The role of Notch signalling in mid-hindbrain bounda formation and maintenance

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Declaration

I, Kyoko Hashimoto, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicate in this thesis.

Kyoko Hashimoto

Abstract

During embryonic development, organiser centres form at compartment boundaries and provide a source of graded signals that instruct cells to specific identities in a concentration-dependent manner. The mid-hindbrain boundary (Geling et al., 2004) is an organiser that arises in the developing neural tube at the interface between midbrain and hindbrain, and is crucial for the formation of tectum and cerebellum.

Local organisers at boundaries have been best studied in invertebrates (Ahmed et al., 2003) where the Notch signalling pathway is important. Recent studies suggest that this signalling cascade may also control boundary and organiser formation in vertebrates. In this thesis I have tested the hypothesis that Notch signalling is important during MHB development.

I have found that genes involved in the Notch signalling pathway are specifically expressed in domains within the chick neural tube that demarcate the MHB from early somite stages, implicating Notch signalling in the establishment of this organiser.

I examined the effect on cells of experimentally altering Notch activity levels. Activation of the Notch pathway regulates cell affinity properties, as cells containing activated Notch are excluded from rhombomeres 1 and 2. I tested the hypothesis that *LFng* acts as a switch to activate Notch only on the midbrain side of the boundary. Perturbation of *LFng* expression leads to disruption of the boundary and cell lineage restriction is lost.

Ectopic activation of the Notch signalling pathway perturbs expression of key MHB organiser genes, *Fgf8* and *Wnt1*. In contrast, in the absence of Notch signalling, the MHB fails to form properly. I propose that Notch signalling through the ligand *Serrate1* is sufficient for the generation of boundaries in this region of the CNS.

Together, these data suggest a role for Notch signalling both in the formation of the MHB, and also in the regulation of cell affinity differences necessary to stabilise and maintain the organiser.

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Chapter 1 Introduction: MHB formation and the Notch signalling pathway

1-1 Development and segmentation of the CNS

1-1-1 Segmentation during embryonic development

The establishment of cell lineage compartment boundaries is seen to be mandatory during embryonic processes. In the 1970s, it was demonstrated that both the abdomen and wing disc of insects are segregated into cellular compartments, and that cells do not cross these boundaries (Garcia-Bellido et al., 1973; Lawrence, 1973; Morata and Lawrence, 1975): reviewed (Kiecker and Lumsden, 2005). By merit of this cell lineage restriction, the cellular compartments prevent cells from intermingling allowing them to accept different fates. At the same time, the cellular compartments provide positional information to maintain the cell population. Therefore, together with growth and patterning, the formation of the cell lineage restricted boundaries is essential for correct body development within the developing embryo.

1-1-2 Embryonic CNS development

The gastrulation process gives rise to three germ layers, ectoderm, mesoderm and endoderm (Pander, 1817). The central nervous system (CNS) develops from neural plate. As development proceeds, the ectoderm becomes thickened at the midline of the embryonic disc and forms a layer of tissue called neural plate. Cells at the lateral margins of the neural plate grow and accumulate to form neural folds. As the embryo develops, the neural folds extend to meet each other across the midline, forming a neural tube along anteroposterior axis. Following closure of the neural tube, or neurulation, neural crest cell migration takes place, and the neural tube separates from the surface ectoderm and lies beneath it (Smith, 2001; Wurst and Bally-Cuif, 2001). Importantly, the immature neural tube consists of a single layer, which only later differentiates into three layers with separated fates; ependymal layer, mantle layer and marginal layer. As the CNS develops further, dorsal and ventral regions of neural tube form roof plate and floor plate. At the same time, neural crest cells are migrating adjacent to neural tube (Smith, 2001; Wurst and Bally-Cuif, 2001). At this time, spinal cord is also formed. Neural tube at this point is expanding from the cranial end, and this

expansion divides CNS to three vesicles anteroposteriorly; forebrain (FB), midbrain (MB), hindbrain (HB), spinal cord (Kiecker and Lumsden, 2005; Lumsden, 2004).

1-1-3 CNS subdivision in the early development

Transient segmentation is a crucial event both cellularly and molecularly that determines the early specification of the developing CNS. The developing neural tube becomes subdivided into repeated bulges or neuromeres, which are separated from one another by transverse constrictions (Lumsden, 2004; Lumsden and Guthrie, 1991). As early as the neural plate stage, the developing CNS prepares to divide into several domains longitudinally and anteroposteriorly. Longitudinally, the neural tube is divided symmetrically into four domains: the floor plate, the basal plate, the alar plate and roof plate. These domains are characterised by the distinct combinations of genes expressed, genes including secreted factors *Sonic hedgehog (Shh)*, *Noggin*, *Wnt1*. Meanwhile, the neural tube elongates along the anteroposterior axis, and is subdivided successively into smaller and more complex regions. Various subsets of neural cell types differentiate within these specialised domains (Kiecker and Lumsden, 2005). Further development gives rise to telencephalon and diencephalon from FB, metencephalon and myelencephalon from HB. Importantly, only the MB remains undivided while FB and HB subdivide in multiple neuromeres.

There have been several perspectives on FB subdivision. In 1994, Rubeistein et al. proposed that the FB is divided into six prosomeres, three from diencephalon (P1-3) and three from secondary prosencephalon (P4-6) (Rubenstein et al., 1994). Figdor and Stern, however, proposed that the diencephalon is subdivided into four neuromeres (D1-4) based on morphological analysis, axonal architecture, lineage restriction and gene expression experiments (Figdor and Stern, 1993). More recently, fate mapping and further cell lineage analysis suggest that there are only three boundaries in the FB: pallial-subpallial boundary (PSB) in the telencephalon, diencephalon-midbrain boundary (DMB) and ZLI in the thalamus (Larsen et al., 2001).

The HB divides into seven rhombomeres, and these compartments are well-defined segments (Cambronero and Puelles, 2000; Lumsden and Keynes, 1989). Fraser and his colleagues demonstrated that all seven rhombomeres have cell lineage restriction (Fraser et al., 1990). Every segmental boundary is also a boundary of specific gene

expression. Neurogenesis, axonal projection and proliferation are found to be restricted in individual rhombomeres (Heyman et al., 1993; Kiecker and Lumsden, 2005). Notably rhombomere 1 (r1) gives rise to the entirety of the cerebellum (Millet et al., 1996; Wingate and Hatten, 1999). Recently Zervas and his colleagues demonstrated that there is a clear cell lineage restriction between MB and r1 by using detailed genetic fate mapping and by tracking cell behaviours (Zervas et al., 2004).

1-2 The role of organisers in CNS patterning

Each of the compartments arising within the developing CNS displays a distinct character and organised neural cell lineage. This regionalisation of the developing CNS is created by signals which regulate restricted gene expression patterns. The gene expressions in turn specify the regional identities of each compartment. In the developing CNS, there are several regions which have been identified as secondary signalling centres or local organisers orchestrating the formation of these compartments.

1-2-1 A signalling centre: organiser

An organiser is a signalling centre which instructs cells to specific identities and controls the growth and development of the neighbouring cells (Bouwmeester, 2001; Harland and Gerhart, 1997). In 1924, Spemann and Mangold carried out the explant assay of the dorsal lip of donor frog to ventral side of host frog embryo in order to understand the cell and tissue fate (Spemann and Mangold, 2001). This experiment resulted in the formation of a secondary axis which consisted of cells from both donor and host. The donor tissue induced neuralisation by changing the ectodermal cells of the host to form neural tissues, and dorsalisation by causing ventral mesoderm to dorsalise. This was the first report to demonstrate that cells, when instructed by other specialised groups of cells, have the ability to adopt developmental fates dependent on their position in the embryo. A specific group of cells in the dorsal lip capable of providing this type of instruction were identified and called the organiser (Sander and Faessler, 2001; Spemann and Mangold, 2001).

The organiser was subsequently found to be conserved between species; Spemann's organiser in *Xenopus*, the shield in zebrafish, Hensen's node in chick, and the node in mouse. For example, when Hensen's node of donor quail is grafted to the inner part of

the extra-embryonic region (area opaca) of the host chick embryo, a new axis is formed alongside the host axis (Storey et al., 1992).

For decades, various studies were carried out to identify the molecular mechanisms which induce both neural and dorsal mesodermal fates (Kessler and Melton, 1994; Slack, 1994). The homeodomain protein *Goosecoid* (*gsc*) is an organiser-specific gene which has the capability of reproducing embryonic twinning when overexpressed (Blumberg et al., 1991; Cho et al., 1991). Following the discovery of *gsc*, several secreted factors such as *Noggin*, *Chordin* and *Follistatin* have been found to be released from the organiser, and are able to induce secondary embryonic axes (Harland and Gerhart, 1997; Smith and Harland, 1992). They mimic organiser activity by antagonising bone morphogenetic proteins (BMPs), which are members of the transforming growth factor β (TGF-β) superfamily (Harland and Gerhart, 1997; Lamb et al., 1993; Smith et al., 1993). The antagonists act to inhibit BMPs by silencing BMP proteins into inactive complex (Harland and Gerhart, 1997; Zimmerman et al., 1996). Thus, the central molecular mechanism underlying induction of the organiser is the inhibition of BMP signalling.

1-2-2 Secondary signalling centre: local organisers

During the development process, local signalling centres, or local organisers, have been reported to be required for the correct subdivision of the CNS. Dorsoventrally there are two signalling centres, the roof plate and the floor plate, while anteroposteriorly, the anterior neural ridge (ANR) at the anterior end of the neural plate, the zona limitans intrathalamica (ZLI) in the middle of the diencephalon and the mid-hindbrain boundary (MHB) are known to refine the anteroposterior specification of three main domains; FB, MB, HB (Gallera, 1971; Ruiz i Altaba, 1994; Storey et al., 1992) Reviewed (Echevarria et al., 2003). In the hindbrain, r4 and each of the boundaries between rhombomeres are also reported as signalling centres (Amoyel et al., 2005; Maves et al., 2002; Riley et al., 2004; Walshe et al., 2002).

Local organisers express secreted factors and transcription factors that lead to the patterning and further development of functional structures. The ZLI is one example of a local organiser in the CNS (Kiecker and Lumsden, 2004). The ZLI, which is situated between the ventral thalamus and the dorsal thalamus shows cell-lineage restriction

(Figdor and Stern, 1993; Larsen et al., 2001). Whole mount *in situ* hybridisation analysis showed that *Shh* expression is strongly expressed within the ZLI (Zeltser et al., 2001). *Shh* has been identified as a secreted signalling molecule which acts as a morphogen in a number of developmental systems (Currie and Ingham, 1996; Herzog et al., 2003; Lewis et al., 2001; Scholpp et al., 2006). In particular, *Shh* expression in both notochord and the floor plate is sufficient for motor neuron development in vertebrates (Cambronero and Puelles, 2000; Lewis and Eisen, 2001). In chick explant assays, ectopic *Shh* induced ectopic expression of motor neuron markers (Ericson et al., 1996; Marti et al., 1995; Roelink et al., 1994), whereas loss-of-function *Shh* mutant mice exhibit downregulation of *Islet1* expression, a marker of primary and secondary neurons (Chiang et al., 1996). Recently, Scholpp et al. demonstrated by genetic ablation of the basal plate in zebrafish that *Shh* in the ZLI alone is sufficient for diencephalic differentiation (Scholpp et al., 2006). This evidence strongly suggests that *Shh* plays a key morphogenetic role at the ZLI during establishment of this boundary.

1-2-3 The MHB, a local organiser

The MHB, another signalling centre, is the best studied local organiser in the developing CNS. The mesencephalon and cerebellum arise from the MB and HB under the influence of signals emanating from the MHB. Chick-quail chimera transplantation experiments initially identified the MHB as a region with organiser activity. When the MHB of donor quail is transplanted into the caudal FB of a host chick embryo, the host tissue is transformed into an ectopic MB or MHB (Marin and Puelles, 1994; Martinez et al., 1991). When the MHB of donor quail is transplanted into the HB of host chick, ectopic cerebellum is induced (Martinez et al., 1991). Furthermore, when MHB tissue of quail is transplanted into the diencephalic region in the host chick, the transplants induce a region of caudal MB character (Alvarado-Mallart, 1993). These experiments show not only that the MHB has organiser activity, but also the neural tube has an underlying competence to respond to the MHB signals depending on the axial level of the graft.

1-2-3-1 Gene expression at the MHB

Decades of studies on the MHB have demonstrated the complex genetic architecture of this boundary. The expression patterns of MHB marker genes showed three separate

phases for the MHB development. Several transcriptional factors (*Engrailed (En)*, *Pax*, *Otx*, *Gbx* families) and secreted factors (*Wnt*, *Fgf* families) were reported to be expressed within the MHB during the early development. Importantly, these molecules are highly conserved amongst species (Rhinn and Brand, 2001; Wurst and Bally-Cuif, 2001).

Of these factors, the ones expressed earliest in an MHB related manner are *Otx2* and *Gbx2*. *Otx2*, a homologue of *Drosophila orthodenticle* (*otd*), is initially expressed throughout the epiblast before gastrulation. However its expression becomes limited to the anterior of the embryo by the headfold stage (Simeone et al., 1993). *Gbx2*, a homologue of *Drosophila unplugged* (*unp*), is first detected during gastrulation and its expression extends anteriorly, complimentary to *Otx2* expression (Bouillet et al., 1995; Wassarman et al., 1997). Upto HH stage 10, the *Otx2/Gbx2* interface is reported to be indistinct (Hidalgo-Sanchez et al., 1999a).

Expression of *Wnt1*, a homologue of *Drosophila wingless (wg)*, appears in a broad domain at the prospective MB region at HH stage 7 in chick (Shamim et al., 1999). *En1* and *En2*, homologues of *Drosophila Engrailed*, are expressed in an inverted gradient across the MHB with a peak at the boundary. *En1* and *En2* gene expressions are upregulated at the mid-neural plate level, at HH stage 7-8, slightly later than *Wnt1* expression (Alvarado-Mallart et al., 1990; Joyner et al., 1985; Martinez et al., 1991; Shamim et al., 1999). A homologue of *Drosophila paired-box* gene, *Pax2* is first observed at the MHB at almost the same time as *En1* and *En2*, from posterior MB to r1 across the MHB (Shamim et al., 1999). In contrast, *Fgf8*, a member of Fibroblast Growth Factor (FGF) family, is expressed on the caudal side of the *Otx2/Gbx2* interface in r1 at HH stage 8, later than other MHB genes like *Pax2, En1 and En2* (Logan et al., 1996; Shamim et al., 1999).

By HH stage 10 in chick, the expression of *Otx2* and *Gbx2* abut, and the MHB is formed at the interface of these two genes (Figure 1.1 A). Expression of other transcription factors and secreted factors are observed by this stage at/around the *Otx2/Gbx2* interface. Importantly, detailed analysis of *Fgf8* expression using double *in situ* hybridisation, confirmed that the anteriormost border of *Fgf8* expression in the HH stage 10 chick sits level with the caudal limit of *Otx2* expression (Hidalgo-Sanchez et

al., 1999a). By HH stage 20, the expression of *Wnt1* and *Fgf8* abut and are found in narrow rings in the region of the MHB (Figure 1.1 B). Likewise, *En1/2* and *Pax2/5* maintain their expression patterns at the MHB, but are found in narrower regions by this stage.

Although these molecules are well conserved between species, there are slight differences in the temporal order of expression at the MHB. In mouse, for instance, expression of *Pax2* appears after *Otx2* and *Gbx2* (Rowitch and McMahon, 1995). *Wnt1* and *En1* expression follows, and *Fgf8*, *Pax5* expression is initiated immediately after that (Crossley and Martin, 1995; Rowitch and McMahon, 1995) reviewed (Gomez-Skarmeta and Modolell, 2002; Rhinn and Brand, 2001).

Figure 1.1 Schematic of the developing chick anterior CNS and gene expression patterns of the MHB organiser markers.

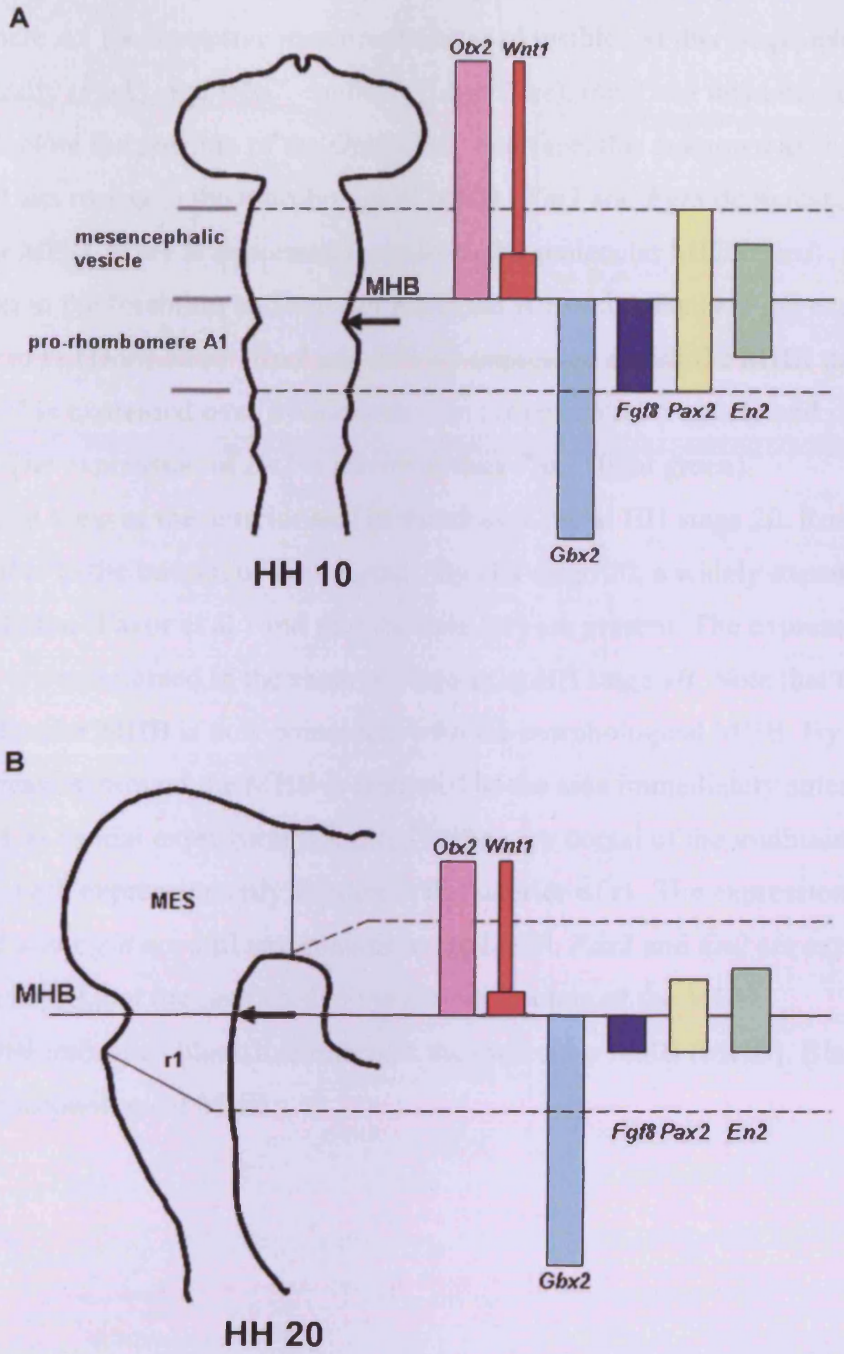


Figure 1.1 Schematic of the developing chick anterior CNS and gene expression patterns of the MHB organiser markers.

