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GENE EXPRESSION AND REGULATION OF HINDBRAIN AND SPINAL CORD DEVELOPMENT

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1. ABSTRACT

The formation of the central nervous system is one of the most fascinating processes in biology. Motor coordination, sensory perception and memory all depend on the complex cell connections that form with extraordinary precision between distinct nerve cell types within the central nervous system. The development of the central nervous system and its intricate connections occurs in several steps. During the first step known as neural induction, the neural plate forms as a uniform sheet of neuronal progenitors. Neural induction is followed by neurulation, the process in which the two halves of the neural plate are transformed into a hollow tube. Neurulation is accompanied by regionalisation of the neural tube anterior-posteriorly into the brain and spinal cord and dorso-ventrally into neural crest cells and numerous classes of sensory and motor neurons. The proper development of the vertebrate central nervous system requires the precise, finely balanced control of cell specification and proliferation, which is achieved through the complex interplay of multiple signaling systems. Bone morphogenetic proteins (BMPs), retinoic acid (RA) fibroblast growth factors (FGFs), Wnt and Hedgehog proteins are a few key factors that interact to pattern the developing central nervous system. In this review, we detail our current knowledge of the roles of these signaling factors in the development of the vertebrate nervous system in terms of the mechanisms underlying the formation and specification of the hindbrain and spinal cord.

2. INDUCTION AND SPECIFICATION OF THE NEURAL PLATE

The neural plate forms initially as a uniform, layer of columnar epithelium derived from uncommitted ectoderm around the time of gastrulation (formation of the mesoderm or third germ layer; Figure 1). The ectoderm cells that are not incorporated into the neural plate give rise to the epidermis of the skin. In 1924, Hans Spemann and Hilde Mangold famously discovered that differentiation of the neural plate from uncommitted ectoderm in amphibian embryos depends upon signals from a specialised group of mesoderm cells underlying the ectoderm, called the blastopore lip, or Spemann's organiser (1). Transplantations of the dorsal blastopore lip in amphibians results in the formation of a duplicated body axis that included an almost complete second nervous system. Only cells from the organiser region have this neural inducing capability and this result provided the first evidence that the nervous system is induced by signals from non-neural cells. Recent studies primarily in amphibian and avian embryos have uncovered a number of secreted molecules that are expressed by the organiser, which provides a molecular mechanism for understanding the process of neural induction. These include *noggin* (2), *chordin* (3) and *follistatin* (4), which mediate neural induction by binding to and inhibiting a subset of bone morphogenetic proteins (BMPs) (reviewed in (5)). These secreted factors all have potent neural inducing activities in *Xenopus* ectodermal explants and mimic the ability of the organiser to induce and pattern a secondary axis.

In *Xenopus* embryos, prior to neural induction, *Bmp4* is widely expressed in the ectoderm (6). During gastrulation, however, *Bmp4* expression is repressed by signals from the organiser in the portion of the ectoderm fated to become the neural plate (7). Therefore, the inhibition of BMP signaling in the ectoderm represses epidermal fate and induces neural differentiation (Figure 1)

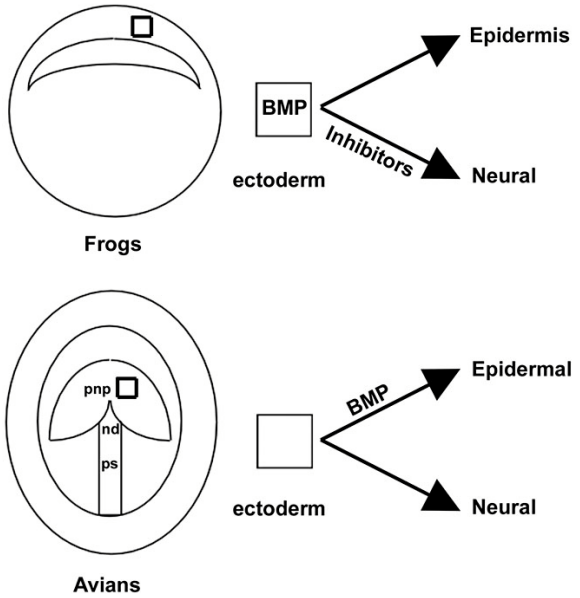

Figure 1. Classic neural induction models in vertebrate gastrula. (A) In frog gastrula, ectoderm cells (square) subjected to BMP signaling differentiate into epidermis. However, ectoderm cells in which BMP signaling is blocked by mesoderm expression of BMP inhibitors such as *noggin*, *chordin* or *follistatin*, differentiate into a neural fate. In contrast in avian embryos, medial prospective neural plate (pnp) explants (square) differentiate into neural tissue in vitro unless they are exposed to BMP signaling in which case they differentiate into epidermis. Therefore the absence of *Bmp* expression in prospective neural tissue during the induction process is consistent between anamniotes and amniotes. However it is now believed that neural induction occurs prior to gastrulation and involves complex interactions between the FGF and Wnt signaling pathways. ps. primitive streak; nd node. (adapted from (210)).

(2,3,7,8). In support of this idea, single ectoderm cells taken from gastrula stage *Xenopus* embryos and cultured in the absence of any additional factors (ie BMP4) will differentiate into neural tissue. These findings led to the proposal of the "default model" for neural induction in which ectodermal cells by default adopt a neural fate when removed from the influence of extracellular signals during gastrulation (9,10). BMP antagonists are expressed by the organiser during normal embryonic development and are sufficient to suppress the epidermis promoting activity of BMP4 (11). In *Xenopus*, therefore, inhibition of BMP signaling in the ectoderm is critical for induction of prospective neural plate tissue. BMPs also seem to play similar roles in patterning the ectoderm of teleosts such as zebrafish (12,13). Although, the "default model" for neural induction adequately accounts for the majority of the experimental data obtained to date in amphibians, difficulties arise when attempts are made to extrapolate this model to amniotes and mammals.

In avian embryos, *Bmp4 and Bmp7* are expressed at low levels in the epiblast prior to gastrulation, however the expression of theses genes is downregulated coinciding with the acquisition of neural character (14). Although the presence of BMP signaling in the chick embryo at pre-gastrulation stages is capable of inducing epidermal fate, by late gastrula stages this effect is lost (Figure 1) (14). In chick embryos, the organiser (Henson's node), similar to its equivalent, the blastopore lip in *Xenopus*, expresses the BMP inhibitors *noggin* and *chordin*. In contrast however, *noggin* and *chordin* fail to induce neural cell differentiation in avian embryos (15). The temporal expression of BMP inhibitors in chick embryos does not coincide with the induction of neural cells, suggesting that the roles of BMPs and their inhibitors in chick neurulation are not as clear-cut as that found in *Xenopus* (16). Further complicating the story is the observation that a neural plate stills forms despite surgical removal of the organizer in chick, frog, zebrafish and mouse embryos (17). In addition, gene-targeting experiments in mouse have shown that proper neural differentiation can take place in the absence of BMP inhibitors arguing that BMP signaling is not required for neural induction (18-20). This suggests firstly, that the organizer is not required for neural induction, secondly, that necessary neural inducing signals originate in tissues other than and in addition to the node/organizer and thirdly that neural induction occurs prior to the onset of gastrulation. Although it cannot be completely ruled out that BMP signaling plays a role in neural induction in chick and mouse embryos, currently it appears to be insufficient to induce differentiation of neural cells on its own.

Although, these differences between *Xenopus* and amniotes may reflect the distinct embryonic morphologies of each species, it is important to note that the avian organiser can substitute for the blastopore lip of *Xenopus* in neural induction assays (21). It's unlikely therefore that fundamentally different molecular mechanisms for specifying neural fate have evolved in amniotes versus anamniotes. However the results described above imply that the organizers in avians and mammals produce additional neural inducers that are not BMP antagonists. FGF signaling has recently been implicated as one of these additional signals. Avian neural induction appears to be initiated prior to gastrulation by FGF signals emanating from precursor cells destined to form the organiser (Henson's node) (14,22). *Fgf8* is expressed during gastrulation in the anterior of the primitive streak, including the node, however its expression is downregulated as the node begins to lose its neural-inducing ability. Consistent with this observation, the inhibition of FGF8 signaling, using either dominant negative FGF receptors or FGF specific inhibitors (SU5402) downregulates the expression of these neural plate specific markers. Interestingly this downregulation occurs without affecting BMP signaling (22) indicating that avian neural induction requires the action of FGFs in addition to BMP signaling.

These results raise the question of whether BMPs, which alone are insufficient for neural induction, play a combined role with FGF signaling. Recent in vitro studies have demonstrated that exposure of epiblast cells to BMP antagonists is sufficient to promote induction of neural cells when FGF signaling is attenuated (14). These results suggest that one possible function for FGF signaling

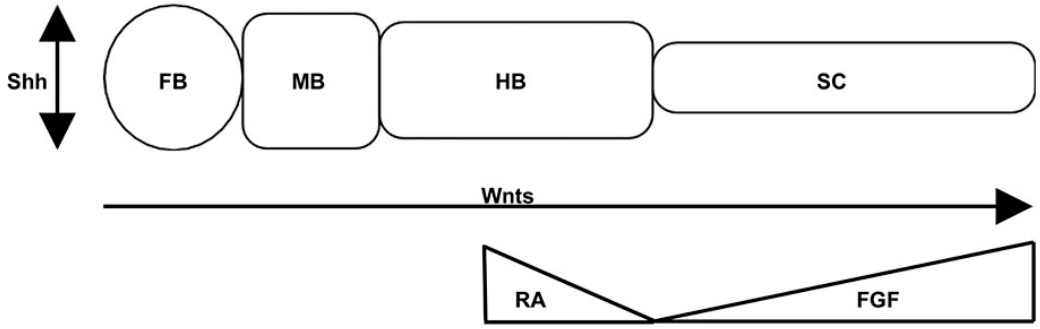

Figure 2. Global growth and patterning of the brain and spinal cord. Following neurulation, the brain and spinal cord undergo extensive differential cell growth and regional patterning giving rise to the forebrain (FB), midbrain (MB), hindbrain (HB) and spinal cord (SC). On a global scale Shh signaling has been implicated in mediating the overall growth of the brain vesicles. Anterior-posterior patterning of the brain and spinal cord however, depends firstly upon signaling centers within the neural tube such as the forebrain-midbrain junction and the midbrain-hindbrain (isthmus) junction and secondly upon the complex interactions between graded Wnt, RA and FGF signaling. Graded Wnt signaling is believed to function along the entire length of the neuraxis inducing progressively more posterior neural fates. *Hox* genes also play important roles in establishing regional cell identity in patterning the hindbrain and spinal cord and this is achieved via opposing gradients of RA and FGF signaling.

maybe to attenuate BMP signaling in prospective neural cells. In support of this idea, inhibition of FGF signaling using SU5042 results in maintenance of *Bmp4* and *Bmp7* expression, which are normally downregulated in epiblast cells of prospective neural character, implying a role for FGF in the repression of BMP signaling. Thus as in *Xenopus*, acquisition of neural fate may require the repression of *Bmp* expression, while epidermal cell fate may require maintenance of *Bmp* expression. This implies that the expression of *Fgf8* by some of the precursor cells of the organiser is important for the early induction of the neural plate. As the organiser (node) forms, other signals, including BMP antagonists begin to be expressed, initiating further genetic cascades capable of inducing later neural markers (22). It is important to note that neither FGF signaling alone or in combination with BMP antagonists, is sufficient for the induction of *Sox2* or later neural markers, which implies that while both FGF signaling and BMP inhibition play roles in neural cell fate specification, neither is sufficient to complete neural induction (9,14,22,23).

