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REVIEW

Segmentation and patterning of the vertebrate hindbrain

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ABSTRACT

During early development, the hindbrain is sub-divided into rhombomeres that underlie the organisation of neurons and adjacent craniofacial tissues. A gene regulatory network of signals and transcription factors establish and pattern segments with a distinct anteroposterior identity. Initially, the borders of segmental gene expression are imprecise, but then become sharply defined, and specialised boundary cells form. In this Review, we summarise key aspects of the conserved regulatory cascade that underlies the formation of hindbrain segments. We describe how the pattern is sharpened and stabilised through the dynamic regulation of cell identity, acting in parallel with cell segregation. Finally, we discuss evidence that boundary cells have roles in local patterning, and act as a site of neurogenesis within the hindbrain.

KEY WORDS: Anteroposterior patterning, Boundary formation, Cell segregation, Hindbrain segmentation, Neurogenesis

Introduction

During embryonic development, many tissues are regionalised into sub-divisions, each with a distinct identity that underlies formation of a specific set of cell types. The boundary of adjacent sub-divisions becomes sharp, and in certain tissues forms a signalling centre that regulates local patterning. Important examples of such regionalisation and boundary formation occur in arthropods and in specific tissues in vertebrates, in which repeated segments with distinct anteroposterior (A-P) identity form along the body axis. There has thus been much interest in elucidating the mechanisms that underlie segmentation, A-P specification and the formation and roles of boundaries (Batlle and Wilkinson, 2012; Dahmann et al., 2011; Kiecker and Lumsden, 2005; Pujades, 2020).

Anatomical studies revealed that segmentation of the hindbrain is central to the organisation of craniofacial tissues. The hindbrain neuroepithelium is transiently sub-divided along the A-P axis at an early stage to form a series of rhombomeres that each generate distinct neuronal cell types (Chandrasekhar, 2004; Clarke and Lumsden, 1993; Cordes, 2001; Gilland and Baker, 2005; Kimmel et al., 1988; Lumsden and Keynes, 1989). Rhombomeres become lineage-restricted cellular compartments, with limited cell mixing between neighbouring segments (Calzolari et al., 2014; Fraser et al., 1990; Jimenez-Guri et al., 2010). Concurrently, distinct boundary cells are formed at the interface of hindbrain segments (Guthrie and Lumsden, 1991; Lumsden and Keynes, 1989). The process of segmentation establishes an early ground plan that plays a crucial role in specifying the pattern underlying formation of the neural circuitry associated with the diverse functions of the hindbrain (Briscoe and Wilkinson, 2004; Davenne et al., 1999; Di Bonito et al., 2013; Pasqualetti et al., 2007; Pattyn et al., 2003). Analyses in jawed and jawless vertebrates have revealed that this segmental organisation is a shared trait, with its origins at the base of vertebrates (Alexander et al., 2009; Lumsden, 2004; Lumsden and Krumlauf, 1996; Moens and Prince, 2002; Parker et al., 2014, 2016). At the molecular level, the formation and A-P identity of hindbrain segments are established by a gene regulatory network (GRN) of cell signalling and transcription factors, which is initiated by graded morphogen signalling along the neuroepithelium. This leads to an initially imprecise pattern of segmental gene expression, which is refined to form segments with a homogeneous regional identity demarcated by sharp borders.

The hindbrain is an excellent model with which to address questions that have broad relevance in developmental biology; studies of hindbrain segmentation have led to the discovery of mechanisms that also act in many other tissues. In this Review, we first provide an overview of the functional anatomy of the hindbrain, and then focus on molecular and cellular mechanisms that underlie its patterning along the A-P axis. We present current understanding of the gene regulatory networks that underlie the formation and A-P identity of hindbrain segments. We then discuss how the initially imprecise segmental gene expression is transformed into a sharp pattern, and how this is stabilised despite potential disruption by cell intermingling. Finally, we discuss how distinct boundary cells form at the segment borders, and emerging evidence for the roles that these cells play in hindbrain development.

Functional anatomy of the hindbrain in craniofacial development

The hindbrain is a key coordination centre in the vertebrate central nervous system (CNS) that serves as an important relay hub for control of sensory and motor functions of the head. Cranial somatic and branchiomotor nerves emanating from the hindbrain transmit motor impulses to head muscles, and receive input from cranial sensory organs through their tight association with cranial sensory ganglia (Fig. 1A-C). Through this network of neuronal circuits, the hindbrain relays sensory information from the perception of hearing, touch, taste and balance, and controls facial expressions and jaw, tongue and eye movements. Areas of the hindbrain transfer signals from the spinal cord to higher brain centres and coordinate functions of the autonomic nervous system, such as control of heart rate, respiration, digestion and swallowing. The hindbrain also contains a wide variety of interneurons and relay neurons, including a network of reticulospinal neurons that regulate alertness, sleep, posture and fine-grained locomotor activities. A series of neuronal circuits are organised in a modular manner in the hindbrain and serve as central pattern generators, producing rhythmic pacemaker-like signals that drive stereotyped behaviours, such as breathing and swallowing (Chatonnet et al., 2002; Fortin et al., 1999, 1995). Hence, from a functional perspective, the vertebrate hindbrain contains a complex network of dedicated neural circuits that play essential roles in controlling

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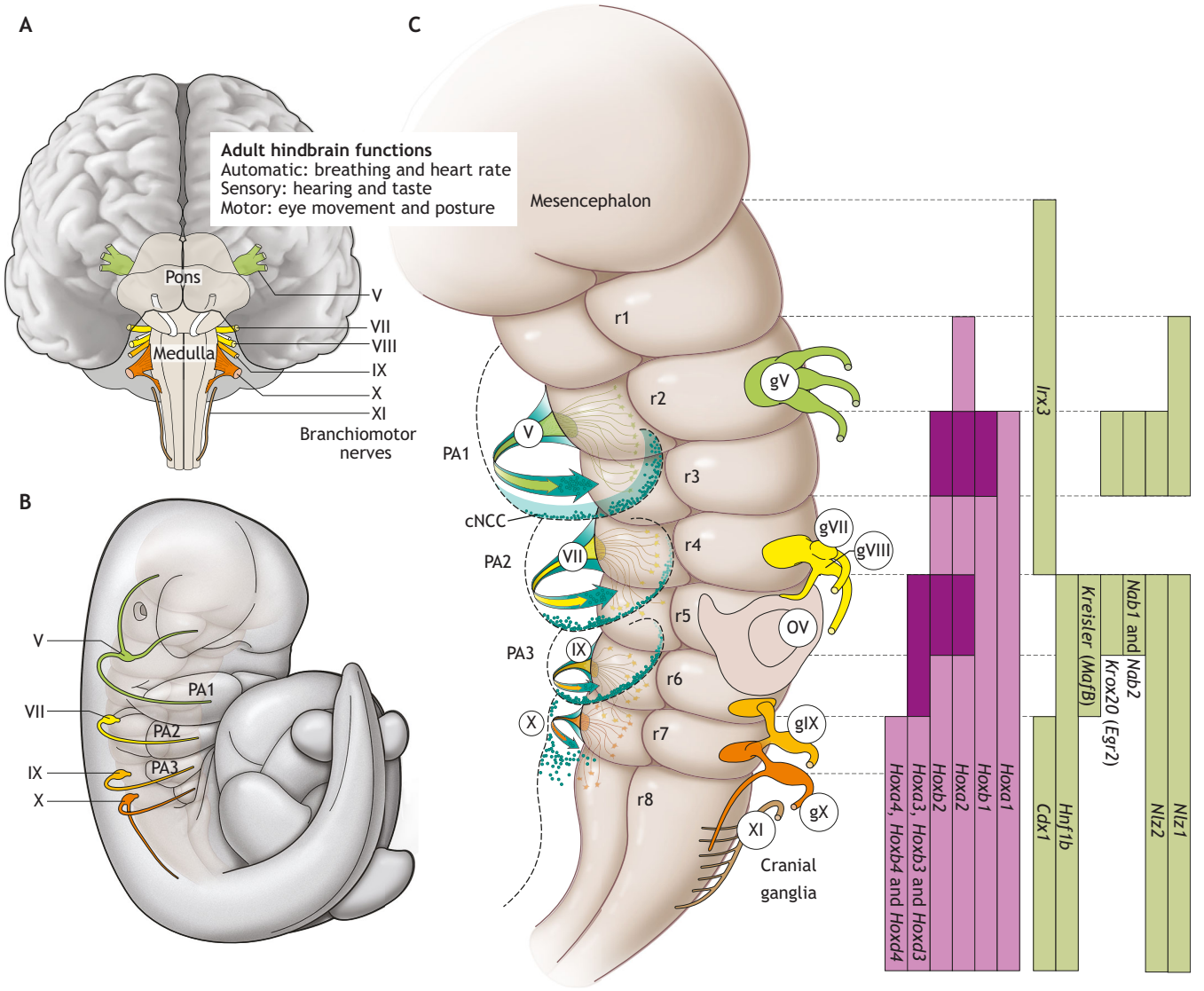

Fig. 1. Anatomy and segmental organisation of the hindbrain. (A) Diagram of ventral aspect of an adult mouse hindbrain indicating the relative positions of branchiomotor nerves and several of its functional roles. (B) Drawing of a lateral view of an E10 mouse embryo showing the paths of branchiomotor nerves and their relationship to the pharyngeal arches (PA). (C) Diagram of a ventral view of a developing mouse hindbrain depicting individual rhombomeres (r) and their relationship with the positions of cranial ganglia (gV-XI), motor nerves (V-X), migrating streams of cranial neural crest cells (cNCCs) and pharyngeal arches. There is a two-segment periodicity in the segmental organisation, as cranial ganglia and major streams of migrating neural crest cells (blue arrows and dots) are associated with even-numbered rhombomeres. Each branchiomotor nerve is derived from alternating pairs of even- and odd-numbered segments, exiting to the periphery from the dorsal aspects of only the even-numbered segments. Rhombomere-restricted domains of gene expression of key transcription factors involved in segmentation and segmental patterning are indicated on the right. The gene symbols correspond to mouse and may vary in other vertebrates. Darker shades of colour (purple, Hox genes; green, segmental sub-division genes) in the expression domains indicate higher levels of expression in specific rhombomeres.

many physiological processes and behaviours. This array of core functions, and the underlying neuroanatomy of their networks, are a common feature of the vertebrate CNS, making the hindbrain one of the most evolutionarily conserved regions of the vertebrate brain (Alexander et al., 2009; Gilland and Baker, 1993, 2005; Kiecker and Lumsden, 2005).