(A) A dorsal view of the anterior half of the chick CNS at HH stage 10. Anterior is to the top and posterior to the bottom. By HH stage 10, the mesencephalic vesicle and pro-rhombomere A1 (presumptive metencephalon) are visible. At this stage, expression of *Otx2* rostrally (*Pink*), and *Gbx2*, caudally (*Light blue*), meet and this interface positions the MHB. Note the position of the *Otx2/Gbx2* interface, this is known as the molecular MHB and sits rostral to the morphological MHB. *Wnt1* and *Fgf8* demarcate the molecular MHB. *Wnt1* is expressed rostrally to the molecular MHB (*Red*). *Wnt1* expression in the forebrain and anterior midbrain is broad dorsally. *Fgf8* expression is restricted to r1 (*Dark blue*). *Pax2* and *En2* are expressed across the MHB domain at this stage. *Pax2* is expressed over a wide area - the mesencephalic vesicle and r1 and 2 (yellow). The expression of *En2* is narrower than *Pax2* (light green).
(B) A lateral view of the anterior half of the chick CNS at HH stage 20. Rostral is right and caudal is to the bottom of the diagram. By HH stage 20, a widely expanded mesencephalon (Favor et al.) and rhombomere (r1) are present. The expression of *Otx2* and *Gbx2* are maintained in the same position as at HH stage 10. Note that the position of the molecular MHB is now coincident with the morphological MHB. By this stage, *Wnt1* expression around the MHB is restricted to the area immediately anterior to the MHB, and its caudal expression is limited to the very dorsal of the midbrain and forebrain. *Fgf8* expression only appears at the anterior of r1. The expression borders of both *Wnt1* and *Fgf8* are still antagonistic at the MHB. *Pax2* and *En2* are expressed across the MHB, but are restricted to the narrow domain of the MHB.
A horizontal unbroken black line indicates the molecular MHB (MHB). Black arrows show the morphological MHB.

1-2-4 MHB molecules and their role at the MHB

1-2-4-1 Positioning and establishment phase of the MHB

The expression patterns of MHB molecules suggest that creation of the *Otx2/Gbx2* interface in the correct place is significant for the positioning of the MHB organiser. *Otx2* and *Gbx2* are expressed in a complementary manner. *Otx2* expression is limited to rostral in developing vertebrate CNS, and it reaches to the MHB (Simeone, 1998). By HH stage 10 in chick, *Gbx2* expression occupies a domain from the MHB to the r3/4 boundary (Shamim and Mason, 1998). *Otx2* and *Gbx2* null mice show that *Otx2* and *Gbx2* are necessary for positioning the MHB. Coincident with the expression patterns, homozygous *Otx2* mice lose the entire brain anterior to rhombomere 3 (r3) (Acampora et al., 1996; Acampora et al., 1995; Ang et al., 1996; Matsuo et al., 1995), while, homozygous *Gbx2* mutant mice show a lack of anterior HB and a caudal expansion of the posterior MB (Wassarman et al., 1997). Remarkably, *Otx2* expression in the *Gbx2* -/- mouse is expanded caudally (Millet et al., 1999; Wassarman et al., 1997). Consequently, *Wnt1* and *Fgf8* expression domains are shifted caudally (Millet et al., 1999). Gain of function studies have demonstrated that antagonism between *Otx2* and *Gbx2* determines the position of the MHB. Misexpression of *Gbx2* anterior to the MHB leads to the repression of *Otx2* in the posterior MB. Furthermore, misexpression of *Otx2* in the HB resulted in repression of *Gbx2* in the metencephalon (Broccoli et al., 1999; Katahira et al., 2000; Millet et al., 1999). In both cases, *Fgf8* expression was shifted to the new *Otx2/Gbx2* interface. These results support the hypothesis that *Otx2* and *Gbx2* could lead to differential specification of MB and HB cells, and that the interactions between these two populations of cells leads to induction of MHB organiser genes such as *Fgf8* at their interface.

The timing of onset of MHB organiser gene expression, however, suggests that the induction of MHB organiser genes is initially independent of one another. Indeed, MHB genes are still induced in the absence of *En1*, *Pax2*, *Pax5*, *Fgf8* and *Wnt1* function. In *Wnt1* null mice for example, *En1* expression at the MB and MHB was observed at 6 somites, only to disappear later in development (McMahon et al., 1992). The *acerebellar* (*ace*) zebrafish mutant, which lacks *Fgf8* function, also showed normal induction of *engrailed1*(*eng1*) and 2 (*eng2*) and *Pax2.1* at the MHB, which was gradually lost and had completely disappeared by the 24hpf stage (Reifers et al., 1998).

In contrast, the lack of *Pax2* and *Pax5* does not affect the expression of *En1*, *Fgf8*, *Wnt1* or *Otx2* (Schwarz et al., 1997).

In 1999, Shamim and her colleagues showed that *Fgf4* can induce MHB markers, such as *Fgf8*, *En1*, *En2*, *Pax2* and *Wnt1*, within the chick neural plate (Shamim et al., 1999). Implantation of Fgf4 soaked beads in the mesoderm has been used to demonstrate that the Fgf4 protein itself can induce transcription of neural markers, including *Otx2* (Alvarez et al., 1998; Storey et al., 1998). In contrast, dominant negative *Fgf4* misexpression blocks neural development (Hongo et al., 1999). A neural induction factor, *Fgf4* is expressed in the anterior notochord for a short period, between HH stage 5+ to HH stage 7 (Shamim et al., 1999). In mouse, *Fgf4* expression was not found in notochord, however ectopic *Fgf4* expression in chimeric mice induced foliation of the MB (Abud et al., 1996). Taken together, notochord-derived *Fgf4* is sufficient for induction of the MHB molecules. However, conditional *Fgf4* mutant mice which lack all *Fgf4* encoding sequences show relatively normal development, including the MHB area (Moon et al., 2000).

1-2-4-2 Maintenance phase of the MHB

At the MHB, expression of all the Fgf8 subfamily members (Fgf8, Fgf17, Fgf18) was found (Crossley and Martin, 1995; Reifers et al., 2000; Xu et al., 2000). Conditional loss of *Fgf8* in mutant mice leads to dramatic cell death in the MHB domain, and most of the MB and cerebellar structures are lost (Chi et al., 2003; Jaszai et al., 2003). Zebrafish mutants lacking *Fgf8*, *acerebellar* (*ace*), also showed loss of cerebellum, although in this case, the tectum was enlarged (Reifers et al., 1998). These indicate that *Fgf8* plays a significant role in MHB development, a hypothesis confirmed by experiments in which Fgf8-soaked beads implanted into the MB or posterior to r1 induced MHB genes. When Fgf8-soaked beads were placed into the posterior diencephalon or MB, expression of *En1/2*, *Pax2* and *Wnt1* was induced (Crossley et al., 1996; Martinez et al., 1999; Shamim et al., 1999). Similarly, Fgf8-soaked beads implanted into the HB, posterior to r1, induced *En1/2* expression (Irving and Mason, 2000). Fgf8 beads also induced ectopic *Gbx2* expression in the MB where *Otx2* is normally expressed (Liu et al., 1999). In contrast, *Fgf8* is able to repress *Otx2* expression in the absence of *Gbx2* (Liu and Joyner, 2001b). These experiments support strongly that *Fgf8* can mimic MHB organiser activity.

Fgf17 and *Fgf18* are expressed after *Fgf8* at the MHB. The *Fgf17* null mouse exhibits a smaller cerebellum later in development (Xu et al., 2000). This phenotype was more severe in the *Fgf8* heterozygous mutant background. *Fgf17* and *Fgf18* are induced when *Fgf8* is misexpressed in chick and zebrafish (Ohuchi et al., 2000; Reifers et al., 2000). These data suggest that members of the *Fgf8* subfamily cooperate to maintain their expression and activity at the MHB. In 1995, MacArthur and colleagues reported an isoform of *Fgf8* (MacArthur et al., 1995). At the MHB in chick, at least two alternative splicing isoforms of *Fgf8*, *Fgf8a* and *Fgf8b*, are expressed (Sato et al., 2001). Misexpression of *Fgf8a* in the MB showed expansion of diencephalon and MB region along with the ectopic expression of *En2* (Sato et al., 2001; Sato and Nakamura, 2004). The expression of *Otx2* and *Gbx2* were not changed. In contrast, *Fgf8b* misexpression leads to a shift of the *Otx2/Gbx2* interface, and an ectopic cerebellum was observed (Sato et al., 2001; Sato and Nakamura, 2004). *Wnt1-Fgf8b* transgenic mice in which the *Wnt1* regulatory region is used to express *Fgf8b* ectopically showed ectopic expression of *Gbx2*, *En1*, *En2* and *Pax5* in the MB, and *Otx2* expression was repressed (Lee et al., 1997; Liu et al., 1999). Furthermore, these embryos demonstrated fate changes of the MB and caudal FB to anterior HB. In short, only *Fgf8b* can shift the *Otx2/Gbx2* interface at the MHB and the *Fgf8b* phenotype is coincident with the phenotype seen in Fgf8 bead implantation assays.

At the MHB, *Pax2* has been reported as a key inducer of *Fgf8* as well as of the expression of *Pax5* and *Pax8* (Ye et al., 2001). Misexpression of *Pax2* in chick neural tube leads to the ectopic expression of *Fgf8* in posterior HB. However, this *Fgf8* induction through *Pax2* requires the presence of *Gbx2*; co-electroporation of *Pax2* and *Gbx2* results in the cell-autonomously ectopic induction of *Fgf8* (Ye et al., 2001).*Otx2* is also required for the *Fgf8* induction, possibly through *Pax2*. *Otx2* misexpression leads to *Fgf8* induction within an adjacent *Pax2* positive domain, in a non-cell autonomous manner, while inhibiting *Fgf8* expression in a cell-autonomous manner (Ye et al., 2001). Most recently, it is reported that *Pax2* regulates the level of *Fgf8* expression through its *cis*-regulatory region (Inoue et al., 2008). In zebrafish, *Pax2a* directly binds to the *Fgf8* *cis*-regulatory region downstream of the *Fgf8* encoding region, and controls its expression both actively and repressively (Inoue et al., 2008). Thus, *Pax2* is a key regulator of *Fgf8* expression at the MHB.

Another secreted molecule, *Wnt1* is expressed at the MHB in a manner complementary to *Fgf8*. Wnts, homologues of *Drosophila* wingless (wg), are highly conserved secreted molecules which regulate cell-cell interaction through the Wnt signalling pathway. One of the Wnt family, *Wnt1*, is reported to be expressed at the MHB (Bally-Cuif and Wassef, 1994; Davis and Joyner, 1988; Fung et al., 1985; Parr et al., 1993; Wilkinson et al., 1987). At E9.5, the expression of *Wnt1* is found in a narrow ring in the caudal MB, adjacent to the HB. Its expression is also detected at the dorsal midline from caudal FB to spinal cord. *Wnt1* knockout mutant mice show a lack of MB (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Furthermore, loss of *Wnt1* activity at the MHB resulted in the gradual loss of *Fgf8*, *En1* and *En2* during development (Lee et al., 1997; McMahon et al., 1992).

Interestingly, the *Wnt1* knockout phenotypes were rescued when *En1* was driven from the *Wnt1* promoter at the MHB which indicates that En1 is a target of the Wnt1 leading signals in the MB (Danielian and McMahon, 1996). Meanwhile, *En1/2* double mutants showed a loss of *Wnt1* expression suggesting *Wnt1* and *En1/2* tightly regulate each other (Araki and Nakamura, 1999). Indeed, in both *En1* knockout and *En1* and *En2* double knockout mice, the mesencephalon and cerebellum are absent, while the absence of *En2* does not affect severely to the dorsal MB (Joyner, 1996; Joyner et al., 1991; Wurst et al., 1994). When *En2* is knocked into the *En1* coding region, it rescues all of the *En1* defects (Hanks et al., 1995). This suggests that the two *En* genes are functionally equivalent as well as crucial in establishing a correct MB. Importantly, it is reported that mouse *En2* regulatory sequence has two Pax binding sites (Song et al., 1996). The deletion of these domains causes downregulation of *En2* expression in mice (Song et al., 1996). Members of the *Pax* gene family (*Pax2, 5, 8*), homologues of *Drosophila paired-box* gene, are also expressed at the MHB. As is the case with other the MHB genes, lack of *Pax* genes cause deletions in the MHB region. *Pax5* mutant mice show a lack of posterior MB and anterior cerebellum (Urbanek et al., 1994). However, the result of *Pax2* mutation ranges from almost normal development to a complete lack of the posterior MB and cerebellum, dependent on genetic background (Favor et al., 1996). Unlike *En1/2*, *Pax2* and *Pax5* have a dose-dependent cooperation between each other (Schwarz et al., 1997).

1-2-4-3 Negative feedback at the MHB

Gene expression regulatory systems usually involve a feedback regulation. A feedback regulation is a process by which the product of a system influences its own production by controlling the amount and activity of molecules involved within the system. This is often inhibitory, which is described as a negative feedback loop. To ensure tight regulation of organiser genes, there are at least two negative feedback loops in operation at the MHB. A number of genes that act to directly regulate FGF expression have been identified, for example MKP3, Sef and the *Sprouty* (*Spry*) gene family. The expression of these molecules are both induced by FGF8, but they act to inhibit *Fgf8* expression in a negative feedback loop (Chambers and Mason, 2000; Klock and Herrmann, 2002; Minowada et al., 1999). *Spry* encodes a unique cysteine-rich domain which localises to the cell membrane (Casci et al., 1999; Hacohen et al., 1998). The phosophorylated *Spry* family inhibits FGFR-induced growth factor-meditated mitogen-activated protein kinase (MAPK) activation by binding to Grb2 (Cabrita and Christofori, 2008; Fong et al., 2003; Gross et al., 2001; Lao et al., 2007). In *Drosophila*, *Spry* functions as a target gene and feedback inhibitor of Fgf and EGF signalling (Placzek and Skaer, 1999). In mammalian embryos, expression of *Spry* family members is found to coincide with Fgf signalling (Chambers and Mason, 2000; Minowada et al., 1999). In mouse, *spry1*, *spry2* and *spry4* are expressed in the rostral FB to anterior HB, and chick *spry1* and *spry2* appear to be similarly distributed (Minowada et al., 1999). Ectopic induction by FGF bead implantation as well as expression analysis under *Fgf8*-negative conditions demonstrated that Fgf signalling is sufficient to induce *Spry* expression (Minowada et al., 1999). In contrast, misexpression of dominant-negative form of *Spry2* across the MHB in chick leads *Fgf8* misexpression rostrally (Suzuki-Hirano et al., 2005). Thus, Spry inhibits Fgf signalling to maintain the level of this signalling at the MHB.

Groucho (*gro*) is a neurogenic gene which encodes a nuclear protein and can act as an active transcription repressor by interaction with the helix-loop-helix (HLH) protein (Fisher and Caudy, 1998b; Parkhurst, 1998). In the *Drosophila* wing disc, *gro* inhibits expression of *en* (de Celis and Ruiz-Gomez, 1995). *gro* mutants show ectopic *en* expression in the wing disc. In *Xenopus, Groucho 4* (*Grg4*), one of *gro* homologues, interacts with *Tcf1* and *Tcf3* at the N-terminal region, and inhibits the WNT signalling pathway (Cavallo et al., 1998; Roose et al., 1998). *Tcfs* are known to bind the *En2* promoter in *Xenopus*, thus *Grg4* is able to repress *En2* expression by interacting with

Tcfs (McGrew et al., 1999). In mouse, *Grg4* is expressed from the diencephalon to MB (Koop et al., 1996). Expression of chick *Grg4* is detected from the diencephalon to r1 at the 5 somite stage. By the 10 somite stage, *Grg4* expression appears in a gradient which is complementary to *En2* (Sugiyama et al., 2000). Misexpression of *Grg4* across the MHB downregulated *En2*, *Pax5* and *Fgf8* expression, but induced *Pax6* (Sugiyama et al., 2000). This could be through primary inhibition of *Wnt1*, loss of which would lead to successive loss of other MHB genes in the positive maintenance loop, or *Grg4* could be acting directly. Thus, *Grg4* negatively regulates the MHB molecules during the development.

