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Cell segregation and boundary formation during nervous system development

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ABSTRACT The development of multicellular organisms involves three main events: differentiation, growth, and morphogenesis. These processes need to be coordinated for a correct developmental program to work. Mechanisms of cell segregation and the formation of boundaries during development play essential roles in this coordination, allowing the generation and maintenance of distinct regions in an organism. These mechanisms are also at work in the nervous system. The process of regionalization involves first the patterning of the developing organism through gradients and the expression of transcription factors in specific regions. Once different tissues have been induced, segregation mechanisms may operate to avoid cell mixing between different compartments. Three mechanisms have been proposed to achieve segregation: (1) differential affinity, which mainly involves the expression of distinct pools of adhesion molecules such as members of the cadherin superfamily; (2) contact inhibition, which is largely mediated by Eph-ephrin signaling; and (3) cortical tension, which involves the actomyosin cytoskeleton. In many instances, these mechanisms collaborate in cell segregation. In the last three decades, there have been several advances in our understanding of how cell segregation and boundaries participate in the development of the nervous system. Interestingly, as in other aspects of development, the molecular players are remarkably similar between vertebrates and invertebrates. Here we summarize the main concepts of cell segregation and boundary formation, focusing on the nervous system and highlighting the similarities between vertebrate and invertebrate model organisms.

KEY WORDS: *nervous system, boundary formation, cell segregation, differential affinity*

Introduction

A striking feature of multicellular organisms is that they have an intrinsic order in their structure; in other words, they are not a homogeneous mixture of cells. In a broad sense, there are three kinds of processes that shape the development of multicellular organisms: growth, differentiation, and morphogenesis. The mechanisms of morphogenesis were very hard to decode at the dawn of the discipline of developmental biology. Today, the unraveling of developmental mechanisms is possible due to the high quality of the available microscopy techniques. It has been known for a long time that intercellular interactions are responsible for the phenomena of morphogenesis that allow the rearrangement of tissues. However, what prevents the mixing of different cell populations during these processes? Cell segregation mechanisms that inhibit cell intermingling to separate distinct cell populations with different properties within a tissue have been described. Furthermore, the formation of boundaries, which can be helped by cell segregation, is also critical to couple growth and patterning in a rapidly growing embryo that is increasing its complexity. These boundaries can have different properties. In some cases there are interfaces between populations of cells, while in other cases, these borders are formed by cells that can also act as signaling centers that contribute to the patterning of tissues (Batlle and Wilkinson, 2012). Models that consider only gene regulatory networks and cellular signaling would

Abbreviations used in this paper: AP, anteroposterior; Cad, cadherin (*Drosophila*); Cdh, cadherin (vertebrates); CNS, Central Nervous System; DE, deutocerebrum; DMB, diencephalon–midbrain boundary; DTB, deutocerebral-tritocerebral boundary; DV, dorsoventral; ECM, extracellular matrix; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; FGF, fibroblast growth factor; IPC, inner proliferation center; LB, labial; LMC, lateral motor column; MHB, midbrain-hindbrain boundary; MN, mandibular; MX, maxillary; OPC, outer proliferation center; PR, protocerebrum; PSB, pallium-subpallium boundary; r, rhombomere; RA, retinoic acid; TF, transcription factor; TR, tritocerebrum; VNC, ventral nerve cord.

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fall short when we consider the many processes involved in the morphogenesis of an organism, such as cell division, change in cell shape and cell migration. All of these processes are standard phenomena that occur during the development of any multicellular organism (Fagotto, 2014).

Seminal work from Townes and Holtfreter (Steinberg and Gilbert, 2004, Townes and Holtfreter, 1955) set the foundations for understanding the underlying mechanism of cell segregation. They found that dissociated embryonic cells segregate and cluster following their layer of origin. Using these observations as a starting point, Steinberg and colleagues introduced the differential adhesion hypothesis in 1960s to explain why groups of cells are segregated into distinct regions during embryo development. More than thirty years later, it was shown that the differential expression of cadherin proteins could lead to segregation of transfected cultured cells into aggregates (Nose *et al.*, 1988). Furthermore, the expression of different quantities of the same cadherin can have the same effect (Steinberg and Takeichi, 1994). More recently, it was shown that other molecular entities could also participate in cell segregation (see below).