Programs of neural differentiation are tightly coupled to the segmental architecture of the hindbrain. For example, a set of projection interneurons display an iterative pattern in every segment (Clarke and Lumsden, 1993; Lumsden, 2004). Neurons that form the trigeminal (V), facial (VII) and glossopharyngeal (IX) branchiomotor nerves arise first in even-numbered rhombomeres (r) r2, r4 and r6. Neurons subsequently differentiate in odd-numbered segments (r3, r5 and r7) and they send axons unidirectionally to the anteriorly adjacent even-numbered rhombomere (Chandrasekhar, 2004; Lumsden and Keynes, 1989). This establishes a two-segment periodicity in the formation and organisation of branchiomotor nerves, whereby each nerve is derived from alternating pairs of even- and odd-numbered segments, exiting to the periphery from only the even-numbered segments (Fig. 1C). In line with this two-segment repeat pattern, the modular neuronal circuits of the GABAergic rhythmic central pattern generators are formed in even-numbered segments (Chatonnet et al., 2002; Fortin et al., 1995, 1999). This illustrates that spatially and temporally controlled patterns of neurogenesis are coupled to segmentation of the early hindbrain. This relationship

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leads to the elaboration of a conserved segmental pattern in the organisation of neurons, cranial nerve roots and neuronal connectivity between the hindbrain, peripheral targets and other brain centres (Chandrasekhar, 2004; Clarke and Lumsden, 1993; Cordes, 2001; Gilland and Baker, 2005; Kimmel et al., 1988; Lumsden and Keynes, 1989).

Beyond the central nervous system, the hindbrain also makes important contributions to head development and craniofacial patterning through the formation of cranial neural crest cells (cNCC). cNCCs delaminate from the neural epithelium and migrate in discrete streams to populate the pharyngeal arches where their differentiated derivatives form most of the bone and connective tissue of the head (Green et al., 2015; Knecht and Bronner-Fraser, 2002; Le Douarin and Kalcheim, 1999; Santagati and Rijli, 2003; Trainor et al., 2004; Trainor and Krumlauf, 2000b). The formation and patterns of migration of cNCCs are also linked to hindbrain segmentation and its interactions with adjacent tissues (Minoux and Rijli, 2010; Trainor and Krumlauf, 2000b). Major streams of cNCCs emanate from r2, r4 and r6 (Fig. 1C), while smaller numbers of cNCCs migrate from r3 and r5, moving rostrally and caudally to merge with streams from the even-numbered segments (Birgbauer et al., 1995; Couly et al., 1996; Golding et al., 2000; Kontges and Lumsden, 1996; Trainor et al., 2002). This segmental registration between cNCC migration and two-segment periodicity of neuronal differentiation serves to align branchiomotor nerves with their peripheral targets in the pharyngeal arches (Fig. 1C). Thus, although the process of hindbrain segmentation is an early transient state, it establishes a crucial ground plan of regional specification that is progressively elaborated during later development to generate craniofacial structures and neural circuits that underlie functions of the adult hindbrain (Briscoe and Wilkinson, 2004; Di Bonito et al., 2013; Geisen et al., 2008; Pasqualetti et al., 2007).

Gene regulatory networks underlying segmentation and A-P identity

At the molecular level, hindbrain segmentation is coupled to mechanisms that regulate A-P identity in a broad spectrum of animals and tissues (Alexander et al., 2009; Carroll, 1995; Frank and Sela-Donenfeld, 2019; Lowe et al., 2015). An early segmental plan can be visualised through spatially restricted expression of key developmental genes encoding transcription factors (TFs) that regulate steps of the segmentation process (Fig. 1C) (Alexander et al., 2009; Lumsden and Krumlauf, 1996; Parker and Krumlauf, 2017; Schneider-Maunoury et al., 1993). For example, the zinc-finger transcription factor Krox20/Egr2 is segmentally expressed in r3 and r5, and many members of the Hox homeobox gene family are coordinately expressed in nested segmental domains of the hindbrain (Hunt et al., 1991; Murphy et al., 1989; Wilkinson et al., 1989a,b). The segmental patterns of *Krox20* and Hox expression in the hindbrain are highly conserved across vertebrates (Godsave et al., 1994; Nieto et al., 1991; Parker et al., 2014, 2019a; Prince et al., 1998).

Because of the conserved segmental organisation of the vertebrate hindbrain, comparative studies between species have facilitated our understanding of GRNs that govern the process of hindbrain segmentation and A-P patterning (Parker et al., 2016; Parker and Krumlauf, 2020). Gene expression, functional perturbation and regulatory analyses in vertebrate models (primarily mouse, chicken, zebrafish and *Xenopus*) have helped to identify and characterise many of the key genes, signals and regulatory interactions that control the process of segmentation in early hindbrain development (Alexander et al., 2009; Frank and Sela-Donenfeld, 2019; Moens and Prince, 2002; Tumpel et al., 2009). These experimental findings have been integrated to generate a hypothetical hindbrain GRN that depicts the dynamic and progressive steps underlying segmentation and A-P patterning (Fig. 2A) (Parker and Krumlauf, 2020). This provides a useful logistical framework for interpretation of functional studies and evolutionary comparisons between species. The framework of the GRN may be broadly depicted as a series of hierarchical steps associated with cell and developmental processes, each with their own components and layers of regulatory circuits (Fig. 2A). Many of the genes and signals play important roles in multiple steps of the segmentation and patterning process. The components, data and logic used to formulate the structure of the GRN have been previously reviewed in detail (Frank and Sela-Donenfeld, 2019; Moens and Prince, 2002; Parker et al., 2016; Parker and Krumlauf, 2017, 2020; Tumpel et al., 2009) (Fig. 2A).

The first step of the GRN relates to A-P signalling, which is initiated by inputs and cooperative interactions between the fibroblast growth factor (FGF), Wnt and retinoic acid (RA) signalling pathways (Frank and Sela-Donenfeld, 2019). Regulatory interactions in the A-P signalling module create temporally and spatially dynamic domains of signalling (Fig. 2B,C). In mouse embryogenesis at ~E7.25, Wnt3a activates Meis and Pbx genes (Fig. 3A), which induce expression of Fgf3/8 and synthesis of RA by Raldh2 in somitic mesoderm, adjacent to the posterior hindbrain (Fig. 2B). RA spreads anteriorly in the hindbrain, whereas Fgf and Wnt signals restrict *Cyp26a1* to the anterior hindbrain, where it degrades RA in r1 and r2, establishing an initial RA signalling domain with an anterior limit at the future r2/3 border at E7.5. *Cyp26c1* is induced in r4 at E7.9, and *Cyp26b1* induced in r3 and r5 by E8.5. This progressive activation of *Cyp26a1*, *Cyp26b1* and *Cyp26c1* leads to dynamic changes in degradation of RA that eventually establish a gradient with an anterior limit at the r5/6 boundary at E9.0 (Fig. 2B) (Hernandez et al., 2007; Schilling et al., 2012; Sirbu et al., 2005; White et al., 2007b; White and Schilling, 2008).

Collectively, this cascade of events establishes the primary signals and initial nested domains of TF expression in the A-P signalling module (Fig. 3A) that in turn activate a network of spatially restricted TFs in the segmental sub-division module (Fig. 3B). Through an extensive network of auto- and cross-regulatory interactions, segmentation gene expression domains become progressively refined to generate a pattern of sharply restricted domains of expression. This provides the transcriptional code that divides the hindbrain into rhombomeres (Figs 2 and 3B).