1-2-4-4*Irx2* is a prepattern factor for cerebellum

Recent studies have demonstrated a crucial role for *Irx2* in chick MHB formation. *Irx* is an *Iroquois* homeobox (*Irx*) gene which was firstly identified as a prepatterning gene, regulates proneural genes in *Drosophila* (Gomez-Skarmeta and Modolell, 2002). In *Xenopus* and zebrafish, *Irx* genes are reported to be required for the formation of the MHB (Glavic et al., 2002; Itoh et al., 2002). Misexpression of *Xiro1* activates the expression of *Otx2*, *Gbx2*, *Fgf8* and *En2* during the introduction and the positioning of the MHB (Gomez-Skarmeta and Modolell, 2002). Similarly, *ziro1* and *ziro7* plays an over-lapping role in the establishment of the MHB (Itoh et al., 2002). In chick, *Irx3* was found to be expressed posteriorly in the brain from the ZLI (Kobayashi et al., 2002; Ogura et al., 2001). *Irx3* expression in the FB meets with the *Six3* domain from rostral FB to the ZLI, and it has been shown that their interaction is mutually repressive. *Irx3* misexpression also perturbs the expression of *Fgf8* and *Shh* at the ZLI (Kobayashi et al., 2002). *Irx2* misexpression induces ectopic cerebellum development (Gomez-Skarmeta and Modolell, 2002); (Lebel et al., 2003). In mouse, *Irx2* expression is found from the posterior MB to HB (Lebel et al., 2003), but the *Irx2* deficient mice showed normal MHB formation (Lebel et al., 2003). Although RT-PCR analysis revealed that lack of *Irx2* in mice does not strongly affect levels of other *Irx* genes, *Irx* genes may show redundancy here (Lebel et al., 2003). Indeed, ectopic *Irx3* expression appeared at the MHB in *Irx2* null mice.

1-3 Boundary and organiser formation - Meinhardt's model

Meinhardt proposed a model in 1983 whereby the establishment of organising centres requires the prior specification of two distinct, adjacent cell populations (Meinhardt, 1983). In this model, one axis region is first subdivided into two distinct regions by region-specific gene activation. These two genes have self-enhancement regulation and may compete with each other directly or by a common repressor. Subsequently, the cooperation between these two populations produces signalling molecules, morphogen, at the interface (Gierer, 1981; Meinhardt, 1983). The concentration of morphogen provides a good measurement for the distance from the border. The morphogen has a positive regulatory manner generally, and an inhibitory regulation for gene activation at high concentration. The morphogen shows gradient expression, and target genes are specifically activated depending on their distance from the morphogen source. Therefore, when one gene property is lost experimentally, neighbouring properties on both sides may expand their expressions to fill the area (Gierer, 1981; Meinhardt, 1983).

The gene expression patterns at the MHB during early development are consistent with patterning of this boundary and the regulatory manner is similar to the model which was proposed by Meinhardt. Firstly, as the earliest event of CNS development, the CNS is subdivided into an anterior *Otx2*-positive and posterior *Gbx2*-positive domain (Wassarman et al., 1997). During late gastrulation stages, *Otx2* is expressed from the anterior limit of the neural plate to a posterior border at the presumptive MHB. By contrast, *Gbx2* is expressed in a complementary fashion in the posterior of the embryo. Subsequently, MHB organiser molecules, like *Pax2*, *En1*, *Wnt1* and *Fgf8* are activated around the *Otx2/Gbx2* interface. This is consistent with the notion that the *Otx2/Gbx2* interface positions the primordial MHB. Molecular analysis in chick-quail chimera transplantation demonstrated that the MHB appears where *Otx2*- and *Gbx2*-expressing neuroepithelial cells interact (Irving and Mason, 1999). Studies using genetically modified mouse embryos have demonstrated that a lack of *Otx2* causes clear defects of rostral neural structures to r3, and embryos lacking *Gbx2* fail to develop anterior HB structures including r1-3 (Acampora et al., 1995; Matsuo et al., 1995; Wassarman et al., 1997). Despite this fact, a lack of both *Otx2* and *Gbx2* genes does not completely disrupt the initiation or maintenance of the MHB organiser marker genes in mouse embryos (Acampora et al., 1998; Li and Joyner, 2001; Rhinn et al., 1998; Wassarman et al., 1997). Mice with a double knockout of *Otx2* and *Gbx2* still express all of the MHB

organiser genes in the rostral CNS, albeit over a much broader domain. This strongly suggests that other signals are required to position these genes leading to their tightly restricted expression at the MHB organiser.

1-4 Dorsoventral boundary formation in the *Drosophila* wing disc.

Experimental evidence for the establishment of boundaries with local signalling centre function comes from pattern formation studies in *Drosophila* imaginal discs. In the wing discs, signalling centres are formed along both the AP and DV axis boundaries. Both of these boundaries function as local organisers to pattern surrounding tissues by expression of long-range graded morphogens. During DV compartment boundary formation in the *Drosophila* wing disc, the Notch receptor is activated along the border between dorsal and ventral cells, leading to specification of specialized cells that express *Wingless* (*wg*), a molecule subsequently responsible for organising wing growth and patterning. At the DV boundary, the Notch signalling pathway also creates a morphological 'fence' to restrict cell intermingling between DV compartments. Differences in DV cell affinity are also specified by cell adhesion molecules, each of which is expressed in only one of the DV compartments (Milan et al., 2005; Milan et al., 2001).

1-4-1 The molecular cascade of Notch signalling during DV boundary formation.

The formation and maintenance of the DV boundary in *Drosophila* wing discs is regulated by the locally restricted activation of Notch signalling. Establishment of the DV compartment starts when two cell populations are divided by the expression of *apterous* (*ap*) only in dorsal cells (Garcia-Bellido et al., 1973; Milan and Cohen, 2003). *ap* is a LIM-homeodomain transcription factor (Jurata and Gill, 1998). *ap* activity in the dorsal compartment is essential for the positioning of the local signalling centre along the DV boundary, for maintaining the lineage restricted boundary and specifying the dorsal cell identity (Blair et al., 1994; Garcia-Bellido et al., 1973). *ap* activates the expression of both the Notch ligand *Serrate* (*Ser*) and modulator *Fringe* (*Fng*) in the dorsal compartment (Blair et al., 1994; Cohen et al., 1992; Diaz-Benjumea and Cohen, 1993). *Fringe* modifies the Notch receptor in the dorsal cells to a form which is insensitive to *Ser,* thus *Ser* becomes effective and capable of activating Notch present only in ventral cells (Micchelli and Blair, 1999; Panin et al., 1997). Another Notch ligand *Delta* (*Dl*) is required in ventral cells to activate modified Notch in the dorsal

compartment. *Ser* and *Dl* regulate and maintain expression of one another through a positive feedback loop whereby *Dl* signals to dorsal cells to induce *Ser* expression and *Ser* signals back to ventral cells to induce *Dl* (de Celis and Bray, 1997; Panin et al., 1997). The restricted activation of Notch along the DV boundary induces expression of *wg*, a long-range morphogen, around the DV boundary (Diaz-Benjumea and Cohen, 1995; Kim et al., 1995; Rulifson and Blair, 1995). *wg* expression in the DV boundary then induces *Ser* and *Dl* expression in neighbouring cells (Micchelli et al., 1997). *Ser* and *Dl* signal back to the boundary cells to induce *wg* expression, thus wg expression at the DV boundary is strictly maintained. Importantly, these Notch ligands also induce the expression of *Cut*, a homeobox gene (Micchelli et al., 1997; Neumann and Cohen, 1996). *Cut* maintains *wg* expression at the DV boundary by promoting *wg* expression and inhibiting *Ser* and *Dl* in the DV boundary cells (de Celis and Bray, 1997). By this mechanism, a high level of *wg* expression is tightly restricted to the DV boundary and is maintained throughout wing disc development in *Drosophila*, and thus this boundary acts as a local organiser centre for the further wing growth and patterning.

1-4-2 Formation of a morphological 'fence' at the DV boundary

At the DV boundary, Notch activation is required not only for formation of the molecular boundary, but also the morphological boundary in which a distinctively smooth and sharp boundary forms, preventing cell intermingling between two compartments. Non-muscle Myosin II has been found to be concentrated near the adherens junctions, and elevated along the DV boundary of the *Drosophila* wing disc. Expression of F-actin is also concentrated at adherens junctions throughout the wing disc epithelial cells, but is significantly more intense along the DV boundary in a manner complementary to Myosin II (Major and Irvine, 2005; Major and Irvine, 2006). Importantly, F-actin forms a continuous cable at the DV interface, and Myosin II appears discontinuous with strongest expression found in cells closest to the edge of the DV boundary (Major and Irvine, 2006). F-actin polarisation at the DV boundary does not require the DV interface, but does require a stripe of Notch activation. Both ectopic Notch activation and *Fng* misexpression in the wing disc disrupt F-actin organisation and induce an ectopic DV boundary-like border, as well as the upregulation of Enabled *(Ena),* a member of the Ena/VASP family of actin regulators (Gertler et al., 1995; Major and Irvine, 2005). A lack of Notch caused a decrease in the high-level Myosin II expression at the DV boundary, and *wg* expression is lost (Major and Irvine, 2006). In

contrast, ectopically induced *Delta* expression leads to upregulation of Myosin II along the edges of such clones (Major and Irvine, 2006). Interestingly, Bazooka/Pac-3 (Baz) serine/threonine kinase which is generally required for actin-myosin interaction is downregulated at the edges of ectopically induced *Delta* clones in wing disc (Major and Irvine, 2006). Baz binds to the transmembrane immunoglobulin domain-encoding gene *echinoid* (*Ed*) (Laplante and Nilson, 2006; Wei et al., 2005). Loss of function analysis showed that a lack of *Ed* decreases Notch effecter gene *E(spl)m8* expression in proneural clusters (Escudero et al., 2003). Overexpression of *Ed* leads to a decrease not only of *E(slp)m8* expression, but also *Delta* expression (Ahmed et al., 2003; Rawlins et al., 2003). Furthermore, *Ed* mutant clones, introduced ectopically into the wing disc, become surrounded by an F-actin cable and Myosin II staining, followed by downregulation of Baz (Wei et al., 2005). Baz accumulates at the adherens junction at the DV boundary (Major and Irvine, 2005; Major and Irvine, 2006). Thus, these data suggest that in the DV boundary of *Drosophila* wing discs, Notch signalling leads the formation of a cytoskeletal 'fence' at the interface of DV compartments.

1-4-3 The LRR proteins, Tartan and Capricious

Signalling by Notch ligands on the both sides of DV boundary is limited to those cells close to the boundary, leading to high-level Notch activity either side of the DV boundary. Altering *Fng* expression to the ventral cells allows cells to cross the boundary, thus modulation of *Fng* plays a key role in the establishment of the DV boundary (Rauskolb et al., 1999). However, it has been shown that Notch activation is not sufficient for the cell lineage restriction at the boundary. When *ap* activity is reduced, *wg* expression at the boundary is lost (Milan and Cohen, 1999). Additional *ap* activity in this mutant rescues DV boundary formation. Despite this, misexpression of *Ser* and *Fng* in *ap* mutant rescues *wg* expression and Notch activation, but fails to reform the regular DV boundary (Milan and Cohen, 1999). Thus additional *ap*-dependent factors are required for cell lineage restriction at the DV boundary.

Cell lineage restriction is proposed to be caused by cell affinity differences between compartments (Garcia-Bellido, 1975). Cell affinity is defined as a compartment specific property regulated by selector factors. In 2001, Milan and his colleagues showed that two of *ap* target genes, *capricious* (*cap*) and *tartan (trn)*, regulate the DV cell affinity boundary (Milan et al., 2001). Cap and Trn are transmembrane proteins which encode

14 leucine-rich repeat (LRR) domains. The LRR domain is thought to mediate protein interactions and cell-cell interactions (Raghavan and White, 1997; Rothberg et al., 1990; Shishido et al., 1998). In the *Drosophila* wing disc, *cap* and *trn* are expressed in dorsal cells during boundary formation (Milan et al., 2001). Ectopic expression of both genes in the ventral compartment led cells to sort across the DV boundary. In a loss-of-*ap*-activity background, restricted *cap* and *trn* expression in dorsal cells reinstalled the DV affinity boundary (Milan et al., 2005). Furthermore, when *cap* and *trn* expression was induced along with *Fng*, both DV affinity boundary and *wg* expression were rescued (Milan et al., 2005). Cap and trn may act as adhesion molecules to maintain cell numbers in the dorsal compartment via short-range signalling, and to maintain the DV boundary (Milan et al., 2002). However, in vitro experiments showed that neither *cap* or *trn* are homophilic adhesion molecules (Milan et al., 2001; Shishido et al., 1998). It could be the case that these two LRR proteins regulate, directly or indirectly, other homophilic adhesion molecules. Or they may act as ligands in an as yet unknown signalling pathway. However, the exact function and molecular pathway of these LRR proteins is not yet clear.

1-5 The molecular core of the Notch signalling pathway

1-5-1 CSL-dependent Notch signalling pathway

The Notch signalling pathway is mediated by direct cell-cell interactions, the core of which is the 300kDa transmembrane receptor Notch. In mammals, four Notch receptors (Notch1-4) have been reported (Gallahan and Callahan, 1997; Lindsell et al., 1996). These receptors are activated by direct interaction with the transmembrane ligands, Delta and Serrate, expressed on the surface of adjacent cells (Figure 1.2 (Parks et al., 2000). The receptor-ligand interaction causes a series of three cleavages, releasing the Notch intracellular domain (NICD). Freed Notch extracellular domain (NECD) is taken into the signal-sending cells along with ligand, and is degraded (Nichols et al., 2007; Parks et al., 2000). In the signal receiving cells, NICD contains nuclear localization signals (NLS) allowing the freed NICD to enter the nucleus where it interacts directly with co-factors, such as CSL (CBF1/Suppressor of Hairless [Su(H)]/Lag-1) protein, SMRT (silencing mediator of retinoid and thyroid hormone receptor) and Skip (ski-related protein) (Huppert and Kopan, 2001; Pursglove and Mackay, 2005). In the absence of active Notch, these co-factors are present in a complex, and act as a transcriptional repressor (Dou et al., 1994; Hsieh and Hayward, 1995; Kao et al., 1998).

NICD antagonises the CSL/co-repressor interaction, converts the co-repressor complex to transcriptional activator, and regulates the expression of down stream target genes (e.g. *HES* [*hairy and E(spl)*]). After NICD initiates transcriptional activation in the nucleus of signal receiving cells, it is removed so that daughter cells can receive fresh Notch signalling to determine their fate (Kopan, 1999). Proteasome plays a role in the degradation of Notch effectors, while E3 ubiquitin ligases, such as Suppressor of Deltex [Su(Dx)] directly interact with NICD to ubiquitinate (Campos-Ortega, 1996; Schweisguth, 1999). Nedd4 family member, Suppresser of Deltex (Su(dx)) together with Nedd4 inactivate Notch proteins by removing them to intracellular compartments for degradation (Sakata et al., 2004; Wilkin et al., 2004). Nedd4 is a HECT-containing E3 ubiquitin ligase. In *Drosophila*, overexpression of *Nedd4* and *Su(dx)* caused inactivation of Notch signalling (Wilkin et al., 2004). Sakata et al. showed that Nedd4 ubiquitinates Notch proteins at the intracellular PPSY motif (Sakata et al., 2004). In mice, *Su(dx)* homologue *Itch* directly ubiquitinates the NICD (Qiu et al., 2000).

Hairy and *Enhancer of split* [*E(spl)*] in *Drosophila, HES* [*hairy and E(spl)*] in mouse, *her* and *him* in zebrafish, which encode basic helix-loop-helix (bHLH) motifs were the first Notch target genes to be identified (Delidakis and Artavanis-Tsakonas, 1992; Sasai et al., 1992). Notch signalling is involved in the differentiation of many kinds of cells, including neurons, blood cells, and tumours. Endogenous expression of mouse *Hes1* and *Hes5*, lead by Notch activation, inhibits neuronal differentiation (Ohtsuka et al., 1999). Similar results were observed in *Hes1* null mice and *Hes5* null mice. Importantly, Hes1 and Hes5 activities are only upregulated in the presence of both Notch1 and RBP-Jk(RBP-Jk and CBF1 are two different names for the same CSL protein) (Nishimura et al., 1998). Hes1 promoter has the CSL (RBP-Jk) site which allows binding of the NICD-CSL (RBP-Jk) complex (Honjo, 1996; Jarriault et al., 1995). Thus it is clear that some Hes members are direct targets of the CSL-dependent Notch signalling pathway. However, it has been shown that Hes family genes are not the only molecules to be directly activated by CSL-dependent Notch signalling. Mammalian homologue of *fringe*, *LFng* has two CSL(CBF1) binding sites in its cyclic promoter (Cole et al., 2002; Morales et al., 2002). Indeed, cyclic expression of *LFng* in the posterior presomitic mesoderm (PSM) is severely disrupted in *Dll-/-* and *Dl3-/-* mice, as well as CSL (CBF-1) -/- mice (Morales et al., 2002).

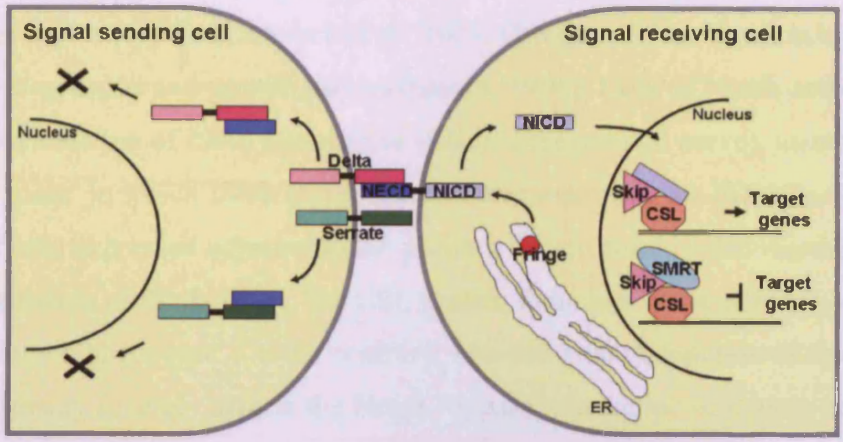


Figure 1.2 The core of the CSL-dependent Notch signalling pathway.