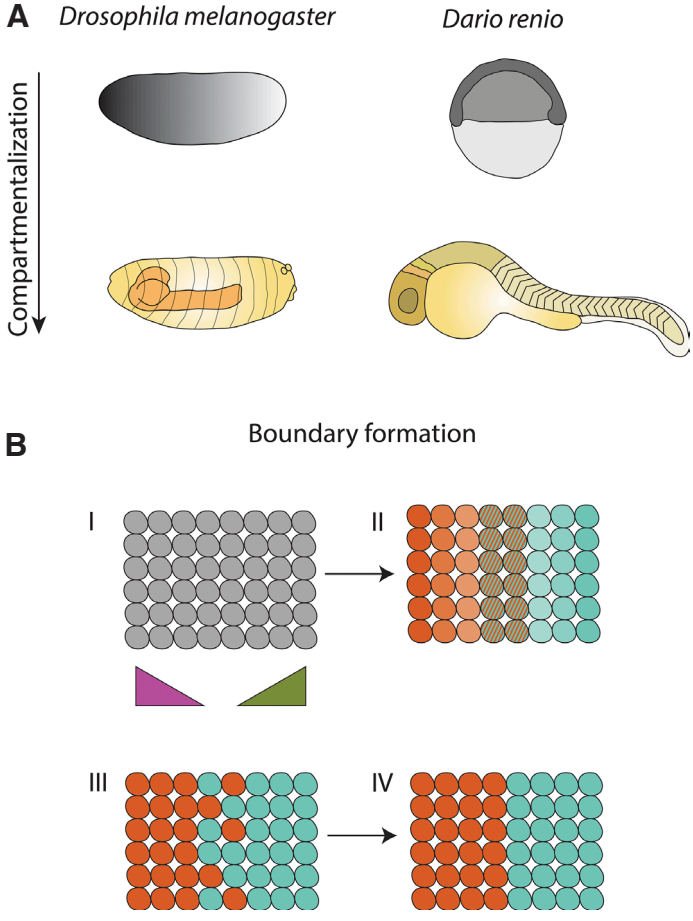

Fig. 1. Steps in organism compartmentalization. (A) *Schematics showing the steps of compartmentalization in animals. Left,* Drosophila *and right represent zebrafish. Early in development, morphogen gradients establish the axial pattern of the embryo, this leads to the expression of transcription factors in specific regions of the embryo. TF can control the expression of distinct molecules that will control tissue separation such as cell adhesion molecules.* **(B)** *Steps in boundary formation, first an initially homogeneous tissue responds to gradients (I). These gradients will generate changes in the expression profile along the tissue making adjacent regions distinct of each other. TFs will be switch on in different regions to establish territories and cross-repression between them will resolve mixed identity (II). Initially, boundaries will be fuzzy (III). Finally, cell-sorting mechanisms will produce a sharp interface between the two regions (IV).*

The first boundaries described in detail were those seen during embryogenesis and wing imaginal development of the fruit fly *Drosophila melanogaster*. These studies revealed that tissues are organized into compartments (Batlle and Wilkinson, 2012, Bryant, 1970, Garcia-Bellido *et al.*, 1973) which allows each territory to acquire a discrete identity (Irvine and Rauskolb, 2001). Compartments are understood as subdivisions of embryonic tissues formed by cell populations that do not intermingle with their neighbors.

During development, the embryonic tissue is initially patterned by long-range signals that induce specific regional territories. Then, the original pattern is translated into differential expression of transcription factors (TFs) that give identity to each region. Cells at the border may acquire mixed identities which are resolved by cross repression of the participant TFs to ensure mutually exclusive identities. In general, the borders between these territories are fuzzy at the beginning. Later, local interactions between border cells generate a sharp interface (Dahmann and Basler, 1999). Additionally, developing tissues have a high rate of cell division, and undergo morphogenetic movements such as convergent extension. Both processes can lead to cell intercalation and therefore impose important challenges to compartment separation (Dahmann *et al.*, 2011). A basic sequence of boundary formation is depicted in Fig. 1.

Several boundaries can be recognized in the nervous system. Although most of the attention has been given to vertebrate models, such as Chick, Zebrafish, and Mice, recent papers showed striking compartmentalization in the fly brain. Additionally, cell segregation mechanisms that do not form boundaries, operate in the nervous system to separate brain nuclei in vertebrates and are also present in the *Drosophila* visual system.

In this review, we start by describing the general principles of cell segregation and boundaries. Next, we give some examples of how these events are achieved in the vertebrate and invertebrate nervous system. Thus, our aim is to give a glimpse into the fundamental mechanisms behind these processes that are highly conserved during animal evolution.

General principles of cell segregation and boundary formation

In this section we will address basic aspects of cell segregation and boundary formation that have been identified in several organisms and tissues. A first important aspect is that cell segregation does not necessarily imply the formation of a boundary, although cell segregation is required in specific types of boundaries. Another crucial aspect of boundaries is that they will not necessarily produce a direct anatomical readout of their initial position. For instance, in *Drosophila*, the parasegments of the embryo do not give rise to anatomical separations similar to the anteroposterior boundary of the wing disc. On the other hand, the boundaries of vertebrates, although they can go unnoticed at the beginning, give rise to physical separation between embryonic tissues. This aspect is seen, for instance, in the early separation between ectoderm and mesoderm (Fagotto, 2014).

Gene expression and tissue boundaries

Two terms have been used to describe territories in the embryo. First, gene expression boundaries that can be separated into two categories: lineage boundaries and non-lineage boundaries. Lineage boundaries are those that restrict clone expansion (here the identity is stably inherited by each daughter cell) and are also called "compartment boundaries". In these boundaries, signaling pathways that control differentiation do not need a constant input, because the fate of cells on either side of a border is stably inherited. Lineage boundaries include the classic examples delimiting compartments in insects such as the wing disc and the hindbrain segments in vertebrates (Tepass *et al.*, 2002, Umetsu *et al.*, 2014). Examples of lineage boundaries will be discussed in the next sections. On the other hand, when a clone could contribute to both sides of the boundary, the term non-lineage boundary is used. In this case, non-autonomous mechanisms will maintain the identity using patterning signals. Therefore, if a cell moves to another compartment, it switches its fate to match that compartment. Thus, in non-lineage boundaries, cell plasticity of the differentiation state is a fundamental property. A classic example of this type of border is the notum/wing boundary in *Drosophila* (Diez del Corral *et al.*, 1999). Unlike boundaries of the wing territory, which are lineage boundaries, cells can freely move through the notum/wing territory. It has been demonstrated that the EGF-like ligand Vein signals through its receptor EGFR to induce notum cells by antagonizing wing development and by activating notum-specifying genes (Wang *et al.*, 2000). Furthermore, EGF signaling is required persistently in the notum region to maintain expression of notum- genes (Zecca and Struhl, 2002), this is a landmark of this type of boundary, in which signals are required continually to maintain the fate of a given compartment. In the case of vertebrates, somite boundaries are examples of this type of boundary (Kulesa and Fraser, 2002). Fig. 2 shows a comparison between these two concepts. A second concept is tissue boundary, which refers to an absolute limit to cell intermingling, and therefore supported by physical constraints. However, this term is used for every kind of physical boundary that includes not only those derived from the separation of two distinct cell populations, but also from the apposition of two previously formed populations (Fagotto, 2014).

