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Abstract

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Brains Emerging: On Modularity and Self-organisation of Neural Development In Vivo and In Vitro

Paul Gottlob Layer

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Introduction: Biologic Determinism Revisited

Preformation and *epigenesis* as mutually exclusive ideas have over centuries dictated the quest for understanding of how organisms come into living. Epigenesis (not to be mistaken for *epigenetics*), as was first formulated by Aristotle, postulates new formation of the entire organism in each generation from scratch, i.e. envisions concepts of development. On the other side, ideas of preformation hold that the final organism is already somehow preformed in the egg (or, alternatively the sperm head; Malphigi 1672; see in Jahn 2000; Gilbert 2016), which then has only to be unrolled during embryonic growth. Preformationism, which has never vanished in biology

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completely, belongs to the category of determinism, while concepts of epigenesis rely on processes of emergence. AQ4

As biologists in the nineteenth century tried to advance their science to a more "exact science", determinism became a common position of eminent figures in biology. Ernst Haeckel presented hundreds of newly discovered protozoa in his famous plates not only as shiny colourful beauties, but also in perfect geometrical symmetry, certainly trying to make the point that a mathematical precision was behind their making (Haeckel 1904, 1998). Haeckel, certainly a shiny figure himself in many respects, was reductionist, monist and determinist. August Weismann, after having detected the early separation of germ and somatic cell lines in embryos, concluded that certain distinct (chemical) "determinants" would predetermine the fate of all cell types, and that only germ cells contained all determinants for the entire future body of a next-generation organism ("mosaic development"). Accordingly, each and every feature (morphologic, physiologic, etc.) would be completely determined by its respective determinants. Supporting this concept, Wilhelm Roux in 1887 had achieved half frog larvae (hemi-embryos), after having killed experimentally one cell of the two cell-staged frog embryos (an experiment which was hampered by methodological flaws). Hans Driesch, in trying to provide support of Roux' findings, managed to separate a four cell-stage sea urchin embryo into its four cells. To his surprise, four little but quite normal sea urchin larvae developed in his culture dish. What became to be called *developmental regulation*, was at the same time the discovery of stem cell totipotency. By then, embryologists had revealed good reasons to conceive development of an organism not as a mere unrolling of a prefixed programme.

During the same period, however, deterministic concepts in biology received strong support through great progress of the upcoming genetic era. Works of Beadle and Tatum in the early forties on the ascomycete *Neurospora grassa* (co)-founded the so-called *dogma of molecular biology* (see Strauss 2016), which stated that one gene codes for one (and only one) protein, and that each protein subserves one distinct function (e.g. enzymatic, structural, etc.). Although these early geneticists themselves were quite cautious in interpreting their findings one-dimensionally, genes then became more and more considered as completely autonomous, autocratic players ("determinants" in Weismann's words), each one sitting on top of a hierarchical cascade.

The development of Neo-Darwinism during the first half of the last century as a standard theory of evolution was much influenced by this concept. It led Ernst Mayr and colleagues to their famous saying "nothing comes between genotype and phenotype"; in fact stating that in order to understand evolution we do not have to bother with development and/or morphologies of embryos (phenotypes), but only with the genomes of adult organisms (capable of reproduction). What presumptuous, exclusive misconceptions, which have come to be called *gene-centrism* and *adultocentrism*: biologic determinism at its best! As a rather new subbranch of *Developmental Biology* now *EvoDevo* (idiom. for *Evo*lutionary *Develo*pmental Biology) has developed, which for the first time provides reasonable clues to mechanisms of macroevolutionary change (Gilbert 2016).

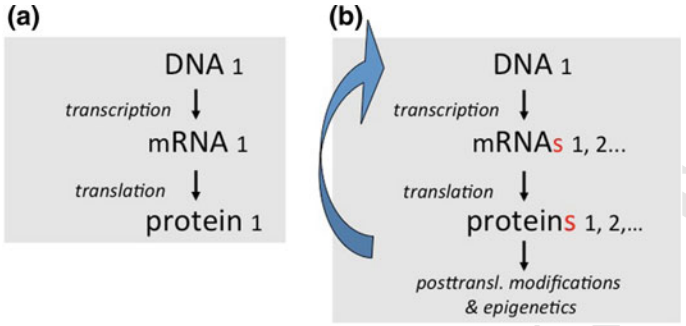

Fig. 1 Classic (**a**) and modern (**b**) concepts of gene realizations. According to (**b**), one gene ("DNA_1") can code for many different proteins, and proteins can feedback on gene activities. Further see text

Time was waiting for the rise of molecular developmental biology from the seventies onwards to achieve a new concept of development. As more and more model organisms were studied, minds of researchers were opened. Actions of genes became conceived as embedded within widely distributed networks, regulated by complex signalling cascades (Fig. 1). Thereby, feedback mechanisms between proteins and genes (transcription factors) can lead to prominent autocatalytic amplifications, or, as well, to silencing of particular genes (inhibition). *Time* and *space* of gene expression became decisive aspects of their actions, revealing the insight that one particular gene can affect many different things. Strict determinism in biology lost its appeal.

Concepts of biological emergence take a decisive anti-deterministic stand; they decline exclusive *gene-centrism*, and favour concepts of "nature *and* nurture". Emergence has been defined as the appearance of a new property in a system at a higher level of organisation, which is not explained by properties of a lower, more fundamental level. Such new properties are not predictable by, and not reducible to the more fundamental properties. Emergence deals with dynamic processes, e.g. processes of appearance (and disappearance), by the insight that "…something comes out from something …" (Fromm 2005; see other contributions in this book). Typically, *weak emergence* is distinguished from *strong emergence* (Chalmers and Jackson 2001). Thereby, "weak" means that the emerging properties are unexpected based on the lower-level properties, while "strong" defines new properties which—even in principle—are non-deducible and unpredictable from the given lower-level properties.

Clearly, the field of *Developmental Biology* is governed by emerging properties. As in all fields, features of emergence in biology are difficult to grasp. Nonetheless, are there means to characterise such processes for a developing organism? What are distinguishable levels of development of an animal? What are building modules, which level is lower, and which is above, if these levels are interrelated by complex feedback mechanisms? What means self-organisation, is it predictable; if not, why not? Such are the questions which are tackled in this chapter, which is divided into three parts.

1. A description of general aspects of normal (e.g. in vivo) animal development from a fertilised egg until—exemplarily—the formation of a vertebrate brain, thereby trying to define building modules and morphological levels of organisation.
2. Considerations on mechanisms of self-organisation (generation) of organised tissue/organ structures in vitro (as nowadays emanating into stem cell regeneration biology), demonstrating that normal developmental paths are not the only possible ones to achieve a certain goal ("many roads to Rome") and
3. A discussion on "genetic backbones" of modules in relation to "environmental constraints" (physical, chemical and ecological) that could drive emergent processes during development, independently from a particular causative gene action.

Modules Governing Normal Development

For long periods in the prehistory of life on our planet, life existed only in the form of unicellular organisms (3.5–1.8 Gya, giga years ago, or, billion years ago). The so-called prokaryotic cell was a "simple" molecular bag, having—as one of its notable features—no real nucleus. A major change occurred with the invention of an entirely new form of cell. Besides other essential novel organelles, the *eukaryotic cell* was equipped with a complete nucleus containing the genetic information and a double-layered outer cell membrane (plus a cell wall in the plant cell). Illustrious, spectacularly shaped unicellular organisms, called Protista, began to populate our planet (1.8–1.4 Gya). Only now the scene was set for the evolution of higher life, which—as we should have understood by now—certainly never was, and still is not possible without continuous mutual interactions with the prokaryotic world (McFall-Ngai et al. 2013).