The four core elements of the Notch signalling pathway, Notch receptor, Notch ligands, Notch modulator and Notch co-factors are shown here in diagram form. Notch receptor is a transmembrane protein which consists of NICD and NECD. NECD contains EGF-like repeats. When these repeats interact with the extracellular domain of Notch ligands Delta and Serrate on the signal sending cells, Notch signalling is activated and the Notch protein is cleaved. The released NECD, bound to ligands, continues its endocytosis and is degraded in the signal sending cell. On the other hand, released NICD translocates to the nucleus. Once in the nucleus, NICD converts CSL from a transcriptional repressor to a transcriptional activator. In the nucleus, CSL binds SMRT and Skip. These proteins normally facilitate CSL localisation to the nucleus, and together with other co-factors, repress transcription of target genes. The direct protein-protein interactions between NICD, CSL, SKIP lead to the dissociation of SMRT and other co-factors. Notch can be modified in its sensitivity for ligands in the Golgi by Fringe. NICD: Notch intracellular domain, NECD: Notch extracellular domain, CSL: CBF1/RBPkj, Suppressor of hairless, Lag-1, SMRT: silencing mediator of retinoid and thyroid hormone receptor, Skip: ski-related protein.

1-5-2 CSL-independent Notch signalling pathway

1-5-2-1Notch/Abl signalling

It has been suggested that CSL-independent Notch signalling is involved in axon guidance in *Drosophila* (Crowner et al., 2003; Giniger, 1998). Notch is highly localised in extending axons and growth cones (Giniger, 1998). Lack of Notch activity disrupts the defasciculation of ISNb axons from ISN (intersegmental nerve), instead, performs axon' bypass' in which ISNb axons remain associated with the ISN (Crowner et al., 2003). *Delta* expressed adjacent to the primary choice point is also required for the defasciculation of ISNb axons, but CSL protein induction alone does not rescue the Notch mutant phenotype. On the contrary, loss and gain of function of the Abelson PTK (Abl) pathway severely affects the Notch 'bypass' phenotype (Crowner et al., 2003). Overexpression of *Abl* rescues the Notch 'bypass' phenotype, while misexpression of the *Abl* antagonist, *Enabled* (*Ena*) accelerates the Notch phenotype. *Abl* is highly concentrated in axons, as is the adapter protein *Disabled* (*Dab*), which is known to interact directly with *Abl* - these two molecules are thought to control the axonal cytoskeleton (Gertler et al., 1993; Hoffmann, 1991; Howell et al., 1997). Interestingly, *Dab* can bind directly to Notch *in vitro* (Giniger, 1998). Thus Abl/Notch signalling is sufficient for axon growth and formation of the axonal cytoskeleton in a CSL-independent Notch signalling pathway. This may be a key to the role of Notch signalling in the cytoskeletal 'fence' formation at the DV boundary in *Drosophila*. *Abl* recruits Rho family GTPase and controls local actin assembly and myosin activity (Hakeda-Suzuki et al., 2002; Luo, 2000; Newsome et al., 2000). *Drosophila* cyclase-associated protein, *capulet* (*capt*), binds with *Abl* to influence axon pathfinding, and restrict apical actin polymerization in *Drosophila* epithelium along with *Ena* (Baum and Perrimon, 2001; Wills et al., 2002). Moreover, analysis using ectopically induced *capt* mutant clones in wing and reduction-of-function analysis demonstrated a specific requirement of *capt* at DV boundary (Major and Irvine, 2005). As well as an actin regulator, Ena is known to be a substrate of tyrosine kinase which is negatively regulated by Abl by a direct interaction (Gertler et al., 1995). Modulating Notch activation and *Fng* sharply upregulates *Ena* expression and disrupts F-actin organisation at the DV boundary (Gertler et al., 1995; Major and Irvine, 2005).

1-5-2-2 Notch/Deltex signalling

A cytoplasmic protein, Deltex (Dx) positively regulates Notch signalling pathway through direct interactions with ankyrin repeats at NICD (Busseau et al., 1994; Matsuno et al., 1995; Xu and Artavanis-Tsakonas, 1990). At the DV boundary, a lack of *dx* downregulates or distrupts both *wg* and *cut* expression (Hori et al., 2004). Furthermore, *dx* overexpression leads ectopic *wg* and *Dl*, as well as Notch activation (Hori et al., 2004). Another Notch signal target gene, *vestigial* (*vg*), is an essential regulator of cell growth and differentiation (Kim et al., 1996). A CSL binding site in the DV boundary enhancer of *vg* (*vg*BE) is required for Notch-dependent activation (Kim et al., 1996). This CSL binding site of *vg*BE is essential for the Dx-mediated Notch activation (Hori et al., 2004; Kim et al., 1996). Under a lack of CSL, NICD overexpression fails to activate vgBE, in contrast to that of Dx overexpression which successfully induces *vg*BE (Hori et al., 2004). Thus, Notch/dx signalling occurs in a CSL-independent manner.

A RING-E3 ubiquitin ligase, Dx binds to Kurtz, the β-arrestin homologue, to promote ubiquitination and degradation of Notch protein (Mukherjee et al., 2005; Veraksa et al., 2005; Xu and Artavanis-Tsakonas, 1990). β-arrestin is an adaptor protein which regulates signalling and trafficking of different classes of target receptors (Shenoy and Lefkowitz, 2003). In order to release NICD, endocytosis followed by γ-secretase mediated cleavage at the transmembrane domain of Notch is required (Gupta-Rossi et al., 2004). Dx is believed to be responsible for this process. Enforced expression of Dx causes the disappearance of Notch proteins intracellularly via endocytic vesicles from the cell surface, and leads to the accumulation of NICD in the nucleus (Hori et al., 2004). *Dx* assists in ubiquitination of the Notch receptor, and cleavage of NICD, and Kurtz acts as an E3 adaptor. Indeed, loss of function of *Kurtz* increases Notch protein levels in the *Drosophila* wing disc, while misexpression of both *Kurtz* and *Dx* significantly downregulates Notch protein levels (Mukherjee et al., 2005). *In vitro* assays show that all three components, Dx, Kurtz and Notch form a complex. Thus, *Dx* is a positive regulator of the Notch signalling pathway, and Kurtz acts as an adaptor to maintain the level of Dx-Notch interaction. Importantly, Su(dx) and Nedd4 antagonise Dx, and Nedd4 negatively regulates Dx levels. Accumulation of Notch in Dx-positive vesicles is limited by the presence of Su(dx) and Nedd4 (Sakata et al., 2004). Thus, these two E3 ubiquitin ligases play a part not only in the degradation of NICD, but also

in the recycling and degradation of Notch to prevent inappropriate activation of unstimulated Notch receptors.

1-6 Notch signalling in vertebrates

1-6-1 The inhibitory function of LFng during somitogenesis

Somites are formed by segmentation of presomitic mesoderm (PSM). In normal development, somites are perfectly aligned symmetrically, and form a regular configuration along the anteroposterior axis. The formation of these somites is periodically regular; a pair of somites is formed approximately every 90 mins in chick (Forsberg et al., 1998; Palmeirim et al., 1997). Oscillatory expression of a number of cyclic genes is observed once during each somite formation. Amongst the genes involved in this process are Notch targets including members of the Hairy/E(*spl*) family, *LFng* and *Delta* (Forsberg et al., 1998; Palmeirim et al., 1997).

In the mouse posterior PSM, *Notch1* activation through *Delta-like 1* (*Dll1*) positively regulates transcription of both *LFng* and *Hes7* (Barrantes et al., 1999). *Hes7* is a strong transcriptional repressor of *LFng* and of *Hes7* itself (Bessho et al., 2003; Bessho et al., 2001; Ishibashi et al., 1995; Kokubo et al., 2005; Ohtsuka et al., 1999). *LFng* expression in the PSM is absent in *Hes7* null mice. However, stabilisation of *Hes7* cyclic expression through a proteasome inhibitor inhibits *LFng* expression (Bessho et al., 2003; Bessho et al., 2001). An *In vitro* study demonstrated that *Hes7* can negatively regulate its own promoter (Bessho et al., 2003). Thus, *Hes7* plays a role in a negative feedback loop at the PSM. Interestingly, *LFng* acts as a negative modulator of the Notch receptor in the posterior PSM (Dale et al., 2003; Morimoto et al., 2005). This positive and negative feedback regulation creates oscillation of Notch activation (Morimoto et al., 2005). In the anterior PSM, *Hes7* expression is downregulated, thus does not negatively regulate *LFng* and Notch1 activity (Bessho et al., 2001). In contrast, a basic helix-loop-helix transcription factor *Mesoderm posterior 2* (*Mesp2*) is expressed at the anterior PSM and regulates *LFng* activation and suppression of *Dll1* (Buchberger et al., 1998; Saga et al., 1997; Sawada et al., 2000; Sparrow et al., 1998). *Mesp2* directly activates *LFng* translation by binding to its enhancer region (Morimoto et al., 2005). *Mesp2* regulation of *LFng* and *Dll1* creates a clear boundary between the high *Notch1* activity domain and the *Mesp2* expressing domain, and this produces a new segment (Morimoto et al., 2005).

1-6-2 Neurogenesis and lateral inhibition

1-6-2-1 Lateral inhibition

A significant role of Notch signalling is to control cellular differentiation by lateral inhibition; a mechanism by which a group of initially equivalent cells can develop into a salt-and-pepper mosaic consisting of two different cell types in the correct spatial arrangement and numbers (Greenwald and Rubin, 1992; Raible and Eisen, 1995). A key role of lateral inhibition by Notch signalling is the inhibitory regulation of Notch-ligand expression by Notch activity. This creates a feedback loop which generates the salt-and-pepper mosaic of cells containing either high or low levels of Notch ligand (Lewis, 1998). A bHLH transcription factor called *achaete-scute* complex (AS-C) in *Drosophila* and MASH in mammals is expressed throughout the cluster of equivalent cells (Artavanis-Tsakonas et al., 1999; Muskavitch, 1994). The AS-C proteins are increased in the cells become committed to a neural fate, but decreased in the neighbour cells (Lewis, 1996; Skeath and Carroll, 1992). AS-C/Ash is a positive regulator of *Delta* expression (Heitzler et al., 1996). A cell in the initially equivalent clusters which express slightly higher levels of *Delta* becomes a signal sending cell, and induces high levels of *E(spl)/HES* expression in neighbouring cells via Notch signalling activation (Jennings et al., 1994). Within the neighbouring cells, high levels of *E(spl)/HES* expression represses expression of *AS-C* and subsequently of *Delta* (Heitzler et al., 1996; Paroush et al., 1994; Schrons et al., 1992). This low level of *Delta* in neighbour cells maintains these cells in a state whereby they send back fewer stimuli than the signal sending cells. Thus, this leads to lower levels of *E(spl)/HES* expression in the signal sending cells, which increases the *AS-C* activity and *Delta* expression (Lewis, 1996). It is not yet known exactly how the initial small differences between receptor and ligand expression that trigger this process occur. However, Delta signalling within the signal sending cells in a cell-autonomous manner is believed to support a rapid selection of two different cell fates (Beatus and Lendahl, 1998).

1-6-2-2 The role of Notch signalling during neurogenesis

In the developing vertebrate CNS, all four Notch receptors (Notch1-4) are reported to be expressed and are linked with undifferentiated cells (de la Pompa et al., 1997; Del Amo et al., 1992; Williams et al., 1995). *Notch1* is expressed throughout the proliferative zone, while *Delta1*(*Dll*) is expressed only in the outer part of this zone (Myat et al.,

1996). Lateral inhibition regulates neuron production in the neuroepithelium. The *Dll*-expressing cells differentiate to neurons (Henrique et al., 1995). Activation of Notch signalling in neighbouring cells promotes *Hes* expression; differentiation of these cells is thereby inhibited, and they remain as proliferating cells.

1-6-3 The Notch signalling in a choice of neural/glia cell fates

In studies of neural stem cells both *in vivo* and *in vitro*, Notch signalling has been shown to play a significant role in the maintenance of correct neuron number during development (Hatakeyama et al., 2004; Hitoshi et al., 2004; Nakamura et al., 2000). Neural stem cells initially only self-renew and give rise to neurons, however, later they produce glial cells. Thus, the maintenance of neural stem cells at later stages becomes important in the maintenance of the correct number of neurons. *Notch1* null mice lose the proper differentiation from primitive to definitive neural stem cells (Hitoshi et al., 2004). A lack of *Hes* genes progressively results in cell differentiation (Hatakeyama et al., 2004). In both *Hes1* and *Hes5* mutant mice, neuroepithelial cells are not maintained and glial cells are prematurely differentiated into neurons. *In vitro* studies showed that *Delta-like gene 1* (*Dll1*) inhibits differentiation of neurons into neurospheres (Grandbarbe et al., 2003).Mutation in *Dll1* does not affect the generation and maintenance of neural stem cells *in vitro*, but causes an increase in neurons at the expense of both oligodendrocytes and astrocytes. In contrast, Notch activation inhibits neuronal specification and differentiation. At later stages, Notch activation inhibits the further differentiation of mature oligodendrocytes, even if oligodendrocyte precursor cells are promoted (Grandbarbe et al., 2003). Thus, Notch controls the choice between neuronal fate and glial fate by inhibiting neuronal fate, and promoting glial fate. However, at later stages, Notch inhibits the differentiation of both neurons and oligodendrocytes, promoting the differentiation of astrocytes (Grandbarbe et al., 2003).

1-6-4 Notch signalling in the spinal cord stem zone

In the chick spinal cord stem zone, Notch signalling is required for cell proliferation. *Notch1* and *Hes5-1* expression are reported in the stem zone in the tail bud. It is possible to block this Notch signalling by dominant negative *Dll1* expression (Akai et al., 2005). In the same domain, there is also expression of Fgfs and *cash4*, a chick homologues of *AS-C* (Akai et al., 2005; Henrique et al., 1997). Interestingly, *Dll1* and *Notch1* are

expressed in a broad and uniform domain in the caudal stem zone, which is a pool of undifferentiated cells. Expression of *Dll* and *cash4* are regulated by Fgf signalling at the caudal stem zone, and Notch signalling maintains cell proliferation to keep the stem zone cell population in the stem zone of the chick tail bud (Akai et al., 2005). Loss of Fgf signalling caused a lack of both *Dll* and *cash4* expression, while gain of function analysis demonstrated that *cash4* actively regulates *Dll*, but not the proneural genes, *neurogenin1* (*ngn1*) and *neurogenin2* (*ngn2*), which are the cell autonomous regulators of neurogenesis. Misexpression of dominant negative *Dll* downregulates *Hes5-1* expression (Akai et al., 2005). Thus, in the presence of Fgf signalling, Notch and Delta manually inhibit one other to maintain cell proliferation levels at the stem zone. In contrast, within the transition zone of the tail bud where the level of Fgf signalling is lowered, cells mix with recently arrived stem cells from stem zone and *Dll* expressing single cells are found (Akai et al., 2005; Mathis et al., 2001).

1-6-5 Notch signalling in the inner ear

In the inner ear, lateral inhibition through Delta-Notch signalling appears to organise the choice between hair-cell and supporting cells. In the CNS, the E3 ligase *mind bomb* (*mib*) zebrafish mutants produce greater numbers of primary neurons; they also overexpress *Delta* genes (Jiang et al., 1996; Schier et al., 1996). Misregulation of *Delta* is seen in inner ear, along with upregulation of *Serrate* (Haddon et al., 1998). These changes cause the *mib* mutant to develop a phenotype in which their sensory patches, normally consisting of hair cells surrounded by supporting cells, develop solely hair cells with a complete absence of supporting cells (Haddon et al., 1998). In the vertebrate inner ear, *Notch1* (*N1*) and *Serrate1*(*Ser1*) are expressed throughout the progenitor cells, while *Delta1*(*Dll*) and *Serrate2* (*Ser2*) are expressed in a scattered subset of the population in the nascent hair cells (Adam et al., 1998; Cole et al., 2000; Morrison et al., 1999). *Dll* and *Ser2* are negatively regulated by Notch activity, while *Ser1* is positively regulated by Notch activity (Haddon et al., 1998). Blocking Notch activity by using *dn-Dll* and *dn-Su(H)* constructs results in a downregulation of *Ser1* expression, while activation of Notch activity by misexpression of full-length *Dll* does not interfere with *Ser1* expression (Eddison et al., 2000). Contrary to lateral inhibition, the lateral induction may act cooperatively, allowing cells to select their fate and preventing a salt-and-pepper mosaic outcome across the tissue. In the inner ear, supporting cells maintain a contact with one other, as well as with hair cells, so that both lateral induction and

lateral inhibition signals maintain high Notch activation, high *Ser1* and low expression of *Dl1* and *Ser2* (Eddison et al., 2000). Moreover, hair cells contain *Numb* expression which downregulates *N1* within the cells (Eddison et al., 2000; Jacobsen et al., 1998). As a result, the cells which are exposed to low levels of Notch activity differentiate into hair cells; while the cells containing high levels of Notch activity differentiate into supporting cells. Interestingly, *LFng* is also expressed in the supporting cells (Irvine, 1999). However, neither *LFng* knockdown nor *LFng* overexpression in mice results in abnormal development of the inner ear (Eddison et al., 2000; Zhang et al., 2000). Hair cell development is only repressed in the *LFng/ Ser2* double knockout (Zhang et al., 2000). Thus, it is not yet clear how *LFng* affects Notch activity during inner ear development.

1-7 The role of LFng at the ZLI

The ZLI is a local organiser formed during vertebrate CNS development. *Lunatic Fringe* (*LFng*) demarcates the ZLI by the boundaries of its expression (Zeltser et al., 2001). The expression of *LFng* in FB is selectively excluded from the ZLI during development of the chick CNS. Both *Lfng* and *Manic Fringe* (*MFng*), another vertebrate homologue of *Drosophila Fringe*, are excluded from the mouse ZLI (Zeltser et al., 2001). Unlike the case in the *Drosophila* wing disc, Notch ligands *Delta1* and *Serrate1* are expressed in the same domain as *LFng*, both sides of the ZLI. However, ectopically introduced *LFng* expressing cells in the ZLI prefer to group at *LFng* positive domains anteriorly and posteriorly, in the same was as *Drosophila Fng* does (Rauskolb et al., 1999; Zeltser et al., 2001). Misexpression of *LFng* across the ZLI also caused the downregulation of *Shh* expression at the ZLI, thereby perturbed the ZLI formation (Zeltser et al., 2001). Taken together, restricted *LFng* expression drives the formation and maintenance of the anterior and posterior limit of ZLI. It is not yet clear how other Notch molecules are related to the *LFng* function or ZLI formation.