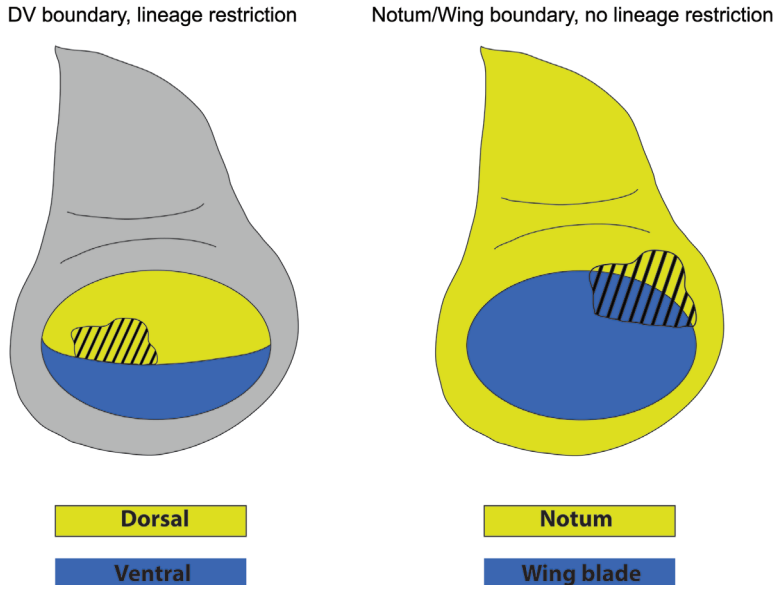

Fig. 2. Lineage and non-lineage boundaries. *The wing disc of* Drosophila *gives rise to the wing and the notum (the dorsal part of the fly thorax). While in the wing blade region (left, blue and yellow area) the boundary between the dorsal and ventral compartments is a lineage boundary, the notum/wing (right) is a non-lineage boundary (see text for details).*

Generation and maintenance of boundaries

In vitro re-aggregates used by Townes and Holtfreter were isolated from the embryo after the tissues are formed. In this condition cell types segregate after being completely intermingled. Although this is not the situation in normal development in which cells are spatially organized, segregation can occur at the borders where there is local intermingling.

Several mechanisms have been proposed to be involved in the generation and maintenance of different compartments. One of the models proposed to drive cell segregation involves the behavior of two cellular populations that do not mix due to mechanical forces and tension, in analogy to what occurs at the molecular level after mixing oil and water. Thus, different affinities between the two cell populations will give rise to tension at the interface. Hence, cohesive forces are stronger between similar cells (homotypic interactions) and weaker between the different populations (heterotypic interactions) (Batlle and Wilkinson, 2012). This model is supported by the assays in which cells from different embryonic layers were dissociated, mixed and later allowed to re-aggregate. This experiment did not only produce segregation of cells from different layers to distinct regions but also the segregation of cells with more cohesive forces to the center of the tissue, something that has been observed in several studies (Batlle and Wilkinson, 2012, Townes and Holtfreter, 1955). A mathematical framework was derived from theoretical work by Malcolm Steinberg, to explain the sorting of a group of cells from aggregates (Steinberg, 1963) leading to the differential affinity hypothesis. The underlying explanation for the model is based on affinity differences that could exist between compartments (Irvine and Rauskolb, 2001). The molecular nature of the proteins involved in establishing these differences may vary, but the cadherin family of cell adhesion molecules is a strong candidate (Halbleib and Nelson, 2006). Consistently with this hypothesis, overexpression of *Drosophila* E-Cadherin (E-Cad) in a clone of cells in the wing disc produced segregation from the neighboring cells, resulting in circular clones (Dahmann and Basler, 1999). This observation, however, does not prove the involvement of E-Cad in wing compartmentalization since E-Cad mutant cells do not trespass the border. On the other hand, the expression of a dominant-negative form of E-Cad did not preclude cell segregation in the notochord-somite boundary. Therefore, cadherins are not always responsible for cell segregation and border formation (Batlle and Wilkinson, 2012, Reintsch *et al.*, 2005). Other examples of cadherin participation in these phenomena will be discussed later.

An additional mechanism, not mutually exclusive with cell adhesion, is contact inhibition. This mechanism is generated by interactions between populations of cells at the interface, preventing intermingling. Interestingly, this type of mechanism seems to be involved in cancer (Abercrombie, 1979, Batlle *et al.*, 2002). Contact inhibition is elicited by heterotypic interactions, and the

most commonly observed molecular system involved is the classic axon guidance pair Eph receptor and ephrin ligand (Cayuso *et al*., 2015, Kania and Klein, 2016, Pasquale, 2008, Wu *et al*., 2019, Xu *et al*., 1999). An Interesting possibility suggested initially was that Eph-ephrin signaling could lead to cell segregation through the modulation of cell adhesion (Steinberg, 2007). Studies in cell culture and in the intestinal epithelium showed that EphB3-ephrinB1 binding gives rise to the redistribution of E-Cad (Cortina *et al*., 2007). Additionally, Eph receptors can interact with the metalloproteinase ADAM-10 leading to the cleavage of E-Cad and loss of adhesion (Solanas *et al*., 2011). However, work in cell culture and *Xenopus* embryos, including mathematical modeling of cell segregation and boundary formation, do not support a model in which cell adhesion has a major contribution to Eph signaling. Instead, cell repulsion and high heterotypic interfacial tension between tissues are the main mechanisms (Canty *et al*., 2017, Taylor *et al*., 2017). A classic example of Eph action in nervous system boundary maintenance is discussed below.

A third well-studied mechanism involves cortical tension generated at the border. The molecular system responsible for this is actomyosin-mediated contraction (Batlle and Wilkinson, 2012, Murrell *et al*., 2015). Although the upstream signaling pathways are still under investigation, there is evidence in vertebrates, that Eph signaling can increase actomyosin contraction (Calzolari *et al*., 2014, Cayuso *et al*., 2019), while in *Drosophila*, signaling pathways such as Wingless and Notch can lead to actomyosin accumulation (Becam *et al*., 2011, Major and Irvine, 2005, Major and Irvine, 2006, Monier *et al*., 2010).

Boundaries in nervous system development

As noted above, boundaries have been identified in different structures of the animal body, including the nervous system. Although anatomical observations indicate that the nervous system may have several boundaries, scientists have focused on a few models to investigate the mechanisms behind nervous system compartmentalization. It is important to note that the mechanistic knowledge is variable depending on the system studied. In some cases, there is in-depth knowledge on mechanisms of the specification between compartments but not on the downstream effectors that allow cell segregation or physical separation. In other cases, research has focused mainly on effector pathways that drive cell segregation, but little is known about the upstream transcription factors that regulate their expression in specific locations. Consequently, there are only a few examples in which patterning and cell segregation and/or boundary formation have been identified for a given system. Finally, segregation can be observed at several levels. We will focus on gross segmentation of the nervous system and we will also give examples of the formation of nuclei or neuropils that also require cell segregation mechanisms.