Wnt proteins represent one of the additional signals required for the regulation of neural and epidermal fates in avians (17). In the chick embryonic ectoderm, lateral or prospective epidermal tissue expresses *Wnt3* and *Wnt8,* while medial or prospective neural tissue does not. The lack of exposure to Wnt signaling in the medial ectoderm permits *Fgf8* expression, which in turn represses BMP signaling and activates an independent pathway specifying neural fate. Conversely, high levels of Wnt signaling in lateral epiblast cells inhibits FGF signaling, allowing increased BMP expression, which in turn directs cells to an epidermal fate (17). These results help clarify why exposure to BMP antagonists alone do not induce neural differentiation in avians. Cells in the lateral ectoderm are normally exposed to high levels of Wnt activity which blocks the BMP-independent pathway of FGF transduction required for neural differentiation. Hence exposure to Wnt signaling is a key constraint for lateral epiblast to differentiate into a neural fate. Similarly, in *Xenopus* embryos, Wnt signaling has been implicated in neural induction via the downregulation of *Bmp4* in prospective neural plate cells (24).

Analyses of the function of Wnt signaling have helped to clarify the distinct roles played by FGFs and BMPs during neural induction and collectively these results demonstrate that neural induction occurs prior to gastrulation in amniote and anamniote embryos, which is much earlier than previously thought. Neural induction involves the coordinated interaction of three different gene families, FGFs, BMPs, their associated antagonists, and Wnts, all of which play significant and distinct roles in the differentiation of neural versus epidermal fate in the developing CNS. Overall, a key feature that remains conserved between vertebrate embryos is the exclusion of *Bmp* expression from the neural induced territory. Vertebrate neural induction therefore is a far more complex process than described in the classic "neural default" model.

One of the current hypotheses for BMP involvement in neural plate development is in regulating the size of the neural plate. Mutations in the zebrafish genes *chordino* and *bozozok* result in smaller neural plates. Conversely, mutations in the *Bmp2b* gene (*swirl*), *Bmp7* (*snailhouse*) and Smad5 (*somitabun*) are associated with an expanded neural plate. Similar studies have also reported an expansion of the avian neural plate in response to exposure to BMP inhibitors. These results are consistent with the idea that BMP antagonists play a role in maintaining neural fate.

3. GENERATING REGIONAL IDENTITY IN THE CNS

Neural plate induction is followed by neurulation, the process in which the neural plate is transformed into a hollow neural tube. In amphibians, mice and chicks, the neural tube arises through the uplifting and fusion of the

two halves of the neural plate. In contrast in fish, the formation of the neurocele occurs via cavitation of the neural plate. At the cephalic or anterior end, the neural tube partitions via differential cell proliferation into a series of swellings and constrictions that define the major compartments of the adult brain: forebrain (prosencephalon), midbrain (mesencephalon), and hindbrain (rhombencephalon) (Figure 2). The forebrain becomes further regionalised anteriorly into the telencephalon and posteriorly into the diencephalon. The telencephalon develops into the cerebral hemispheres and the diencephalon gives rise to the thalamic and hypothalamic brain regions. Similar to the forebrain, the hindbrain also becomes further sub-divided anteriorly into the metencephalon and posteriorly into the myelencephalon. The metencephalon gives rise to the cerebellum, the specific part of the brain responsible for coordinating movements, posture and balance, while the myelencephalon forms the medulla oblongata, the nerves of which regulate respiratory, gastrointestinal and cardiovascular movements. In contrast to the forebrain and hindbrain, the midbrain is not subdivided further. However, the lumen of the midbrain gives rise to the cerebral aqueduct. Posterior to the head, neural tube which extends the remaining length of the body axis, ultimately develops into the spinal cord.

The early morphogenesis of the brain is characterised by differential expansion of the dorsal neural epithelium and such rapid morphogenesis implies a tight co-ordination of cell proliferation and survival (25). The balloon shaped forebrain, midbrain and hindbrain vesicles provide much of the structural basis of the adult brain and although it is largely unknown mechanistically how the three dimensional morphogenesis of the vertebrate brain is co-ordinated, a recent study implicates *Shh* as being crucial in driving the rapid expansion of the early forebrain and midbrain vesicles (Figure 2) (25). Ablation of local Shh signaling by surgical removal of the notochord resulted in the overall collapse and abnormal folding of the vesicles and is accompanied by reduced cell proliferation and increased cell death. These abnormalities could be rescued by implantation of Shh secreting cells in a dose dependent manner. Therefore the notochord plays a key role in regulating brain vesicle expansion by promoting cell survival proliferation through a Shh mediated signaling mechanism.

An important question is how cells in the neural plate become regionalised and specified into forebrain, midbrain, hindbrain and spinal cord domains since immediately following induction, the neural plate is assumed to have a uniformly rostral character. Is there a mechanism that can account for the anterior-posterior specification of individual cells along the entire neural axis? Recent evidence suggests there is (Figure 2). In chick embryos, medial epiblast cells from blastula stage embryos generally express *Sox2*, *Sox3*, *Otx2* and *Pax6* a combination of markers characteristic of the forebrain. In addition, these cells do not express *En1/2*, *Krox20*, or *Hoxb8* which are typical markers of the midbrain, hindbrain and spinal cord respectively (17). This implies that neural progenitors initially possess a rostral "forebrain-like" character and that midbrain, hindbrain and spinal cord characteristics are generated by subsequent reprogramming. New data indicates that posteriorising the early neuroepithelium, at least in chick embryos, involves the convergent actions of FGF signaling with graded concentration-dependent Wnt signals to specify cells of caudal forebrain (*Otx2+, Pax6+*), midbrain (*Otx2+, En1+*), rostral hindbrain (*Gbx2+, Krox20+, Pax6+*) and caudal hindbrain (*Krox20+, Gbx2-, Pax6-*) character (26). Higher concentrations of Wnt signals induce progressively more caudal character in the neural tube (Figure 2). Conversely, in the absence of Wnt signaling, caudal neural cells grown in vitro revert to a rostral forebrain character. These studies support a model whereby Wnt signaling plays distinct roles in nervous system development at the blastula and gastrula stages. Prior to gastrulation, Wnt signaling promotes epidermal fate and blocks neural fate by preventing epiblast cells from responding to the neuralising activity of FGFs. During gastrulation the generic committed neural plate cells become regionalized more and more posteriorly in response to an increasing gradient of Wnt activity in combination with FGF signaling.

Wnt signaling can establish basal anterior-posterior patterning of the CNS. However localized regionalisation within the brain that generates the distinct forebrain, midbrain hindbrain and spinal cord sub regions of the central nervous system involves local signaling or organizing centers within the neuroepithelium itself, such as the isthmic junction which separates and demarcates the midbrain and hindbrain and also the diencephalic-mesencephalic junction which separates the forebrain from midbrain (Figure 2) (27-31). It would be impossible in the scope of this paper to review in sufficient depth the patterning mechanisms underlying the specification of each and all these regions that constitute the central nervous system. Therefore, for the purpose of this review we concentrate below on the anterior-posterior regionalisation of the hindbrain and the dorso-ventral regionalisation of the spinal cord as examples of the mechanistic complexity of patterning in the brain and spinal cord.

4. HINDBRAIN REGIONALISATION AND SPECIFICATION

The hindbrain is perhaps the most comprehensively analyzed region of the brain in terms of cell patterning and compartmentalization. It has long held a fascination for embryologists due to the fact that the hindbrain becomes visibly subdivided into seven clearly demarcated, cell lineage restricted compartments called rhombomeres (r) (32-34). The rhombomeric organization of the hindbrain provides an important cellular and genetic blueprint for establishing many of the characteristic features of the central and peripheral nervous systems (Figure 4). For example, each rhombomere gives rise to a unique region of the adult brain such as the cerebellum which is derived from rhombomere 1 (35,36). The hindbrain also presages the periodic organization of the cranial sensory ganglia and cranial motor nerves (Figure 3).