Cells Forming Spheres

At some later point of evolution (1.4 Gya), particular eukaryotic cells developed a tendency to form small cell clusters, as a first sign of development of multicellular organisms. As still nowadays can be observed with green algae new species emerged step by step that would form larger and larger cell aggregates (here not considering that some prokaryotes also can associate to large biofilms). There are multiple hypotheses how multicellularity was achieved during evolution (Grosberg and Strathmann 2007), one of them suggesting colony-forming signals from bacteria onto eukaryotic cells (Alegado et al. 2012). Such colonies could still disaggregate under certain circumstances, and each individual cell would multiply by normal cell division (mitosis). Eventually, much larger, more organised species emerged. Presenting themselves under the microscope as splendid translucent spheres, they steadily rotate in their water habitat; that is why they became named "Volvox" (order

of *Volvocales*, name from *Latin* "volvere", to roll, rotate). Their individual cells were not identical any longer, but began to show signs of specialisation (e.g. flagella for motion), revealing the evolutionary onset of differentiation. Besides so-called somatic cells, they also produced reproductive cells. Their progeny was kept inside the spherical body, there forming *spheres within spheres*, until the outer body would release them and the original parent sphere would disintegrate and die. Along with the invention of multicellularity, cell and tissue differentiation, sexual reproduction and cell death had entered the living world. Hence, aggregation of cells into more and more regular spheres characterised this period.

This is not the place to engulf further into the spectacle of early evolution, but only to point out that the first multicellular shape within which cells organised themselves during *phylogeny* was the *cellular sphere*. Amazingly, a similar sequence of early events happens during the development of nearly each and any individual animal, during their *ontogeny*. After fertilisation of the egg, fast cell divisions amplify cell numbers (*cleavage divisions*), thereby forming a spherical ball of cells, a *blastula*. As in phylogeny, the sphere is the earliest and simplest multicellular structure in each individual´s life. Such an assembly of cells could be considered the simplest developmental module, with which new capacities/functionalities can and will emerge (e.g. communication between cells; see below). Sphere formation is an ever-recurring theme in biology: for instance, during the development of kidneys, liver, lungs and testes; in brain formation, cellular spheres will form brain nuclei or ganglia (e.g. dorsal root ganglia, DRG). Not to forget, as tissues disintegrate during cancerogenesis, tumours grow in the shape of spheres.

From Hollow Spheres to Planar Tissues

As we follow the developmental paths in different animals, patterns of development become more difficult to generalise. As blastulae in model animals like sea urchin or frogs grow bigger, a fluid-filled space emerges in their interior (blastocoel). Nearly in all animals, the following process of *gastrulation* represents a real cellular revolution. Spherical blastulae become quite abruptly transformed by an invagination of their outer parts (note: shapes of blastulae and types of *morphogenetic movements* differ greatly, depending on species). A distinction between inner and outer parts emerges with entoderm and ectoderm representing the first two *germ layers*. In most animal branches, the mesoderm as an intermediate germ layer pushes itself in between the other two (in fact, the—future—mesoderm appears to exert an initiating and driving force during gastrulation). Notably, along with these transformations creating three novel modules, cells transit from a more globular to a layered arrangement. Concomitantly, in some animal groups (Coelomata) a secondary fluid-filled bodily space forms the so-called *coelom* (abdominal cavity, dt. *sekundäre Leibeshöhle*). That is, from now onwards cells are not assembled any longer within a spherical volume, but they have become organised within planar cell layers, which marks the beginning of tissue formation.

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The Epithelium, the Most Basic Tissue

In histology, several types of tissues are distinguished (epithelium, blood, fat, nerve, muscle and bones/supportive). The only one that is relevant here is the epithelium. Epithelia are widespread in all animal bodies, covering outer and inner bodily surfaces, like skin, gut and capillaries, in embryonic and mature organisms alike. In an epithelium, many cells of a particular type are arranged "side-by-side", forming (in its simplest form) a one cell-wide layer in planar register. Along with their integration into a compound tissue, cells attain the same cell shape (e.g., cylindrical, cuboidal, etc.). Driven partially by active as well as passive forces, formation of epithelia represents an emergent process. The cell plane as a whole is polarised by a basal and an apical side, representing its inner and outer surface, respectively. The basal side is endowed with an extracellular matrix for optimal contact; the apical side presents protrusions (e.g. cilia, microvilli) for secretion, transport of fluids, etc. Several types of cell-to-cell junctions connect neighbouring cells, to stabilise the whole tissue and allow communication between all cells of the tissue. Each epithelium will subserve specialised functions, such as mechanical protection, containment of fluids and gases, ingestion or glandular secretion. Planar epithelia of diverse morphologies (simple, stratified and pseudostratified) will form tubes as essential parts of intestines, lungs, blood circulations (called *endothelia*) and heart. Each one tissue type represents an organismic building block, a module, which only as such (not the individual cells) can fulfil its distinct function(s).

Brain and Eyes Emerging from the Body Surface Epithelium

The initial step of neurogenesis is nothing but formation of an epithelial tube, derived from the ectoderm, a process called *neurulation*. Shortly following gastrulation, a mesodermal rod-like structure, the *chorda dorsalis*, is formed along the length of the embryo and becomes an *organising centre* for the steps coming. Chemical factors secreted from the chorda induce the overlying ectoderm to form an inwardly oriented, longitudinal groove. The groove closes dorsally to form a tube and separates from the overlying ectoderm. Then, the tube enlarges and differentiates in rostral–caudal direction, e.g. the future head is always farther developed than trunk and tail regions. Notably, some features that could be marginalised as "inevitable side products" will be indispensable for development of the nervous system. A population of cells that "accidentally" escapes during the process of tube closure, called *neural crest cells*, will migrate on defined paths out into the body space. The *neural crest* represents a major building module to find—besides other parts—the entire peripheral nervous system. Due to invagination of the ectoderm during neural tube formation, its inside-out polarity becomes reversed, e.g. the basal side will become the outside of the neural tube (see Fig. 2, and further below on eye development). As the tube extends in length and thickness, space restrictions within the future head

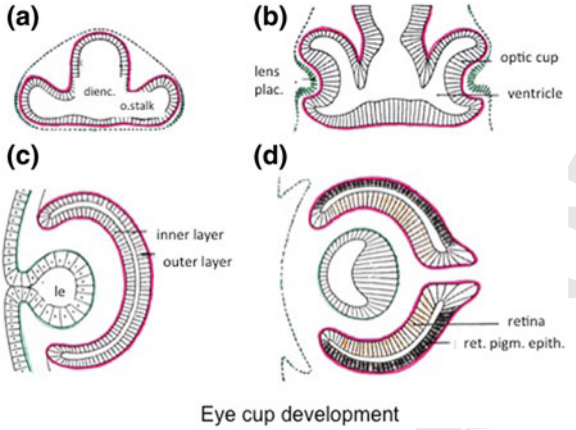


Fig. 2 Schematics of vertebrate eye cup formation. **a** Stage of optic stalk evagination from diencephalon. **b** Invaginating neuroepithelium after contact with ectoderm; lens placode is induced; **c** an inner and an outer layer of the neuroepithelium form the eye-cup; lens vesicle has enlarged; **d** inner layer forms retina, outer layer forms pigmented retinal epithelium (RPE), lens differentiates

will cause tube flexures, bends and partial rotations (note: this result is an excellent example for a mechanic rather than genetic causation). Along with it, the rostral (front) end of the tube is constricted into first three, then five brain vesicles (front-, mid- and hindbrain vesicles, or *Latin*, tel-, mes- and rhombencephalon), representing the first subdivisions of the rostral tube. All brain vesicles will be further subdivided into *neuromeres*. These become most evident in the hindbrain (rhombencephalon) as a series of numbered *rhombomeres* (Fig. 3). The number one rhombomere will later develop into the cerebellum. Following differentiation of the tube towards more caudal parts, the future trunk and tail regions will be segmented. Thereby, a close interplay between neural tube structures and mesodermal tissue (e.g. somites), muscular and skeletal anlagen is strictly controlled by a rostro-caudal clockwork (not further detailed here). Modularity of brain development is overtly demonstrated by these longitudinal subdivisions of the frontal neural tube since from each and every neuromere a distinct part of the future brain will develop (Lumsden and Keynes 1989; Layer and Alber 1990; Puelles 2001).