The network of segmentation TFs also provides input into the cell segregation and boundary cell formation module through regulation of Eph receptors (Theil et al., 1998) and establishes secondary signalling centres for Fgfs and Wnts in specific rhombomeres, which play an important role in elaborating segment formation (Fig. 2A,C). As rhombomeres begin to form, signalling between them plays a role in regulating segmental identity and cell segregation. The segmentation sub-division module begins to restrict *Hoxb1* expression to r4, which leads to expression of Fgf3/8 and the formation of a secondary signalling centre (Figs 2C and 3B). In zebrafish, r4 serves as an early signalling centre that patterns the posterior hindbrain by regulating the expression of *Hoxb1*, *Krox20* and *Kreisler* (also known as *Mafba* and *valentino*) (Marin and Charnay, 2000; Maves et al., 2002; Walshe et al., 2002; Wiellette and Sive, 2003, 2004). In combination with *vhnf1* (*Hnf1b*) and FGF signalling, these transcription factors specify individual rhombomere identities in the caudal hindbrain (r4-r7) (Parker and

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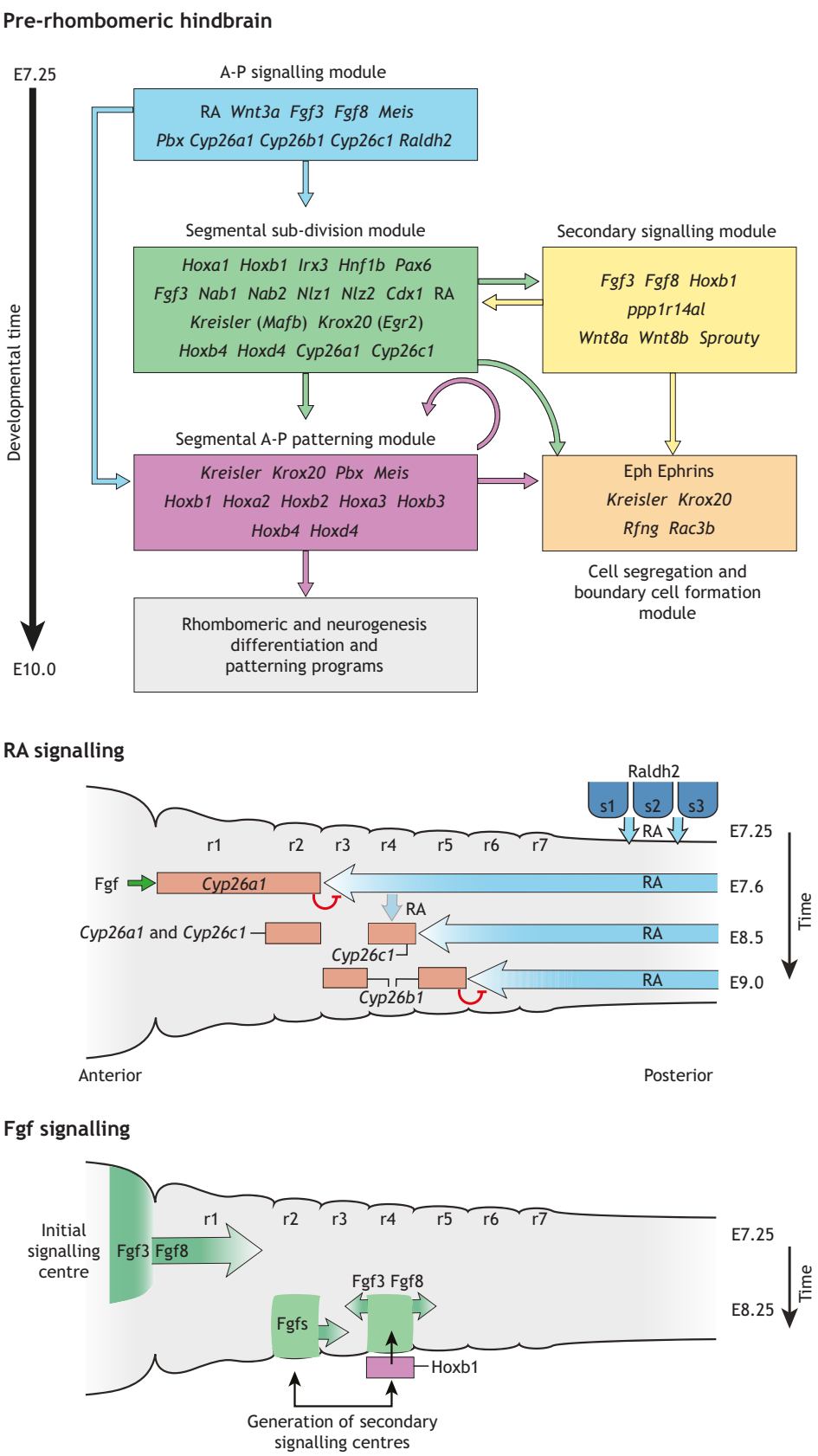

Fig. 2. Hierarchical structure of the gene regulatory network (GRN) governing hindbrain segmentation. A framework of the GRN model depicted as a progressive series of modules/steps associated with underlying cell and developmental processes in the mouse hindbrain between E7.25 and E10.0. Each module (coloured box) has its own layer of regulatory circuits and components, as indicated by the key genes and signals inside each box. The gene names correspond to mouse and may vary in other vertebrates. The arrows indicate the flow of regulatory information between modules. Many genes and signals have fundamental roles in multiple steps of the segmentation and patterning process. Regulatory interactions in the A-P signalling module set up temporally and spatially dynamic domains of signalling. (B) Diagram of shifting RA gradients involved in regulating early events in mouse hindbrain segmentation at stages between E7.5 and E9.0. RA is generated by Raldh2 in somitic mesoderm and diffuses anteriorly in the hindbrain. Fgf signals from the midbrain isthmic organiser induce Cyp26a1, which degrades RA in r1 and r2. This establishes an initial RA signalling domain with an anterior limit at the future r2/3 border at E7.5. Cyp26c1 is expressed in r4 at E7.9, and slightly later (E8.5) Cyp26b1 is induced in r3 and r5. This progressive activation of Cyp26 genes (orange rectangles) and degradation of RA create shifting gradients of RA (blue arrows) that eventually establish an anterior limit at the r5/6 boundary by E9.0. (C) Diagram of the dynamics of initial and secondary Fgf signalling centres. At E7.25, an initial Fgf signalling centre forms at the isthmic organizer in the mid/hindbrain border region and patterns the anterior hindbrain. At E8.5, initiation of Hoxb1 expression in r4 leads to activation of Fgf3 and Fgf8, and the formation of a secondary signalling centre in r4 that is important for patterning the posterior hindbrain. In chick embryos, there is evidence for a secondary Fgf signalling centre in r2. r, rhombomere; s, somite; RA, retinoic acid.

Krumlauf, 2017; Sun and Hopkins, 2001; Wiellette and Sive, 2003). In chick embryos, there is evidence for secondary Fgf signalling centres in r2 and r4 that participate in regulating *Krox20*-independent *EphA4* expression (Cambronero et al., 2020). Collectively, this illustrates the presence of crosstalk and regulatory feedback between modules in the GRN.

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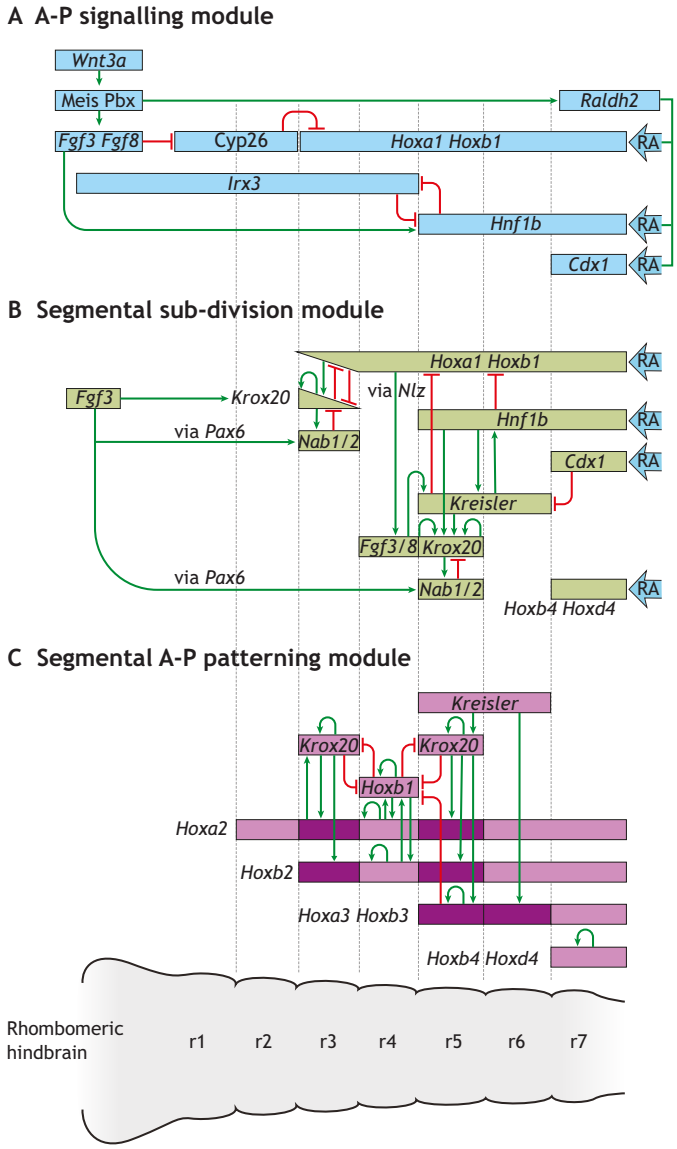

Fig. 3. Regulatory circuits and interactions between genes and signalling components in three modules governing successive steps in the GRN for hindbrain segmentation. (A) The A-P signalling module initiates the process of segmentation in the pre-rhombomeric hindbrain through combined inputs and cooperative interactions between the fibroblast growth factor (Fgf), Wnt and retinoic acid (RA) signalling pathways. This establishes nested domains of spatially restricted enzymes, signals and transcription factors (blue rectangles) that then trigger the next step. (B) The segmental sub-division module represents a series of regulatory interactions that establish sharply defined expression domains of segmentation genes (green rectangles) through extensive auto- and cross-regulatory interactions, involving mutual repression and activation. This provides a transcriptional code that sub-divides the hindbrain into rhombomeric compartments. (C) Circuits in the segmental A-P patterning module activate rhombomere-restricted domains of Hox expression (purple rectangles) to specify regional identity of individual segments. Darker shades of colour in the Hox expression domains indicate higher levels of expression in specific rhombomeres. The colours of the domains of expression and activity of genes and signals in each of the three modules correspond to that of the GRN structure in Fig. 2A. In A-C, the interactions depicted within each module are not intended to imply a specific temporal or hierarchical order, and represent cumulative interactions associated with each module. r, rhombomere; RA, retinoic acid.