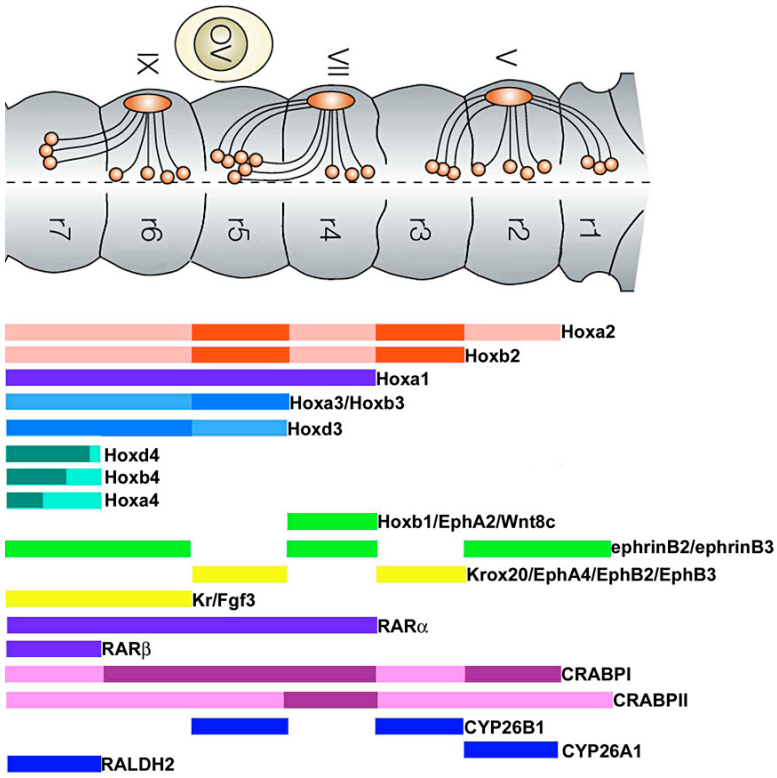

Figure 3. Rhombomeric blueprint for the hindbrain and nervous system. The hindbrain is segmented into seven cell lineage restricted units called rhombomeres (r) which exert a profound influence on brain and nervous system patterning. There is a clear registration between individual rhombomeres and the spatial distribution of the cranial nerves (V, trigeminal; VII, facial: IX, glosso-pharyngeal), the axons of which leave the neural tube only from exits points contained within the even numbered rhombomeres. Concomitant with hindbrain segmentation is the establishment of sharp domains of gene expression within individual rhombomeres, or pairs of rhombomeres or alternating rhombomere segments. The domains of expression are incredibly dynamic during the 7.5-10.5dpc of embryonic development and only the principal domain of expression is represented here. The combinatorial gene expression exhibited by an individual rhombomere, ultimately determines its identity and fate and removing specific genes such as *Hoxb1* can transform r4 into an r2 like identity and fate.

Cell bodies of individual sensory ganglia exhibit a precise relationship to specific rhombomeres as do the motor nerves innervating the first three branchial arches (V trigeminal, VII facio-acoustic and IX glosso-pharyngeal) which are respectively derived from nuclei confined primarily within rhombomeres 2, 4 and 6 (33,37). The facio-acoustic, glossopharyngeal and trigeminal motor nerves are all augmented by neurons developing in adjacent rhombomeres. Despite each nerve being derived from neurons born in multiple rhombomeres, the axons leave the hindbrain only through exit points contained within the even numbered rhombomeres as they project to the branchial arches and peripheral tissues. Therefore the segmental organisation of the hindbrain clearly underpins the metameric character of the nervous system and represents a conserved strategy used by vertebrates for establishing domains of cellular differentiation.

Hindbrain morphogenesis and the generation of rhombomeric segements coincides with the establishment of precise expression domains of a number of transcription factors (*Krox20*, *kreisler*, *Hox*), signaling molecules (*Eph/ephrins*), membrane and nuclear receptors (*RAR/RXR*), and enzymes involved in the retinoid biosynthetic pathway (Figure 3). Some genes are expressed in single rhombomeres, or pairs of rhombomeres, but all exhibit extremely dynamic patterns of activity during neurulation. The *Hox* genes, which comprise a set of transcription factors characterised by the presence of a DNA binding domain sequence motif called the homeobox (38), are among the earliest markers of presumptive hindbrain neuroepithelium. From a single invertebrate cluster, the murine *Hox* gene family has evolved via duplication and divergence into 4 distinct chromosomal clusters (*Hoxa-Hoxd*) comprising some 39 genes, although no cluster contains members of all 13 paralagous groups presumably due to evolutionary gene loss (39). Spatial and temporal colinearity is the most striking feature of the organization of the *Hox* gene transcription factor family such that gene order within the complex is transposed directly in embryonic time and space conferring anterior-posterior positional information (39-43). Genes located at the 3' end of the cluster are expressed earlier and more anteriorly during development than those found at the 5' end, such that *Hox* genes exhibit nested domains of anterior-posterior expression within the neuroepithelium of the hindbrain and spinal cord.

5. REGULATION OF HOX GENE EXPRESSION IN THE CNS

Hox genes display incredibly dynamic expression patterns in the hindbrain (Figure 3). Initially, the borders of *Hox* gene expression are diffuse, but they subsequently sharpen as the morphological boundaries demarcating individual rhombomeres develop. The establishment of rhombomeric-restricted domains of expression involves a conserved sets of genetic interactions and considerable effort has been spent in identifying upstream regulatory factors that govern *Hox* gene expression. The regulation of *Hox* gene expression in the hindbrain involves an initiation phase followed by a maintenance phase (44). Initiation occurs during gastrulation, in the mesodermal tissue arising from the primitive streak and in the overlying neuroepithelium in response to retinoic acid and FGF signaling (Figure 2). The maintenance phase which follows sustains and fine-tunes *Hox* gene expression within the characteristic rhombomeric boundaries. This is achieved through a combination of auto-, cross- and para-regulation along with direct regulation by segment specific genes such as *Krox20* and *kreisler*.

6. RETINOIC ACID INITIATION OF HINDBRAIN HOX GENE EXPRESSION

Retinoic acid (RA), a metabolic derivative of vitamin A, is enriched in the primitive streak of amniotes, which is consistent with its role as a key initiator of *Hox* gene expression in the hindbrain (45-47). The expression of *Raldh2*, a major RA biosynthetic enzyme and the RA-catabolizing enzymes *Cyp26A1* and *Cyp26B1* implies the existence of a RA gradient diffusing from the rostral spinal cord into the caudal hindbrain (Figure 2) (48,49). Exogenous RA treatments during the late gastrulation phase of mouse embryo development rapidly induces *Hox* gene expression, promoting anterior shifts in *Hox* gene expression domains and effecting anterior to posterior transformations of cell fate (50-52). Interestingly, the response to retinoic acid is co-linear, with the 3'-most *Hox* genes being induced earlier, and more rapidly at lower concentrations than genes located more 5' in each *Hox* cluster. RA treatment at 7.25-8.0 days post coitum (dpc) rapidly induces the 3' genes *Hoxa1*, *Hoxb1* and *Hoxa2* in the hindbrain without any corresponding response in the group 4 genes, *Hoxa4*, *Hoxb4* and *Hoxd4*. However if RA is administered between 8.5 and 9.5dpc then *Hoxa4*, *Hoxb4* and *Hoxd4* respond rapidly within the hindbrain while upregulation of the 3' genes is no longer inducible (53-55).

The retinoid signal is transduced primarily by two distinct ligand-activated transcription factors: the retinoic acid receptors (*RAR*) and retinoid receptors (*RXR*) (56) of which there are three genes (a, b and g) in each family. In addition to the nuclear retinoic acid receptors, the mechanism of RA action is also believed to involve cellular retinol and the retinol binding proteins *CRBPI, CRBPII and CRABPI, CRABPII* respectively. The expression domains (Figure 3) of these RA receptors and binding proteins are thought to regulate the availability of free versus bound retinoids thereby controlling the regional concentration of RA along the AP axis (57).

Robust support for the function of retinoids in *Hox* gene activation came primarily from the discovery that the retinoid nuclear receptor family comprises heteromeric DNA binding proteins that recognize specific motifs within target genes called retinoic response elements (RAREs) (57,58). Numerous analyses have identified RAREs in the regulatory control regions of paralagous groups 1 and 4 *Hox* genes, which enables them to respond directly to retinoid signaling (59-65). The most comprehensively characterised RAREs are those associated with the group 1 paralogs *Hoxa1* and *Hoxb1* (Figure 4). In the *Hoxa1* gene a single RARE is located within the 3' regulatory region of the gene (59). This enhancer mediates the RA-inducibility of *Hoxa1* and is required for establishing neuroectodermal expression up to the presumptive r3/r4 boundary in the hindbrain (66). This is particularly evident in mice lacking the 3' *Hoxa1* RARE which display reduced levels of *Hoxa1* transcripts and fail to establish r3/4 boundary restricted expression in the hindbrain (67). Two RAREs have been identified within the 3' flanking sequence of the *Hoxb1* gene (Figure 4) (62,64,68). Similar to *Hoxa1*, these enhancers initiate *Hoxb1* during gastrulation and establish an anterior limit of expression up to the r3/4 boundary in the hindbrain. An additional RARE was identified in the 5' flanking region of the *Hoxb1* gene which is of particular importance as it allows RA to make a second regulatory input into *Hoxb1* and it is essential for restricting *Hoxb1* expression to r4 by repressing its activity in r3 and r5 (63,64). The targeted disruption of the 3' *Hoxb1* RARE, reduces its anterior expression limit and levels thereby demonstrating its importance in initiating the expression of *Hoxb1* (69). Although *Hoxd1* is not expressed in the neuroectoderm of the mouse (70), studies in *Xenopus* have shown that RA is involved in regulating *Hoxd1* (71,72). This indicates that all three vertebrate *Hox* group 1 members contain RAREs that are required to activate their early expression in the neuroepithelium. The initiation or activation of *Hoxa4* expression in neuroectoderm up to the r6/7 boundary also requires an RARE (65). Other similar RAREs have been described for the *Hoxa4* (73) and *Hoxd4* (54,55,61,74-76) loci, which mediate induction and establish the proper rostral expression boundaries of these genes. Therefore, similar to the group 1 genes, RA signaling is clearly involved in regulating the expression of the group 4 paralogs, *Hoxa4*, *Hoxb4* and *Hoxd4*. A surprising corollary to these observations is that to date no RAREs have been reported for the group 3 paralogous genes (77).

