_C and I_B, respectively. A, B, and C outputs, denoted A′, B′, and C′, are then described as follows:

$$C' = C \tag{15}$$

$$B' = B \times \left(1 - I_C \times \left(\frac{C'}{C'_{max}}\right)\right) \tag{16}$$

$$A' = A \times \left(1 - I_b \times \left(\frac{B'}{B'_{max}}\right)\right) \tag{17}$$

C'_{max} and B'_{max} are the maximal values of C′ and B′ for WSS varying from 0 to 20. **Figure 4D** shows the resulting profiles of A′, B′, and C′ for $I_C = 100\%$ and $I_B = 80\%$. These profiles are similar to that presented by Baeyens and Schwartz (2016). The curves show indeed a "set point" value, corresponding to the WSS value for which B′ is maximal and superior to A′ and C′. This set point does not exist for each pathway taken individually in the absence of inhibitory interactions, but is the consequence of the dynamic equilibrium between A, B, and C and their inhibitory interactions. As illustrated in **Figure 4E**, change in the activation profile of one pathway results in change in the set point value. This may explain how similar pathways may lead to local differences in set point values through local changes in their activation profile. Additionally, if we define the robustness of the system by the amplitude of the difference between B′ (quiescence) and A′ and C′ (remodeling), the model explains how the robustness of the quiescent state can vary locally. The limit case is illustrated in **Figure 4F**. If $I_B = 40\%$, half of its initial value, then A′ is always superior or equal to B′. This shows that change in these interactions can lead to the loss of the quiescent state, and hence loss of the physiological equilibrium of the vessel.

Such a model can be adapted to vessel regression/stabilization, considering that vessel stabilization corresponds to a quiescent state for EC migration. One possible model is presented in

Figure 5. In this case, only two pathways denoted D and E are considered, E inhibiting D. D and E are described by two sigmoidal equations.

$$D = \frac{D_m}{1 + 10^{(\delta + WSS)hd}} \tag{18}$$

$$E = \frac{E_m}{1 + 10^{(\varepsilon + WSS)he}} \tag{19}$$

The parameters D_m, E_m, δ, *hd*, and *he* have been chosen to obtain the desired profiles presented in **Figure 5A**. D and E outputs, denoted D′ and E′, resulting from the inhibition of D by E, are expressed as follows:

$$E' = E \tag{20}$$

$$D' = D \times \left(1 - I_E \times \left(\frac{E'}{E'_{max}}\right)\right) \tag{21}$$

I_E is the inhibitory coefficient and E'_{max} are the maximal value of E′ for WSS varying from 0 to 20. E′ and D′ profiles for $I_E = 100\%$ are given in **Figure 5B**. According to E′ and D′ profiles, there exists a threshold value for WSS sensing. Considering that D′ determines cell migration against blood flow, the model predicts that cells migrate from low WSS regions to higher WSS regions, until they reach a region with a WSS value corresponding to the complete inhibition of migration. If E′ induces cell polarization, the model also predicts that cells polarization increases with WSS until it reaches the same value. This WSS value can be said the "set point" value, with the same semantic limitations previously notified. In these set point models of vessel remodeling and vessel regression/stabilization, the WSS "set point" value is the consequence of a dynamic equilibrium, not the determinant of an algorithmic process. It is also locally determined, being the consequence of local interactions between several pathways.

Clearly, the algorithmic representation is also inadequate for WSS-induced sensitivity. Schematically, the consequence of EC WSS sensing is an enhanced production of vasorelaxant agents (VA) such as NO that induce smooth muscle cell relaxation and subsequent increase in vessel diameter. This can be ensured by a positive (e.g., linear) relationship between WSS and VA production, which induces a proportional increase in vessel diameter, and subsequent drop in WSS (**Figure 6**). This does not pretend to describe the precise mechanisms of WSS-induced vasoreactivity, but just to illustrate its general principles.

This representation is a simplified but relevant one of the cellular processes identified as responsible for WSS-induced vasoreactivity. These processes constitute a feedback loop that limit WSS fluctuation, but there is no set value for WSS that determines VA production. The range of WSS sensitivity has boundary values, but, within these limits, there is no threshold value for VA production that corresponds to the "normal" steady state WSS. Hence, within the sensitivity range, WSS equilibrium is ensured by threshold-free mechanisms. The "set point" value is a consequence of the existence of the processes involved in WSS sensing, but not a causal element of any of these processes. Similarly, in the developmental model of flow-dependent vascular remodeling proposed by Franco et al. (2016), we should be aware that the threshold value present in the model does not correspond to a set point value for WSS, but to the lower limit of WSS sensitivity. The representation of WSS regulation as a series of instructional steps that include measurement of WSS and comparison with a reference value does not correspond to the real operating processes. The classical terminology of "standard value," "error," and "error monitoring" is purely metaphoric and hence misleading. The formulation of "set point" is also ambiguous. When used, it should be interpreted as a steady state level of WSS resulting from the dynamic equilibrium of mutually interacting processes, what can be called the "dynamic set point" theory.