Neural Tube Evagination, Invagination and Widening to Form an Eye

The eye, in particular, retina and pigmented epithelium (RPE) are derived from the neural tube also. From the first brain vesicle, the neuroepithelium evaginates laterally to eventually touch the ectodermal surface (Fig. 2; eye formation). Being stopped at a point that marks the origin of the lens, the so-called optic stalk once again

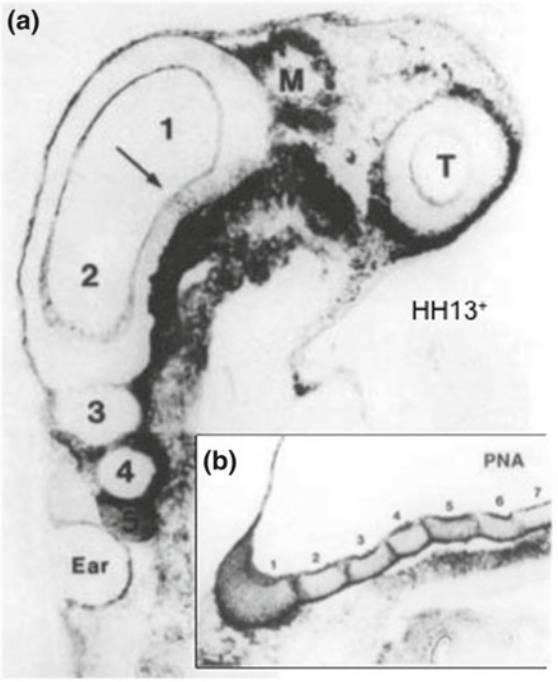


Fig. 3 Emergence of molecular boundaries in hindbrain of chicken embryos. **a** Sagittal section of a 2 day-old (HH13$^+$) chicken head and **b** a more horizontal section of a 3 day-old hindbrain, both stained by PNA lectin (black). Rhombomeres of hindbrain are numbered 1–7. Note diffuse emergence of staining between R1 and R2 at HH13$^+$ (arrow in **a**). By HH17 (**b**), all boundaries in between rhombomeres 1–7 are strongly stained. Further see text. Pictures taken from Layer and Alber (1990)

invaginates to form a double-layered optic cup; the outer layer will soon turn into the black RPE, the inner will differentiate into the retina. Similar to movements during gastrulation, evagination and invagination of epithelial tissues lay the grounds for eye-cup formation.

The neural tube presents some unique epithelial features that found later formation of neuronal cell layers and networks during brain development (*lamination* or *stratification* of brain regions). As cells heavily divide within the neural tube, individual cell bodies shift back and forth between inside (apical) and the outside (basal) side, while their radial processes remain anchored to both epithelial surfaces. Each transversal (radial) position of a cell body correlates with a specific state within the cell cycle. Due to these *interkinetic migrations*, the neuroepithelium is wider than other unistratified epithelia. Under a microscope, it appears as if it would be stratified; therefore, it is called *pseudostratified neuroepithelium*. After a dividing cell undergoes its last mitosis, one of the emerging two daughter cells will continue to divide, while the other cell, which has now become "postmitotic", will migrate to the outer surface and begin to differentiate, e.g. it will send out a neuronal process. Consequently, a mantle layer forms on the outside of the tube, which marks the beginning of cell layer formation (lamination and stratification; see Weikert et al. 1990). In different areas of the future brain, lamination will follow different schemes (e.g. inside-out scheme in cortex, lamination of cerebellum or retina, etc., see below). Now, future network formation will set in: neuritic outgrowth, path and target finding

of neurites to/into distant brain areas (e.g., eye/retina to tectum), thereby establishing connections between neurons of different layers and areas. Synapse formation, refining of connections by their use, according to *fire-and-wire* mechanisms (see Glossar), only are some of further emerging steps of a maturing complex brain (here not further discussed).

Retinogenesis is comparable in all vertebrates, forming three nuclear (ONL, INL and GCL, see Abbrev.) and two plexiform layers (OPL, and IPL); of course, in detail, there are many species-specific differences not dealt with here (Fig. 4). In the forming eye-cup, the inner layer widens, since interkinetic cell migrations are prominent in the future retina. The first cells begin to differentiate at the inner border of the retina (e.g. basal side). The retina differentiates gradually from central to the eye periphery near the lens. As a rule, big cells are born before small cells, e.g. ganglion cells and photoreceptors, then amacrine and horizontal cells, and finally bipolar and radial glial cells (for different retinal cell types, see below and legend to Fig. 4). Vertebrate photoreceptors, which are considered the most complex cells in nature, become located at the outer interface next to the RPE. Their well-being during development and adult functioning depends heavily on mutual relationships with the RPE. During the first phase, photoreceptors in some species target directly on to ganglion cells, the terminal retinal cell type which will send an axon to the brain. Only as the network further matures, entrance (PRs) and exit cells (GCs) will become interconnected through interneurons. As amacrine ("without process"), horizontal and bipolar cells are born, they become located in an intermediate "inner" nuclear layer (INL). All neurons become wired together at the level of two synaptic layers, called inner and outer plexiform layers: first the inner plexiform layer (IPL) will emerge, followed by the outer OPL. Precursors of radial glial cells (Müller cells) spanning through the entire retina, stabilise the tissue during development (Reichenbach and Bringmann 2013). Being last to differentiate, they retain hidden features of stem cells, rendering them with capacities for retinal homeostasis and regeneration.

In summary, formation of cellular spheres, their transformation into planar epithelia, followed by tube formations are decisive steps leading to the development of nervous systems, which—as is dealt with in section "Decoding Self Organisation of Brain Tissue Formation (Genetic Backbone Versus Non-genetic Constraints)"—can be conceived as developmental modules. Thereby, morphogenetic movements (e- and invaginations), mechanic forces, inductive events between epithelia, polarity reversal, widening of epithelia are driving forces of emergent laminated neural tissues, like the retina.

Self-organisation of Neural Tissues In Vitro from Stem Cells

When development of a tissue or organ is being studied in its normal in vivo environment, effects due to cell-autonomous factors often cannot be clearly distinguished from external factors. Thence, causes of self-organisation or emergence of tissues remain ambiguous or occluded. One way to overcome this drawback relies on per-

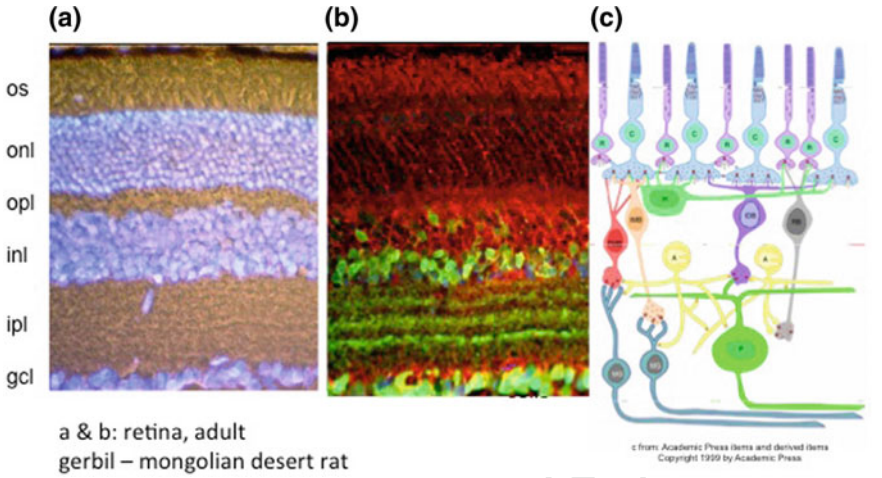


Fig. 4 Stratified (laminar) structure of vertebrate retinae, as represented by DAPI- (**a**), and Pax6-stained (green in **b**) retina sections of an adult Gerbil. Note three layers of cell bodies (ONL, INL, GCL in **a**), and synaptic sublaminae formed by $Pax6^{+}$ neurites from neurons in INL and GCL. **c** Network scheme of vertebrate retinae, consisting of five major neuronal cell types (photoreceptors, horizontal, bipolar, amacrine and ganglion cells), interconnected in OPL and IPL; radial Müller glial cell is not shown