Thus, RAREs convey the ability to drive early abundant expression of the 3' *Hox* genes within the neurectoderm. Most importantly, they establish the rostral limits of Hox gene expression and regulate the interpretation of positional information supplied by posteriorising signals from either the mesoderm or neural plate. Surprisingly the cis-regulatory mechanism for controlling *Hox* gene expression in the neuroepithelium, including a dependence upon retinoic acid signaling, has

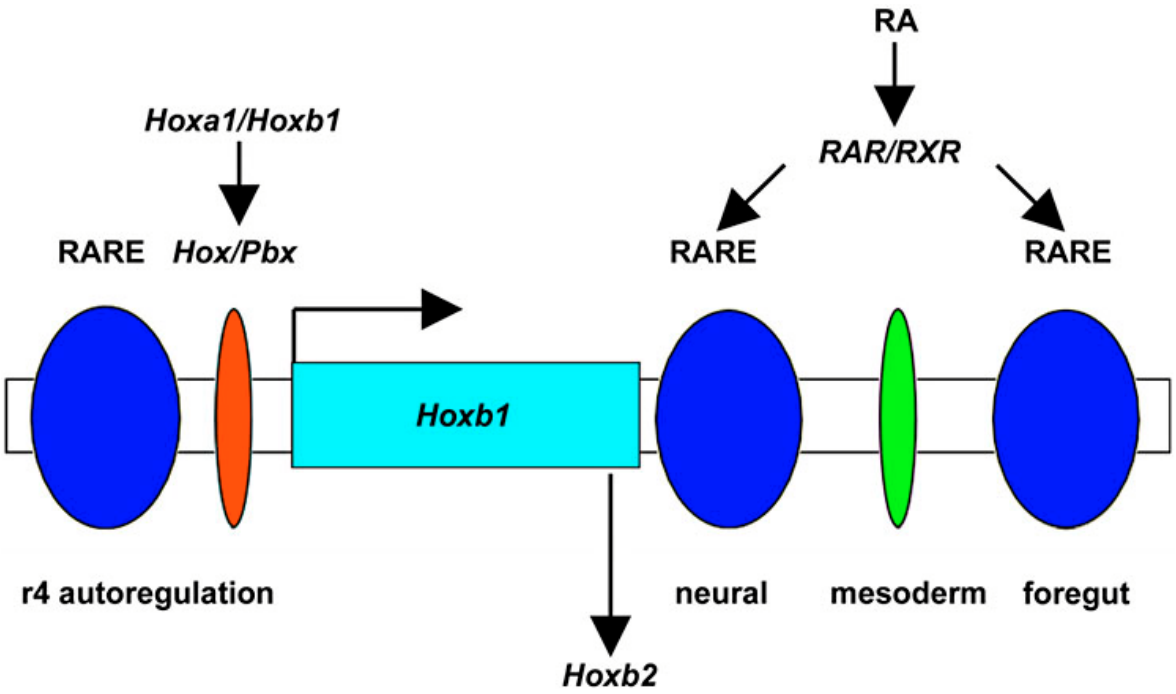

Figure 4. Regulation of *Hoxb1* expression in the hindbrain. The complexity in regulating *Hoxb1* expression in r4 of the hindbrain is dependent upon *Hox/Pbx* (red oval) and retinoic acid response element (RARE) binding sites in the 5' autoregulatory region of the *Hoxb1* locus that mediates auto-, para- and cross regulatory interactions. In addition, two other RAREs are locate in the 3' regulatory region of *Hoxb1* which bind retinoic acid receptor heterodimers and are required to initiate the early broad expression of *Hoxb1*. *Hoxb1* not only feeds back on itself in an autoregulatory loop but cross regulates the expression of *Hoxb2* in r4.

been conserved during half a billion years if evolution (78). In a study designed to examine how *Hox* gene regulation evolved, the cis-regulatory regions of 3' genes in the amphioxus *Hox* cluster which are homologous to the 3' *Hox* genes in vertebrates, were linked to a *lacZ* reporter and assayed in transgenic mouse and avian embryos. Two regions designated *Amphihox-1A* and *Amphihox-3b* stimulated expression in the central nervous system. In particular *Amphihox-3b* mediated expression in mouse and avian embryos up to an anterior limit that coincided with the r5/6 boundary. Given the importance of retinoic acid in regulating basal *Hox* gene expression, this study also investigated whether the *Amphihox* elements were dependent upon and could respond to RA signaling. Sequence analysis of *Amphihox-1A* revealed the presence of many putative RAREs and exogenous RA induces an anterior shift in *Amphihox-1A* expression in the CNS. Conversely, blocking RA signaling with a dominant negative *RAR*a downregulates *Amphihox-1A* driven reporter expression. *Amphihox-3B* is also dependent upon RA signaling in the CNS as reporter activity is downregulated by dominant negative *RAR*a. Retinoid signaling therefore is an important mechanism conserved throughout evolution for governing the spatial and temporal regulation of *Hox* gene expression during embryonic development

7. FGF INITIATION OF SPINAL CORD *HOX* GENE EXPRESSION

The most 5' genes in each *Hox* cluster are refractory to RA, which is consistent with a direct role for retinoid signaling in relation to *Hox* genes at the 3' end only of each cluster. This raises the question of what regulates the expression of 5' *Hox* genes posteriorly in the spinal cord. A recent study has now demonstrated that 5' *Hox* gene expression is activated in response to graded FGF signaling (Figure 2). The exogenous application of FGF (FGF2 or FGF4) induces and expands the expression domains of several 5' *HoxB* members (*Hoxb6-Hoxb9*) anteriorly into the caudal hindbrain of avian embryos, a territory where they are not normally expressed (79). Furthermore, this effect appears to be mediated in part through *Cdx* transcription factors. The ectopic expression of an activated Cdx protein throughout the chick neuropeithelium drives *Hoxb9* expression up to the level of the otic vesicle, although this effect has to be promoted by exogenously supplied FGF. In contrast using dominant negative Cdx protein blocks FGF-mediated *Hox* gene induction. *Cdx* genes are not expressed in the hindbrain and this possibly accounts for the lack of expression of *Hox* paralog groups 5 to 9 in this region (80-82).

Thus, the 5' *Hoxb* genes are refractory to RA treatment in the chick neural tube, while the 3'-most *Hoxb* genes fail to respond to FGF signaling. This indicates that *Hox* gene complexes can be broadly classified into two regions with distinct regulatory mechanisms governing their expression: A retinoid-responsive domain encompassing *Hox* paralog groups 1 through 4 at the 3'end of the complex, and a FGF-sensitive domain that includes *Hox* groups 5 to 9 in the 5' region of the cluster. In light of analyses detailed earlier suggesting that Wnt signaling is

also involved in establishing anterior-posterior fate in uncommitted neuroepithelium, these results imply that collectively Wnts, RA and 3' *Hox* genes specify hindbrain character whereas Wnts, FGF, *Cdx* and 5' *Hox* together specify more caudal spinal cord character.

8. MAINTENANCE OF *HOX* GENE EXPRESSION BY AUTO-, CROSS- AND PARA-REGULATION

The initiation of *Hox* gene expression in the neural tube in response to retinoic acid, FGF and Wnt signaling (Figure 2) is followed by a maintenance phase in which the domains of *Hox* gene expression are sustained and refined (Figure 3). The fine-tuning and continuance of *Hox* gene expression within characteristic rhombomeric boundaries is achieved through a combination of auto-, cross- and para-regulation as well as direct regulation by segment specific genes such as *Krox20* and *kreisler*.

Auto-, cross- and pararegulatory interactions are critical for regulating *Hox* gene expression in the hindbrain. For example, *Hoxb1* expression which is initially activated by RA, subsequently maintains high levels of expression only in r4 of the hindbrain via a conserved autoregulatory mechanism (83,84). Three sequence related motifs located 5' to the *Hoxb1* gene constitute a bipartite recognition element of overlapping sites for *Hoxb1* and for a mouse homologue of the *Exd* homeobox gene, *Pbx* (85). *Hoxb1* together with mouse Pbx/Meis proteins are crucial for r4 specific expression and identity (Fig 4).

A number of *Hox* genes such as *Hoxb2* exhibit variation in their relative expression levels within specific rhombomeres which hints at differential modes of regulation. *Hoxb2* is expressed in the neural tube up to the r2/3 boundary but exhibits higher levels of expression in r4. Transgenic deletion analyses of the 5' flanking region of *Hoxb2* identified a 181bp element capable of mediating the upregulation of *Hoxb2* expression specifically in r4. This element contained no consensus RARE, however it did have a single motif high in homology to the *Hox*/Pbx autoregulatory motifs identified in the *Hoxb1* locus (86). Through in vitro binding assays it was demonstrated that *Hoxb1* binds to *Hoxb2* in an interaction necessary for normal r4 activity. Deletions of the *Pbx/Hox* motif abrogates expression. In addition, the *Hoxb2* motif was able to distinguish between itself and Hoxb1 proteins which is indicative of the fact that r4 specific upregulation of *Hoxb2* occurs via cross regulatory interactions with Hoxb1 and not as a consequence of it's own autoregulation (Figure 4) (86).

Further evidence for the importance of *Hox* auto- and cross-regulation has come from analyses of the *cis*-acting regions of the group 4 paralogs (65,87). Within the 3' flanking region of *Hoxb4* and *Hoxd4* are enhancers that establish the r6/7 anterior limits of neural expression (55,65,88-91). The neural enhancer of the *Hoxb4* gene is located next to a distal promoter of the *Hoxb3* gene and this *cis*-element is shared by the *Hoxb4* and *Hoxb3* genes. Through sequence comparisons of pufferfish, chicken and mouse, a highly conserved region designated CR3 was identified, which alone is capable of directing expression with a sharp r6/r7 anterior boundary. Mutations in the CR3 element abrogate the normal expression patterns indicating that *Hoxb4* and *Hoxd4* autoregulation are mediated through the CR3 fragment (88). Therefore in a manner analogous to *Hoxb1*, RA signaling directly initiates the early r6/r7 expression domain of *Hoxb4* expression which is then maintained by a *Hoxb4* autoregulatory loop (65).

The *Hoxa3* and *Hoxb3* genes exhibit subtle differences in regulation of their segmental expression in the hindbrain up to the r4/5 boundary. An auto-regulatory element consisting of two *Hox/Pbx* binding sites mediates *Hoxa3* expression in the neuroectoderm (77). Similary, neural expression of *Hoxb3* is also dependent upon an auto-regulatory element containing two *Hox* binding sites. Interestingly, both *Hoxb3* and *Hoxb4* can interact with these binding sites in vitro indicating that *Hoxb3* also uses cross-regulation for the establishment and maintenance of its segmental expression in the hindbrain (92).