In the segmental A-P patterning module, TFs directly regulate segmental expression of Hox genes (Fig. 3C). This generates nested and segment-restricted domains of Hox expression, which form a combinatorial code for specifying distinct A-P identities to each segment. Through the Hox genes, the segmental A-P patterning module also provides input into cell segregation and boundary cell identity, in part through regulation of Eph receptor genes (Prin et al., 2014) (Fig. 2). These progressive steps are dynamic and parts of the regulatory logic may be used in multiple steps. For example, the GRN that underlies Hox PG1 gene expression in r4, and *Egr2* expression in r3 and r5, are relevant not only for segmentation, but also for the dynamic regulation of cell fate that stabilises the segments in the cell segregation and boundary cell formation module (Fig. 2A).

Conserved network of A-P patterning

Although many of the proposed functional and regulatory interactions have not been independently validated in each jawed vertebrate species used to formulate the GRN model of hindbrain segmentation, it provides a useful regulatory framework for considering hindbrain evolution. Studies in the sea lamprey, a jawless fish, have provided insight into its ancestry at the base of the vertebrate family tree (Shimeld and Donoghue, 2012). Key genes involved in the hindbrain segmental sub-division and A-P segmental patterning modules (Fig. 2A) are present in lamprey and display segment-restricted patterns of expression coupled to hindbrain segmentation and patterning of cNCCs (Jimenez-Guri and Pujades, 2011; Parker et al., 2014, 2019a,b; Smith et al., 2018). Many *cis*-regulatory elements associated with segmental expression in the GRN of jawed vertebrates can direct analogous rhombomere-restricted domains in the lamprey hindbrain, and conserved *cis*-regulatory elements that mediate segmental expression have been identified in similar positions in and around lamprey genes (Parker et al., 2014, 2019b). These analyses show that essential aspects of the hindbrain GRN, including upstream regulatory factors and *cis*-regulatory circuits underlying segmental expression, form part of an ancient regulatory circuit already present in the common ancestor of lamprey and jawed vertebrates. Therefore, hindbrain segmentation appears to be a fundamental innovation that, along with the ability to form neural crest cells, is wired into conserved GRNs for developmental programs governing head development at the base of the vertebrate tree (Parker et al., 2016; Sauka-Spengler et al., 2007).

In light of the high degree of conservation of the hindbrain GRN and its A-P Hox code, it is interesting to consider how diversity in hindbrain and craniofacial development has evolved in vertebrates. One possibility is that diversification between species may have been achieved through differences in downstream targets of TFs in the GRN, impacting elaboration of rhombomeric and cNCC differentiation programs. The genome-wide duplications associated with evolution of vertebrates has generated families of paralogous genes that can partition ancestral activities and evolve new roles. The Hox PG1 genes are a good example, where the expression and function of the three mammalian PG1 genes (*Hoxa1*, *Hoxb1* and *Hoxd1*) in the hindbrain have been differentially distributed among the paralogues in zebrafish and *Xenopus* (Frank and Sela-Donenfeld, 2019; Kolm and Sive, 1995; McClintock et al., 2002; Moens and Prince, 2002; Studer et al., 1998; Tvrdik and Capecchi, 2006). Furthermore, analyses of mouse HOX PG1 proteins have uncovered functional divergence between paralogues and found that downstream targets of HOXA1 and HOXB1 are associated with different biological processes (De Kumar et al.,

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2017; Singh et al., 2020, 2021). This illustrates how specific functional components and regulatory circuits in the hindbrain GRN can vary between species as a result of diversification in the roles and outputs of paralogous genes.

Another input into diversity between species may arise through differences in roles for genes in the GRN and their targets after hindbrain segmentation is completed. There is evidence for continued roles of these genes in later steps of hindbrain development and we know very little about the GRNs governing these processes (Santagati et al., 2005). In the future, it will be important to explore the nature of similarities and differences in downstream Hox target genes and pathways in different species during head development to extend the hindbrain GRN into stages that govern processes underlying morphological and neuroanatomical diversity.

As hindbrain segmentation arose during the evolutionary transition to vertebrates, this raises the question of how it became coupled to ancient A-P patterning networks. Comparative regulatory analyses in tunicates (*Ciona*) and cephalochordates (amphioxus) indicate that the *cis*-elements responsible for the segmental expression of Hox genes in jawed vertebrates do not appear to be present in these chordates (Manzanares et al., 2000; Natale et al., 2011). Studies in a hemichordate, the acorn worm *Saccoglossus kowalevskii*, have surprisingly uncovered deep similarities in A-P axis formation and organisation in embryonic stages across deuterostomes. The A-P expression domains of key developmental TFs, including Hox genes and components of signalling pathways (FGF, Hh and Wnt) are similarly aligned along the bodies of hemichordates and chordates, suggesting a deeply conserved axial patterning system (Gerhart et al., 2005; Lowe et al., 2003, 2015; Pani et al., 2012). Functional studies have shown that Wnt signalling is important for regulating Hox genes in hemichordates (Darras et al., 2018) and also provides multiple inputs into coordinated regulation of mouse HoxA genes during gastrulation and primitive streak formation (Neijts et al., 2016, 2017; Neijts and Deschamps, 2017). RA-responsive enhancers embedded in mouse Hox clusters are involved in coordinated regulation of multiple genes in each cluster (Ahn et al., 2014; Gould et al., 1997; Nolte et al., 2013; Oosterveen et al., 2003; Qian et al., 2018; Sharpe et al., 1998) and there is experimental evidence for several highly conserved retinoic acid response elements (RAREs) in neural enhancers of amphioxus and mouse Hox clusters (Manzanares et al., 2000; Wada et al., 2006). This suggests that major axial signalling centres evolved long ago in chordate evolution, and an ability to coordinately respond to RA and Wnt signalling may be part of an ancient regulatory mechanism that underlies the generation of nested domains of Hox expression along the A-P axis. Vertebrates may then have co-opted this ancient patterning system and coupled it to hindbrain segmentation through changes in existing control modules and/or the emergence of new *cis*-regulatory elements.

Generation of sharp and homogeneous hindbrain segments

At early stages, the expression domains of genes specifying regional identity are not precise, but are refined to form sharp borders. Studies of hindbrain segmentation have shown that several molecular mechanisms act together to sharpen segment borders. In particular, important insights have come from studies of how complementary and sharp borders of *Krox20* and *Hoxb1* gene expression are established in r3-r5. At the onset of *Krox20* and *Hoxb1* upregulation, the borders of their segmental expression are ragged, but once hindbrain boundaries can be seen at the morphological level, the borders have become sharp and straight (Cooke and Moens, 2002; Irving et al., 1996; Murphy et al., 1989; Murphy and Hill, 1991; Prince et al., 1998; Sundin and Eichele, 1990; Wilkinson et al., 1989b). Furthermore, some cells at the borders initially co-express *Krox20* and *Hoxb1*, which specify distinct identities (Zhang et al., 2012). For example, in zebrafish, the fuzzy expression of *Krox20* seen at 11 hpf (hours post fertilisation) becomes sharpened by 13 hpf (Fig. 4A). The initial imprecision of gene expression domains is likely due to two factors: first, that the generation and interpretation of morphogen gradients that regulate gene expression is not precise; and second, that cell intermingling challenges the formation and maintenance of a sharp border. Studies in chick (Fraser et al., 1990) and zebrafish (Addison et al., 2018) found that some cells intermingle between segments at early stages, owing to cell intercalation concomitant with cell proliferation and convergent-extension of the neural epithelium (Kimmel et al., 1994). Subsequently, cell intermingling is restricted across segment borders (Calzolari et al., 2014; Fraser et al., 1990; Jimenez-Guri et al., 2010). This progressive establishment of cell segregation mechanisms during hindbrain segmentation reflects the fact that Eph receptors and ephrins, which restrict intermingling, are regulated downstream of TFs that underlie segmentation. For example, *Epha4* is a direct target of *Krox20* (Theil et al., 1998) and is repressed by *Hoxa3* and *Hoxb4* (Prin et al., 2014); *Ephb4* is regulated downstream of *Kreisler* (Cooke et al., 2001), and ephrin B2 (*Efnb2*) is regulated by *Hoxb4* and *Hoxd4* (Prin et al., 2014). Consistent with this, some isolated *Krox20*-expressing cells are observed in r2+r4+r6 during the period of border sharpening (Cooke and Moens, 2002; Irving et al., 1996) (Fig. 4A). Potentially, such ectopic cells could switch identity to match their neighbours or segregate back to r3 or r5, such that segments maintain a homogeneous identity (Cooke and Moens, 2002; Pasini and Wilkinson, 2002). Recent studies support the idea that both dynamic regulation of cell identity and cell segregation contribute to form a precise segmental pattern.