1-8 The role of Notch molecules during hindbrain segregation

At the HB, neural epithelium segregates into rhombomeres, a process crucial to the proper segmental specification of the neurons and neural crest cells (Kiecker and Lumsden, 2005). Well-conserved *Hox* cluster gene expression regulates AP identity formation, and *val/Kr* and *Krox20* regulates formation of particular segments

(Manzanares et al., 1999; McKay et al., 1994; Schneider-Maunoury et al., 1997; Waskiewicz et al., 2002). *Krox20* expression in r3 and r5 regulates Eph receptors and ephrins which are required for the cell restriction between even and odd- numbered rhombomeres (Seitanidou et al., 1997; Theil et al., 1998). Each rhombomeric segment is restricted by boundary cells; cells with large intercellular spaces and distinctive shapes (Guthrie et al., 1991; Guthrie and Lumsden, 1991; Heyman et al., 1995; Heyman et al., 1993; Mellitzer et al., 1999; Xu et al., 1995; Xu et al., 1999). Importantly, neurogenesis is delayed in rhombomere boundary cells compared to the segmental cells. Neural differentiation first occurs at the centre of each rhombomeres, and later at the boundary region (Trevarrow et al., 1990).

In 2004, Cheng and his colleagues demonstrated that the Notch signalling pathway plays a similar role in the regulation of rhombomere boundary cell specification in zebrafish HB as it does in the *Drosophila* wing disc (Cheng et al., 2004). In the zebrafish HB, *deltaA* and *deltaD* are expressed in rhombomere segments, while *rfng* is expressed in boundaries (Cheng et al., 2004; Qiu et al., 2004). At the same time, expression of proneural genes such as *ngn1* or *ash* is restricted to the cells adjacent to the rhombomeric boundaries, and *wnt1* expression appears in the boundaries themselves (Amoyel et al., 2005; Cheng et al., 2004). As in *Drosophila*, the modulation of Notch by *rfng* at the boundary upregulates *wnt1* expression (Cheng et al., 2004). Cells expressing active Su(H) selectively migrate into the rhombomere boundaries, while cells in which Notch/Su(H) activity is downregulated selectively migrate out of the boundaries (Cheng et al., 2004). Blocking *rfng* via morpholino antisense oligonucleotides results in a loss of restriction of *wnt1* expression in boundaries (Cheng et al., 2004). Moreover, blocking proneural genes or *delta* expression leads to a significant expansion of the boundary markers (Amoyel et al., 2005). In contrast, *delta* was downregulated in the absence of the proneural genes, and these proneural genes were in turn downregulated when *wnt1* expression was blocked (Amoyel et al., 2005), Thus, unlike *wg* in *Drosophila*, *wnt1* in rhombomere boundaries is able to act as a long-range signal to support neurogenesis in rhombomeres, and *delta* and proneural genes may prevent boundary cells from expanding throughout the hindbrain (Amoyel et al., 2005).

1-9 *Hairy/E(spl)* family in the MHB

The MHB is another boundary which shows delayed neuronal differentiation (Bally-Cuif et al., 1993; Vaage, 1969; Wullimann and Knipp, 2000). Notch target genes, bHLH transcriptional factors *Hairy/enhancer of split [E(spl)]* act as repressors for proneural factors in neurogenesis (Fisher and Caudy, 1998a). At the MHB, *Hes1* and *Hes3* are expressed in the mouse and *her5* and *him (her11)* in the zebrafish (Bally-Cuif et al., 2000; Hirata et al., 2001; Muller et al., 1996; Ninkovic et al., 2005). *Hes1-/-; Hes3-/-* mice develop an abnormally small MHB region with partial absence of MHB marker gene expression, as well as a lack of midbrain-specific neurons (Baek et al., 2006; Hirata et al., 2001). Hes family members and Pax genes regulate each other at the MHB. Zebrafish *Pax2.1* mutants (*no isthmus*) do show initiation of *her5* expression, but do not maintain it over development (Lun and Brand, 1998). *Pax* expression is lost in the absence of *Hes1* and *Hes3* (Hirata et al., 2001). Furthermore, *her5* and *him* block neuronal genes *ngn1* and *coe2* at the medial MHB in dose-dependent manner (Geling et al., 2004; Ninkovic et al., 2005). Thus, expression of Hes family members at the MHB prevents neuronal differentiation across the medial MHB by inhibiting neurogenesis.

Hes family members are known to be Notch target molecules, however, the expression and activity of *her5* at the MHB is independent of the Notch signalling pathway. In zebrafish, *Her5* expression was not perturbed in a *notch1a* deficient mutant, *deadly-seven (des)*, and in this mutant, *ngn1* expression at the MHB was blocked (Geling et al., 2004; Holley et al., 2002; Kane et al., 1996). Loss of Notch activity did not severely affect *her5* expression at the MHB (Geling et al., 2004). Furthermore, misexpression of a dominant negative form of delta did not affect *her5* expression or MHB formation (Geling et al., 2004). However, ectopic expression of NICD blocked both *her5* and *ngn1* expression. Thus *her5* expression and function at the MHB is independent of the Notch signalling pathway. Recent studies demostrate some evidence of which not only *her5* but other Hes family members are expressed and function in Notch-independent manner. Amongst these, only *Hes1* shows any severe effect on its expression under both *Notch1* mutation and a lack of *Su(H)* (de la Pompa et al., 1997). Thus, it is also possible that *Hairy1* and *Hairy2* may be independent of Notch at the MHB in chick.

1-10 Aims of this thesis

The visible boundary between MB and HB (the morphological MHB) starts to form from HH stage 9 in chick. This morphological MHB coincides with the molecular MHB by HH stage 16 (Figure1.1). In this study I firstly characterise the expression patterns of all the major Notch related genes in the chick embryo at HH stage 10 when the molecular MHB is set. Genes involved in Notch signalling are expressed in restricted domains in the chick neural tube during the time period that the MHB forms. Sustained ectopic activation of the Notch signalling pathway perturbs expression of genes associated with the MHB organiser. Furthermore, cells containing ectopically activated Notch sort out of the MHB. *LFng* is specifically expressed in the domains that the activated Notch cells sort preferentially into- out of the metencephalic vesicle (rhombomeres 1 and 2) into the adjacent MB and HB compartments. In *Drospohila*, Fng influences Notch activation at boundaries of *Fng* expressing and non-expressing cells (Rauskolb et al., 1999). I predict that activated Notch cells acquire distinct affinity properties and move within the neural tube to a "like" environment, where Notch is normally active.

The MB and anterior HB form distinct lineage compartments from early stages of their development (Zervas et al., 2004). I propose that Notch signalling provides an affinity difference that is required to maintain midbrain and hindbrain as distinct compartments. How does Notch signalling mediate this affinity difference?
Cap and *Trn* are LRR proteins which are required to maintain DV compartments in *Drosophila* through the Notch signalling pathway. I show that chick *Trn* homologue, *Lrrn1*, is a candidate for mediating the active Notch cell affinity differences, as its expression demarcates the domain into which activated Notch cells sort. I will test the hypothesis that *Lrrn1* is mediating the cell affinity differences in Notch expressing cells through ectopic expression *in ovo*.

Gain of function analysis suggest a role for Notch signalling both in regulating cell affinity differences necessary to stabilise and maintain the organiser, and also in the formation of the MHB organiser. I find that Notch signalling is required for correct positioning of the MHB, and expression of *Fgf8* and *Wnt1* there. In the absence of Notch signalling, the MHB organiser does not form properly. The Notch ligands *Ser1* and *Ser2* have striking complementary expression profiles at the MHB, defining the

boundary. I predict that activation of Notch by *Ser1* or *Ser2* positons/maintains the boundary by promoting MB-specific MHB genes anteriorly and repressing r1-specific MHB genes posteriorly. I will test this hypothesis through functional analysis of *Ser1*.

Chapter 2 General materials and methods

2-1 Materials and solutions

In all cases, solutions were autoclaved at 121 °C for 30min.

2-1-1 Molecular Biology

50x TAE (pH8.5)

The stock TAE (pH8.5) was made in 50x concentration. 2M of Tris-base (Sigma) and 500mM of EDTA (Sigma) were dissolved in the Ultra-Pure water. In order to adjust pH of the solution, 5.71% of Acetic acid was added. The stock solution was autoclaved and stored at room temperature.

LB broth

20g of LB broth (Sigma) was dissolved within 1L of Ultra-Pure water. The solution was autoclaved and stored at room temperature. The relevant antibiotics were added after the broth had cooled down suitably.

LB Agar plates

37g of LB agar (Sigma) was dissolved in 1L of Ultra-Pure water. This mixture was shaken well and autoclaved. As soon as this agar had cooled sufficiently, the relevant antibiotics were added, and mixed well. This mixture was poured into 90mm Petri dishes before the agar had set.

2-1-2 Embryology

1x Phosphate Buffered Saline (PBS)

1 PBS tablets (Calbiochem) per 100ml were dissolved in the Ultra-Pure water. This buffer was autoclaved and stored at room temperature.

10x MEM (pH7.4)

1M of MOPS, 20mM of EGTA and 10mM of $MgSO4 \cdot 7H_2O$ were dissolved in autoclaved Ultra-Pure water. The pH was adjusted to 7.4 with NaOH, and sterilised through 0.22 μm Millex filter (Milllipore). The stock solution was stored at 4°C in the dark.

1x MEMFA fixative

10% of 10x MEM and 3.8% of Formaldehyde solution (BDH) were mixed in pre-autoclaved ultra-pure water. This solution was freshly made before each use.

4% Paraformaldehyde (PFA) fixative

4% of PFA (Sigma) was added to pre-autoclaved 1x PBS and heated to 65 °C to dissolve completely. The stock solution was aliquoted and stored at -20 °C.

10x ink solution

Tusche A Drawing ink A (Pelikan) was autoclaved and sterilised with 0.22 μm Millex filter (Milllipore). The stock solution was stored at room temperature.

1x ink solution

10x ink solution was diluted with 1x PBS solution which was sterilised with 0.22 μm Millex filter (Millipore) before use. This mixture was made by fresh each time to avoid contamination.

10x Fast Green

5% Fast Green FCF (BDH: 340304) and 50% Sucrose were dissolved in autoclaved Ultra-Pure water and push-filtered trough 0.45 μm Millex filter (Millipore). The stock was aliquoted and stored at 4 °C.

Electroporator

Electro Square Porator™ ECM 830 (BTX) was used for *in vivo* and *in vitro* electroporation.

The settings were as follows;

Voltage	25 volts
Pulse	4 pulses
Pulse length	50msec
Pulse interval	950msec

2-1-3 Whole mount in situ hybridisation

1M Tris-HCl buffer (pH7.5, pH8.0, pH9.5)

Tris base (Sigma) was dissolved in two thirds of the total required amount of Ultra-Pure water, and the pH was adjusted to 7.5, 8.0 and 9.5 with HCl. The volume was altered to the total required amount with Ultra-Pure water. The stock solution was autoclaved and stored at room temperature.

20xSSC (pH4.5)

3M of NaCl and 300mM $C_6H_5O_7Na_3 \cdot 2H_2O$ were dissolved in ultra-pure water. The pH was adjusted to 4.5 with Citric Acid. The stock solution was autoclaved and stored at room temperature.

MABT

100mM Maleic acid and 150mM of NaCl were dissolved in ultra-pure water.
The pH of the MAB stock solution was adjusted to 7.5 with NaOH, and autoclaved.
This MAB solution was stored at room temperature. 0.1% Tween20 (Sigma) was added before the solution was used.

BBR solution

10% Boehringer blocking reagent (BBR) was added to MAB and heated to 65°C to dissolve completely. This stock solution was autoclaved and stored at -20°C.

Hybridisation Buffer

2% of BBR was added to a mixture of 50% Formamide, 5x SSC (pH4.5), 5mM EDTA and 0.1% CHAPS. This mixture was heated to 65°C to dissolve the BBR completely. 50 µg/ml of Heparin, 50 µg/ ml of yeast tRNA and 0.1% of TritonX100 (Sigma) were added to the BBR/Formamide mixture and the volume was altered with autoclaved Ultra-Pure water. This buffer was made to be RNase free as far as possible. The stock solution was stored at -20°C.

Wash solution

50% Formamide and 1x SSC (pH4.5) were mixed. This solution was freshly made when required. 0.1% Tween 20 (Sigma) was added before use.

NTMT (pH9.5)

100mM NaCl, 100mM Tris-HCl (pH9.5) and 50mM $MgCl_2$ were freshly mixed when required to use. 0.1% Tween20 (Sigma) was added before use.

NTMT (pH8.0)

100mM NaCl, 100mM Tris-HCl (pH8.0) and 50mM $MgCl_2$ were freshly mixed when required. 0.1% Tween20 (Sigma) was added before use.

2-1-4 Histology

1x Saline

0.72% of NaCl was dissolved and autoclaved. The stock solution was stored at room temperature.

Gelatin embedding solution

7.5% Gelatin (Bovine Bone: Sigma), 15% Sucrose and 0.05% Sodium azide were dissolved into 1x PBS and heated up to 60°C. This solution was stored at room temperature.

2-2 Methods

2-2-1 Embryology

Animal handling

Chicken eggs were set pointed end down and humidified by sprinkling trays with pure water. The egg incubator was set at 38 °C with humidity. Eggs were incubated for the required length of time and staged according to Hamburger and Hamiliton.

Fixation of chick embryos

Embryos were explanted into cooled 1xPBS and were nicked at the anterior most of the neural tube. They were then fixed in MEMFA fixative or 4% PFA at 4 °C overnight.

Dehydration of chick embryos

Embryos were dehydrated through a 25%, 50%, 75% methanol series in PBST (1x PBS, 0.1%Tween 20). Each step was performed on ice for 15min. Embryos were then washed with 100% methanol once for 20min on ice, transferred to fresh 100% methanol and stored at –20 °C overnight.

2-2-2 Whole mount in situ hybridisation

2-2-2-1 Probe synthesis

Preparation of DNA

Plasmid DNA was cut to linearise at the 5' end of the gene of interest with appropriate enzyme. After cutting, linearised DNA was checked on an agarose gel. Restriction endonucleases were removed by using Qiagen Qiaquick Gel extraction kit (cat no. 28706) following Qiaquick Spin Handbook for PCR purification. DNA was eluted in 30µl nfH_20 (BDH).

Transcription

Linearised DNA was transcribed with appropriate RNA polymerase (T3, T7 or SP6). Exact proportions of reaction solution were mixed up carefully on ice.
1 µg of linearized DNA was mixed into 1x transcription buffer, 1x DTT, 10% DIG-nucleotide mix/ FITC-nucleotide mix, 5 % RNAsin RNase inhibitor and 5% RNA polymerase (T3, T7 or SP6) along with nfH_20 (BDH).
DNA was transcribed at 37°C for 2 hours, and checked on an agarose gel.

For *in situ* hybridisation with two probes:
The second probe was transcribed exactly as above, except that FITC-labelled UTP (Roche) was used in place of DIG-nucleotide mix.

Purification

The mRNA probe was purified by using ProbeQuant™ G-50 Micro Columns (GE Healthcare) according to manufacturers instructions and eluted in 50µl nfH_2O (BDH). 3 µl of purified probe was run on a 0.8 % agarose gel and checked for quality. 0.5 µl of RNAsin RNase inhibitor was added to each probe and they were stored at -20 °C.

2-2-2-2 *in situ* hybridisation (single colour: anti-DIG)

Embryos which had been stored in 100% MeOH at -20 °C were rehydrated by washing with 75%, 50% and 25% MeOH in PBT for 10min each. 25% MeOH/ PBT was replaced with PBT for 10min. After another wash in PBT for 10 min, the embryos were treated with 10µg/ml of Proteinase K in PBT according to embryonic stage: e.g. 7 min treatment up to HH14 stage, then 1 min extra per stage. In order to remove the

Proteinase K completely, this solution was carefully replaced with a large volume of PBT at room temperature. The embryos were refixed with 0.2% gluteraldehyde in MEMFA for 20 min at room temperature and washed three times in PBT with the aim of removing all fixative.

Hybridisation buffer was pre-warmed to 65 °C. The embryos were rinsed once with pre-warmed hybridisation buffer, which was replaced with fresh pre-warmed hybridisation buffer and incubated at 65-70 °C for more than 1 hour. This buffer was replaced with pre-warmed hybridisation solution containing approximately 1ug/ml of mRNA riboprobe and the embryos were incubated at 65-70 °C overnight. Post hybridisation, embryos were washed once with pre-warmed hybridisation solution for 30 min at the same temperature used for the hybridisation step. An excess of wash solution over the volume of the embryonic tissue was used for all washes. Embryos were then washed with pre-warmed Wash solution for 30min at 65-70 °C, followed by 50% Wash solution / 50% MABT for 20min at 65-70 °C. This solution was replaced with MABT for 10 min at room temperature. After another 10 min washing with MABT, the embryos were blocked for 2 hours in MABT/ 2% BBR/ 20% heat inactivated sheep serum. At the same time, anti-DIG alkaline phosphatase (Roche) was pre-incubated in same blocking buffer on ice at a 1:2000 dilution. After the incubation, the blocking solution was replaced with antibody solution overnight at 4 °C. The embryos were washed 6x 1hr in MABT and incubated within MABT overnight at 4 °C. This buffer was replaced with NTMT (pH9.5) 2x for 20 min at room temperature. Embryos were then incubated in NTMT (pH9.5) containing NBT/BCIP (NBT 4µl/ml, BCIP 3.5µl/ml) in the dark until colour had developed. After the colour had developed, the embryos were washed in MABT three times and post-fixed with MEMFA or 4% PFA for 20 min. This fixative was replaced with PBT. Following several washes with PBT, the embryos were transferred to 80% glycerol/PBT, and stored at 4 °C.