Collectively, these studies demonstrate the importance of auto-, cross- and para-regulatory mechanisms for the functional maintenance of *Hox* gene expression during vertebrate hindbrain development. The fact that many elements are shared implies a necessity for keeping genes clustered, such as in the *Hox* complex, in order to maintain appropriate spatio-temporal expression patterns.

9. *KROX20* AND *KREISLER* REGULATION OF *HOX* GENE EXPRESSION

During hindbrain development, *Hoxa2* and *Hoxb2* exhibit marked upregulation in rhombomeres 3 and 5. By virtue of its early segmentally restricted expression in r3 and r5, the zinc finger transcription factor *Krox20* was considered to be a prime candidate for regulating *Hox* genes (Figure 3). The pivotal regulatory roles of *Krox20* in hindbrain segmentation have now been clearly demonstrated by transgenic analyses in mice. Two Krox20 binding sites were found in the 5' flanking region of the *Hoxa2* gene (93,94). Similarly, three Krox20 binding sites were identified within the 5' flanking region of the *Hoxb2* gene (95). Although the *Krox20* binding sites are insufficient to induce r3 and r5 specific expression of *Hoxa2* and *Hoxb2*, they are essential for their upregulation in the odd numbered rhombomeres. Therefore *Hoxa2* and *Hoxb2* are direct targets of *Krox20* but other cis-regulatory elements which remain to be identified are required to regulate their basal expression levels. *Krox20* is similarly required for *Hoxb3* activity in r5, providing further evidence that it functions upstream in the *Hox* gene regulatory cascade (96).

Hoxb3 is also directly regulated by *kreisler*, a gene identified in mice due to their circling behaviour (97). *Kreisler* is specifically expressed early in r5 and r6 suggesting it too might play a role in transcriptional regulation during hindbrain development (Figure 3) (98). Two binding sites in the 5' flanking region of *Hoxb3* are necessary and sufficient for an interaction with *kreisler* (99) in r5. In contrast to *Hoxb3*, *kreisler* directly regulates the

expression of *Hoxa3* in both r5 and r6 through the presence of a unique *kreisler* binding site in the 5' flanking region of *Hoxa3* (100). Mutations in the *kreisler* site abolish its activity. Additional factors serve to regulate the expression of *Hoxb3* in r6. Further deletion and mutation analyses identified a second cis-regulatory element that corresponded to an activation site for *Ets*-related transcription factors and which is necessary to potentiate and restrict *kreisler* enhancer activity to r5 (99). Therefore transgenic analyses have revealed that both *Hoxa3* and *Hoxb3* are directly regulated by *kreisler* and that *Hoxa3* and *Hoxb3* are distinctly regulated in the hindbrain (99,100).

Collectively these results indicate that there are intricate, interactive loops between the *Hox* genes, *Krox20* and *kreisler* in the developing hindbrain, all of which are crucial for the control of the segmentation and specification process. Based on the detailed information that has been generated from the cis-regulatory analyses of *Hox* genes, the dynamic expression patterns and interactions have been integrated and modelled *in silico* which can predict the outcomes of disruptions to the *Hox* gene signaling network and such phenotypes are described below (101).

10. MUTATION ANALYSES UNDERSCORE THE MECHANISTIC REGULATION OF GENE EXPRESSION IN THE HINDBRAIN

Retinoid signaling mediates the nested expression of *Hox* genes during hindbrain development and has the potential to modify the anterior-posterior character of the central nervous system. Unsurprisingly, altering the retinoid gradient perturbs hindbrain development. Indeed, the loss of RA signaling via null mutation of the major RA biosynthetic enzyme *Raldh2* results in the down-regulation of the rostral expression domains of several 3' *Hox* genes such as *Hoxa1*, *Hoxb1*, *Hoxa3*, *Hoxb3* and *Hoxd4* (102,103). Similarly, avians and rodents raised on a vitamin A deficient diet (VAD) exhibit equivalent hindbrain abnormalities (104-106). Interestingly, the severity of hindbrain abnormalities in *RAR*a/*RAR*g double mutants is equivalent to that observed in *Raldh2* null mutant and VAD embryos (107). Furthermore, antagonizing retinoid signaling in mouse and avian embryos leads to a progressive reduction of posterior rhombomeres along with an enlargement of anterior rhombomeres (108,109).

In contrast, increases in retinoid signaling posteriorise the hindbrain. Inactivation of *Cyp26A1* in the mouse, a RA-catabolizing enzyme expressed in r2, leads to a mild posterior transformation of the anterior hindbrain. Concomitant with a rostral expansion of *Hoxb1* are enhanced levels of *Hoxb1* expression in r4 (110,111). These analyses are consistent with a graded patterning role for retinoid signaling during hindbrain development (Figure 2). Blocking retinoid signaling results in a truncation of the caudal hindbrain concomitant with respecification of the remaining rhombomeres to a more anterior identity. Conversely upregulation of retinoid signaling results in a posteriorisation of the hindbrain.

The targeted deletion of *Krox20* results in a progressive loss of r3 and r5 and their derivatives (112-114). Consequently, there is no upregulation of *Hoxa2*, *Hoxb2*, *Hoxb3* or *EphA4* in the null mutants and *Hoxb1* is downregulated in presumptive r4 (115). Fusions of the trigeminal ganglion with the facial and vestibular ganglia arise due to the profound perturbation of hindbrain development and *Krox20* null mutants die shortly after birth. *Krox20* therefore plays a pivotal role in hindbrain segmentation and in the maintenance of segment identity.

Kreisler is a classical mutant that was identified by virtue of its circling behaviour (116). *Kreisler* is not a null allele, rather it is a mutation affecting the regulatory elements responsible for its r5 and r6 domains of expression in the hindbrain. Consequently, the hindbrain in *kreisler* mutant embryos is unsegmented posterior to the r3/r4 boundary (98,117-119) and the mutation causes a specific loss of r5. This is demonstrated by the absence of the normal expression domains of *Krox20*, *Hoxa2*, *Hoxb2*, *Hoxb3* and *Hoxb4* in r5. Although r6 is formed, the absence of *Hoxa3* in r6 indicates it is not properly maintained (100). *Kreisler* mutants exhibit inner ear abnormalities as well as defects in neural crest derived skeletal elements such as the hyoid (116,117). *Kreisler* therefore, similar to *Krox20* is a true segmentation gene which regulates multiple steps during hindbrain development, including the proper formation of r5 and the regulation of the segmental identity of r5 and r6.

Evidence obtained in *Drospohila*, suggested that homeobox genes were regulators of body segmentation (120,121) and it was hypothesised that the mammalian homologs of these genes also functioned as homeotic selectors, conferring segment identity in the hindbrain. This idea has now been extensively tested via mutational analyses in mice. Two distinct null alleles have been generated for *Hoxa1* and defects in the early hindbrain phenotype differs slightly between the two alleles, ranging from a reduction in the r4 and r5 territories (122-124) to a complete absence of presumptive r5, as assayed by the reduced expression of *Hoxb1* in r4 and *fgf3* and *Krox20* in r5 (125,126). Thus *Hoxa1* is required for the formation of the r4/r5 territory which accounts for the severe defects in inner ear development and the loss of facial and abducens nerve motor neurons in homozygous mutants (122-124,126). Interestingly, the exogenous application of RA during a very narrow time window is able to rescue the inner ear defects associated with the *Hoxa1* null mutation (127). This again highlights the intimate relationship between retinoid signaling and *Hox* gene function in the developing hindbrain. *Hoxa1* also synergises with *Krox20* in a dose dependent manner in patterning r3 as evidence by *Hoxa1/Krox20* double null mutations (128). These results are consistent with *Hoxa1* functioning as a homeotic selector gene that is not only required for hindbrain segmentation, but also confers identity to r4 and r5. It also suggests that *Krox20* and *Hoxa1* may interact to distinguish odd-numbered rhombomeres from even-numbered rhombomeres and are required together to precisely define the r3 territory.

In contrast to the *Hoxa1* mutants, *Hoxb1* null mutants do not display any gross morphological defects in hindbrain segmentation. Although r4 patterning and development is initiated, r4 identity fails to be maintained as upregulation of r4-specific markers, such as *wnt8c* and *CRABP1*, is compromised. Interestingly, *EphA4*, an r2-specifc marker, is abnormally expressed in the presumptive r4 territory of *Hoxb1-/-* hindbrains, suggesting r4 is transformed into an anterior r2-like segment phenotype (129). Consequently, the facial brachiomotor and contralateral vestibular acoustic neurons which arise in r4 are incorrectly specified and fail to migrate to their correct efferent positions, eventually leading to their loss (129,130). Thus in addition to conferring segment identity to r4, *Hoxb1* may be required for proper neuronal migration. Evidence supporting this comes from the demonstration that the ectopic activation of *Hoxb1* within the r2 territory of avian embryos transforms it to an r4-like character (131).

Extensive functional overlap and synergy between *Hoxa1* and *Hoxb1* during hindbrain development has been demonstrated through the generation of double *Hoxa1/Hoxb1* null muants (69,132,133). Although a presumptive r4 territory forms in *Hoxa1/Hoxb1* double null mutants, *EphA2*, the earliest r4 marker fails to be induced which is indicative of improper segment specification. Consequently, the organization of brachiomotor neurons VII to XI is grossly perturbed (133).

Hoxa2 null mutants do not exhibit any severe hindbrain segmentation abnormalities (134,135). They do, however, display alterations in the anteroposterior and dorsoventral patterning of neuronal derivatives. These changes in the anterior hindbrain are consistent with a transformation of r2 to an r4-like character (136,137). This is evident by the loss of r2-specific gene expression and a reduction in r2 and r3 territories accompanied by a concomitant expansion of r1. Consequently, trigeminal nerve axons exit more caudally in these mutants; i.e. from r4 instead of normally exiting from r2 (136). *Hoxa2* null mutants also display alterations in motor neuron projections from r2 and r3, suggesting that the dorsoventral patterning of the anterior hindbrain is affected. Thus, in addition to specifying r2/3 segment identity in the anterior hindbrain, *Hoxa2* also plays a role in dorsoventral patterning of the neural tube.