Roles and mechanisms of dynamic regulation of cell identity

Hoxb1 and *Krox20* are expressed in, and have key roles in the specification of, r4 and r3+r5, respectively. Segmental expression of these genes is directly (*Hoxb1*) or indirectly (*Krox20*) regulated by a gradient of RA in the hindbrain that is established by a counter-gradient of the RA-degrading enzyme Cyp26a1 (White et al., 2007a). At early stages, some cells at the borders of r4 co-express *Hoxb1* and *Krox20* (Zhang et al., 2012), which is resolved through reciprocal repression (Alexander et al., 2009) (Fig. 4B). Direct visualisation of RA reveals that the gradient is noisy at single-cell resolution (Sosnik et al., 2016), and computer simulations suggest that this noise contributes to the initial overlap of *Hoxb1* and *Krox20* expression (Zhang et al., 2012). Experiments and simulations support an important role of the RA-binding protein Crabp2a, as well as Cyp26a1, which dampen noise in the regulation of RA target genes (Sosnik et al., 2016). Interestingly, the simulations suggest that an appropriate amount of noise in the level of *Hoxb1* and *Krox20* expression has a positive role in enabling the transition from overlapping to mutually exclusive expression of these genes (Zhang et al., 2012).

In addition to resolving mixed identity at the border, there is dynamic regulation of segmental gene expression in cells that have intermingled between segments (Fig. 4C). Whereas single cells transplanted between hindbrain segments change identity to match their new location, cells transplanted as a group do not switch identity (Schilling et al., 2001; Trainor and Krumlauf, 2000a). This suggests that there is a community regulation of segmental identity, which in classical models is mediated by positive feedback between TFs and intercellular signalling (Bolouri and Davidson, 2010).

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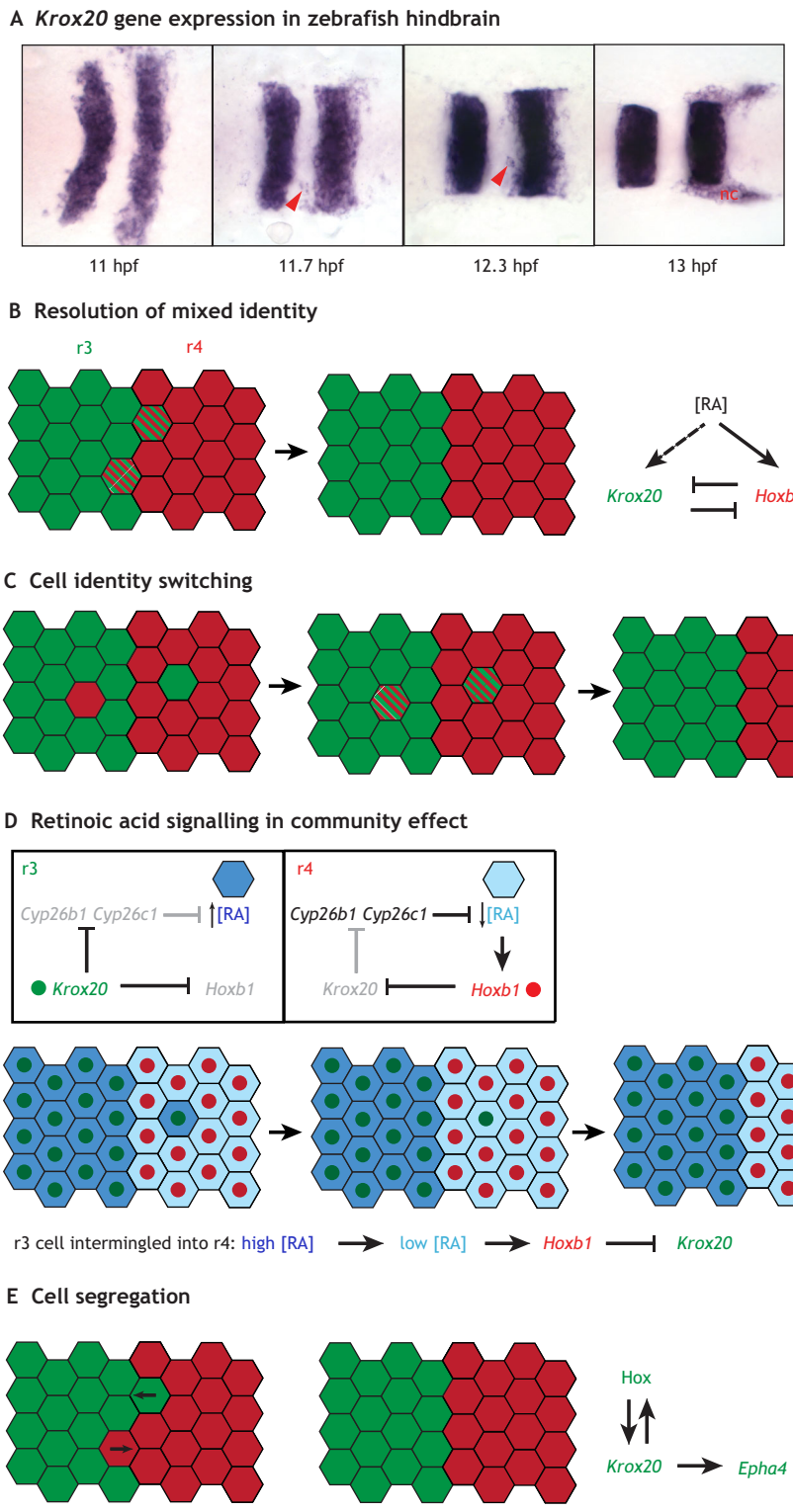

Fig. 4. Mechanisms of border sharpening in the hindbrain. (A) Time course of *Krox20* gene expression in the zebrafish hindbrain. From 11 to 13 hpf, the initial 'wiggly' borders of expression in r3 and r5 become sharp and straight. Some isolated *Krox20*-expressing cells (red arrowheads) are present in adjacent segments. nc, Krox20 expression in neural crest. (B) At early stages, some cells at the borders of r3-r4-r5 co-express *Krox20* and *Hoxb1*, indirectly or directly downstream of retinoic acid (RA) signalling, respectively. The overlapping expression is resolved by mutual repression. (C) Intermingling of cells between segments occurs at early stages, when cell segregation mechanisms are not fully established. Ectopic cells switch identity to match their new neighbours. (D) Cell identity switching is regulated by retinoic acid signalling, illustrated for r3 and r4. In r3, *Krox20* reduces expression of *Cyp26b1* and *Cyp26c1*, and consequently the RA level is high. In r4, there is a higher level of *Cyp26b1* and *Cyp26c1* expression, and the RA level is lower. Following intermingling of an r3 cell into r4, its RA level is decreased, leading to upregulation of *Hoxb1* and repression of *Krox20* expression. (E) At later stages, cell segregation mediated by Eph-ephrin signalling has been established and drives reorganisation of cells to sharpen borders. *Epha4*, which contributes to cell segregation, is directly regulated by *Krox20*, and thus coupled to Hox gene expression. (A) Adapted, with permission, from Addison (2016). (D) Adapted, with permission, from Addison et al. (2018) where it was published under a CC-BY 4.0 license.

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Krox20 may participate in such feedback, because its mosaic overexpression induces *Krox20* expression in adjacent cells (Giudicelli et al., 2001).

Recent studies have revealed an RA-mediated mechanism that underlies community regulation of *Krox20* expression (Fig. 4D). Two Cyp26 family members, *Cyp26b1* and *Cyp26c1*, have dynamic segmental expression in the zebrafish hindbrain that contributes to A-P patterning in parallel with graded *Cyp26a1* (Fig. 2B) (Hernandez et al., 2007). Collectively, *Cyp26b1* and *Cyp26c1* are expressed at a lower level in r3+r5 than in r2+r4+r6, owing to their repression downstream of *Krox20* (Addison et al., 2018). As Cyp26 expression influences the level of RA, segmentation genes thus regulate a difference in RA signalling between adjacent segments. Furthermore, high levels of Cyp26 can act as a sink to non-autonomously decrease RA in adjacent cells (Rydeen et al., 2015; Rydeen and Waxman, 2014; White et al., 2007a). Supporting a role for the segmental regulation of RA levels, *Cyp26b1* and *Cyp26c1* are required for the identity switching of *Krox20*-expressing cells that have intermingled into adjacent segments (Addison et al., 2018). In r4, the identity switching also requires *Hoxb1*, which is a direct target of RA signalling (Studer et al., 1994). These findings suggest that coupling of the level of *Cyp26b1* and *Cyp26c1* expression to segment identity mediates a community effect that switches the identity of *Krox20*-expressing cells that intermingle into adjacent segments (Fig. 4D). These cells move from a high RA (low Cyp26) to lower RA (higher Cyp26) environment, which, by non-autonomously decreasing RA levels, leads to upregulation of *Hoxb1* and downregulation of *Krox20* expression.

These findings suggest a two-step model for the relationship between RA signalling and segmental identity. At early stages, a gradient of Cyp26a1 expression underlies a gradient of RA that regulates segmental identity. Cyp26a1 is RA inducible and acts in self-enhanced RA degradation that is crucial for gradient formation (White et al., 2007a). Subsequently, Cyp26b1 and Cyp26c1 are expressed in segmental patterns that are downstream of segment identity genes. Consequently, positive feedback can occur in which segment identity regulates the level of RA, which in turn can reinforce segment identity. The findings also provide an explanation of the relationship between cell organisation and regulation of segmental identity. When cells are surrounded by others with a distinct identity, they regulate *Krox20* expression to match their neighbours, as occurs following mosaic overexpression of *Krox20* (Addison et al., 2018; Giudicelli et al., 2001). In contrast, non-autonomous induction of *Krox20* expression does not occur once cells have segregated (Addison et al., 2018). Thus identity switching depends upon how many neighbours have the same or different identity, which mechanistically can be explained by the short range of the non-autonomous effect of Cyp26 on RA levels (Rydeen et al., 2015; White et al., 2007a).