forming tissue culture experiments. With standard procedures, cells isolated from a specific tissue (e.g. embryonic, brain part, diseased organ, etc.) are raised in a tissue culture dish, whereby the cell environment (atmosphere, media supplements, temperature, etc.) can be fully controlled. Depending on chosen culture conditions, cells will settle on the surface of the dish and proliferate. Cell division stops as soon as a more or less densely populated cell carpet is formed, and cells begin to differentiate. For instance, conditions of neurite outgrowth from embryonic neurons and of synapse formation between them can be studied at ease. In such two-dimensional (2D), or "flat" cell cultures, however, a cellular compound resembling a normal tissue formation is never achieved (except for some clustering of cells, in particular so with malignant cancer cells).

Emergence of Tissues In Vitro: Cell Reaggregation and Sphere Formation

As at the phylogenetic base of multicellular organisms (see above), formation of cellular spheres from the fertilised egg represents the most basic module of each individual development. In this respect, the postulate of *a recapitulation of phylogeny in ontogeny* fits well (ascribed to Haeckel, but in fact, was already formulated by Johann Friedrich Meckel 1821 and Fritz Müller 1864; see Jahn 2000, p. 373). Hence

not surprisingly, 3D cell cultures provide a superior approach over 2D cultures to demonstrate and probe self-organisational cellular capacities to form distinct tissues. In applying 3D cell culture techniques, fully dissociated stem cells from embryonic organ anlagen, or from some other source are constantly kept under rotation during their culturing (suspension cultures). Thereby, dispersed cells quickly reaggregate and form more or less regular cellular spheres. Under defined and optimal in vitro conditions, they can form tissue-specific structures. Besides improved nutritional and oxygen supplementation of cells, a major advantage of using 3D over 2D cultures are enhanced interactions between aggregating cells, which are promoted through constant movements of dispersed cells.

Self-organisation of a Chicken Retina from Precursor Cells

To form an organised "histotypic" tissue in vitro needs more than initial reaggregation and sorting-out processes. To this end, the chicken embryonic retina had proven an ideal study model already in the forties, not only because the retina is easily reachable within the eye, but also because retinal cells can be instantly distinguished from black cells of the retinal pigmented epithelium (RPE). Earlier work had revealed that RPE cells sort out in the centre of mixed retina/RPE reaggregates. Since RPE and retinal cells mutually influence each other (reviewed in Layer and Willbold 1994; Layer et al. 2010), in the early eighties we added RPE cells to retinal 3D cultures of the chick. Immediately, we could detect highly ordered spherical structures (Fig. 5; Vollmer et al. 1984). The histology of *stratospheroids* reveals an almost complete threefold retinal lamination, much comparable with the normal retinal lamination (Fig. 5c). This experiment demonstrated for the first time in history that formation of a nearly complete neuronal tissue can be experimentally reconstituted through self-organisation from stem cells in vitro (we called these structures *retinal stratospheroids*). Before their formation can be analysed in more detail, a more basic type of retinal reaggregate, which we have called *rosetted spehroids*, needs to be explained (Figs. 5b and 6).

Spheres Within Spheres: Rosettes and Clonal Cell Columns as Modules

As cells have been sorted out within spheres, their initial random distribution has much diminished. As a next step of tissue organisation, sorting-out is directly associated with emergence of rosettes (note: with murine cells, different processes apply; see below "many roads to Rome"). Groups of few segregated cells form several small cell rosettes within a much larger spheroid (within hours for chick cells). Thereby, rosettes are dividing stem cells that have—in principle—formed a small

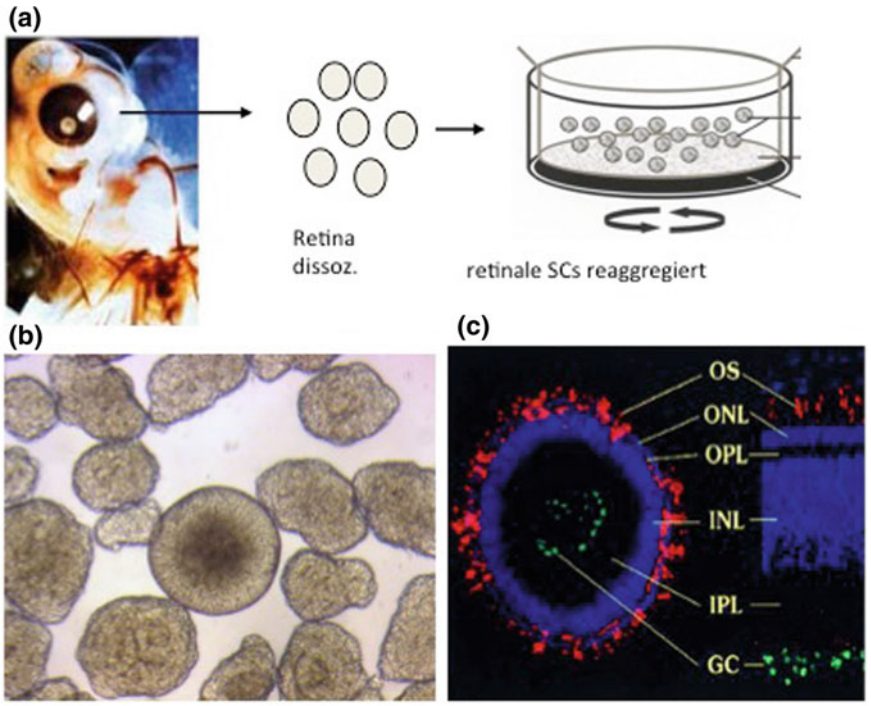


Fig. 5 Production (**a**, **b**) and histologic structure of correctly stratified retinal spheroids (called *stratospheroids*, **c**; see one in centre of **b**) from retinal precursor stem cells of the chicken embryo. **a** The retina is isolated from the eye and dissociated into single cells. Cultured under constant rotation, cells reaggregate into more or less regular cellular spheres (**a**, **b**). The potato-shaped spheres in (**b**) are *rosetted spheroids* (see Fig. 6)

circular, but already epithelial compound (Fig. 6a, equiv. to spheres within a sphere; cf. *Volvox*). Through cell division newborn mitotic cells are integrated laterally into this rosette, which thereby enlarges; internally, a fluid-filled space inflates. At the same time, clones of postmitotic cells are produced from precursor cells within the rosette (Fig. 6a, b). These daughter cells are stacked upon each other to present transversally oriented cell columns, which are stabilised by processes of radial glial precursor cells. Columnar cell clones become neatly stacked one-by-one, thereby surrounding each one rosette (see Fig. 6a, b). Cells within columns then differentiate into various retinal cell types, e.g. photoreceptors, amacrine, horizontal and bipolar cells. Therefore, by the two processes of *rosette enlargement* and *column formation* (*lateralisation* and *radialisation* of rosettes), modules of laminar retinal tissues have emerged within a larger spheroid.