The generation of *Hoxa1/Hoxa2* double mutants further illustrates the importance of *Hox* gene cross-regulatory interactions in hindbrain segmentation and patterning (138). As with *Hoxa1* single null mutants, *Hoxa1/Hoxa2* double mutants display a failure to maintain the anterior *Hoxb1* expression limit at the r3/r4 boundary, resulting in aberrant hindbrain specification from r2 to r5. These defects are generally more severe in the *Hoxa1/Hoxa2* double mutants relative to *Hoxa1-/-* embryos, and demonstrate the importance of cross-regulatory interactions in setting *Hoxb1* expression levels up to the r3/r4 boundary.

Similar to *Hoxa2*, *Hoxb2* null mutants do not exhibit any hindbrain segmentation defects. However, the maintenance of r4 identity is severely compromised which is consistent with *Hoxb2* being a direct target of *Hoxb1* in r4 (137,139). In the absence of *Hoxb2*, the r4 territory is transformed into an r2 like identity as evidenced by alterations to several dorsoventral neural tube markers, such as *Mash1*, *Math3*, *Nkx2.2*, and *Phox2b* (137). *Hoxb2-/-* embryos also display defective development of the facial motor nucleus exiting from r4, as well as a reduction in migrating r4 brachiomotor neurons. Therefore similar to *Hoxa2*, *Hoxb*2 is also required for both the anteroposterior and dorsoventral patterning of neuronal derivatives.

The combined loss of both *Hoxa2* and *Hoxb2* leads to exacerbated dorsoventral patterning defects in r2 and r3 in comparison to single null mutations (137). *Hoxa2* and *Hoxb2* synergize in establishing rhombomere boundaries in the anterior hindbrain, since these are missing between r1-r4 in the double mutants. The combined *Hoxa2*/*Hoxb2* mutations revealed a previously unsuspected role for *Hox* genes in dorsoventral patterning as evidenced by the absence of *Pax6*-positive ventral interneurons in r3. These results demonstrate that *Hox* genes can couple anteroposterior specification of the hindbrain with the dorsoventral generation or birth of neurons.

Neuronal defects in *Hoxa3* null mutants involve the IXth cranial nerve and range from a loss of the glossopharyngeal branch of the IXth ganglion to a fusion between the IXth and Xth ganglia. Similar cranial ganglion defects are observed in *Hoxb3-/-* mutants, although less severe and at lower penetrance relative to *Hoxa3-/-* mutants (140,141). In contrast, *Hoxd3-/-* embryos do not show any defects in cranial ganglia formation, implying genetic redundancy with *Hoxa3* and *Hoxb3* (142,143). Indeed the generation of double null mutants (*Hoxa3/Hoxb3*, *Hoxa3/Hoxd3*, and *Hoxb3/Hoxd3*) demonstrates extensive functional overlap within this paralogous group in the patterning of the cranial ganglia (141). Both the penetrance and the severity of IXth cranial nerve patterning defects are increased in the double null mutants

Based on the co-linear expression of vertebrate homeobox genes in the developing hindbrain it was hypothesised that mammalian homologues would also function as homeotic selector genes. Collectively, these mutational analyses highlight the conserved role of *Hox* genes in hindbrain patterning and segmentation and demonstrate the remarkable patterning properties *Hox* genes have evolved in order to sculpt the unique characteristics of vertebrate head.

11. RHOMBOMERE SEGMENTATION MECHANISM

Segmentation and the formation of compartments is an integral component of embryonic brain development and is essential for generating cell lineage restrictions and discrete domains of gene expression. The hindbrain is composed of seven transient rhombomeres and cell transplantation experiments have demonstrated that cells within one hindbrain compartment are generally unable to mix with those from adjacent rhombomeres (144,145).

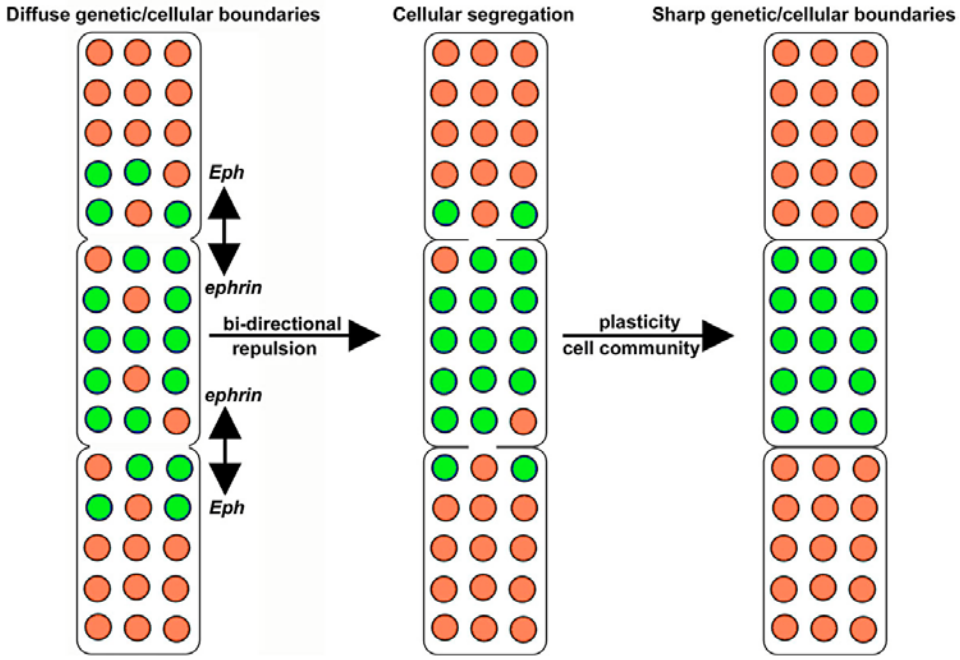

Figure 5. Mechanism for hindbrain segmentation. The formation of hindbrain rhombomeres is characterised by the generation of morphological boundaries and sharp restricted domains of gene expression. This occurs in vertebrates in a two step process. Initially, hindbrain domains of gene expression are diffuse. Firstly, bi-directional repulsive signaling mediated via the Eph/ephrin gene families leads to the sorting of cells in the hindbrain based on their gene expression characteristics. Individual cells caught on the wrong side as the morphological boundaries are forming, exhibit plasticity by responding to the signals from the cell community surrounding them and altering their gene expression. By doing so, restricted domains of gene expression sharpen concomitantly with rhombomere boundary formation.

Compartment-specific cellular restriction constitutes the basis of boundary formation in the hindbrain and it has been hypothesized that a hierarchy of cell adhesion molecules facilitates cell segregation (146). During early hindbrain development, gene expression boundaries are generally diffuse, however these expression domains refine and sharpen concomitantly with the generation of rhombomere boundaries (Figure 5). Therefore the establishment and maintenance of segment identity in the developing hindbrain, both morphologically and genetically is dependent upon the restriction of intermingling between rhombomeres.

The *Eph* tyrosine kinase receptors and their membrane bound *ephrin* ligands are intimately involved in mediating the hindbrain segmentation process (147,148). The *Eph* receptors can be subdivided into two general classes such that *EphA* receptors only interact with glycosyl phosphatidylinositol (GPI) linked *ephrinA* ligands while *EphB* receptors only interact with transmembrane bound *ephrinB* ligands (149). The *Eph* receptors and their *ephrin* ligands are generally expressed in alternating segments which is consistent with a role in defining boundary interfaces within the hindbrain (Figure 3). With the exception of *EphA2*, which is expressed in r4, hindbrain expression of the *Eph* receptors is restricted to odd-numbered rhombomeres. In contrast, *ephrin* transcripts are usually found within even-numbered segments. The mosaic activation of *ephrinB2* in the hindbrain results in the sorting of cells to the boundaries of even rhombomeres.

Conversely the mosaic activation of *EphA4* in rhombomeres 3 and 5 of the hindbrain leads to the sorting of cells to the boundaries of the odd rhombomeres. These experiments clearly demonstrate that *Eph* receptors and their *ephrin* ligands are required for the segmental restriction of cell intermingling during hindbrain development (150,151). The demonstration that activation of either *Eph* receptors or *ephrin* ligands alters cell sorting properties, indicates that bi-directional signaling at rhombomere boundaries restricts cell intermingling between adjacent segments (147).

The generation of morphological boundaries by mutual cell repulsion within the hindbrain correlates with the refinement and sharpening of *Hox* gene expression domains. Classically, it has been hypothesised that a direct correlation also exists between restricted domains of *Hox* gene expression in the hindbrain and the commitment to rhombomere specific fates. This idea is supported by numerous gain and loss of function analyses in several vertebrates. For example, in the *Hoxb1* mutants, r4 is transformed into an r2 like character (129). Furthermore, rhombomeres transplantated to ectopic locations within the avian hindbrain generally display molecular and cellular autonomy (152). In contrast however, grafts of avian neural plate stage tissue can alter their fate and acquire the *Hox* identity and neuroanatomy characteristic of their new location (153). This implies that during the process of hindbrain segmentation, the progression towards rhombomere autonomy occurs concomitantly with the