Roles and mechanisms of cell segregation

Initial evidence for how cell intermingling is restricted between rhombomeres came from transplantation experiments in chick, which found that it involved cell affinity properties that distinguish r3+r5 from r2+r4+r6 (Guthrie et al., 1993). The identification of segmentally expressed Eph receptors (Becker et al., 1994; Nieto et al., 1992) led to functional analyses (Calzolari et al., 2014; Cayuso et al., 2019; Cooke et al., 2001, 2005; Xu et al., 1995, 1999) that revealed key roles in cell segregation that establish and maintain sharp borders in the hindbrain. Eph receptor tyrosine kinases are clustered and activated upon interacting with membrane-bound ephrins, which also mediate signal transduction, leading to 'forward' and 'reverse' signalling, in the Eph-expressing cell and ephrin-expressing cell, respectively (Kania and Klein, 2016; Pasquale, 2008). Eph receptors and ephrins that have a high affinity are expressed in complementary hindbrain segments, and consequently bidirectional signalling occurs at segment borders. For example, in zebrafish, this is seen for *Epha4* and *Efnb3*, and for *Ephb4* and *Efnb2a* (Chan et al., 2001; Cooke et al., 2001; Xu et al., 1995) (Fig. 5A). Mosaic gain or loss of Eph or ephrin function leads to cell segregation within segments (Cooke et al., 2001, 2005; Kemp et al., 2009; Xu et al., 1999), suggestive of a role in the regulation of cell affinity. Furthermore, *Epha4* loss of function in zebrafish (Cayuso et al., 2019; Cooke et al., 2005; Xu et al., 1995) and chick (Sela-Donenfeld et al., 2009) increases cell intermingling and disrupts sharpening of the borders of r3 and r5; only the r4/r5 border

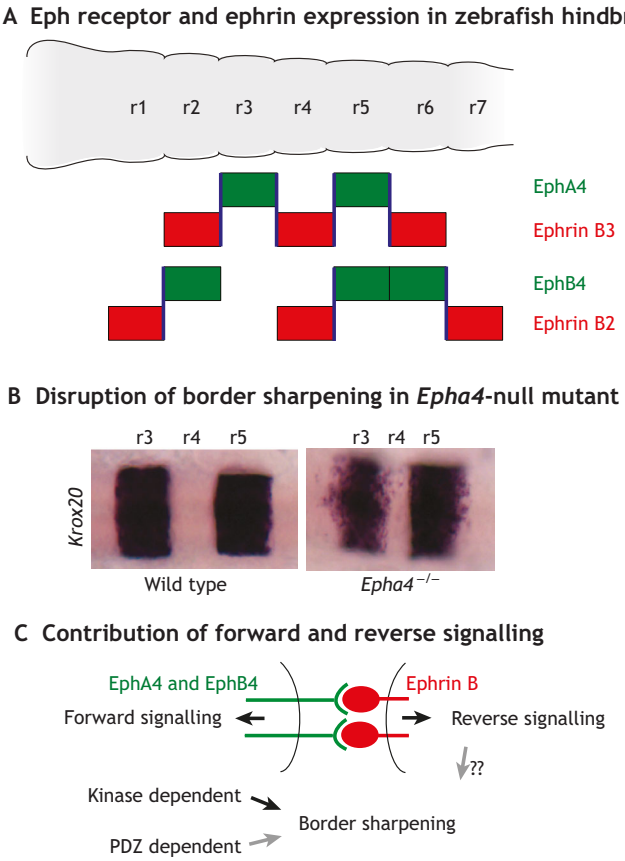

Fig. 5. Eph-ephrin signalling in hindbrain border sharpening. (A) Depiction of the segmental expression of Eph receptors and ephrins in the zebrafish hindbrain. EphA4 has high affinity for ephrin B2 and ephrin B3, whereas EphB4 binds selectively to ephrin B2. The segmental expression of high-affinity Eph-ephrin pairs is complementary, such that strong activation (blue lines) occurs at segment borders. (B) *Krox20* expression in wild-type and *Epha4* null mutant zebrafish embryos. Sharpening is disrupted at r3 and r5 borders, except for the r4/r5 border, where there is functional redundancy with EphB4 and ephrin B2, as depicted in A. (C) Signalling through Eph receptors occurs through tyrosine phosphorylation following activation of the kinase domain, and also through interaction of PDZ domain proteins with a C-terminal-binding motif. Use of truncation and point mutations of *Epha4* reveals a major input of kinase-dependent forward signalling and minor input of PDZ-dependent signalling to border sharpening. Further studies are needed to determine whether reverse signalling also contributes.
(B) Adapted from Cayuso et al. (2019) where it was published under a CC-BY 4.0 license.

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remains sharp, which is likely due to functional redundancy with *Ephb4* (Cooke et al., 2001) (Fig. 5B).

It is now well established that Eph-ephrin signalling is a major player in cell segregation and border formation in many tissues in vertebrates (Batlle and Wilkinson, 2012; Fagotto et al., 2014; Kania and Klein, 2016; Pasquale, 2005). As in the hindbrain, expression of high-affinity Eph- and ephrin-binding partners occurs in complementary domains (Gale et al., 1996), and there is overlapping expression of lower affinity partners (Rohani et al., 2014). Consequently, there is strong activation of Eph and ephrin signalling at the border of the adjacent domains, but also weak activation within each domain. Studies of cell responses and biochemical targets of Eph-ephrin signalling suggest that it can drive cell segregation by decreasing cadherin-mediated adhesion, and/or through increased cell repulsion or cortical tension mediated by actomyosin contraction (Fagotto et al., 2013; O'Neill et al., 2016; Rohani et al., 2011, 2014; Solanas et al., 2011). The use of quantitative measurements in computer simulations suggest that the principal mechanisms are heterotypic repulsion or cortical tension, which are more efficient than differential adhesion in driving cell segregation (Canty et al., 2017; Taylor et al., 2017). However, cadherin-mediated adhesion has a crucial role in counter-balancing the repulsion or tension response to low level Eph-ephrin signalling that occurs within each tissue or regional domain (Taylor et al., 2017). This latter finding potentially explains the requirement for cadherin function in segregation of cells from different rhombomeres (Wizenmann and Lumsden, 1997).

In the zebrafish hindbrain, increased levels of actomyosin and phosphorylated myosin light chain (pMLC) are detected at rhombomere boundaries from 15 hpf, which for r3 and r5 are dependent upon EphA4 (Calzolari et al., 2014; Cayuso et al., 2019). Furthermore, actomyosin contraction underlies increased tension and the distinctive shape of hindbrain boundary cells (Gutzman and Sive, 2010), and inhibition of myosin function leads to 'wiggly' hindbrain borders (Calzolari et al., 2014). These findings suggest that Eph and/or ephrin activation leads to increased cortical tension required for border sharpness. As pMLC and increased actomyosin are first detected at segment borders several hours after they have sharpened, increased cortical tension may maintain rather than generate sharpness, but it remains possible that there is a dynamic regulation of tension at early stages that underlies segregation. An important question is whether cell segregation requires forward and/or reverse signalling; however, in null Eph or ephrin mutants, signalling is disrupted in both directions. To address this problem, deletion and point mutations of EphA4 were generated that disrupt all or specific pathways of forward signalling but leave reverse signalling intact (Cayuso et al., 2019). It was found that border sharpening in the hindbrain requires kinase-dependent forward signalling (Cayuso et al., 2019) (Fig. 5C). A similar picture has come from studies in other tissues, with forward signalling having the dominant role in cell segregation and border sharpening (O'Neill et al., 2016; Rohani et al., 2014). However, studies in a cell culture model suggest that reverse signalling can contribute to sharpening (Wu et al., 2019), but this has yet to be directly tested *in vivo*.

Interplay of cell identity regulation and cell segregation

The relative contribution of cell identity regulation and cell segregation shifts during the progression of hindbrain border sharpening. Intermingling between segments occurs at early stages, before expression of *Epha4* downstream of *Krox20* (Theil et al., 1998) has been sufficiently upregulated. Consequently, an early transgenic reporter of *Krox20* gene expression detects cell intermingling followed by identity switching (Addison et al., 2018), whereas cells marked by a later *Krox20* reporter segregate rather than switch identity (Calzolari et al., 2014). During this progression, hindbrain cells transition from a plastic to a more committed state (Schilling et al., 2001), by which time cell segregation mechanisms have been fully established. Further insights have come from computer simulations revealing that cell identity regulation alone, or cell segregation alone, is not able to generate a sharp border, but in combination they lead to efficient sharpening (Wang et al., 2017). The simulations suggest that these mechanisms are synergistic because they make distinct contributions to border sharpening: cell segregation is not efficient if the transition zone of intermingled cells is too wide, and cell identity regulation serves to create a narrow transition zone (Wang et al., 2017). Further simulations suggest that cell reorganisation during convergent-extension, and identity regulation by Fgf signalling, also contribute to border sharpening (Qiu et al., 2021 preprint). Recent studies have found a similar interplay of cell segregation and identity regulation at the midbrain-hindbrain boundary (Kesavan et al., 2020). Some intermingling of cells between midbrain and hindbrain occurs prior to formation of the compartment boundary, and sharp gene expression borders are formed through cell identity switching and cell segregation, which requires Eph-ephrin function (Kesavan et al., 2020). Interestingly, midbrain-hindbrain border sharpening also requires N-cadherin (Kesavan et al., 2020), which, as discussed above, may be due to an interplay between adhesion and cell responses to Eph-ephrin signalling (Taylor et al., 2017).