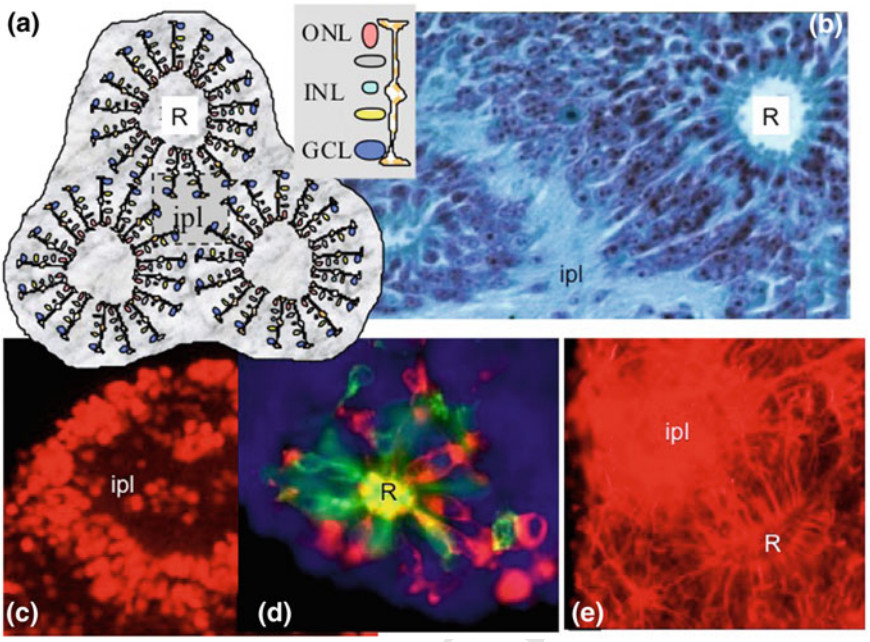


Fig. 6 Rosette ("R") and cell-column formation in *rosetted retinal spheroids*. **a** Schematic of internal structure of rosetted spheroids; note that photoreceptors point inside the rosette; insert in middle represents one cell column, consisting of all major cell types as a basic construction module. **b** HE-stained section of a rosette; coherent cell columns are evident. **c** Shows Pax6-stained amacrine cells of INL and GCL; **d** rod (rot) and cone photoreceptors (green) are located in rosette; **e** radial glial cells emanate from rosette towards IPL-like space

From Spherical Compounds to Planar Tissue: Fusion and Tissue Inversions

How can transformation from a *rosetted* into a *planar* arrangement of cells be achieved? At the outset of retinal spheroid formation, development of *stratospheroids* follows a similar path as that of *rosetted spheroids*. As their modular units (rosettes, see above) have reached a certain size, several of them will fuse. Often, these larger structures present an inverted laminar arrangement, e.g. future photoreceptors tend to be found internally and amacrine cells on the outside (note: in vitro ganglion cells quickly will die, due to absence of growth factors). Only after a complete reversal of the entire spheroid, a correctly layered retinal sphere, the retinal *stratospheroid* will be achieved. Thus, formation of rosettes and of cell columns represent spatial in vitro preconditions for further cell-layer differentiation, followed by the establishment of interconnecting networks (synaptic layers of IPL and OPL; not further discussed here). These different retinal spheroid models became the most instrumental to learn about self-organisational tissue formation

from isolated cells (see below in section "Decoding Self Organisation of Brain Tissue Formation (Genetic Backbone Versus Non-genetic Constraints)").

Brains Emerging In Vitro—Brain Organoids Have a Great Future

Having been neglected for a long time, only with the recent rise of stem cell biology the advantages of three-dimensional suspension cultures were again fully recognised. In particular, the availability of human *induced pluripotent stem cells* (iPSCs), highly structured retinal spheroids derived from human iPSCs now can be produced, called *organoids* (Meyer et al. 2009; Eiraku et al. 2011; Lancaster et al. 2013; Zhong et al. 2014). Organoids from hiPSCs resembling human *gastrulae*, so-called *Gastruloids*, are spectacular since they can form a *primitive streak* (area of gastrulation and onset of neurulation). After some authors considered these structures as "synthetic human embryos" (*sheefs*), a public dispute came up as to whether sheefs may become endowed with a human mind and consciousness. At any rate, organoids from retina or from other organs clearly have a great future in regenerative and transplantation medicine (Huch et al. 2017). The present hype on human organoids is based on two envisioned fields of applications: 1. human organoids could possibly be used for transplantation purposes to replace diseased organs, e.g. to cure blinded people. For some organs, e.g. skin, pancreas and liver, applications may become feasible soon, while for others there are still huge obstacles to be mastered (brain, retina, etc.). Successful first trials are ongoing. 2. At least as important, human organoids are already much applied as test models to analyse causes and possible cures of certain diseases. For instance, causes for congenital microcephaly disorders were analysed in cerebral organoids (Lancaster et al. 2013). Their applications will provide pharmacological and toxicological assay systems, which will help to drastically replace animal experiments. In fact, patient-specific (autologous) assays should become feasible, which would allow to test drugs and their side effects directly on a patient's in vitro tissue. Thus, 3D stem cell cultures form the basis of modern *Tissue Engineering* (Huch et al. 2017). Its present progress would not have been possible without extensive basic analytical research on construction principles of spheroids from different embryonic tissues, which will be described below.

Decoding Self Organisation of Brain Tissue Formation (Genetic Backbone Versus Non-genetic Constraints)

Section "Modules Governing Normal Development" has briefly outlined the development of animals by sequential processes from a fertilised egg to the cellular, then to histological (tissue) and organismic levels. Using retinal in vitro tissue regeneration as

an example, section "Self Organisation of Neural Tissues In Vitro from Stem Cells" documented that a population of dispersed stem cells can find ways to rearrange, multiply and eventually form a tissue that is highly comparable to its in vivo counterpart, a result apparently favouring autonomy of retinal tissue formation. However, particular details of in vitro retinal development were clearly dependent on specific features of the provided culture conditions. Can these findings help to analytically resolve to what extent emergent features contribute to brain development?

Each developmental step is regulated by underlying complex genetic-molecular networks. At the same time, each completed step brings with it novel environmental conditions, which in turn exert *constraints* on possible future (genetic) steps. On all organisational levels, from molecular up to organismic (including most decisive interactions with microbioms; see excellent review by McFall-Ngai et al. (2013), and ecological), such constraints bring about situations of needs or even stress that necessitate some reaction(s). Constraints upon genetic activities can be of purely physical nature (e.g. traction, pressure, gravitation, shape, sorting-out, temperature and pH) or chemical nature (cytokines, paracrine factors, hormones and nutritional status). Constraints can also originate from restricted time windows, limited spatial options, evolutionary relicts and more. Recent *EvoDevo* research defines these constraints as heterochronic, heterotopic and phyletic, respectively (Gilbert 2016). The following section attempts to decipher how much of retinal development can be assigned to genetic determination (is predictable), and how much to non-genetic constraints (not reducible and not predictable)?

Common Genetic Backbone In Vivo and In Vitro

Progress of modern molecular biology brought tremendous novel insights into the *nature versus nurture* dispute; whereby "nature" refers to the genetic backbone of a system, while "nurture" points to non-genetic (environmental) actions upon it. In fact, understanding of modular developments—as analysed above histologically—has now achieved molecular and genetic bases. To mention just a few examples: a spatial gradient of a fibroblast growth factor (FGF) and a counter-gradient formed by retinoic acid together balance segmentation of the neural tube in rostro-caudal dimension. Then, codes of *Hox (master)genes* define the identities of hindbrain *rhombomeres*, as well as those of cell layers and cell types in several brain areas (example eye development, see Meyer et al. 2009). Notably, the so-called *Wnt signalling pathway* is one of the most relevant molecular regulators of early development. Briefly, a cell-external *Wnt protein* binds to its cell-surface receptor. Receptor activation then initiates an intracellular molecular cascade, eventually regulating the expression of particular nuclear genes. This cascade is involved in a multitude of developmental processes (e.g. cell movements, axis specification and regionalisation of tissues), including the organisation of planar epithelia. In case of retinal spheroids, the molecular basis of tissue reversal remained obscure for a long time; although we had detected that it can be induced by RPE and also by Müller glial cells. Several groups including

ours searched for a lamina-inducing factor in retinal spheroids. Some growth factors, such as FGF, PEDF and GDNF (see Abbrev.) affected the ratio of rods to cones in both types of spheroids; however, they did not promote a laminar retinal structure. Eventually, a Japanese group found that Wnt-2b could induce the transformation of chicken rosetted into laminar stratospheroids (Nakagawa et al. 2003). Supporting this finding, supplementation of retinal cells from the Mongolian desert mouse (Gerbil) with Wnt-3b led to production of the first mammalian retinal *stratospheroids* (Rieke et al. 2018). Up to date, several reports have concluded that genetic networks that regulate retinal development in vitro and in vivo are basically comparable.