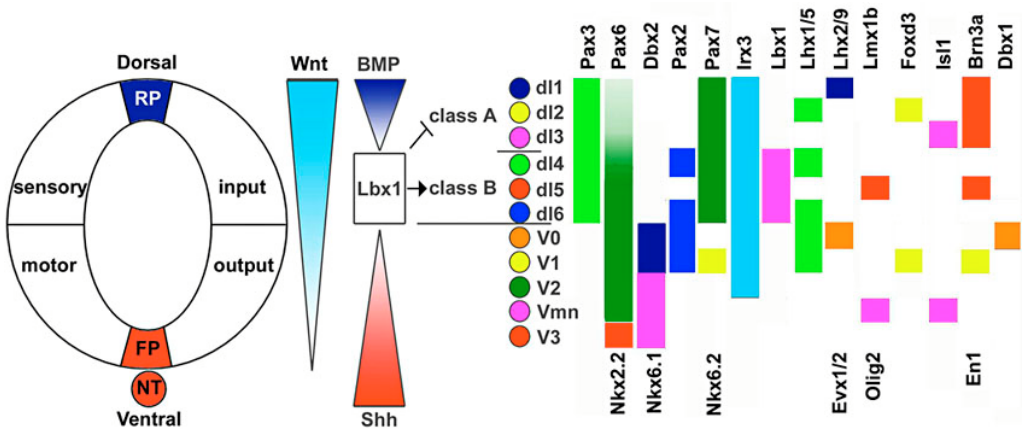

Figure 6. Specification of dorso-ventral neuronal cell fate in the spinal cord. Schematic diagram showing the sensory and motor subdivision of the spinal cord and the primary expression patterns of a number of genes which determine the progenitor domains and direct the differentiation of neuronal subtype. There are 6 classes of dorsal neurons (d1-d6) that form in response to the influence of Wnt and BMP signaling gradients and which can be subdivided into class A and class B neurons based on their response to *Lbx*. In contrast there are 5 classes of neurons that form in the ventral neural tube (V0-V3 and Vmn) in response to a gradient of Shh. Expression of the homeodomain factors that specify a distinct ventral progenitor domain is modulated by Shh signaling. Class 1 genes such as *Pax7, Dbx1, Dbx2, Irx3 and Pax6* are repressed by Shh, whereas genes including *Nkx2.2* and *Nkx6.1* are activated in response to Shh signaling. Furthermore cross-repressive interactions by homeodomain factors (ie between *Pax6* and *NKx2.2* and also between *Dbx2* and *Nkx6.1* refine and maintain progenitor domains and direct neuronal subtypes during cellular differentiation. Neuronal subtype in the spinal cord therefore can be distinguished by the combinatorial expression of numerous homeodomain transcription factors which determines the progenitor domain in which they form and then their post-mitotic specificity.

restrictions in cell mixing and the refinement of *Hox* gene expression domains (Figure 5).

Recent analyses involving transplantations of small sub-rhombomeric and single cell populations in mouse (154) and zebrafish (155) embryos have uncovered the mechanistic link between the restrictions in cell movement and the sharpening of gene expression domains which generate segment identity. When genetically marked cells isolated from rhombomeres 3, 4 or 5 were heteroptically transplanted into rhombomere 2, the majority of the transplanted cells remained as a tight cohort in their new location and autonomously maintained their original anterior-posterior *Hox* identity (154). Surprisingly, a small number of cells, which dispersed from the graft, exhibited clear cellular plasticity by downregulating inappropriate *Hox* gene expression in their new location. In zebrafish embryos, transplanted single cells were observed to upregulate *Hox* genes appropriate for their new location (155). These studies highlight the inherent plasticity within individual rhombomeric cells with respect to *Hox* gene expression and cell fate (156). The observation that rhombomeric autonomy is maintained in cells that stick together but not in cells that intermingle with surrounding populations indicates that the reinforcement of regional identity depends upon cell-community effects. This also has important implications for the mechanism that generates the sharp segmental rhombomere boundaries of *Hox* gene expression during normal hindbrain development.

The current mechanistic model for generating hindbrain segments and establishing restricted *Hox* gene expression domains is believed to be a two-step process (Figure 5) (154,156). Firstly, *Eph* receptors and their *ephrin* ligands mediate repulsive signaling that establishes the distinct rhombomeric territories as units of cell lineage restriction. Secondly, plasticity at the level of the individual hindbrain cells allows cells caught on the wrong side of the border during rhombomere boundary formation to alter their identity and fate. Collectively, the two processes of cell repulsion and plasticity provide a mechanism for the progressive generation of precise rhombomeric domains of gene expression in the hindbrain. The morphological and molecular segmentaion of the hindbrain subsequently functions to maintain the appropriate anterior-posterior register between the neural tube, sensory ganglia and cranial motor nerves, which underscores the functional importance of the blueprint provided by the hindbrain during central and peripheral nervous system development.

12. SPECIFICATION OF THE NEURONAL POPULATIONS WITHIN THE SPINAL CORD

The proper wiring and hence function of the mature spinal cord requires the correct establishment of regional neuronal identities during embryogenesis. The development of the vertebrate central nervous system is the consequence of a complex genetic programme that begins with the establishment of specific neuron types in defined positions along the dorsal-ventral axis of the neural tube (Figure 6). The mature spinal cord in vertebrates is characterised by two prominent structures, the ventral and dorsal horns, which are populated by neurons that respectively process motor and sensory information. The neurons that integrate proprioceptive input and motor output are located in the ventral half of the spinal cord, while neurons that relay cutaneous sensory information to

the brain are confined to the dorsal half of the spinal cord (157). Classical birthdating analyses of the CNS have demonstrated that there is a ventral to dorsal gradient of histogenesis in the neural tube or presumptive adult spinal cord (158) and the organisation of cell types in the spinal cord along the dorso-ventral axis is a fundamental feature that reflects the major flow of signal information and processing from sensory input to motor output. This ventral to dorsal gradient of differentiation and dorsal to ventral gradient of proliferation appears to correlate with a gradient of mitogenic Wnt activity (26). Wnt signaling can act via a *B-catenin/TCF* pathway to positively regulate cell cycle progression and negatively regulate cell cycle exit of spinal neural precursors, in part through transcriptional regulation of *cyclinD1* and *cylcinD2* (159). *Wnt1* and *Wnt3a* are produced in the dorsal midline and they can promote proliferation whilst inhibiting the differentiation of neural precursors by directly impinging upon the cell cycle. Hence it has been suggested that a Wnt signaling gradient regulates the dorso-ventral growth of the developing spinal cord (Figure 6) (26,159).

The neurons of the spinal cord are distinct from each other both functionally and anatomically. Numerous studies suggest that the identity of individual neural cells is determined by two independent signaling systems that intersect along the dorsoventral and rostrocaudal axes of the neural tube, creating a grid of positional cues. The positional identity of cells within this grid of signals determines the type and concentration of inductive signals to which they are exposed, ultimately specifying individual cell fate and regional development (23,160,161). Distinct neuronal cell types are derived from a specific group of progenitor cells in restricted domains at defined positions along the dorsoventral axis of the neural tube and can be identified by subtype specific gene expression (Figure 6) (162). Ventrally, floor plate cells form at the midline, with motor neurons and three type of interneurons (V0-V3) forming progressively more dorsally. Conversely, roof plate cells form at the dorsal midline of the neural tube, and six classes of interneurons (dl1-dl6) differentiate progressively ventrally. The notochord and floor plate are required for ventralizing the neural tube, while dorsal specification involves dorsal tissues such as the roof plate and flanking ectoderm. Each of these tissues expresses multiple sets of regulatory genes that are required for proper dorsal-ventral patterning.

13. SPECIFICATION OF VENTRAL NEURONS

Ventralization of the spinal cord and development of the five ventral cell types occurs in response to the production of *Shh* and the BMP antagonists *chordin* and *noggin* by the notochord (163). *Shh* ligand is bound by *Ptc1* (*Patched*), a 12 pass transmembrane receptor. In the absence of *Shh* ligand, *Ptc1* inhibits the activity of *Smo* (*Smoothened*) a seven-pass membrane protein, which blocks transduction of the active signal. Activation or repression of *Shh* targets is mediated in vertebrates by *Gli1*, *Gli2* and *Gli3,* each of which has distinct transcriptional properties and functions (164). *Shh* is initially expressed in the notocord and then later in the floor plate which in turn generates a secreted gradient of *Shh* signaling necessary for neural differentiation (Figure 3). The presence of a graded *Shh* signal is key to the specification of distinct neuron classes in the ventral spinal cord and the cholesterol modification of *Shh* has been shown to be critical in facilitating the diffusion of *Shh* to create such a long-range signal (165,166).

Neurons that differentiate in more ventral regions of the neural tube, including floor plate cells and motor neurons, require higher concentrations of Shh for their induction, while lower Shh concentrations induce the more dorsally located interneurons V0-V3 (167,168). It is evident that Shh secretion from the notochord initially, and later from the floor plate cells, creates a long-range ventral to dorsal gradient of signaling activity which directs neuronal cell fate (Figure 6). Both *Shh* and *Ptc1* are expressed in a ventral to dorsal gradient along the neural tube. Although *Shh* expression is restricted to the ventral-most populations of the neural tube, *Ptc1* extends more dorsally, suggesting *Ptc1* mediates the function of *Shh* as a long-range morphogen (169,170). The ectopic expression of *Shh* results in the ectopic formation of floor plate cells, motor neurons and interneurons (171-173). When *Shh* signaling is blocked using antibodies or by gene targeting in mice, or following notochord ablations in chick, differentiation of the three most ventral cell types does not occur (171,173,174). V0 and V1 neurons still form when *Shh* expression is blocked, which indicates that although the more dorsal interneurons can be induced by Shh, they may not be dependent on Shh expression for induction. It has been suggested that a Shh-independent pathway may be sufficient for their induction (175) but alternatively and perhaps more likely it seems that genetic redundancy is involved and that induction is achieved via low level expression of *Ihh* secreted by the aortic endothelium (176).

Shh signaling establishes distinct progenitor domains by regulating the spatial domains of expression of various homeodomain proteins that comprise members of the *Pax*, *Nkx*, *Dbx* and *Irx* families (Figure 3) (167,175,177-179). Two specific classes of homeodomain proteins – Class I and Class II proteins – have been identified based on their regulation by *Shh* (178). Class I proteins (*Pax7*, *Pax3*, *Dbx1*, *Dbx2*, *Irx3* and *Pax6*) are expressed by neural progenitor cells in the absence of Shh signaling, and their expression is repressed by Shh at specific concentrations (168,174,175,178). Conversely, expression of Class II proteins (*Nkx6.1*, *Nkx6.2*, *Olig2*, *Nkx2.2* and *Nkx2.9*) is enhanced by specific concentrations of Shh (167,177-180). At a common neural progenitor domain boundary where two pairs of homeodomain proteins meet each other due to their expression at a given Shh concentration, such as in the case of *Pax6* and *Nkx2.2* as well as *Dbx2* and *Nkx6.1,* the pairs repress the expression of each other (167,178,181). Thus, specific homeodomain proteins are activated or inhibited at each concentration of Shh, and it is the resulting pattern of these homeodomain proteins that specifies the identity of the neuron classes (Figure 6). These domains of homeodomain protein expression can be ordered in such a pattern that specifies each specific class of motor neuron and interneuron. In agreement with this model, predictable

changes in neural progenitor domains and neural identity occur when homeodomain protein expression is extinguished by gene targeting (167,177,181,182).