2-2-2-3 *in situ* hybridisations using two probes (blue and red colours)

For double colour *in situ* hybridisation. DIG- and FITC- labelled probes were added simultaneously at the hybridisation step. The embryos were then processed first with anti-DIG alkaline phosphatase as above. However, after the BCIP/NBT staining, the embryos were rinsed several times with MABT, and incubated in MABT at 70 °C for 30min. This MABT was replaced immediately with MABT at room temperature in

order to cool the embryos quickly. Following fixation with MEMFA or 4% paraformaldehyde for 20 min, the embryos were washed three times with MABT for 10 min each, and were pre-blocked again for at least 1 hour in MABT/ 2% BBR/ 20% heat inactivated sheep serum. At the same time, anti-FITC alkaline phosphatase was pre-incubated in MABT/ 2%BBR/ 20% heat inactivated sheep serum on ice at a 1:2000 dilution. After the blocking, embryos were incubated overnight at 4 °C. Post-antibody washing steps were carried out as for anti-DIG alkaline phosphatase. The embryos were rinsed in freshly made NTMT (pH8.0) and washed twice for 20 min each and incubated in freshly made FAST TR/Naphtol AS-MX solution (Sigma) in the dark until colour had developed. After the colour had developed, the embryos were washed in MABT three times and post-fixed with MEMFA or 4% PFA for 20 min. This fixative was replaced with PBT. Following several washes with PBT, the embryos were moved in 80% glycerol/PBT for photography and storage.

2-2-3 Whole mount Immunohistochemistory (anti GFP)

Fixed embryos were washed twice with PBT for 5 min, and were incubated in PBT with 20% heat-inactivated sheep serum for at least 1 hour at room temperature. This blocking buffer was replaced with PBT with 10% heat-inactivated sheep serum which containing 1:1000 dilution of anti-eGFP antibody (rabbit polyclonal: Clontech) at 4 °C overnight. The length of incubation can be extended in order to get a stronger signal. In particular, embryos already processed for double *in situ* hybridisation often require longer incubation (up to 7 days). Following incubation with primary antibody, embryos were washed for 3 hours in PBT/ 5% heat-inactivated sheep serum (6 times for 30min) and incubated in the 1:1000 dilution of HRP-conjugated anti-rabbit antibody (NEB) in 10% heat-inactivated sheep serum /PBT for overnight at 4 °C. Embryos were washed for 3 hours with PBS (3 times 1 hour) and developed according to manufactures instructions using FAST™ 3,3'-DIAMINOBENZIDINE tablets (Sigma) in the dark. After colour development, the colour reaction solution was removed, and the embryos were quickly rinsed with PBS several times. Following at least 5 washes with PBS, the embryos were post-fixed with MEMFA or 4% PFA for 20 min. This fixative was replaced with 3x PBT washes and embryos were transferred into 80% glycerol/PBT for photography and storage.

2-2-4 in ovo electroporation

Chicken eggs were set pointed end downwards 2 days prior to electroporation for HH8-9 stage. Using blunt #5 biological forceps, a small hole was made at the pointed end of the egg and 3ml of thin albumen was removed with 16 gauge needle. Also using blunt #5 biological forceps, a window to access the embryo was opened. Occasionally, it was necessary to inject 1x ink solution with 25 gauge needle underneath the embryo to visualize the embryos. DNA was injected using a Picospritzer III (Intracel) into the lumen of the neural tube. One to two drops of sterile room temperature PBS were added on the top of the embryo to moisten them well and allow them to recover quickly from the electro pulse. The electrodes were settled 1-2mm either side of the anterior half of the embryo, mainly targeting the MHB domain. Both micro-pipette and electrodes were held by a micro-manipulator. Electrodes were carefully removed and eggs were sealed with Sellotape. The manipulated eggs were put back into the incubator as quickly as possible to allow them to recover from electro pulses.

2-2-5 Histology

If embedding was carried out straight after collection and fixation, the embryos were washed in PBS or Saline once. If this was after colour reaction, the embryos were washed in PBS or Saline twice for 10 min each. If this was after mounting in glycerol, the sample was washed in PBS or Saline four times for 30 min each.

2-2-5-1 O.C.T. embedding and frozen section

The embryos were washed 3 times for 10 min each in PBS, and then 10% sucrose/ PBS, 20% sucrose/ PBS and finally 30% sucrose/ PBS. In each case, the embryos were incubated in those solutions at 4 °C or on ice until the sample sinks. Following this incubation with sucrose, the embryos were moved into O.C.T (Sakura) and incubated for 30min at 4 °C. The O.C.T was replaced with fresh O.C.T and samples were left overnight at 4 °C to allow O.C.T to be absorbed by tissues. O.C.T was poured cautiously to prevent bubbles into the disposable base moulds (7x7x5mm) and a single embryo was placed into an individual mould. Each embryo was carefully orientated toward the necessary direction and the mould was then moved immediately on top of dry ice in order to freeze rapidly. Sectioning was carried out using a Cryostat set to 10-

25 μm thickness. Each section was picked up onto a Polysine coated slide glass (VWR) individually. Cover glasses were mounted using Aquamount (BDH) solution.

2-2-5-2 Gelatin embedding

In order to achieve better orientation of more difficult samples, some embryos were embedded in gelatine before O.C.T. These embryos were incubated in 15% sucrose/ PBS overnight. They were then moved to pre-heated gelatin embedding solution and incubated for 3-4 hours at 37°C in order to permit gelatin to be absorbed into cells absolutely. Gelatin embedding solution was cautiously poured into the disposable base moulds (7x7x5mm) and a single embryo was placed into each mould. This mould was moved onto ice and embryos were re-orientated. After the gelatin had set properly, the block was removed from the mould, and cut with a razor blade to define a straight and parallel surface of the block which accommodates the embryo for subsequent orientation. This block was put into a new mould and covered with O.C.T. Each mould was immediately moved onto dry ice, and frozen rapidly. They were then sectioned as described above.

2-2-5-3 Wax embedding and section

Samples were re-fixed in 4 % PFA for 1 hour, and washed in PBS twice for 15 min each. Following PBS washing, the embryos were moved to 100% MeOH for 5 min, and were incubated in Propan-2-ol for 10 min. The embryos were then incubated with Tetrahydronapthalene for 1 hour. This was then replaced with fresh solution and the embryos were incubated for 30min more. The embryos were then moved to ready-warmed wax and incubated 1 hour at 65 °C. Wax was changed twice for one hour each. During this process, the glass container of each embryo was kept at 65 °C together with glass pipettes and other tools to avoid the wax cooling down and setting. Subsequent to the wax washing (the smell and colour of Tetrahydronapthalene should have gone), the embryos were embedded into the disposable base moulds (16x16x5mm) and orientated with pre-warmed needles. Embryos were sectioned with microtome at 15-20μm. The ribbon of sections was then moved onto the pre-warmed slide glasses covered with clean water, allowing the ribbons to de-crease. The slides were kept on the hotplate until the water on the slides had evaporated completely and the sections had adhered to the glass. The slides were transferred to slide racks and dipped into histoclear for 10 min. Following another wash within the clean histoclear for another 10 minutes, the slides

were drained slightly and carefully mounted with DPX or Canada Balsam. In order to avoid trapping air bubbles between the slide glass and cover slip, in some cases, Canada Balsam was diluted to 50% with histoclear.

2-2-6 Photography

Whole embryos were photographed by using Nikon digital camera (DXM1200F) attached to the Nikon SMZ1500 microscope or Nikon ECLIPSE 80i compound microscope. Photographs were taken with using ACT-1 (Nikon) software in Jpg format. Colour levels and contrast were adjusted with using Adobe Photoshop 7.0 and CS2. All figures were compiled in Adobe Photoshop 7.0 and CS2.

Chapter 3 Gene expression analysis of Notch related genes

3-1 Introduction

Local organisers at boundaries have been best studied in invertebrates (*Drosophila*) where the Notch signalling pathway is important. Recent studies suggest that this signalling cascade may also control boundary and organiser formation in vertebrates. In order to test the hypothesis that Notch signalling may be instrumental in boundary formation at the MHB organiser, I first analysed the expression patterns of a number of key genes identified as involved in the Notch signalling pathway.

3-1-1 The expression pattern of Notch modulator, Fringe.

Fringe (*Fng*), a glycosyltransferase, is one of the key molecules of Notch signalling as it is the glycosylation of Notch receptor by *Fng* that alters the sensitivity of this receptor to its ligands *Delta* and *Serrate* (Yang et al., 2005). In *Drosophila*, it is known that Notch receptor modulated by *Fng* is more susceptible to activation by *Delta* and less susceptible to activation by *Serrate*. As a result of this process, Notch is strongly activated only at the interface between two cells, one co-expressing *Fng*, *Notch* and *Serrate*, and the other co-expressing *Notch* and *Delta*. Therefore, an on/off border of *Fng* expression together with the balance of Notch ligands expressed either side is significant for the process of boundary formation by Notch signalling.

In vertebrates, three *Fng* homologues have been identified; *Lunatic Fringe* (*LFng*), *Manic Fringe* (*MFng*) and *Radical Fringe* (*RFng*). Mouse *Fngs* were reported to be implicated in hindbrain boundary determination due to their expression patterns, however, little is known about their role at the MHB. In mouse, *MFng* and *LFng* are expressed selectively in pre-rhombomeres r3 and r5 in the 10 somite embryo (Johnston et al., 1997). In chick, *cLFng* was first cloned and its expression pattern was described by Sakamoto and his colleagues (Sakamoto et al., 1997). In particular, its expression at the presometic mesoderm (PSM) is well characterised (McGrew et al., 1998; Sakamoto et al., 1997). In the CNS, *LFng* expression in the HB and spinal cord, as well as the selective exclusion from the ZLI are reported (Sakamoto et al., 1997; Zeltser et al., 2001). In contrast, the expression of *cRFng* has only been described in the limb bud, its expression in the CNS is not yet known (Laufer et al., 1997; Rodriguez-Esteban et al.,

1997). In zebrafish, *rfng* is expressed in a boundary restricted manner in the HB (Cheng et al., 2004), and is also found at the MB, but is absent from r1 (Qiu et al., 2004). However, *mRFng* expression is described to appear broadly in the anterior neural tube, and does not become restricted to either the MB nor the HB boundaries (Johnston et al., 1997). With these differences in mind, it is important to analyse *cRFng* expression in the chick CNS. Similarly, like *mLFng*, *mMFng* also shows distinctive localisation in particular rhombomeres. However, chick *MFng* has not yet been described. It is clear therefore that large gaps exist in our understanding of the expression and roles of the fringe homologues in the chick model.

3-1-2 Notch receptor and Notch ligands

In *Drosophila*, Notch receptor has two major ligands, Delta and Serrate. These are transmembrane molecules containing EGF repeated domain, normally present on cells adjacent to those with the Notch receptor at the membrane. In vertebrates, four Notch receptors (Notch1, 2, 3, 4), three Deltas (Delta1, 3, 4) and two Serrates (Serrate1, 2) have been reported (Kopan and Weintraub, 1993; Lardelli et al., 1994; Lindsell et al., 1995; Myat et al., 1996; Weinmaster et al., 1991; Weinmaster et al., 1992). In chick, *cNotch1* has been cloned and its expression pattern characterised (Myat et al., 1996). Like other mammalian homologues, *cNotch1* is expressed broadly in the ventricular zone, except in the floor plate and roof plate in early development (Coffman et al., 1990; Myat et al., 1996; Reaume et al., 1992; Weinmaster et al., 1991). In contrast, the expression patterns of the ligands are different. *cDelta1*(*cDl1*) was first identified by Henrique and his colleagues (Henrique et al., 1995). *cDelta1* expression in the neural plate is first detected at HH stage 6, later developing in the mid and posterior HB (Henrique et al., 1995; Raya et al., 2004). Importantly, its expression coincides with the neurogenesis period (Chitnis, 1995; Henrique et al., 1995). The expression patterns of the *Serrate*s have also been characterised. *cSerrate1* (c*Ser1*) was first cloned by Henrique and his colleagues, and its gene expression was reported later by Myat and her colleagues (Henrique et al., 1995; Myat et al., 1996). Unlike *cDl1*, *cSer1* expression in the CNS is first found in FB and HB at HH stage 8 (Myat et al., 1996). The expression in the MB is gradually found at later stages, but there has been no report of *cSer1* expression in the MHB. Comparison of *cDl1* and *cSer1* confirmed that these two expressions are complementary in the HB and spinal cord (Myat et al., 1996). *cSerrate2* (c*Ser2*) expression is more striking. Initiation of *cSer2* expression is reported at HH

stage 11 in the FB and MB (Hayashi et al., 1996). Expression in telencephalon is strongly maintained throughout the development. Interestingly, in the section of FB, both *cSer2* and *cNotch1* expression appears in all cells, while *cSer1* and *cDl1* expression in the same region is in a scattered manner (Hayashi et al., 1996). The functional reason of this expression difference is not yet clear. However, it will be crucial to identify the pattern of expression that these genes take during MHB formation.

3-1-3 Hairy/E(spl) family members

The downstream targets of Notch signalling, Hairy/E(spl) family members, have been reported to be expressed within the MHB in various species (Bally-Cuif et al., 2000; Hirata et al., 2001; Muller et al., 1996; Ninkovic et al., 2005). In the chick, *cHairy1,* which has high homology to *Xhairy1,* was cloned by Palmeirim and his colleagues in 1997 (Palmeirim et al., 1997). Soon after, another *Hairy* gene, *cHairy2* was identified (Jouve et al., 2000). Both *cHairy1* and *cHairy2* show cyclical expression patterns at the PSM. *cHairy1* and *cHairy2* exhibit a similar anterior expression border; however, the expression in the rostral-most PSM appears to be complementary (Jouve et al., 2000). In zebrafish, both *cHairy1* homologue *her9* and *cHairy2* homologue *her6* are expressed in the MB during early CNS development (Leve et al., 2001; Pasini et al., 2001). In paraxial mesoderm, *her6* expression is reported to be Notch signalling-dependent. Silencing Notch signalling by dn-*Su(H)* injection suppressed/downregulated her6 expression in both the anterior PSM and somites, while da-*Su(H)* misexpression resulted the ectopic expression of *her6* throughout the PSM (Pasini et al., 2004). However, several studies demonstrated that some *Hairy/ E(spl)* family members are regulated and act in Notch-independent manner. *her6* expression is not induced by Notch1a-ICD misexpression in the posterior PSM (Pasini et al., 2004; Takke and Campos-Ortega, 1999). In mice, a *Notch1* mutation does not affect *cHairy2/her6* homologue *Hes*1 expression (de la Pompa et al., 1997). *HES1* is originally thought to be a downstream gene of Notch signalling in the PSM since *HES1* is strongly downregulated in *Dl1* knockout mice (Jouve et al., 2000), however, a lack of *Su(H)* also does not disrupt *Hes1* expression (de la Pompa et al., 1997). More recently, *in vitro* assays demonstrated that *Hes1* is mediated by Notch-independent mechanisms in some cases (Curry et al., 2006; Stockhausen et al., 2005). In both human neuroblastma cells and epithelial cells, human *Hes1* expression was observed in the absence of Notch activation (de la Pompa et al., 1997). Strikingly, *Hes1* was induced by transforming

growth factor (TGF)-α in MAP kinase ERK signalling-dependent manner (Stockhausen et al., 2005). In human epithelial cells, *Hes1* expression is mediated by a Notch-independent mechanism involving c-jun N-terminal protein kinase (JNK) signalling (Curry et al., 2006). *cHairy1* homologue *her9* is also reported to be induced in a Notch-independent manner in proneural region (Bae et al., 2005). Application of DAPT, a gamma secretase chemical inhibitor, blocks Notch activity by preventing Notch cleavage. Zebrafish *mind bomb* (*mib*) mutant lacks Delta ubiquitin ligase and strongly reduces Notch activity (Itoh et al., 2003). Under the Notch activity disruption in both cases, *her9* expression was observed in early segmental stages (Bae et al., 2005). Taken together, both *cHairy1* and *cHairy2* are also likely to be regulated by a Notch-independent mechanism.

3-1-4 LRR protein, cLrrn1

As previously discussed, cell lineage restriction, a pivotal event during boundary formation, requires cell affinity differences between two compartments (Garcia-Bellido, 1975). In the *Drosophila* wing disc, *ap* target genes *cap* and *tartan* are found to provide the D cell affinity (Milan et al., 2001). In mouse, homologues of *tartan*, *Leucine-rich repeat neuronal* (*Lrrn*) 1, 2, 3, have been identified (Taguchi et al., 1996; Taniguchi et al., 1996). All three of the *Lrrn*s have been reported to be expressed in the developing CNS in mouse. Interestingly, in *Xenopus*, *XLrrn1* was reported to restrict its expression in ventricular zone from the diencephalon to the HB (Hayata et al., 1998). Recently, an EST, 2B10, screened by subtractive hybridisation of HB in chick was identified as *cLrrn1* (Christiansen et al., 2001; Garcia-Calero et al., 2006). Remarkably, *cLrrn1* expression is strongly excluded from the MHB throughout the chick development, as well as from the dorsal and ventral midline (Andreae et al., 2007; Garcia-Calero et al., 2006). Furthermore, *cLrrn1* was seen to be downregulated at both the ZLI and HB boundaries in early CNS development (Andreae et al., 2007). *In situ* hybridasation analysis of *cLrrn1* showed the selective exclusion of *cLrrn1* from all major inter-boundary domains in the early CNS, while its mRNA was clearly localised in the neuroepithelium. The function and mechanism of action of *Lrrn1* is not yet clear but within a HeLa cell, *Lrrn1* shows early endosomal localisation, indicating that *Lrrn1* may play a role in vesicle trafficking and signal transduction (Andreae et al., 2007). Furthermore, family members of leucine-rich repeat containing proteins are reported to interact with major signalling pathways. For example, one member of FLRT

(fibronectin leucine-rich transmembrane protein) family binds directly to FGF receptors and positively modulates FGF-MAP signalling (Bottcher et al., 2004; Haines et al., 2006). Thus, it is important to know the precise expression and timing when *Lrrn1* is absent from the MHB in order to understand the function of *Lrrn1* during MHB formation.