Sequence of Gene Activations Is Preserved In Vitro

Importantly, developmental genes have to be activated in the embryo at the right time at the right place. Accordingly, a spatiotemporally appropriate expression of the retinal genetic backbone is indispensable for normal retinal, as well as for retinal spheroid development. Indeed, proliferation and differentiation of cells occur in vitro on a comparable time scale as in vivo, eventually leading to a nearly complete laminar network, presenting all cell types including complex synaptic layers. Within spheroids, the various cell types differentiate quite normally, including expression of specific neuronal genes. As in vivo, in vitro formation of complex retinal connections is established, whereby an inner plexiform layer (IPL) precedes that of an outer (OPL). For instance, IPL sublamination in vitro is detectable in 5–6 days-old rosetted spheroids, corresponding well to completion of lamination around E12 in the normal chick retina. Recent seminal work by David Gamm and colleagues (Madison, WI) has documented that genetic networks that rule normal eye development from the state of a neural tube epithelium until reaching a differentiated retina plus a black RPE compare quite well with in vitro retinal spheroids. Most interestingly, at the earliest onset of aggregate formation of embryonic stem cells (ESCs) or, of induced pluripotent stem cells (iPSCs), *Oct* 4 and *Nanog* genes were expressed. These are genes which characterise the blastula/blastocyst stage, e.g. the earliest spherical multicellular structure following fertilisation. About one week later, genes characteristic of formation of the eye field within the telencephalic brain vesicle, e.g. Pax6, Rx and a.o., and only a couple days later genes characteristic of retina or RPE differentiation became expressed (Meyer et al. 2009). These findings convey important information: irrespective of in vivo or in vitro environments, all development relies on activities of particular genetic networks (with a stress on *networks*, not on *genes*). The fact that most differentiation events occur on a similar time scale as in vivo strongly indicates that differentiation in vitro underlies similar, or even identical regulatory genetic networks. On one side, such networks can be considered as molecular modules (for instance, the Wnt signalling pathway); on the other side they are quite often flexible and/or mutually overlapping (whereby one particular gene can be involved in different modules performing different functions) or can even be exchanged by others. For instance, during eye-stalk formation the Pax6 gene is involved in a different genetic

network than it is during later differentiation of amacrine cells, when this gene fulfils a completely different function within another network. Thus, the same gene can be involved in very different events. Often, it remains uncertain what gene is on top, which one is at the bottom of a molecular network, which gene acts above (master-gene), which protein "downstream", which gene regulates which protein, and which protein acts back on which gene (feedback effects, cf. Fig. 1). But noticeably, gene activities are never non-essential, or dispensable.

Non-genetic Constraints on Tissue Self Organisation

Many features of retinal normal and in vitro development are strongly dependent on non-genetic constraints and self-organisational processes. Even at the subcellular level during the cell cycle, a high local chromatin order within cell nuclei is achieved through self-organisation (Cremer et al. 2014). Also, small chromosomal regions become autonomously arranged according to their chromatin class (van de Werken et al. 2017). Two examples for physical constraints during normal eye development are as follows: (i) as the eye stalk protrudes laterally (Fig. 2), it eventually will contact the outer surface ectoderm, which induces the lens placode, and also—due to expanding growth—pressures the neuroepithelium to bend inwards and thus form the two-layered optic cup; [note that in vitro produced "eye-cups" also bend inwards, which may be due to mechanic instability of an enlarging hollow sphere; cf. conflicting interpretation by Eiraku et al. (2011)]. (ii) As a further consequence, the two tissue layers will now touch each other with their apical sides. The opposition of two apical epithelial surfaces provokes a rare situation, leading to mutual inductive events between future retina and RPE, which in turn will determine differentiation of both photoreceptors and RPE.

A Brief History of Spheroids: Self-organisation in Spheres by Sorting-Out

A brief look into the long history of 3D cultures helps to get a better conception of self-organisation and emergence of tissues from individual cells, in particular, in understanding that tissues can be reconstituted by purely physical means in a culture dish. When kept in suspension, dispersed cells enjoy an additional spatial degree of freedom which allows them during and after their primary aggregation (also called *self assembly*) to find the best suitable locations within a growing cellular sphere. 3D cell culturing has begun with "shaking cultures" ("Schüttelkulturen") at the end of the nineteenth century by using sponges, sea urchins and newt larvae, swiftly unravelling basic concepts of cell biology. As an outstanding example, Henry van Peters Wilson dissociated sponges completely into isolated cells, transferred them

into glass dishes and shook them softly in salt water, to then follow how they grew into cell clusters ("*reaggregates*"). To Wilson's surprise, his reaggregates eventually self-organised into complete viable sponges (Wilson 1905; Fig. 7). Even more surprising, when he used cells from two different sponge species (which were marked by colours), differently stained cells were either found within separate reaggregates, or they were amassed in distinct areas within one reaggregate. If differently stained cells originated from the same sponge species, but from different individual animals, cells were distributed statistically within reaggregates. What became well-known as phenomenon of "sorting-out" was—at the same token—the striking discovery of cell-cell recognition (distinction of self versus non-self). Townes and Holtfreter documented pronounced sorting-out of epidermal cells from neural plate cells of the amphibian embryo, whereby their relative position within the aggregate resembled that within the embryo (review in Layer and Willbold 1994). Moreover, an advanced tissue-specific differentiation was indicated. Based on the same technique, regeneration of complete hydras from isolated cells became an outstanding animal model, revealing significant genetic, molecular and histologic knowledge of stem cell and regeneration biology of hydrozoa (Gierer 2012).

Malcolm Steinberg provided a theoretical explanation of the sorting-out phenomenon, based solely on physicochemical properties of cells. Accordingly, different cell types in a mixture were assumed to segregate as a consequence of differential strength of intercellular adhesion (*differential adhesion hypothesis*). Indeed, cells in a given tissue compound depend largely on their respective cell surfaces and extracellular matrices. Accordingly, emergence of tissue properties primarily depends on purely physicochemical conditions, and not so much on *one* particular gene. Such short distance forces will mediate cell cohesiveness (adhesion), optimal integration of cells into a given space, growth directions of their processes, etc. It is of note that individual contributions to the whole emergent process will be numerous (e.g., including mechanical forces; see Franze 2013); they cannot be deciphered in detail or estimated by precise numbers. The effects even can turn out anti-intuitively. For instance, minute irregularities of similar cell shapes can have positive pattern-forming power (Lenz and Witten 2017). Together with forces acting on distance (e.g. diffusible growth factors, cytokines), attraction and retraction between cells, cell migration and final placement all contribute to tissue self organisation. *In summa,* combined physical forces can direct primary steps of tissue formation in an artificial "in vitro space".