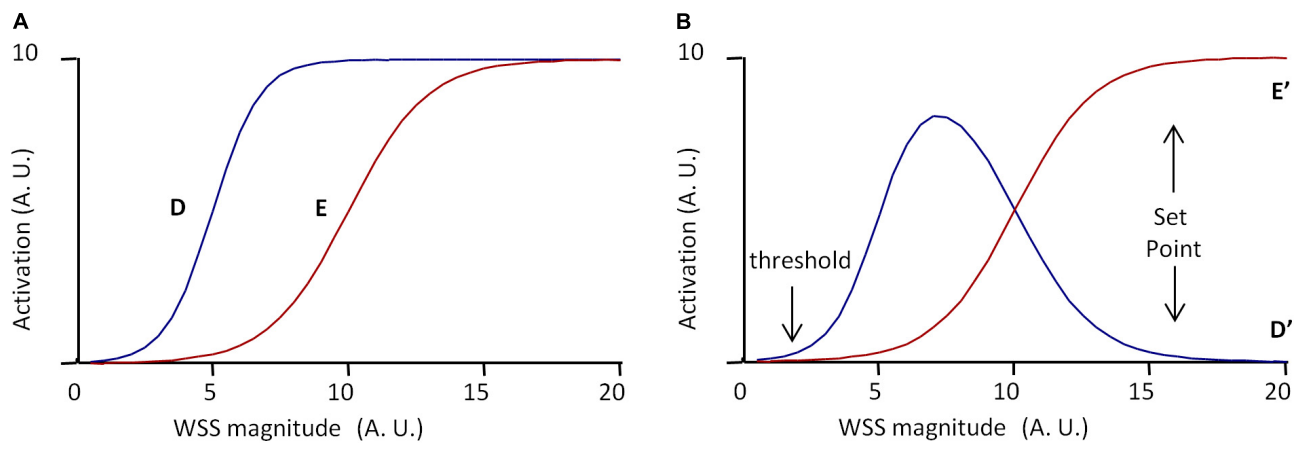

FIGURE 5 | Scheme of the set point model for vessel stabilization. The model is based on two pathways, D and E, defined by Eqs 18–21, E inhibiting D. **(A)** Independent activation profiles of D and E with D_m = 10, E_m = 10, δ= 5, ϵ= 10, *hd* = 0.5, and *hc* = 0.3. **(B)** Predicted activation profiles of D and E outputs (D′ and E′, respectively), for I_e = 100%. Values are expressed in arbitrary units (A.U.). The model predicts a minimal WSS threshold for WSS sensitivity. If D determines cell migration and E cell polarization, and if vessel stabilization is defined by maximal EC polarization and the end of EC migration, the model predicts a set point value for vessel stabilization, cell migration being maximal before the set point value is obtained.

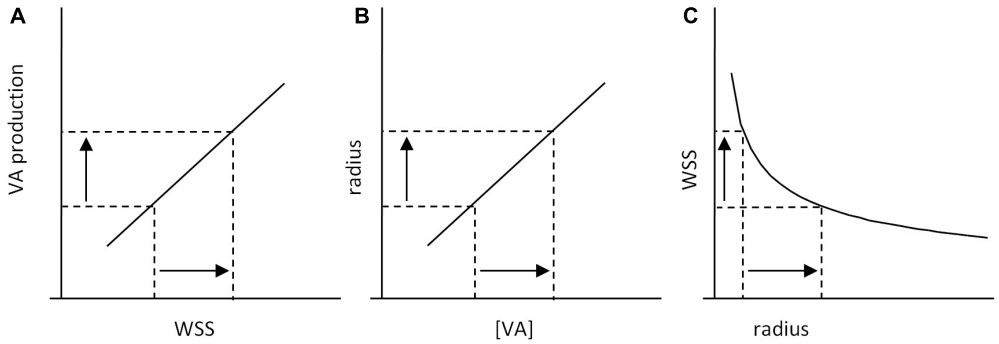

FIGURE 6 | Scheme of WSS-induced vasoreactivity regulation. **(A)** WSS-induced vasorelaxant agents (VA) production by ECs. An increase in WSS induces an increase in VA production, which leads to an increase in VA concentration (VA) in the smooth muscle cells (SMC) of the vessel wall. **(B)** VA-induced vasorelaxation of SMCs induces an increase in vessel radius. **(C)** Because of the physical relationship between vessel radius and WSS, increase in radius induces a decrease in WSS. Taken together, these processes constitute a causal loop that limits WSS variations: the consequence of an initial increase in WSS is WSS decrease, and reciprocally.

CONCLUSION

Wall shear stress sensing by ECs is a complex process that requires the ability of the cells to integrate the spatiotemporal characteristics of the WSS and behave in a differentiate manner to its different patterns. The concept of biotensegrity seems a relevant and fruitful conceptual framework to provide causal links of the stimulus–response coupling. In such a view, the notion of biochemical pathways is embedded in a more general organizational view governed by tensional constraints. The notion of primary sensors is not really relevant, since the whole cell architecture is the sensor, and a predominant explanatory value is attributed to mechanotransducers as a key element of active modification of the tensional equilibrium on the cell.