Boundaries and cell segregation in the vertebrate nervous system

Forebrain

The segmentation in the forebrain, although less evident than hindbrain segmentation (discussed below), was proposed long ago (Figdor and Stern, 1993, Kiecker and Lumsden, 2005). The forebrain (anterior region of the brain) is organized according to the "prosomeric model" into six transverse subdivisions, identified as prosomeres. The three more posterior divisions (p1–p3) subdivide the diencephalon while the anterior divisions (p4–p6) divide the secondary prosencephalon, which includes the hypothalamus and the telencephalon (Rubenstein *et al*., 1994). Unlike the clear divisions in the hindbrain observed with molecular markers, the situation of the forebrain is less clear since the expression domains of various segmentation markers are highly dynamic concerning its anatomical subdivisions (Kiecker and Lumsden, 2005). Furthermore, many proposed boundaries of this model, based on expression patterns are not boundaries of lineage restriction (Larsen *et al*., 2001). Indeed, only a handful of cell lineage restriction boundaries have been found in the forebrain: the pallium-subpallium boundary (PSB), the diencephalon–midbrain boundary (DMB), and the interface between the thalamic and the prethalamic primordia, known as, the zona limitans intrathalamica (Kiecker and Lumsden, 2005, Puelles and Rubenstein, 2003). We will describe the mechanisms involved in the formation of some of these boundaries.

The pallial-subpallial boundary (PSB)

As in hindbrain boundaries (see below), the only region in the PSB where lineage restriction occurs is the ventricular zone. In the mantle zone, the neuronal cell progeny can cross freely (Kiecker and Lumsden, 2005). The PSB boundary is defined early in development by complementary expression of two transcription factors. Pax6 in the cortex and Dlx in the basal ganglia (Stoykova *et al*., 1996). Mutations in the gene Pax6, induce developmental abnormalities in the central nervous system (CNS) and the eye. In the brain, the PSB is disorganized, and the structures of the thalamus are aberrant (Stoykova *et al*., 1997).

Interestingly, cells from the cortex and striatum differ in their adhesive properties. Short-term aggregation assays showed that cells from the cortex segregate from striatum cells during early stages (Gotz *et al*., 1996). Strikingly, this behavior is lost in Pax6 mutants, where the cells do not sort out from the aggregates (Stoykova *et al*., 1997). The reason seems to be an effect on the expression of a member of the cadherin superfamily of cell adhesion molecules. Cadherins were the first molecules to be suspected of underlying cell segregation and boundary formation. However, *in vivo* examples of their function are not abundant. In the developing telencephalon of mice, differential cadherin expression defines neighboring regions. Cadherin-6 (Cdh6) outlines the lateral ganglionic eminence (LGE), while Cadherin-4 (Cdh4) does so with the future cerebral cortex, and then is co-expressed with Pax6. Interestingly loss of expression of Cadherin-4 is observed in Pax6 mutants while no changes are observed in Cdh6 expression. The interface between these two cadherins generates a boundary for cell lineage restriction at embryonic day 10.5 (Inoue *et al*., 2001). In this context, electroporation of Cdh6 at the boundary leads to the movement of cells from the cortex towards the LGE, where normal Cdh6 expression occurs. However, sorting defects are not observed in Cdh6 mutant animals, suggesting the presence of compensatory mechanisms. The sorting of ectopic Cdh6-expressing cells in the cortex is hampered when the experiment is performed in the mutant animals. Hence, the above experiments demonstrate a role of differential expression of cadherins in cell segregation.

The midbrain-hindbrain boundary (MHB)

The boundary between the developing midbrain and hindbrain (known as the MHB) is also known as the isthmus. The MHB has been utilized as a model for local signaling centers in the developing

brain. The MHB is essential for the generation of the midbrain and the cerebellum (Kiecker and Lumsden, 2005). Although the core network that regulates MHB formation has been well defined, novel genetic and mechanistic processes that interact with core components are still under investigation. The MHB can induce surrounding cells to change fate and become ectopic midbrain and hindbrain cells if it is transplanted to other regions of the brain (Joyner *et al.*, 2000). Thus, the MHB is a special kind of boundary that not only divides two compartments but it also has inductive properties. The position of the MHB is determined at the interface of the expression of two transcription factors, Otx2 and Gbx1/2. In this interface, a signaling network, including FGF8 (Fibroblast Growth Factor 8), Wnt1, and a set of transcription factors (Pax2/5/8 and Eng1/2), induces the formation of MHB and is also needed for its maintenance (Dworkin and Jane, 2013).

The importance of the Otx-Gbx transcription factor pair has also been studied in mice. The results indicate that Otx2 mutants show expanded anterior limit expression of Gbx; while Gbx2 mutants display posterior expansion of the Otx2 expression domain. On the other hand, double Otx2 and Gbx2 mutants do not show problems in the expression of MHB markers but they do in the position in which this takes place. In double mutants, the expression of these genes is observed in a wider anterior area of the neuroectoderm (Li and Joyner, 2001).

Initial work in the chick suggested that the MHB was not a lineage restriction boundary. Using ionophoretic labeling, Jungbluth *et al.,* found that cells from adjacent territories intermingle without obstruction (Jungbluth *et al.*, 2001). However, in this study cell movements were not followed directly. On the other hand, in zebrafish, time-lapse experiments demonstrated the existence of lineage restriction, which is probably established during late gastrulation (Langenberg and Brand, 2005). Today, it is accepted that this boundary indeed operates as a lineage restriction border as in other regions of the neural tube (Langenberg and Brand, 2005).