Multiple lines of evidence suggest that Shh acts as a long-range morphogen, with direct effects on distant cells and the mechanism by which homeodomain proteins are activated or repressed by Shh signaling is finally beginning to be clarified. First, extracellular Shh is detectable throughout the ventral neural tube (173) and Shh has been visualized outside of Shh-producing cells (183). Secondly, both ectopic expression of a constitutively-active form of *Smo* (184) and expression of mutant *Ptc*, which does not bind *Shh* but inhibits *Smo* (185), results in a switch in progenitor cell fate, which is consistent with a direct effect of *Shh*. This is supported by a similar phenotype observed in a more recent study of *Smo* null mutants which genetically prevents all *Hh* responsiveness (176). These results demonstrate the existence of a direct long-range requirement for *Hh* in patterning the neural tube and that specification of all ventral progenitors requires *Hh* signaling.

Analysis of *Smo/Gli3* double mutants provides further insights into the neural patterning process. In contrast to *Smo* null mutants which are thought not to generate any ventral progenitors, the *Smo/Gli3* double mutants restore 4 ventral domains (V0, V1, V2, Vmn) but not V3 or floor plate fates (176). Whereas V0 precursors are appropriately positioned, V1, V2 and Vmn precursors in the double mutants now overlap and extend over nearly all the ventral half of the neural tube, suggesting a direct Hh signaling input into the stratification of progenitor populations into separate domains within the neural tube. One of the central roles therefore of Gli3-Shh signaling is to refine the position and size of ventral progenitor pools rather than specifying individual progenitor identity and it has been speculated that Shh may regulate the separation of progenitor cell boundaries along the dorso-ventral axis on the basis of controlling cell affinity. *Gli3* is believed to play no essential role in patterning ventral regions of the spinal cord (186-188), however, *Gli3* repression may in fact regulate the position of dorsal progenitors that reside close to the dorso-ventral boundary (189). *Gli2* is required for floor plate induction, normal numbers of V3 neurons and activation of *Gli1* (186,190). The phenotype of *Smo/Gli3* double mutants therefore is consistent with Shh activation of *Gli2* in the specification of the floor plate and an activator role for *Gli3* in the normal induction of V3 neurons. *Gli3* transcriptional repressor activity is important for patterning the intermediate region of the spinal cord which compliments the requirement for *Gli2* in more ventral regions.

These findings are all consistent with a model in which Gli proteins act as common mediators of spinal cord dorsal-ventral patterning, integrating Shh signals and other sources of positional information throughout the ventral neural tube (189). However these results could also argue for a patterning mechanism independent or parallel to graded Shh signaling. Many other regulators of Shh signaling have been identified, and these may be involved in mediating Shh signaling in a *Gli*-independent manner. The effects of Shh signaling do not merely reflect the concentration levels of Shh protein present in a given region, but also reflect the activity of accessory proteins that modulate Shh signaling. BMP signaling, for example, appears to modulate the response of ventral neural progenitors to levels of Shh. At a given concentration of Shh, the presence of increasing levels of BMP results in a shift from ventral to more dorsal cell types, while the inhibition of BMP signaling through BMP antagonists or dominant negative effects results in ventral transformation of progenitor cells and neuronal fates (191). Several BMP inhibitors are expressed by cells in or around the ventral neural tube, and seem to be restricted to the ventral region (192-194). These antagonists may regulate the level of endogenous BMP exposure within the ventral neural tube, suggesting that specification of ventral neuronal cell types depends on a balance between Shh and BMP signaling. Factors other than BMPs may also regulate neural cell response to Shh activity, but these pathways remain to be defined. Shh signaling has been shown to induce the expression of Hedgehog-interacting protein (Hip), a surface membrane protein which is able to bind Shh and attenuate its signaling, as well as vitronectin, an extracellular matrix glycoprotein that binds Shh and potentiates its activity (195-197). These and other proteins may play a role in the interpretation of Shh signaling for neural patterning and are currently under investigation. Taken together with *Shh* control of brain vesicle growth, these studies demonstrate that *Shh* is intimately involved in the fine regulation and balance of both cell proliferation and differentiation. It will be important in the future to dissect any common or parallel genetic cascades controlling these processes.

14. SPECIFICATION OF DORSAL NEURONS

In the dorsal neural tube, signals necessary for neuronal induction are generated initially by the epidermal ectoderm and are later propagated by the roof plate. The primary mediators of dorsal neural fate are the highly conserved members of the *TGF-b* family, with multiple studies demonstrating a key role for BMPs in the control of dorsal neural cell identity. In the chick, *Bmp4* and *Bmp7* are expressed in the epidermal ectoderm at the time of dorsal differentiation, and ectopic administration of each can induce roof plate cells in neural plate explants (198,199). Alternatively, treatment with the BMP antagonists *follistatin* and *noggin* inhibits the ability of the epidermal ectoderm to induce roof plate cells and neural crest (199,200). Thus, secretion of BMP signals by the epidermal ectoderm appears to be sufficient and necessary for the induction of dorsal midline cells.

BMP signaling also plays an important role in the differentiation of dorsal interneuron specification. Interneurons of the dorsal horn are generated late in development, after the closure of the neural tube, at a time when the epidermal ectoderm is no longer in contact with the neural tube and when BMP expression in the ectoderm has been downregulated (198,201). There are now at least 6 known distinct classes of dorsal interneuron populations (dl1-dl6) that can be defined by unique and overlapping

expression domains of various transcription factors (Figure 6) (202,203). Notably the expression of *Lbx1* subdivides these populations into a dorsal non-*Lbx1* expressing group (class A: dl1-dl3) and a ventral *Lbx1* expressing group (class B: dl4-dl6). In *Lbx1* null mutants, ventral class B interneurons adopt the molecular and projection characteristics of dorsal class A cells. Misexpression studies show that *Lbx1* can suppress dl1-dl3 interneuron fates. Therefore *Lbx1* suppresses class A differentiation programmes while promoting class B dorsal interneuron fates (204). Progenitors in the ventral portion of the alar plate (class B: dl4-dl6) are not thought to be specified by dorsal midline signals (205). However multiple studies have demonstrated that signals secreted by the roof plate are sufficient for promoting dorsal interneuron (dl1-dl3) differentiation. When the roof plate is ablated, the neural tube loses its dorsal identity and becomes ventralised, correlating with an absence of dorsal neural progenitors and an expansion of more ventral interneuron classes (199). Not surprisingly, the roof plate expresses many members of the *TGF-b* family, including *Bmp4*, *Bmp5*, *Bmp7*, *Dsl1*, *Gdf6/7*, and *activin-b* (199,200). These dorsal signals establish progenitor domains that express the bHLH factor genes *Math1*, *ngn1/2* and *Mash1*. Progenitors specified by dorsal signals give rise to the three dorsal neuronal subtypes designated dl1-dl3, which are marked by *Lh2a/b*, *Lim1/2/Brn3a* and *Isl1/2* respectively (Figure 3) (206,207). Treatment of chick neural plate tissue with exogenous BMP4, BMP5, BMP7, DSL1, or GDF6/7 induces dl1 interneurons, while treatment with activin induces dl3 interneurons (199). Similarly, treatment with BMP antagonists *noggin* and *follistatin* inhibits the induction of dl1 and dl3 interneurons by the roof plate (199). Therefore dorsal midline signals, primarily of the *TGF*-b family, specify dorsal cell identity in the neural tube.

Recent evidence suggests that Wnt signaling may also play a role in dorsal interneuron specification (208). The absence of *Wnt1* and *Wnt3a* leads to diminished development of dl1 and dl3 neurons. Furthermore, *Wnt3a* expression is able to induce expression of dl1 and dl3 markers in chick neural plate explants, and noggin does not interfere with this induction, suggesting a role for Wnt signaling that is distinct from BMP signaling.

New factors involved in dorso-ventral patterning of the neural tube continue to be uncovered. Recently a novel molecule, *Tiarin*, which regulates patterning of the anterior-dorsal CNS, has been described (209). This secreted protein, which is expressed by the nonneural ectoderm surrounding the neural plate, appears to act independently of the known dorsal signals such as BMPs and Wnts. Tiarin induces expression of dorsal CNS markers and suppresses expression of ventral markers in vivo, and appears to antagonize the ventralising activity of *Shh*. While recent studies define the role of *Tiarin* in the anterior CNS, it has also been demonstrated that *Tiarin* is capable of promoting dorsal differentiation in the trunk. Thus, this new signaling molecule may have an expanded role in dorsal CNS patterning.

Most factors involved in the development of the spinal cord seem to be segregated in their ability to influence either dorsal or ventral cells. Shh signaling appears to be the primary driver of ventral patterning and BMP and Wnt signaling are the key players regulating dorsal patterning. The enormous number of intricate connections that are necessary for proper functioning of the central nervous system requires the extremely precise integration of spinal cord growth with neuronal cell fate specification. The challenge of the future will be to uncover the mechanisms that are responsible for this precise integration or coupling of the growth and specification processes. One could imagine a scenario in which the Wnt mitogen gradient would regulate the rates of neuronal proliferation and differentiation in conjunction with the opposing gradients of BMP and Shh signaling to appropriately pattern cell type identities in the central nervous system.

15. CONCLUSIONS

The central nervous system develops with incredible precision during embryonic development, and it is the orchestration of cell fate determination, cell proliferation, and cell growth that creates a properly patterned, functional organ. Control of CNS patterning involves the interplay of multiple signaling pathways that interact to mediate the transcriptional events that ultimately specify cell fate. Interestingly, it is being found that a small set of signaling molecules can specify a diverse number of cell types, based on differences in signal concentration or differences in spatial and temporal expression. In recent years, considerable progress has been made in defining the signaling systems that control specification of the CNS during development, but a number of questions still remain. It will be essential in the future to be able to precisely distinguish between the function of BMPs, Shh, FGFs, RA, and Wnts given the extraordinary roles these signaling mechanisms in numerous overlapping patterning events. Determining how these signals that control cell fate interact with those that control cell growth, as well as defining the precise mechanisms by which individual signals act, will help to further our understanding of vertebrate CNS development.

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