Regulation and roles of hindbrain boundary cells

Early studies of hindbrain segmentation revealed that cells at the borders, termed boundary cells, have distinct cellular and molecular properties from cells further away from the border (Lumsden and Keynes, 1989). In chick, boundary cells proliferate more slowly, have less interkinetic nuclear migration (Guthrie et al., 1991) and have larger intercellular spaces that are filled with specific extracellular matrix (ECM) components (Heyman et al., 1995; Lumsden and Keynes, 1989; Weisinger et al., 2011). Boundary cells also express a distinct set of genes from non-boundary cells (Cooke et al., 2005; Letelier et al., 2018; Riley et al., 2004; Tambalo et al., 2020; Xu et al., 1995), but with some differences between species, such as *Fgf3* in chick (Mahmood et al., 1995) and *Rfng* in zebrafish (Cheng et al., 2004). Recent studies have started to uncover how boundary cells are formed at segment borders and the roles that they play in hindbrain development (Pujades, 2020).

Boundary cell induction

Ablation and transplantation experiments revealed that boundary cells are induced when odd- and even-numbered segments are juxtaposed (Guthrie and Lumsden, 1991). This correlates with cell segregation that also involves distinct properties of odd versus even segments, but it was argued that boundary cells are not involved in restricting cell intermingling (Guthrie et al., 1993). This receives support from recent studies that have revealed mechanisms of cell segregation and boundary cell formation, and how they are linked.

Disruption of *Epha4* function leads to loss of boundary cells at the borders of r3 and r5 (Cooke et al., 2005; Xu et al., 1995), suggesting that forward and/or reverse signalling is required for boundary cell induction. Furthermore, there is growing evidence that, in some tissues, Eph-ephrin signalling is involved in the regulation of cell differentiation (Laussu et al., 2014; Wilkinson, 2014). The use of mutants to dissect EphA4 signalling in zebrafish found that hindbrain

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boundary cell induction is mainly regulated by kinase-dependent forward signalling, and thus correlates with border sharpening (Cayuso et al., 2019). Expression of *Rfng* at boundaries is induced by cortical tension generated by myosin activation downstream of EphA4 signalling (Cayuso et al., 2019). Increased cortical tension leads to nuclear translocation of Taz, which, in cooperation with Tead1, induces boundary gene expression (Cayuso et al., 2019). There is thus a coupling in which increased cortical tension both maintains border sharpness and induces boundary cells, ensuring that boundary cells form at a sharp interface (Fig. 6A). Boundary cells also express a modulator of the actin cytoskeleton, *Rac3b*, which is implicated in increasing cortical tension (Letelier et al., 2018). This observation suggests that, in addition to acting directly on actomyosin contraction, Eph receptor signalling maintains border sharpness through transcriptional targets regulated downstream of cortical tension (Fig. 6A). As EphA4 signalling is also required for boundary cell formation in chick (Sela-Donenfeld et al., 2009), it will be interesting to determine whether this likewise involves the increase in cortical tension that occurs at segment borders (Filas et al., 2012).

Role of boundary cells as signalling centres

It is notable that boundary cells express a number of signalling molecules, including members of the Wnt family (Riley et al., 2004), follistatin family (Connolly et al., 1995; Tambalo et al., 2020; Weisinger et al., 2008) and Fgf3 (Mahmood et al., 1995; Sela-Donenfeld et al., 2009). *Fgf3* has been found to act as an autocrine regulator of boundary cells in chick, in which there is also elevated expression of Fgf receptors, ECM components (e.g. HSPG, CSPG) and genes associated with neurogenesis [e.g. *Brn3a* and *NSCL1* (*Nhlh1*)] at hindbrain boundaries (Weisinger et al., 2011). *Fgf3* knockdown abrogates the expression of these ECM and neurogenic genes, although not of follistatin (*Fst*), suggesting that it is required for specific properties of boundary cells (Weisinger et al., 2011). Thus, *Fgf3* has stage-specific roles in the hindbrain (Box 1) that are associated with a switch from segmental to boundary-restricted expression. Interestingly, the downregulation of segmental *Fgf3* expression requires signals from boundaries (Sela-Donenfeld et al., 2009), thus ensuring the appropriate spatial restriction of Fgf signalling at late stages.

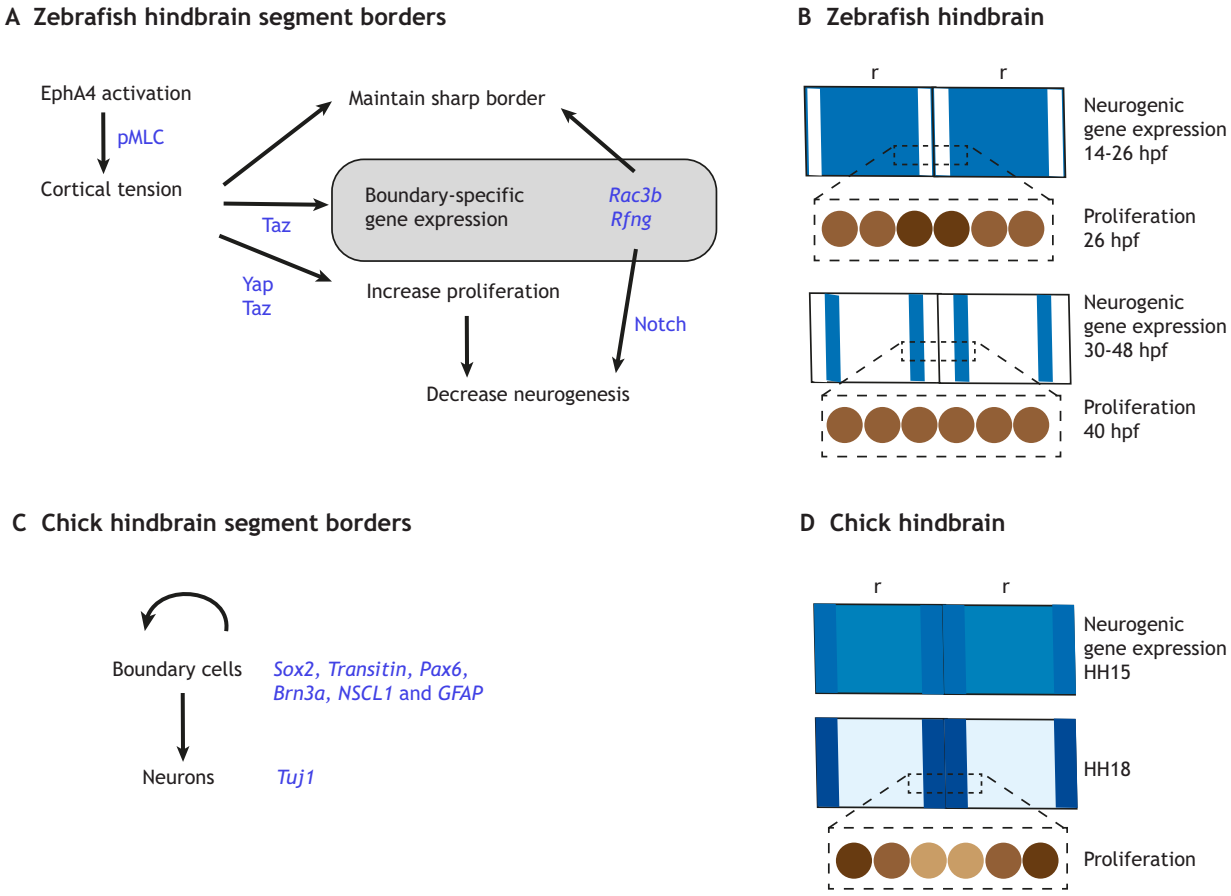

Fig. 6. Regulation of hindbrain boundary cells. (A) Molecular mechanisms of boundary cell regulation in the zebrafish hindbrain. EphA4 activation leads to increased cortical tension at boundaries through myosin light-chain phosphorylation (pMLC). Increased tension maintains border sharpness and, through nuclear translocation of Taz, leads to upregulation of boundary-specific gene expression. Boundary-specific genes include *Rac3b*, which contributes to border sharpness, and *Rfng*, which inhibits neurogenesis by promoting Notch activation. Cortical tension acts through Yap and/or Taz activation to increase boundary cell proliferation, thus decreasing neurogenesis. (B) The pattern of neurogenesis in the zebrafish hindbrain is depicted for two adjacent rhombomeres (r) to illustrate the relationship with boundaries. Neurogenic gene expression is widespread but lower at the boundaries from 14 to 26 hpf. The rate of cell proliferation (light- to dark-brown shading, low to high rate) at 26 hpf is higher at boundaries than in non-boundary cells. Neurogenic gene expression has become confined to zones adjacent to hindbrain boundaries from 30 hpf onwards. By 40 hpf, there is no longer increased cell proliferation at boundaries. (C) In the chick hindbrain, boundary cells express *Sox2* and neurogenic markers, and are self-renewing neural stem cells that can generate neurons. (B) In the chick hindbrain, neurogenic gene expression (blue shading) is widespread at HH15, and has become mainly confined to boundaries by HH18. The rate of cell proliferation is low at the centre of the boundary region and higher adjacent to the non-boundary region.