3-1-5 Notch related genes and MHB formation

During early mammalian development, *Otx2* is initially expressed before gastrulation throughout the epiblast. Its expression then becomes limited to the rostral side of the embryo by the headfold stage (Li and Joyner 2001). *Gbx2* is also first detected during gastrulation in all three germ layers from posterior of embryo to prospective HB, and its expression later extends anteriorly (Wassarman et al., 1997). The anterior limit of *Gbx2* is known to abut the posterior domain of *Otx2* by E7.5 in mouse embryo. *Fgf8* and *Wnt1* expression are observed broadly and complementarily at E8.5, however, by E9.5 the expression domains of these two genes become restricted to two significantly sharp transverse rings which sit next to each other at the MHB. It is important to determine how the expression domains of Notch related genes are linked with MHB molecules at this crucial time period.

In order to examine Notch signalling during formation of MHB, I performed whole mount *in situ* hybridisation analysis of major Notch related genes: Notch receptor *cNotch1*, Notch ligands, *cDll1*, *cSer1*, and *cSer2*, Notch effectors, *cHairy1* and *cHairy2*, and Notch modulators, *cLFng*, *cMFng* and *cRFng*. I also looked at the expression pattern of the LRR protein *cLrrn1*. I particularly focused on HH stage 9-11 - stages when expression of *Fgf8* and *Wnt1* are restricted around the MHB. Interestingly, in the neural tube at these stages, the *Otx2/Gbx2* interface appears rostral to the morphological constriction of the MHB. These two MHBs, molecular and morphological, are reported to meet in the mouse embryo by E9.5 and in the chick by HH stage 20 (Hidalgo-Sanchez et al., 2005; Liu and Joyner, 2001a). My results reveal that Notch ligands, effectors, modulators and cell adhesion molecules have expression boundaries that coincide with molecular MHB at HH stage 10.

3-2 Materials and methods

3-2-1 Cloning of chicken manic fringe

Chicken EST clone ChEST197M8 (received from MRC geneservice) was used. The clone was streaked onto LB agar and grown in LB media which contained ampicillin.

3-2-2 Sequence comparisons and phylogeny analysis

Protein alignments were performed by using GENETIX-MAC Ver. 10.1 (Software Development Co).The GenBank accession numbers of the compared genes are: chicken *mfng*, XM_416278; mouse *mfng*, NM_008595; zebrafish *mfng*, NM_001007788; chicken *lfng*, GGU91849; mouse *lfng*, NM_008494; chicken *rfng*, GGU82088; and mouse *rfng*, NM_009053. A similarity tree was generated with NJ methods by using GENETIX-MAC Ver. 10.1.

3-3 Results

3-3-1 LFng and MFng expression change in MHB related manner.

3-3-1-1 Cloning and sequence analysis of cMFng.

To determine the requirement for *cMFng* during MHB formation, I firstly isolated *cMFng* EST clone. The BBSRC chick EST database (http://www.chick.manchester.ac.uk/) was searched for a potential candidate for the *cMFng* homologue, and a predicted EST clone was identified; ChEST197M8. This partial *cMFng* encodes a protein of 197 amino acids. The protein contains potential N-linked glycosylation sites, one DXD-motif and conserved cysteins which are characteristic of the *Fng* family (Johnston et al., 1997; Qiu et al., 2004). The *cMFng* DNA sequence was compared to the recently reported sequence of full-length *cMFng* (XM_416278), which encodes for a protein of 352 amino acids. The sequence homology between both clones was 100% identical (data not shown), therefore, this full-length *cMFng* was used for the further sequence analysis. The amino acid sequence of the EST partial *cMFng* clone can be seen as a blue line in Figure 3.1A. Sequence comparison indicated that this EST clone is homologous to mMFng with 62.9 % amino acid sequence identity, while it matches cLFng, mLFng, cRFng and mRFng with 49.7%, 50.5%, 48.3% and 45.8% homology respectively. The outcomes of a similarity tree, together with other features of the sequence that are consistent between species, tend to

confirm that this is a chicken homologue of *MFng* (Figure 3.1B). Therefore, we used this clone for further analysis of *MFng* expression in the chick.

3-3-1-2 *cMFng* expression is strongly restricted to r3 and r5 throughout early development.

In order to examine the expression of *cMFng*, I carried out whole mount *in situ* hybridisation in chick embryos at HH stage 6-14. A few *cMFng* positive cells were detected around the neural folds at 4 somite stage (Figures 3.2A, F). Expression in the neural tube was first observed at the 9 somite stage and was strongly restricted to pre-rhombomere 3 (pre-r3) (Figure 3.2B, G and K). From the 10 somite stage, *cMFng* expression was observed in r5 as well as r3 (Figure 3.2C-E, H-J, and L-N). The strong expression at r3 was maintained between 9 and 12 somite stage (Figure 3.2K-M), however, it appeared to weaken at the 14 somite stage (Figure 3.2N). Interestingly, the expression at the MHB which was observed weakly at 9 somite stage, disappeared at the 10 somite stage, and reappeared at the 14 somite stage (Figure 3.2K-N). Transverse sections of the neural tube revealed that *cMFng* expression at both r3 and r5 was limited to the ventral half of the neural tube and excluded from the roof and floor plates (Figure 3.2P). In contrast, expression at the MHB at 14 somite stage appeared to be universally distributed throughout the neural tube, both dorsally and ventrally (Figure 3.2Q). These results describe that *cMFng* is expressed at the MHB at HH stage 9 when the MHB formation begins, but this expression is very short term. The *cMFng* restriction in r3 and r5 is parallel to what was observed in mouse (Johnston et al., 1997). However, *mMFng* is also expressed throughout the anterior neural tube, as well as these particular rhombomeres at the early development (Johnston et al., 1997).

3-3-1-3 *cRFng* is not restricted within the CNS during the stage when the MHB is formed.

cRFng expression was detected in ectodermal tissue from HH stage 7 through to HH stage 10 (Figure 3.3A-E). At HH stage 11, the intense expression previously observed throughout the entire neural tube became more restricted to the anterior CNS (Figure 3.3F, G). Transverse sections of MB and HB indicate that the expression in both areas remains within the neural tube (Figure 3.3H, I). These results are distinctively similar to those documented in the mouse (Johnston et al., 1997).

3-3-1-4 *cLFng* expression is excluded from the MHB.

cLFng expression was observed as early as at HH stage 4 (Figure 3.4A). At the 5 somite stage, expression of *cLFng* is restricted to the neural tube, including the MHB, and presumptive mesoderm (Figure 3.4B-E). Interestingly, this expression becomes weaker at the MHB by HH stage 9 (Figure 3.4F-I). The anterior most edge of this absence moves posteriorly by HH stage 10, but does not cross the morphological MHB (Figure 3.4L-P). Transverse sections of the neural tube show that *cLFng* expression is excluded from both the roof plate and floor plate (Figure 3.4J, K). Between 10 and 13 somite stages, strong mRNA expression was observed in the MB, r3 and r4, but not at the MHB and r1 (Figure 3.4L-O, Q and R). Some weak expression was detected in r2. At HH stage 14, *cLFng* was expressed widely in the head region, except for the ZLI (Figure 3.4S). *cLFng* transcripts were stronger in r1 than r2 at HH stage 16 (Figure 3.4T, U). These results indicate that *cLFng* expression is excluded from the MHB between HH stage 9-11 when the MHB is formed, however, its expression both anteriorly and posteriorly forms a border either side of the organiser.

3-3-2 Notch ligands are expressed within/around the MHB

3-3-2-1 *cDelta1* expression follows the morphological boundary of the MHB

The expression of *cDl1* was strongly detected within the caudal region of the neural tube including the presomitic mesoderm. Expression in the neural tube was only observed after HH stage 8 (Figure 3.5A-E). At the 7 somite stage, *cDl1* mRNA was expressed in the telencephalon and diencephalon, but not in the optic cup (Figure 3.5D, E). Expression in the MB was weaker than that observed in the region caudal to r1 (Figure 3.5E). The same expression pattern was also detected in embryos older than HH stage 9 (Figure 3.5F-M). Flatmount analysis of the neural tube showed that *cDl1* transcripts were localised within the cytoplasm mainly to r1 (Figure 3.5P). *Delta* is expressed in prospective neurons during neurogenesis (Chitnis et al., 1995; Henrique et al., 1995). Interestingly, clear transcript localisation at the nucleus was observed within a number of cells in r3 and other parts of the caudal HB (Figure 3.5K, Q). *cDl1* transcripts are also observed scattered towards the cell surface in the same cell (Figure 3.5Q). The active transcription of *cDl1* at these cells indicates that these cells are neurogenic, thus, committing to neuron. This is supported with the observation of cytoplasmic localisation of *cDl1* mRNA indicating that translation is occurring.

3-3-2-2 *cSerrate1* mRNA is excluded from the MHB domain during early development

The expression of *cSer1* in neurogenic ectoderm was first visible at HH stage 6 (Figure 3.6A). By HH stage 8, *cSer1* mRNA was observed in the cranial ectoderm and posterior part of neural fold (Figure 3.6B, C). At HH stage 9, expression in the posterior of the neural folds started to become restricted mainly within presumptive r3 and r5. Expression of *cSer1* also became visible in the diencephalon at this stage (Figure 3.6D-I). Strikingly, the anterior limit of *cSer1* expression in the HB shifted anteriorly to the presumptive r2 territory, and the posterior limit of the expression in the diencephalon shifted posteriorly to the MHB at 9 somite stage (Figure 3.6H, I). Expression in r2 was excluded from the roof and floor plates, while the neural tube at the MHB contained less *cSer1* transcript than in the adjacent segment r2 (Figure 3.6J, K). By the 11 somite stage, expression within the MB had moved posteriorly, close to the MHB, while the expression in r2 moved anteriorly. There was a gradual downregulation of *cSer1* at the MHB (Figure 3.6L, M). This gradient was replaced by a clear boundary by HH stage 11 (Figure 3.6N, O). At HH stage 14, *cSer1* transcripts were clearly localised within neuromere D2, lens vesicle and otic vesicle (Figure 3.6P). Analysis at higher magnification showed variation in intracellular localisation of *cSer1* mRNA in the neural tube. Transcripts were mainly localised to the cell surface, particularly in the MB, r3 and r5, and at low levels in the MHB. A few scattered cells contained transcripts in the cytoplasm (Figure 3.6Q-T). At HH stage 16, a striking striped expression pattern in the HB was seen, probably correlating to neurogenesis (Figure 3.6U). Moreover, intense expression was observed in the HB boundaries (White arrow heads).

3-3-2-3 *cSerrate2* expression is highly restricted in the telencephalon and the MHB.

Expression of *cSer2* in the neuroectoderm was not observed until the 7 somite stage. At 8 somite stage, *cSer2* was detected in the telencephalon, but not MB or HB (Figure 3.7A, B). By 10 somites, there was expression at the MHB, and levels were maintained in the telencephalon (Figure 3.7C-E). Flatmounting these samples allowed visualisation of a graded expression pattern towards the MB and a clear exclusion from floorplate (Figure 3.7E). Expression at the MHB was seen throughout the entire dorsoventral span of the neural tube, except for the floor plate (Figure 3.7K). At HH stage 11, the expression in the MHB was maintained strongly (Figure 3.7F-I). Interestingly, this *cSer2* expression abutted the anterior border of *Fgf8* expression (Figure 3.7M-O).

Strikingly, *cSer2* expression in the MB was only observed within the ventral half of the neural tube, and was not seen in either the dorsal half or the floor plate (Figure 3.7O).

3-3-3 cNotch1 is broadly expressed in the CNS

cNotch1 mRNA expression was observed as early as HH stage 4 in the primitive folds, but excluded from Hensen's node (Figure 3.8A). By HH stage 7, expression appeared within the neural plate (Figure 3.8B, C and J). This ubiquitous expression in the neural plate was seen at 5 somite stage, but was excluded from the telencephalic area (Figure 3.8D, E). From HH stage 8, expression in the anterior most part of the telencephalon was detected (Figure 3.8F-I). Expression at the MHB was observed to be weakened at HH stage 10 (Figure 3.8H, I, L, M). From HH stage 11, *cNotch1* transcripts appeared within r1 (Figure 3.8N-P). In order to investigate the weakened expression at the MHB further, the neural tube of 10 somite stage was flat mounted. Interestingly, when cells from the MB and r1 were analysed under high-power magnification, highly localised transcripts were found in the MB side of tissue, something that was rarely seen in r1 (Figure 3.8S, T). By HH stage 17, the expression of *cNotch1* was observed throughout the entire embryo. Notably, the expression in cells either side of HB boundaries was stronger than inter-boundary expression (Figure 3.8U). Significantly, HH stage 17 and 18 is the time period of morphological emergence of the hindbrain boundary cells (Heyman et al., 1995; Heyman et al., 1993). This coincidence suggests that Notch1 downregulation may be involved in the HB boundary cell specification or maintenance.

3-3-4 cHairy1 and cHairy2 are expressed at the MHB at HH stage 10.

cHairy1 mRNA was observed only in Hensen's node at HH stage 5 (Figure 3.9A). At HH stage 7, *cHairy1* expression is found throughout the neural tube (Figure 3.9B-E). At HH stage 9, *cHairy1* was downregulated in r1, while the expression in r2 remained strong (Figure 3.9F, G). This expression appeared to be maintained as development proceeded through to HH stage 11 (Figure 3.9H, I, L, M). Interestingly, unlike both 9 and 17 somite stage embryos, 10 somite stage embryos showed a clear restriction of cHairy1 at both the MHB and in r3, and little expression was observed within r2 (Figure 3.9N-P).

cHairy2 mRNA was detected almost exclusively in the primitive streak at HH stage 5 (Figure 3.10A). Strong telencephalic expression was consistent throughout development. In particular, the expression in the optic cups was striking (Figure 3.10G, I, M, R and T). Transverse sections of 9 somite stage embryos indicated that *cHairy2* was expressed in the floor plate, but not in the notochord (Figure 3.10J and K). Stronger expression of *cHairy2* within the neural tube was detected in the MB, r1 and r3 at the 9 somite stage (Figure 3.10N), and subsequently in the MHB at the 10 somite stage (Figure 3.10O). Strong expression was observed in r3, however, this was restricted to the posterior half of r2 (brackets). By the 14 somite stage, expression in the MHB had spread anteriorly into the FB, while the expression in r3 was observed in the whole segment (Figure 3.10P). Interestingly, zebrafish *Hairy2* homologue, *her6*, is also reported to be expressed strongly in r3 and r5 which coincides with *Krox20* expression in similar stage embryos (Pasini et al., 2001). Thus, *Hairy2* may be regulated in a similar manner to *her6,* which is independent of Notch signalling.

3-3-5 cLrrn1 transcripts are excluded from the MHB at HH stage 9.

The mRNA expression of *cLrrn1* is limited to the neurogenic ectodermal tissue during early chick development. Strong expression at the level of the anterior neural folds was observed from HH stage 7 (Figure 3.11A). This expression pattern had not changed by the 4 somite stage (Figure 3.11B). *cLrrn1* transcripts were restricted within the ectodermal tissue, but excluded from both roof and floor plates (Figre 3.11E). From the 5 somite stage, a gradual downregulation at the presumptive MHB was observed (Figure 3.11C, D). At HH stage 9, this downregulation was still strongly evident around the MHB, but was also apparent in the MB (Figure 3.11G-J). A flat mount preparation of the neural tube showed that the downregulation around the MHB did not possess a clear border to its expression (Figure 3.11K). Interestingly, by the 12 somite stage, this gradual downregulation showed restriction to a clear domain corresponding to the MHB region (Figure 3.11N, O). *cLrrn1* expression at HH stage 15 was localised strongly within the MB and rhombomeres, posterior from r2, but not in the MHB (Figure 3.11P). These analyses show a clear co-exclusion of *cLrrn1* and *cLFng* at the MHB from HH stage 9 to HH stage 12.

3-3-6 The expression of Notch related genes changes dramatically within the MHB domain between HH stage 9 and 11.

In order to understand the relationship between the expression patterns of Notch pathway genes and MHB formation, the expression of Notch pathway genes at HH stage 10 were compared with those of known MHB markers, *cOtx2*, *cGbx2*, *cFgf8* and *cWnt1*.

3-3-6-1 *cLFng*, *cLrrn1* and *cSerrate1* are excluded from the MHB domain.

The expression of *cLFng* and *cLrrn1* was excluded from the MHB domain at HH stage 10 (Figure 3.12B-D). Remarkably, the expression of *cSer1* started to accumulate in this domain from HH stage 9, but was restricted to outside of the MHB domain where coincide to the *cLFng* and *cLrrn1* expressing domain. Two colour *in situ* hybridisation of *cSer1* and *cFgf8* revealed that the anterior border of *cFgf8* expression clearly abuts the posterior limit of *cSer1* MB expression (Figure 3.12O, P). *Fgf8* is known to be expressed at the MHB and the anterior limit of its expression is seen to be adjacent to the *Otx2/Gbx2* interface. When compared with the expression of *cOtx2* and *cGbx2*, the posterior limit of these two Notch pathway genes as well as cell adhesion molecule *cLrrn1* appears to be at the same position as *Otx2/Gbx2* interface (Figure 3.12A-D; black arrow heads). Interestingly, intracellular localisation of *Notch1* was also seen at the *Otx2/Gbx2* interface at 10 somite stage, suggests that *Notch1* is actively transcribed at the area (Figure 3.8Q, 3.12E).

3-3-6-2 The expression of *cHairy1* and *cSerrate2* are restricted within the MHB.

It is interesting to note that both the Notch ligand *cSer2*, and Notch effector *cHairy1* are strongly expressed at the MHB at 10 somite stage (Figure 3.12G, H). Comparison with *cFgf8* expression shows that these three genes occupy the same domain. *cHairy2* is also expressed in the MHB, although its range is wider, extending from MB to r1.2 (Figure 3.12I).

3-3-6-3 *cMFng* and *cDelta1* are expressed in HB segments.

cDl1 is expressed strongly in r1 and then posterior from r3 (figure 3.5N, 3.12M). *cMFng* expression at HH stage 10 is restricted within r3 and r5 (Figure 3.2L). Neither of

these genes observes expression boundaries that coincide with the MHB, although both are restricted within HB boundaries.

Figure 3.1 Alignment and phylogeny of the chick Manic Fringe protein with other vertebrate counterparts.