Hindbrain

An extensively studied example of boundary formation is the segmentation of the vertebrate neuroepithelium into the anteroposterior axis, in which a special focus has been given to the posterior part of the brain, the hindbrain. The hindbrain is formed by a characteristic sequence of seven or eight bulges, termed rhombomeres, which are known to be lineage restriction compartments (Fraser *et al.*, 1990, Guthrie *et al.*, 1991, Kiecker and Lumsden, 2005).

During embryonic development, the neuroepithelium is morphologically characterized by a series of constrictions and bulges. Most of these structures appear only transitorily during this stage. However, it should be clarified that this segmentation pattern is incomplete, as no lineage restriction has been detected along the floorplate, where morphological landmarks are not present (Fraser *et al.*, 1990, Kiecker and Lumsden, 2005).

There are boundary cells between rhombomeres. However, formation of boundary cells is not the primary cause of cell lineage restriction between rhombomeres. Removing boundary cells in the chick embryo does not affect cell segregation between adjacent rhombomeres. After surgical ablation, boundaries are reconstructed between opposing rhombomeres, while under conditions of boundary-loss by local application of Retinoic acid (RA), segregation is also maintained (Guthrie and Lumsden, 1991, Nittenberg *et al.*, 1997). Thus, these experiments support the idea that separation is not the product of mechanical restriction by boundary cells in the interface. The use of the classical dissociation-reaggregation assays demonstrates that there are different cell affinity properties among rhombomeres. Indeed, cell-mixing from two even- or two odd-numbered rhombomeres led to homogenously mixed aggregates, while even and odd cells segregate to form separate domains in aggregates derived from two adjacent rhombomeres (Wizenmann and Lumsden, 1997). Furthermore, donor-to-host transplantation experiments between r3/r4 and r4/r5 form new boundaries; in contrast, r3/r5 or r4/r6 grafts form compound rhombomeres without a boundary supporting the same conclusion (Guthrie and Lumsden, 1991). Therefore, different cell properties with two-segment periodicity are responsible for cell segregation in the hindbrain.

As in multiple other systems, an initial graded signal induces specific transcription factors that give identity to distinct regions of the CNS (brain and spinal cord). In the case of hindbrain segmentation, the molecular gradients involved are Wnt, RA and FGF (Addison and Wilkinson, 2016, Schilling *et al.*, 2012). RA generates a noisy gradient that is decreased by expression of intracellular RA binding proteins (Sosnik *et al.*, 2016). This noise generates mixed identities in border cells, leading to the co-expression of Hoxb1 (r4 TF) and Krox20 (r3 and r5 TF, also termed Egr2) which seems to be resolved by mutual repression. This process contributes with the generation of sharp borders between rhombomeres (Sosnik *et al.*, 2016, Zhang *et al.*, 2012).

Once the basic pattern is achieved and molecular distinction has been established between rhombomeres, cell segregation is commanded by the complementary expression of the receptor EphA4 (which is expressed in r3 and r5) and ephrinB3, a ligand involved in contact inhibition (expressed in r2, r4 and r6). Upon activation of the EphA4 in odd rhombomeres by ephrinB3, the forward signaling is triggered (Kania and Klein, 2016). This signal is necessary for cell repulsion and segregation at the boundaries between these different compartments. The maintenance of border sharpness is controlled by the formation of actomyosin cables. Inhibition of these cables using either blebbistatin, an inhibitor of myosin II, or rock-out, a blocker of Rho Kinase, leads to the presence of ectopic cells that cross to adjacent rhombomeres (Calzolari *et al.*, 2014). Strikingly, the same molecular mechanism that regulates border sharpening also regulates the expression of boundary cell markers (Cayuso *et al.*, 2019). In this case, actomyosin contraction leads to the activation of Taz, a protein that connects tension and gene expression (Cayuso *et al.*, 2019). Thus, in this case, the same effector regulates cortical tension and the formation of boundary cells.

Conclusions about the function of boundary cells between rhombomeres have changed over time. In initial studies, it was shown that there was reduced cell proliferation in the boundary region. In the chick embryo, the density of mitotic cells is higher near the center of rhombomeres than in boundary regions (Guthrie *et al.*, 1991). This observation supported the interpretation that this difference may reduce the difficulties during times of extensive tissue growth. However, recent work from several groups has changed this view. Careful examination of neural stem cell markers during chick development, has shown that boundary cells express SOX2 and other stem cell markers, which indicates that these cells constitute a pool of neural progenitors (Peretz *et al.*, 2016). This has also been confirmed in zebrafish, in which boundary cells have higher proliferation rates than rhombomere regions during early stages. Strikingly, boundary cell proliferation is downstream of mechanical cues that act on the

Yap/Taz pathway (Voltes *et al.*, 2019).

In addition to cell segregation mechanisms that sort misplaced cells back to their region of origin, cell plasticity (a concept generally related with non-lineage boundaries) has also been observed during zebrafish hindbrain development. A regulatory loop between Krox20 and RA has been described, in which RA positively regulates the expression of Krox20. However, there is less available RA in even-numbered rhombomeres because of the higher expression of RA-degrading enzymes cyp26b1 and cyp26c1. These enzymes are, in turn, repressed in r3 and r5 by Krox20. This mechanism is part of a community regulation of RA signaling to help the maintenance of segmental identity (Addison *et al.*, 2018).

Another factor that may participate in the maintenance of boundaries is the extracellular matrix (ECM). However, although it is known that ECM accumulates between rhombomeres, its functional significance has not been yet addressed in the hindbrain. On the other hand, the participation of a fibronectin-based ECM has been demonstrated for the maintenance of somite compartments (Dahmann *et al.*, 2011).