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Box 1. Differences in gene expression and patterning in chick and zebrafish

Although many aspects of gene regulation and cell organisation are conserved, there are some important differences between species. An example is the spatial regulation of Fgf signalling and its roles in neurogenesis. In all species studied, early segmental Fgf expression (e.g. *Fgf3* and *Fgf8* in r4) is required for further segmentation of the caudal hindbrain (Hernandez et al., 2004; Maves et al., 2002; Walshe et al., 2002). Following this, the segmental expression is downregulated and, in chick, *Fgf3* is upregulated in hindbrain boundaries where it promotes the expression of neurogenic genes and ECM components (Weisinger et al., 2011). Conversely, late Fgf signalling in the zebrafish hindbrain occurs at segment centres (Esain et al., 2010; Gonzalez-Quevedo et al., 2010), where *Fgf20*-expressing neurons inhibit neurogenesis and are positioned by chemorepulsive signalling from boundaries. What may underlie interspecies differences in the late expression and role of Fgf signalling? A clue comes from the observation that primary reticulospinal neurons are located at each segment centre in zebrafish (Hanneman et al., 1988). These neurons colocalise and may overlap with *Fgf20*-expressing neurons. Reticulospinal neurons are among the earliest neurons generated and form a neuronal circuit that mediates the escape response that enables aquatic embryos to move away from predators (O'Malley et al., 1996). This is not relevant in amniotes and, as for other neuronal cell types, reticulospinal neurons are not localised to segment centres (Cepeda-Nieto et al., 2005). Thus, a requirement for A-P patterning of neurons within segments in zebrafish may have been lost in the evolutionary transition to amniotes, accompanied by changes in the organisation of neurogenesis by Fgf signalling.

The expression of signalling molecules by hindbrain boundaries also suggests potential roles in patterning of cell differentiation within segments, analogous to boundaries in other tissues (Dahmann and Basler, 1999; Kiecker and Lumsden, 2005; Rhinn and Brand, 2001). Direct evidence for a patterning role has come from studies of the spatial regulation of neurogenesis within segments in the zebrafish hindbrain. Expression of proneural genes that initiate neuronal differentiation initially occurs throughout the hindbrain and later becomes downregulated at boundaries as well as in the centre of each segment (Cheng et al., 2004; Gonzalez-Quevedo et al., 2010; Riley et al., 2004) (Fig. 6B). Consequently, by 30 hpf, neurogenesis has become confined to zones adjacent to hindbrain boundaries. Two mechanisms have been implicated in the decrease in neurogenesis in boundary cells (Fig. 6A): first, *Rfng* expression is induced downstream of cortical tension and Taz (Cayuso et al., 2019), and this promoter of Notch activation acts to inhibit neuronal differentiation (Cheng et al., 2004); and second, cortical tension acts through Yap/Taz to increase boundary cell proliferation and thus reduce neurogenesis (Voltes et al., 2019). The inhibition of neurogenesis in segment centres is mediated by Fgf20, which is expressed by a subset of neurons in the adjacent mantle zone (Gonzalez-Quevedo et al., 2010). *Fgf20*-expressing neurons form a cluster in the centre of each segment, which locally inhibit neurogenesis in the adjacent neural epithelium (Gonzalez-Quevedo et al., 2010). As Fgf signalling is required for gliogenesis in the hindbrain (Esain et al., 2010), *Fgf20* may underlie a switch from neuronal to glial cell differentiation at segment centres. The clustering of *Fgf20*-expressing neurons at segment centres is maintained by chemorepulsion mediated by semaphorin family members that are expressed by hindbrain boundary cells (Terriente et al., 2012). Thus, in zebrafish, boundary cells have a role in the patterning of neurogenesis within segments, albeit through an unconventional mechanism in which they organise *Fgf20*-expressing neurons that act as a signalling source. It currently remains unclear whether other signals from hindbrain boundary cells mediate direct paracrine regulation of cell differentiation (Amoyel et al., 2005; Gerety and Wilkinson, 2011; Riley et al., 2004).

Role of boundary cells as neural stem cells

Recent studies have found that hindbrain boundary cells are neural stem cells that are a source of neurogenesis, although with differences between species in timing and spatial organisation. In the chick hindbrain, the boundaries become a slowly dividing population of neural stem cells that are the major source of neurogenesis after HH18 (Fig. 6C). This is reflected by expression of markers of neurogenesis, which is initially widespread in the hindbrain and becomes restricted to boundaries by HH18 (Peretz et al., 2016) (Fig. 6D). In addition to expressing proneural genes, boundary cells express *Sox2*, a key regulator of neural stem cell properties, and can form neurospheres in culture that self-renew and differentiate (Peretz et al., 2016). There is a low rate of cell proliferation at the centre of the boundary cell zone and a higher rate at the outer part of the boundary (Peretz et al., 2016) (Fig. 6D). By analogy with other tissues, the accumulation of ECM at boundaries may contribute to regulation of these properties of boundary cells.

There is a different situation at zebrafish hindbrain boundaries, in which neurogenic gene expression is inhibited downstream of Eph signalling and increased cortical tension. Cortical tension acts through Yap/Taz to increase cell proliferation and upregulate *Rfng* expression (Cayuso et al., 2019; Voltes et al., 2019), leading to a sharp demarcation between non-neurogenic boundary cells and the adjacent neurogenic zones (Fig. 6B). Subsequently, there is a decrease in cortical tension, leading to a decline in the rate of cell proliferation and a shift towards neuronal differentiation of boundary cells after 40 hpf (Voltes et al., 2019).

Although the spatial regulation of cell proliferation and neurogenic gene expression at boundaries seems to differ in chick and zebrafish (Fig. 6B,D), a common feature is that neurogenesis is lower at the segment borders (i.e. the centre of the boundary region) than in flanking regions. The inhibition of neuronal differentiation at the segment borders may serve to maintain a stable cell population that has other functions, such as a signalling source. One interpretation is that the boundary region described in chick is functionally equivalent to the boundary plus flanking neurogenic zones in zebrafish. In both species, boundaries comprise a pool of neural stem cells generating progeny that can move away from the segment border and differentiate. An important difference between chick and zebrafish is that, in the latter, the boundary cells initially have a higher rather than lower proliferation rate than non-boundary regions. This could reflect a much shorter time interval between segmentation and neurogenesis in zebrafish compared with chick, which requires an early expansion of progenitor cells at boundaries (Voltes et al., 2019).

There is currently less understanding of the relationship between boundaries and neurogenesis in the mouse hindbrain. There is sustained high level expression of *Hes1* at boundaries, in contrast to the variable and oscillating expression of this gene away from boundaries (Baek et al., 2006). As high *Hes1* maintains quiescent neural stem cells and inhibits proneural gene expression (Sueda et al., 2019), this suggests that there is decreased neurogenesis at hindbrain boundaries. Consistent with this, *Plzf*, a transcriptional repressor that inhibits neurogenesis (Sobieszczuk et al., 2010), is expressed at hindbrain boundaries in mouse (Cook et al., 1995). It will be interesting to ascertain whether hindbrain boundaries become a source of neurogenic stem cells at later stages in mouse, as occurs in chick and zebrafish.

Conclusions

Studies of hindbrain segmentation have given important insights into how mechanisms of cell segregation and cell identity regulation cooperate to generate sharp and homogeneous regional identity. Such cooperation has also been found in recent studies of the midbrain-hindbrain border (Kesavan et al., 2020) and it is likely that similar principles apply to other tissues in which an initial imprecise pattern is sharpened. It will be important to have a deeper understanding of how these mechanisms are embedded in the GRN of hindbrain patterning. With regard to cell segregation, this requires uncovering of how other segmentally expressed Eph receptors and ephrins are regulated by TFs that underlie segmentation. It will also be important to understand dynamic aspects of segment identity regulation through modelling of the GRN. For example, how do *Krox20*-expressing cells that intermingle into an adjacent segment switch identity? Such plasticity likely involves indirect responses of the initiator and autoregulatory elements of the *Krox20* gene (Labalette et al., 2015) to the lower level of RA that the cell is exposed to in the new environment. As autoregulation increases and maintains *Krox20* expression (Labalette et al., 2015), this potentially contributes to the decrease in cell identity switching at late stages in the hindbrain (Schilling et al., 2001).

Another important area for future work is to understand how the early networks that establish segmental patterning lead to the organisation and coordinated differentiation of neuronal cell types in the hindbrain. Studies have shown that Hox genes have important roles in the trunk in regulating subtype diversity of motor neurons and display multiple functions in diverse neuronal classes to impact neuronal specification and connectivity (Philippidou and Dasen, 2013). In later stages of hindbrain development, Hox genes display dynamic dorsal-ventral (D-V) patterns of expression that correlate with the birth of major classes of neurons (Graham et al., 1991), and mutational studies demonstrate that Hox genes play important roles in patterning hindbrain neurons (Arenkiel et al., 2004; Davenne et al., 1999; Gaufo et al., 2003; Gavalas et al., 2003; Pattyn et al., 2003; Philippidou and Dasen, 2013). Existing studies have generated a rich level of knowledge of the molecular mechanisms and cellular processes regulated by the early roles of Hox and other TFs in the GRN of hindbrain patterning. The rapidly emerging array of genomic approaches for investigating small numbers of cells and single cells in developing tissues holds promise for identifying downstream targets and how the TFs to which they are coupled control neurogenesis. Single-cell transcriptional profiling in the developing zebrafish hindbrain has begun to unravel the D-V and A-P distribution of neuronal cell types as they differentiate (Tambalo et al., 2020). There are major shifts in the transcriptomes of progenitors and differentiating cells over time that provide molecular insights and novel markers for functional analyses on the regulation and patterning of neural differentiation. A systematic application of this approach in multiple species holds promise for expanding the GRN and unravelling how Hox genes and segmentation regulate neurogenesis programs in the developing hindbrain, and have broader roles in circuit formation.

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Competing interests

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