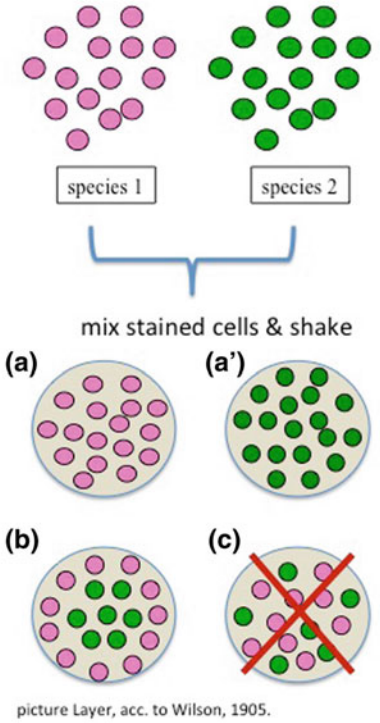
Emergent Borders Are Decisive to Structure Tissues and Organs

In a culture dish, separation of similar cells can be directly followed under a microscope (provided that they are somehow labelled). Their segregation leads to "islands", i.e. to regions of similar cells within a larger sphere. However, the process of physical sorting-out is not as obvious during normal development of tissues, yet in principle

Henry van Peters Wilson
Marine Lab John Hopkins U.
Bild: http://beaufortartist.blogspot.de/2008/03/origins-of-marine-lab.html

Reaggregation experiment of dissociated sponges revealed:

- „sorting out" phenomenon
- recognition of self/non-self



picture Layer, acc. to Wilson, 1905.

Fig. 7 Discovery of cell communication and sorting out in reaggregation experiments of dispersed sponges (Wilson 1905). After reaggregation of dispersed cells from two different sponge species, cells from the two species were found either in different aggregates (**a**), or within segregated areas of the same aggregate (**b**), but were not distributed randomly (**c**)

it also takes place. In fact, it represents a basic process during formation of morphologic/functional subunits. For instance, during subdivision of the early neural tube a series of *rhombomeres* of the early hindbrain become separated by strict (structural) border lines, which can be visualised by appropriate marker molecules (Lumsden and Keynes 1989; Puelles 2001). At onset, some of these markers emerge faintly and are spread quite broadly, to then concentrate more and more towards a focussed border (Layer and Alber 1990; cf. Fig. 3). Eventually, mechanically forced constrictions coincident with these borders further strengthen separation of brain subareas. That thereby sorting-out is involved has been again demonstrated in vitro by mixing and sorting of cells from individual rhombomeres (Götz et al. 1996). Hence, emergence of tissue borders is supported by physical (incl. mechanical, cf. also Franze 2013) means, and without doubt is indispensible for normal embryonic development.

Many Roads to Rome—Plasticity of Tissue Formation

The formation of several distinct types of chicken retinal spheroids highly depends on environmental factors. Retinal spheroids in their most basic form are characterised by internal rosettes and plexiform synaptic regions (*rosetted spheroids*; Fig. 6; their modular structure). Similar rosetted spheroids could be produced from embryonic mouse and rat retinae (e.g., by C. Barnstable, P. Linser, T. Reh; see Layer and Willbold 1994). However, it was most stunning that when retinal spheroids were produced from the Mongolian desert mouse (gerbil), they were not initiated from rosettes, but tissue organisation began at the level of formation of an inner plexiform layer (IPL; Bytyqi et al. 2007). Similarly, retinal spheroids from *Brachydanio rerio* (zebrafish) achieve a laminar structure without being initiated much by rosettes (Eldred et al. 2017). These findings are highly relevant in terms of retinal tissue self-organisation: albeit the basic laminar structure of avian, rodent and fish retinae is very similar (three-layered structure of all vertebrate retinae, see above), to rebuild them from dissociated cells can follow very different paths ("many roads lead to Rome"). Apparently, dispersed cells from different vertebrate origins in a culture dish seem to be determined by an inherent intention of "we are going to build a vertebrate retina" somehow, clearly indicative of a "meta-level" of information above the genetic code that is driving and safeguarding development. The physical nature of this "blueprint" remains widely unclear. At any rate, what becomes instantly clear when working with 3D cultures is that in vitro tissue formation depends to a large extent on culture conditions, e.g. on paracrine factors, on species and many more. Hence, not only particular genes drive formation of a layered neural network tissue, each one performing one specific function (nature versus nurture discussion; indeterminate versus cell-autonomous development), but non-genetic constraints are as decisive.

Conclusions

The idiom of "something comes out of something"—well exemplifying emergence thought—is represented by no other research field more directly than by organismic development (saying this is nearly a tautology). At a first sight, however, normal development appears to follow a determinate one-way road, whereby typically not individual genes, but genetic networks regulate what will happen at a certain place and a certain time in a growing organism. At each given spatio-temporal point in development, distinct *environmental situations* will prevail to cause novel constraints on the genetic backbone. However, as revealed by retinal spheroids, development depends much on environmental conditions. The sequel of any particular "space-time point" under in vivo conditions is only predictable because *the respective constraints themselves are reliably reproduced* during each individual course of normal development. When released from constraints during in vitro development, then development of a

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system (tissue, organ, organism) is liberated from its determinative power. In summary, we conclude that…

- Normal development of organisms (in vivo DoO) is governed by ground-laying developmental genes.
- In vivo DoO appears as if it were determinate, since the result is predictable.
- However, when analysed under in vitro conditions, emergent principles of DoO are readily revealed, rendering DoO as highly regulative and non-predictable.
- During DoO not individual genes, but rather gene–protein networks represent molecular toolboxes which can be used in changing combinations.
- DoO can resort to such tools for regulating formation of recurring modules, such as cellular spheres, planar epithelia, constricted tissue borders and more.
- In vitro analyses of developmental modules of a tissue, more specifically, of their genetic backbone and environmental constraints (as exemplified here for retina) are essential to understand normal as well as aberrant (diseased) development of a tissue (promoting applicability in stem cell-based regenerative medicine).
- Therefore, earlier prevailing deterministic positions in embryology have been much restricted by insights of modern developmental biology.

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Glossary and Abbreviations

- Blastocoel—fluid-filled hollow space of blastula;
- Blastula—cell ball (sphere) formed through cleavage divisions;
- Cleavage—rapid cell divisions after fertilisation;
- Coelom—fluid-filled space surrounded by mesodermal epithelium;
- Constraints—limitations of development through environmental (non-genetic) conditions;
- Differential adhesion hypothesis, see *sorting-out*;
- Ectoderm—outer germ layer;
- Endothelium—epithelium forming blood vessels;
- Endoderm (entoderm)—inner germ layer;
- Epithelium—planar tissue covering internal and external surfaces, e.g., skin, gut, etc.;
- *fire-and-wire* mechanism—refinement and stabilisation of neuronal connectivities by their repeated usage;

- Gastrulation—proces by which three germ layers are established in animals;
- Growth factors (cytokines):
  - FGF, fibroblast growth factor;
  - PEDF, pigment epithelium-derived factor;
  - GDNF, glial derived neurotrophic factor;
- Lamination, see *stratification*;
- Mesoderm—middle germ layer in between ecto- and entoderm;
- Morphogenetic movements—classification of cell migratory mechanisms, e.g., during development, such as e- and invagination, ingression, epiboly, etc.;
- Müller glial cell—radial glial cell of retina, spanning its entire width;
- Neural crest—cell population in most vertebrates emigrating dorsally from closing neural tube, which will found peripheral nervous system (and more);
- Neuromeres—early regional subdivisions of frontal neural tube;
- Ontogeny—course/process of development of an individual organism;
- Organising centre—cells or tissue parts, from which particular steps of development are initiated;
- Organoid—from stem cells in vitro regenerated organ-like tissue;
- Phylogeny—course/process of appearance of all phyla (stems) of organisms (phylogenetic tree) over the entire evolutionary period;
- Primitive streak—tissue structure in developing birds and mammals indicating the onset/course of gastrulation;
- Pseudostratified neuroepithelium—monolayered cellular status of neural tube, which due to its width appears to be stratified, but it is not;
- Retinal cell layers:
  - GCL, ganglion cell layer;
  - INL, ONL, inner and outer nuclear layer;
  - IPL, OPL, inner and outer plexiform layer;
- Retinal cell types:
  - AC, amacrine cell—large axon-less cell positioned at inner border of INL, connecting BPs and GCs in IPL;
  - BP, bipolar cell—interneuron in INL, connecting PRs and HCs in OPL, and with ACs and GCs in IPL;
  - HC, horizontal cell—large cell positioned at outer border of INL, connecting PRs with BPs;
  - PR, photoreceptor cell; comes either as rod or several types of cones;
- Rhombomeres—segmental subdivisions of hindbrain;
- Reaggregate—ball (sphere) of adhering cells formed by reaggregation from dispersed cells;
- RPE—retinal pigmented epithelium;
- Sheefs—"synthetic human entities with embryo-like features": a human organoid made from hiPSCs which presents a primitive streak (see, *gastrulation*);