Cell segregation in the formation of nervous system nuclei

Besides the segmentation of the nervous system into compartments at the macroscopic level, organization can also be observed at smaller scale; neurons with similar functions cluster into discrete structures. For example, the lamination observed in the cerebral cortex is also based on segregation of different cell populations (Price *et al.*, 2002). The most common way of organization of neurons in the nervous system is the formation of nuclei in the brainstem and spinal cord (Pereanu *et al.*, 2010). In the spinal cord, the segregation of functionally related motor neurons of the lateral motor column (LMC) into clusters (termed motor pools) has been investigated in some detail (Price *et al.*, 2002). Distinct motor pools can be recognized by their specific profile of TF expression. Furthermore, it has been shown that type II cadherins are expressed in particular subsets of motor pools (Price *et al.*, 2002). In this context, MN-Cadherin (MN-Cdh) allows the distinction between two motor pools: eF, which expresses MN-Cdh, and A, which does not. Although ectopic expression of MN-Cdh in eF and A motor pools does not change the number of total neurons in each pool, it produces the intermingling of eF with A neurons. Consistently, expression of a dominant negative form of MN-Cdh in A neurons induced their mixing with eF neurons (Price *et al.*, 2002). These findings demonstrate a function MN-Cdh in cell segregation. Although, it is unclear what are the transcription factors responsible for the expression of cadherins in particular motor pools, Hox genes are good candidates. Indeed, double mutant Hoxc10 and Hoxd10 mice, display severe identity defects in motor pools and failure of presumptive lateral LMC neurons to migrate toward their normal position in the spinal cord (Wu *et al.*, 2008).

In summary, several mechanisms participate in the formation and maintenance of boundaries, and in the segregation of distinct cell populations in the nervous system of vertebrates. Interestingly, although some differences seem to arise when investigating different regions, common molecular players have been discovered.

Boundaries and cell segregation in the *Drosophila* nervous system

Drosophila is one of the first organisms where boundaries were observed and studied mainly in the wing disc (Dahmann and Basler, 1999, Vincent, 1998). Initial studies showed that clonally related cells became restricted to specific wing regions during larval development (Bryant, 1970, Garcia-Bellido *et al.*, 1973) suggesting the presence of boundaries of lineage restriction. Further research demonstrated the participation of several signaling pathways and cell-cell interaction proteins in the formation and maintenance of two borders, the anteroposterior (AP) and dorsoventral (DV) boundaries (Blair and Ralston, 1997, Dahmann and Basler, 1999, Milan *et al.*, 2001, Shen and Dahmann, 2005). Interestingly, a recent RNAi-based screening identified Eph as a necessary factor for the maintenance of the AP boundary (Umetsu *et al.*, 2014), supporting a conserved role of this protein in lineage restriction. In contrast to the extensive knowledge about wing boundaries, much less has been explored regarding the fly nervous system. In this section, we begin by describing boundaries in the segmentation of the embryonic brain and later focus on the development of the optic lobe as a novel system to study the cellular and molecular basis of cell segregation during nervous nuclei development.

Embryonic brain

The segmentation program of the *Drosophila* embryo has been extensively studied (Hartenstein and Wodarz, 2013). While the epidermis of the embryo is organized into segments, gene expression data largely supports the view that another segmentation unit, which is out of face with segments, is also present. These units termed "parasegments" were described by Martinez-Arias and Lawrence (Martinez-Arias and Lawrence, 1985) and include the posterior compartment of a segment that expresses the gene *engrailed* (*en*, a transcription factor) and the anterior compartment of the following segment, which expresses *wingless* (*wg*, the fly homolog of Wnt) (Deutsch, 2004).

The insect CNS is composed of the ventral nerve cord (VNC), located in the trunk region and the brain. In the first step of CNS development, the ectoderm is differentiated into neurogenic and non-neurogenic regions by the products of early dorsoventral and segment polarity patterning genes (Hartenstein and Wodarz, 2013, Skeath *et al.*, 1992). There is a ventral neurogenic region, which gives rise to the VNC, and the procephalic neurogenic region, which develops into the brain. The identity of each neuroblast is set mainly by positional information within the neuroectoderm, and temporal cues (Technau *et al.*, 2006). Interestingly, the parasegmental unit is maintained in the VNC. The VNC of *Drosophila* embryo has a clear metameric organization. Each unit called neuromere is formed by the delamination of neuroblasts when the germ band displays parasegmental organization (Deutsch, 2004).

Although the mechanisms maintaining neuroblast segregation after delamination have not been elucidated, recent work described boundaries, which behave as boundaries of lineage restriction in the ectoderm of the embryo and prevent cell mixing between parasegments (Monier *et al.*, 2010). These boundaries occur during the same stages of neuroblast delamination, and therefore defects in their formation could affect the organization of the neuroectoderm before neuroblast delamination. It was shown, using a combination of expression analysis, genetic and pharmacological tools, that actomyosin cables are assembled at the boundaries in which a membrane alignment is observed in border cells. Strikingly, the critical challenge to embryonic boundaries is the division of boundary cells, which deforms the boundary. Inactivation of the actomyosin cable leads to cell sorting defects at parasegment

boundaries during cell division (Monier *et al*., 2010). Thus, as in zebrafish rhombomeres, actomyosin cables are essential for the maintenance of borders in the *Drosophila* embryo.

The *Drosophila* brain is originated from the anterior neuroectoderm, which gives rise to the procephalic or pregnathal neuroectoderm. Developmental and evolutionary evidence demonstrates that the brain comprises six neuromeres (Ito *et al*., 2014): the protocerebrum (PR), deutocerebrum (DE); tritocerebrum (TR); and the mandibular (MN), maxillary (MX), and labial (LB) neuromeres. Two more prominent subdivisions termed cerebral ganglia, which includes PR, DE, and TR, and the gnathal ganglia, which comprises MN, MX, and LB define the position of brain compartments relative to the esophagus (Ito *et al*., 2014). In evolutionary terms, it has been proposed that the compartments of the fly brain in the anteroposterior axis are homologous to vertebrate brain compartments. Thus, the PR would be equivalent to the forebrain, the DE to the midbrain and the TR plus the gnathal ganglia to the hindbrain (Ghysen, 2003, Hirth *et al*., 2003, Urbach, 2007).