The existence of vascular WSS sensing is a general property of vascular systems in Mammals and other animal taxons (LaBarbera, 1990; Kochhan et al., 2013). This means that, though involved in several pathologies, it is first of all a physiological property that contributes to the adequate development and functioning of the vascular system. Involvement of WSS sensing in diseases is the exception, not the rule. Hence, understanding how it can be associated, in some cases, with vascular dysfunction requires first to understand how it contributes to the normal vascular function. Under some restrictions and precisions, both conceptual and terminological, the uniform shear stress principle and the SPT provide a relevant framework to interpret how WSS-induced EC behaviors contribute to the overall organization of the vascular network. Indeed, WSS variation is limited by active processes that regulate WSS value. The set point models for vascular remodeling, vessel stabilization, and vascular reactivity presented in this article are very simplified ones. Their purpose is not to give a realistic description of the precise mechanisms responsible for WSS sensing. It is to demonstrate that interplays between WSS-induced physiological processes can generate a set point toward which the vessels tend to stabilize. The concept of WSS set point value seems thus relevant, as far as it is interpreted as the consequence of a dynamic equilibrium between physiological processes and physical constraints, and not as a reference value in an algorithmic monitoring of WSS. In this view, the "set point" terminology is useful for designating the value to which the dynamic processes tend to equilibrate, but its meaning is more metaphorical than real. The expression of "threshold value" is ambiguous, since it may be used to name two different things that should not be confounded, the WSS set point value, and the boundary values of WSS sensitivity range. Actually, in the range of sensitivity, regulatory processes that contribute to the "set point" value can be threshold-free.

The uniform shear stress principle is a relevant concept, since it is broadly verified in practice. Coupled with the dynamic SPT, and applied to both vascular morphogenesis and vessel diameter adaptation, it explains how local processes of WSS regulation can produce overall optimized design (defined in term of energy expenditure) of the vessel network. The way WSS can shape the vascular architecture is typically a self-organized process, namely, a process in which the overall organization is determined by local behavioral rules without centralized control (Seeley, 2002). The dynamic SPT also provides a possible explanation for local variations of WSS set point values. It also predicts local variations in the robustness of the quiescent state. This may explain why some vascular zones are more susceptible than others to shift from a physiological quiescent state to a pathological permanent inflammatory one. Shift from normal to pathological vessels may also be explained by the loss of the existence of the quiescent state. It can also be due to the inability of WSS-induced vessel modification to restore the initial set point WSS value.

Though the dynamic SPT provides a theoretical possible explanation for local variations of the set point WSS value, it does not fully explain the local variations of WSS normal value. If these variations are due to local differences in WSS-sensitive pathway interactions and activation profile, the question remains of the cause of such variations. It can be hypothesized that other local processes (e.g., O_2 sensing) interplay with WSS sensing. This requires further investigation and implementation of the dynamic SPT.

Very localized variations in WSS normal values, and hence in normal WSS set point, can occur in a given vessel segment, as it happens, for example, in the aortic cross, with zones of laminar blood flow and high WSS, and others with disturbed flow and

low WSS. As seen previously, models based on 3D imaging of the vascular architecture and computational fluid dynamics can predict the spatial distribution of WSS values and their temporal variations due to blood flow pulsatility (Ong et al., 2020). The principle of dynamic set point WSS locally determined remains conceptually valid. If local variations of WSS values are coupled with local variations of WSS set point, the overall stability of the vessel is maintained. Also, local cell–cell interactions in the endothelial layer can contribute to coordinate the local response of the endothelium to WSS. However, at that scale, the validity of the principle of uniform shear stress is questioned. So, though valid in principle, application of the dynamic SPT to localized variations in WSS remains highly speculative. Its theoretical formulation would require coupled computational models of fluid dynamics with modeling of WSS-sensitive cell behavior. The development of such models would be helpful to understand how a vessel segment can shift from its physiological state to a pathological one.

In summary, the concept of biotensegrity provides a relevant explanatory framework for WSS sensing, and the dynamic SPT, coupled with the principle of uniform shear stress, a relevant one to understand how local WSS sensing can lead to the global optimization of the vascular architecture. Both concepts are dynamic ones. The behavior of the cells and the architecture of the vasculature are viewed as dynamic equilibrium of tensional forces and pathway outputs. These concepts can be formulated in a mathematical way. However, realistic models of such processes remain to be developed.

AUTHOR CONTRIBUTIONS

ER contributed to the general conception of the manuscript, presentation of the theoretical principles of WSS, shear stress and vasoreactivity, critical analysis of tensegrity, theoretical formulation and critical analysis of uniform stress principle and set-point theory, calculation of WSS sensitivity from bibliographical data, and overall writing of the manuscript. PB contributed to the review on WSS sensitivity in angiogenesis and vascular remodeling. PD contributed to the general conception of the manuscript, review on WSS sensitivity in angiogenesis vascular remodeling in relation with set-point theory, and overall writing of the manuscript. TC contributed to the general conception of the manuscript and overall writing of the manuscript.

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