- Sorting-out—process by which different reaggregating cells kept under rotation/in motion associate with similar, and separate from different partner cells; see, differential adhesion hypothesis;
- Spheroids, reaggregated from embryonic chicken retinae,
  - rosetted retinal spheroid—reaggregated cell sphere from dispersed embryonic chicken retinal cells, spatially organised by internal cell rosettes;
  - stratospheroid—dto., achieving a (nearly) complete retina-specific lamination (retinal organoid);
- Stem cells—cell with inherent proliferative ability, which in vitro can be amplified and then directed into one or more distinct differentiated cell type(s);
  - ESCs—embryonic stem cell;
  - iPSCs—induced pluripotent stem cell;
  - hiPSCs—human iPSCs;
- Stratification—arrangement of distinct cell types within cell layers, e.g., in brain and retina;
- Tissue Engineering—artificial (in vitro) reconstruction of tissues from stem cells applying engineering technologies;
- Wnt protein—cell-external ligand protein for the Wnt signalling pathway, a major communication pathway between cells during development and disease (*Wnt* stands for "wingless-related integration site").

References

AQ6

Alegado RA, Brown LW, Cao S, Dermenjian RK, Zuzow R, Fairclough SR et al (2012) A bacterial sulfonolipid triggers multicellular development in the closest living relatives of animals. eLife 1:e00013

Bytyqi AH, Bachmann G, Rieke M, Paraoanu LE, Layer PG (2007) Cell-by-cell reconstruction in reaggregates from neonatal gerbil retina begins from the inner retina and is promoted by retinal pigmented epithelium. Eur J Neurosci 26:1560–1574

Chalmers DJ, Jackson F (2001) Conceptual analysis and reductive explanation. Philos Rev 110:315–361

Cremer T, Cremer C, Lichter P (2014) Recollections of a scientific journey published in human genetics: from chromosome territories to interphase cytogenetics and comparative genome hybridization. Hum Genet 133:403–416

Eldred MK, Charlton-Perkins M, Muresan L, Harris WA (2017) Self-organising aggregates of zebrafish retinal cells for investigating mechanisms of neural lamination. Development 144:1097–1106. https://doi.org/10.1242/dev.142760

Eiraku M et al (2011) Self-organizing optic-cup morphogenesis in three-dimensional culture. Nature 472:51–56

Franze K (2013) The mechanical control of nervous system development. Development 140:3069–3077. https://doi.org/10.1242/dev.079145

Fromm J (2005) Types and forms of emergence. Cornell University Library. arXiv:nlin/0506028

Gierer A (2012) The hydra model—a model for what? Int J Dev Biol 56:437–445

Gilbert SF (2016) Developmental biology, 11th edn. Sinauer Ass, MA, USA

Götz M, Wizenmann A, Reinhardt S, Lumsden A, Price J (1996) Selective adhesion of cells from different telencephalic regions. Neuron 16:551–564

Grosberg RK, Strathmann RR (2007) The evolution of multicellularity: a minor major transition. Annu Rev Ecol Evol Syst 38:621–654

Haeckel E (1904, 1998). Kunstformen der Natur. Neudruck der Erstausgabe in Faksimile. Leipzig, Wien, Bibliogr Inst. ISBN 3-7913-1979-5

Huch M, Knoblich JA, Lutolf MP, Martinez-Arias A (2017) The hope and the hype of organoid research. Development 144:938–941. https://doi.org/10.1242/dev.150201

Jahn I (2000) Geschichte der Biologie, 3rd edn. Spektrum Akad. Verl. Heidelberg, Berlin

Lancaster MA, Renner M, Martin CA, Wenzel D, Bicknell LS, Hurles ME, Homfray T, Penninger JM, Jackson AP, Knoblich JA (2013) Cerebral organoids model human brain development and microcephaly. Nature 501:373–381

Layer PG, Alber R (1990) Patterning of chick brain vesicles as revealed by peanut agglutinin and cholinesterases. Development 109:613–624

Layer PG, Willbold E (1994) Regeneration of the avian retina by retinospheroid technology. Prog Ret Res 1994(13):197–230

Layer PG, Araki M, Vogel-Höpker A (2010) New concepts for reconstruction of retinal and pigment epithelial tissues. Exp Rev Ophthalmol 5:523–544

Lenz M, Witten TA (2017) Geometrical frustration yields fibre formation in self-assembly. Nat Phys 13:1100–1104. https://doi.org/10.1038/nphys4184

Lumsden A, Keynes R (1989) Segmental patterns of neuronal development in the chicken hindbrain. Nature 337:424–428

McFall-Ngai M, Hadfield MG, Bosch TC, Carey HV, Domazet-Loso T, Douglas AE et al (2013) Animals in a bacterial world, a new imperative for the life sciences. Proc Natl Acad Sci USA 110:3229–3236. https://doi.org/10.1073/pnas.1218525110

Meyer MS et al (2009) Modeling early retinal development with human embryonic and induced pluripotent stem cells. Proc Natl Acad Sci USA 106:16698–16703

Nakagawa S, Takada S, Takada R, Takeichi M (2003) Identification of the laminar inducing factor: Wnt-signal from the anterior rim induces correct laminar formation of the neural retina in vitro. Dev Biol 260:414–425

Puelles L (2001) Brain segmentation and forebrain development in amniotes. Brain Res Bull 55:695–710

Reichenbach A, Bringmann A (2013) New functions of Müller cells. Glia 61:651–678

Rieke M, Bytyqi A, Frohns F, Layer PG (2018). Reconstructing mammalian retinal tissue: Wnt3a regulates laminar polarity in retinal spheroids from neonatal Mongolian rats, while RPE promotes cell differentiation. Int J Stem Cell Res Therapy. https://doi.org/10.23937/2469-570x/1410051

Steinberg MS (2007) Differential adhesion in morphogenesis: a modern view. Curr Opin Genet Dev 17:281–286

Strauss BS (2016) Beadle and Tatum and the origins of molecular biology. Nat Rev Mol Cell Biol 17:266. https://doi.org/10.1038/nrm.2016.42

Vollmer G, Layer PG, Gierer A (1984) Reaggregation of embryonic chick retina cells: pigment epithelial cells induce a high order of stratification. Neurosci Letts 48:191–196

Wilson HV (1905) On some phenomena of coalescence and regeneration in sponges. J Exp Zool 5:245–258. https://doi.org/10.1002/jez.1400050204

Zhong X et al (2014) Generation of three-dimensional retinal tissue with functional photoreceptors from human iPSCs. Nat Commun 5:4047. https://doi.org/10.1038/ncomms5047

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Chapter 7

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Substitute character or substitute part of one or more word(s)	through letter or through characters	new character or new characters
Change to italics	under matter to be changed	
Change to capitals	under matter to be changed	
Change to small capitals	under matter to be changed	
Change to bold type	under matter to be changed	
Change to bold italic	under matter to be changed	
Change to lower case	Encircle matter to be changed	
Change italic to upright type	(As above)	
Change bold to non-bold type	(As above)	
Insert 'superior' character	through character or where required	or under character e.g. or
Insert 'inferior' character	(As above)	over character e.g.
Insert full stop	(As above)	
Insert comma	(As above)	
Insert single quotation marks	(As above)	or and/or or
Insert double quotation marks	(As above)	or and/or or
Insert hyphen	(As above)	
Start new paragraph		
No new paragraph		
Transpose		
Close up	linking characters	
Insert or substitute space between characters or words	through character or where required	
Reduce space between characters or words	between characters or words affected	