Similar to what is observed in the VNC, boundaries between brain segments are defined by segment polarity genes and dorsoventral patterning genes (Urbach and Technau, 2003). A striking feature of the segmentation of the fly brain is the proposed conservation of the MHB boundary found in vertebrates. As mentioned before for the vertebrate brain, the MHB is defined by the expression of Otx genes expressed in the anterior region, which include the forebrain and anterior midbrain; Hox genes in the posterior region (hindbrain); and Pax2, Pax5 and Pax8 genes in the intervening region (MHB). In *Drosophila*, the most anterior aspect of the brain primordia expresses the Otx-orthologue *orthodenticle*. This protein is located in the protocerebrum and anterior deutocerebrum of the embryonic brain, while the *hox* gene *labial* is expressed in the posterior region (Hirth *et al*., 2003). In *Drosophila,* the *Pox neuro* (*Poxn*) and *Pax2* genes are the orthologues of vertebrate Pax2/5/8 (Fu and Noll, 1997, Noll, 1993). They are expressed in a territory encompassing the posterior part of the DE and the anterior region of the TR. This region has been called the deutocerebral-tritocerebral boundary (DTB).

Interestingly, *unplugged* (*unpg*), which is the orthologue of Gbx2, is also expressed in the fly brain. As in vertebrates, *unpg*/Gbx2 is expressed in a complementary pattern with Otd/Otx, in which the posterior limit of Otx expression coincides with the anteriormost limit of unpg/Gbx2. Another similarity between MHB and DTB was revealed by loss of function experiments of Otd and Unpg. *otd* null mutants fail to generate the PR (Hirth *et al*., 1995). In this context, the anterior border of *unpg* expression extends anteriorly into the anterior deutocerebrum. On the other hand, *unpg* mutants display a shift in the posterior limit of *otd* expression expanding it into the posterior deutocerebrum. Thus, *otd* and *unpg* control each other expression at the boundary of their expression domains, as interactions between Otx and Gbx2 observed in the MHB.

Not surprisingly there are also differences in the gene network operating in MHB and DTB. While in vertebrates the inactivation of Pax2, Pax5, En1 or FGF8 leads to the absence of midbrain and cerebellum, in *Drosophila*, no evident brain defects are observed in mutants of orthologues of some members of this network. Furthermore, their expression timing does not fit with having a function in the specification of this structure. Some of the similarities described between *Drosophila* and vertebrates are depicted in Fig. 3.

Cell segregation in the development of the optic lobe

Besides the compartmentalization into neuromeres in the trunk and brain of the embryo, it has long been recognized that as in vertebrates, the *Drosophila* neuromeres are organized into smaller compartments. In *Drosophila*, the neuropile is subdivided into anatomically discrete regions, which are rich in terminal neurite branching and synapses (Pereanu *et al*., 2010). It is in these regions that signal processing takes place. Layers of glial cells populate the boundaries of these compartments, and the bundles of axon fibers that communicate compartments. For example, in the brain, *en* gene expression is found in neuroblasts located in the posterior boundary of each neuromere; while later in development, *en*-expressing neurons innervated brain regions in the same area of the neuromere of origin (Kumar *et al*., 2009). This pattern of organization is also observed with other neuronal populations in the brain and trunk. The signaling networks dedicated to controlling this type of compartmentalization may be similar to

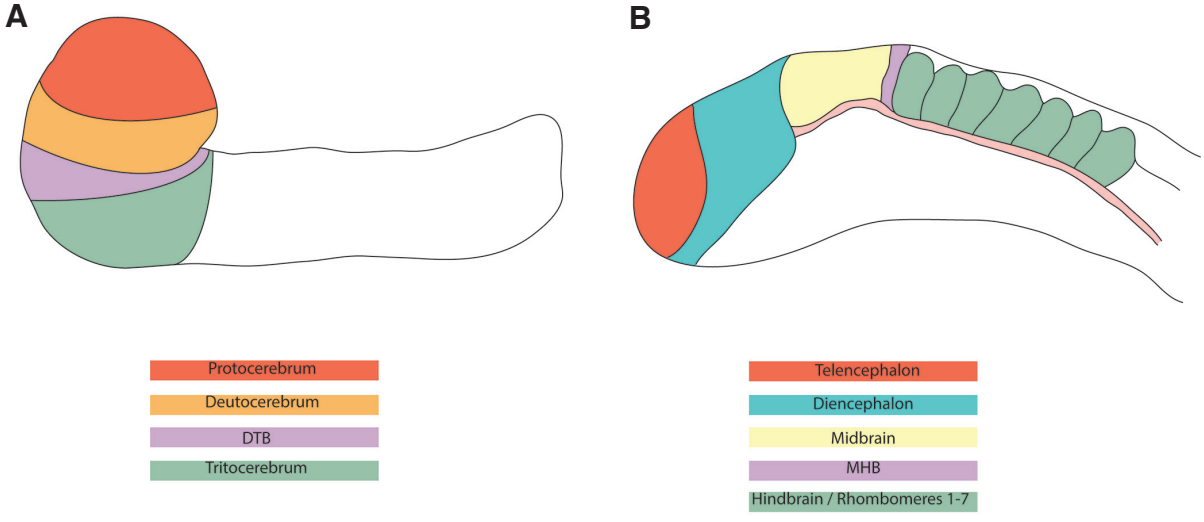

Fig. 3. Proposed homology in the regionalization of the nervous system between *Drosophila* and vertebrates. (A) *The fly embryonic central nervous system and* **(B)** *that of zebrafish. MHB, midbrain-hindbrain boundary; DTB, deuterocerebrum-tritocerebrum boundary.*

the mechanisms discussed above for cell segregation. In the next section, we discuss recent work on the *Drosophila* optic lobe as an example of neuropil segregation.

The optic lobe is unique in the sense that it is not present in the fly embryo. All the structures are developed during the larval stage by neuroblasts generated *de novo*. The optic lobe is the entrance point for the visual information coming from the retina. It is composed of four ganglia lamina, medulla, lobula, and lobula plate. The optic lobes process all the visual information, and then convey this information to the optic glomeruli in the central brain (Apitz and Salecker, 2014, Contreras *et al*., 2019, Ngo *et al*., 2017, Perry *et al*., 2017). The development of this structure begins with the formation of the optic placode, which originates from a group of 30-40 cells located in the posterior region of the procephalic region, around stage 11 of embryonic development (Contreras *et al*., 2019, Hartenstein and Campos-Ortega, 1984). Then, in the larval stage, the developing optic lobe differentiates into two groups of cells, the inner proliferation center (IPC), which gives rise to the lobula and the lobula plate; and the outer proliferation center (OPC) that originates the medulla and lamina. These two structures are in close proximity, and their progeny will connect extensively during development. However, to secure the correct assembly of the optic lobe, segregating mechanisms are crucial for maintaining cell populations from these two regions apart from each other.

To keep OPC and IPC cells separated, the participation of attractive and repulsive molecules is necessary. Two pairs of ligand-receptors involved in axon guidance are required in this context. One is the well-known Netrin-DCC/Unc5 signaling (Hand and Kolodkin, 2017, Lai Wing Sun *et al*., 2011). In *Drosophila* there are two paralogues of Netrin, NetA, and NetB, and double mutants show the invasion of IPC cells into the OPC region, which disrupts neuropil development. This event leads to a disordered architecture of the different neuropils in the adult stage (Suzuki *et al*., 2018). The other pathway involved is Slit-Robo, which as in the development of the embryonic VNC (Blockus and Chedotal, 2016, Dickson and Gilestro, 2006), also collaborates with Netrin signaling in the assembly of the optic lobe. Slit-Robo has been implicated in cell segregation between lamina and lobula, and lobula and medulla. The distal neurons present at the edges of the lobula cortex invade the lamina in *slit* and *robo* loss of function conditions (Tayler *et al*., 2004). Furthermore, cell-specific loss of function experiments demonstrated that Slit expression in glial cells present at the interfaces between lamina and lobula plate, and that of lamina and medulla are required for the optic lobe segregation (Caipo *et al*., 2020, Suzuki *et al*., 2018, Tayler *et al*., 2004). Additionally to glial expression, Slit secretion by neurons from the medulla is essential for cell segregation in this region of the brain (Caipo *et al*., 2020).

Robo proteins have a differential expression pattern in the optic lobe (Tayler *et al*., 2004). Loss of function of *robo2 and robo3* produce ectopic IPC cells in the medulla cortex zone. Consistent with functional data, Robo3 is expressed in medulla cells and Robo2 in lobula complex cells. Interestingly, GPC neurons, which migrate and locate at the interface between medulla and lobula, also secrete Slit and contribute to cell segregation (Suzuki *et al*., 2016). Although Robo1 is expressed without a preference for a specific neuropil, its loss of function has not been addressed in this context.

Eph-ephrin signaling function in boundary formation is well known in vertebrates, and recently it has become more relevant in invertebrates, as it was shown above. However, there is little research in *Drosophila* regarding this signaling pathway in the nervous system. Work on the optic lobe showed that Eph and its ligand ephrin are essential for axon guidance (Dearborn *et al*., 2002, Dearborn *et al*., 2012). Although cell segregation is not directly evaluated, some phenotypes involving defects in the morphology of the medulla seem similar to those reported for *slit* and *robo2/3* loss of function mutants However, additional work needs to be done to determine whether there is an invasion of adjacent neuropil compartments.

The *egghead* gene (*egh*), which encodes a glycosyltransferase, is also required for cell segregation in the optic lobe (Fan *et al*., 2005). *egh* mutants produce a similar phenotype as *slit* and *robo* loss of function mutants (Tayler *et al*., 2004). However, *egh* seems to function in an independent pathway because the *egh* mutation does not change Slit or Robo levels (Fan *et al*., 2005). Thus, further experiments are needed to determine which signaling pathway requires *egh* to work properly.

During cell segregation between neuropils in the brain of *Drosophila*, two signaling pathways act together: Net-Fra/Unc5 and Slit-Robo. This is essential not only for the localization of neuronal cell bodies but also for the navigations of their axons. In this case, the downstream signals have not been discovered yet. However, Slit-Robo signaling, for instance, has been shown to regulate the expression of cadherins in other contexts (Rhee *et al*., 2007, Shiau and Bronner-Fraser, 2009), which are excellent candidates to test in this case.

Conclusions and future perspectives

Biologists first started to think about how zygotes give rise to embryos in a choreography of cell rearrangements in the 1800s. Today, there is a massive body of evidence to support the idea that cell interactions are a driving force during development. These interactions allow cells to differentiate and form tissues, and also to establish and maintain boundaries that will allow the organization of tissues, organs, and the organism itself.

From the data presented here, it is clear that there are some common themes regarding boundary formation and cell segregation during nervous system development in invertebrates and vertebrates, including mammals. In both cases, first there is a patterning of the neuroepithelium that requires the action of morphogen signals and transcription factors, which will often repress each other to generate distinct compartments as mentioned before. There are many transcription factors and signaling pathways shared at this stage. In the step of cell segregation, actin cables are found in *Drosophila* and vertebrate segments. A difference may be the case of cell-cell adhesion since there are no examples showing its importance in the *Drosophila* nervous system development. However, this may be the consequence of lack of research in this area. Besides, there is also the lack of data regarding the participation of the Eph-ephrin signaling in cell segregation during the development of the *Drosophila* nervous system. Thus, Eph and ephrin mutants need to be described in more detail to arrive at a conclusion. This is a very interesting topic to be addressed in future research. Another interesting point to investigate is whether the Slit-Robo and Netrin-DCC signaling pathways, which are in general not mediating cell-cell contact type of interactions, are involved in

boundary formation in the vertebrate nervous system and what would the downstream effectors be in this case. The cytoskeleton is a possibility since both pathways are known to regulate actin dynamics. However, at least in the case of Robo, it has also been shown to regulate cell adhesion, contact inhibition of locomotion and the formation of the basal membrane (Blockus and Chedotal, 2016, Kim *et al*., 2019, Ypsilanti *et al*., 2010).

In summary, many questions are still open in the field of cell segregation and boundary formation. The use of vertebrate and invertebrate models will continue to contribute to our better understanding of the cellular and molecular basis of these processes. We hope that future research will help to fill in the gaps, especially on the molecular mechanisms that are still unclear.

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