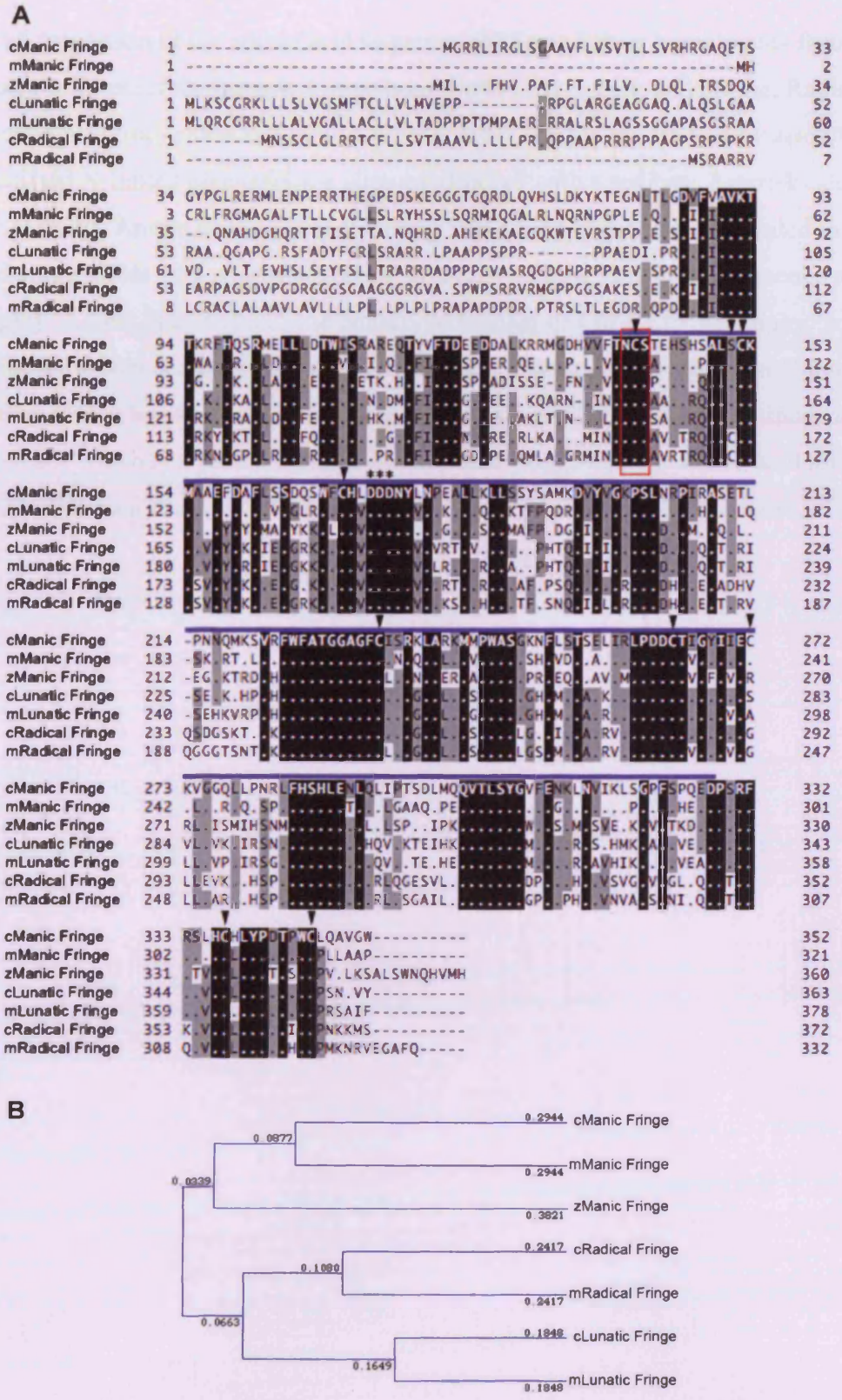


Figure 3.1 Alignment and phylogeny of the chick Manic Fringe protein with other vertebrate counterparts.

(A) Comparison of the amino acid sequence of Manic Fringe homologues from chick, mouse and zebrafish, Lunatic Fringe homologues from chick and mouse, Radical Fringe homologues from chick and mouse. Conserved cysteines are marked by arrowheads and predicted N-linked glycosylation sites are denoted with a red box. Asterisks show the DXD-motif. Amino acids identical among 50% of the sequences are shaded in black and amino acids with a light degree of similarity among 50% of the sequence are shaded in grey. A blue line indicates the conserved domain of ChEST197M8 amino acid sequence which is a partial form of chick Manic Fringe and the amino acid matches 100%. A blue line indicates the conserved domain of ChEST197M8 amino acid sequence which is a partial form of chick Manic Fringe and the amino acid matches 100%. (B) Similarity tree of vertebrate Fringe proteins. c; chick, m; mouse, z; zebrafish.

Figure 3.2(1) c*MFng* expression is limited to the MHB, r3 and r5 during early development.

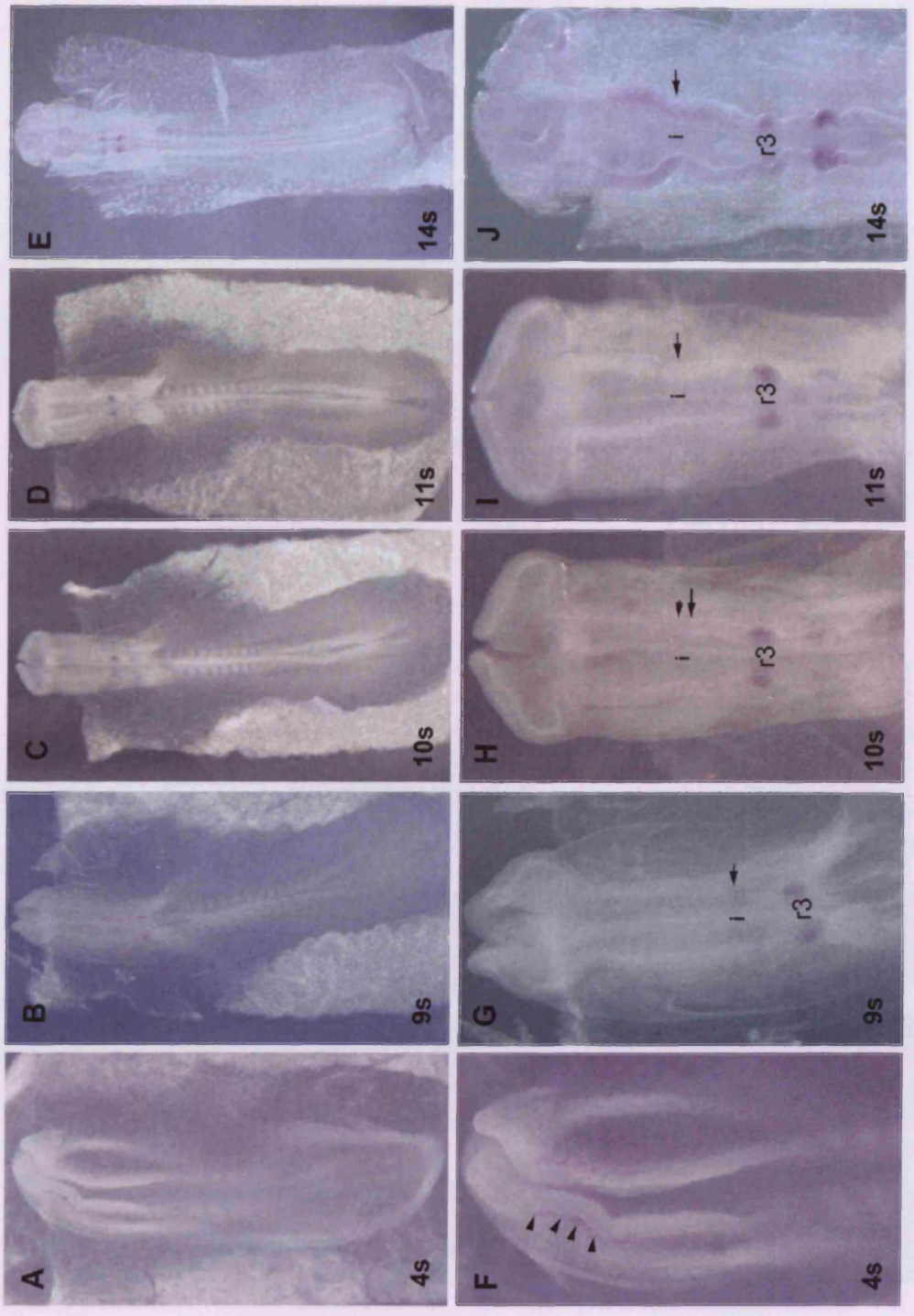
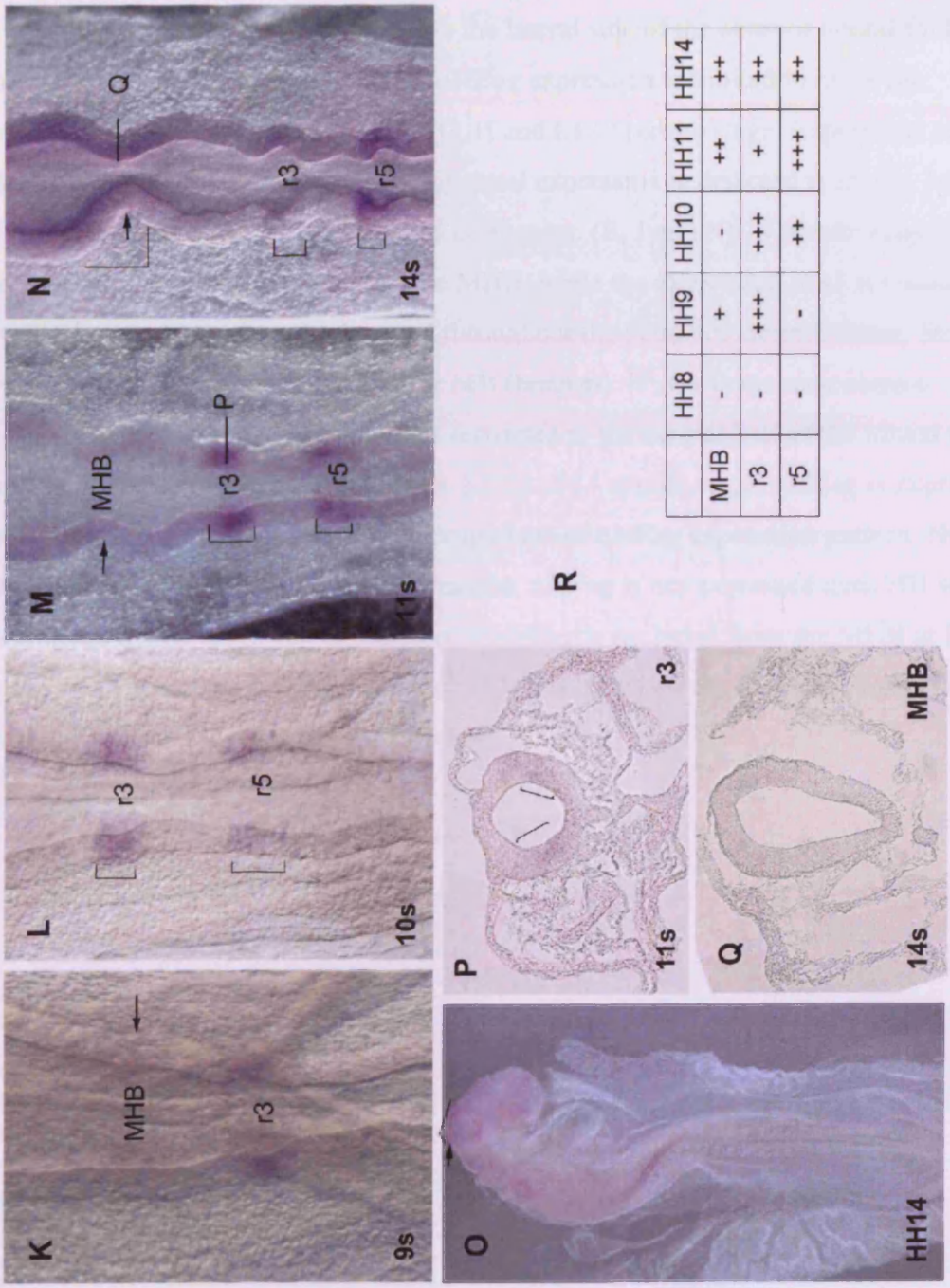


Figure 3.2(2) c*MFng* expression is limited to the MHB, r3 and r5 during early development.



R

	HH8	HH9	HH10	HH11	HH14
MHB	-	+	-	++	++
r3	-	+++	+++	+	++
r5	-	-	++	+++	++

Figure 3.2(1-2) c*MFng* expression is limited to the MHB, r3 and r5 during early development.

c*MFng* whole mount *in situ* hybridisation (Kato et al.) dorsal view: (A-E, O) low magnification view (F-J) 8x magnification (K-N) 17.6x magnification: (A, F) 4 somite stage. Weak expression is observed on the lateral side of the anterior neural fold (arrow heads). (B, G and K) 9 somite stage. c*MFng* expression is limited to r3. Weak expression is observed in the MHB. (C, H and L) 10 somite stage. Expression at the MHB is down-regulated, and new segmental expression is detected in r5. (D, I and M) 11 somite stage. The expression at r5 is stronger. (E, J and N) 14 somite stage. c*MFng* transcripts are up-regulated within the MHB, while the expression in r3 is weakened. (O) HH stage 14. c*MFng* is observed throughout the neuroectodermal tissue. Stronger expression is detected in the posterior MB (bracket). (P, Q) Transverse section (P) r3 of 11 somite stage. c*MFng* expression is restricted to the ventral half of the neural tube, and excluded from the floor plate. (Q) MHB of 14 somite stage. c*MFng* is expressed weakly but ubiquitously. (R) Stage comparison of c*MFng* expression pattern. Number of + indicates the strength of the expression. c*MFng* is not expressed until HH stage 9 within the neural tube. The expression of c*MFng* is excluded from the MHB at HH stage 10. Arrows show the morphological MHB. Brackets show strong expression. i: MHB, r3: rhombomere 3, r5: rhombomere.

Figure 3.3 c*RFng* is expressed within the head region throughout the early development.

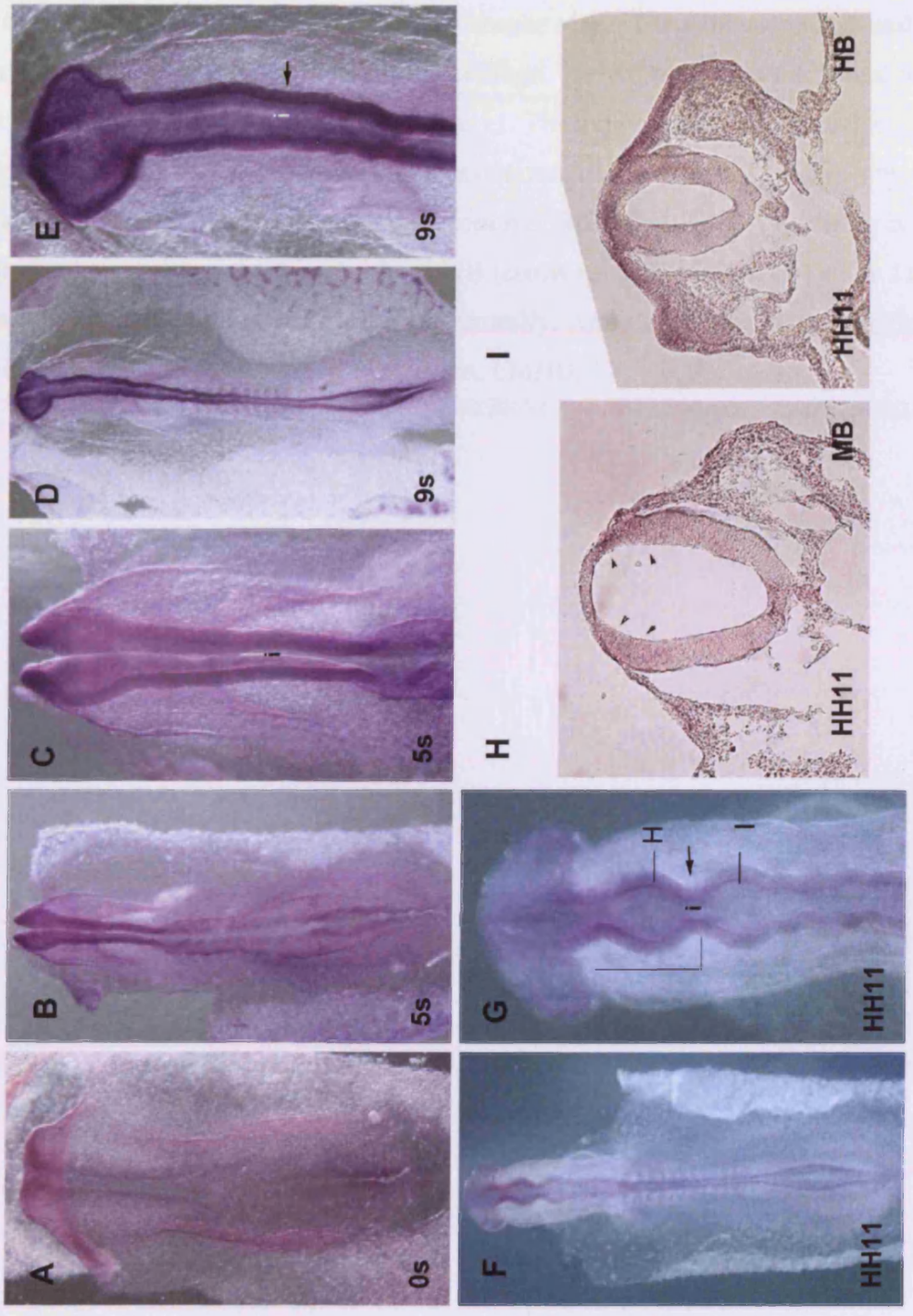


Figure 3.3 c*RFng* is expressed within the head region throughout the early development.

Whole mount *in situ* hybridisation of c*RFng* (A-G) dorsal view: (A, B, D and F) low magnification (C, E, G) 8x magnification (A) 0 somite stage. c*RFng* is expressed throughout the ectodermal tissue. (B, C) 5 somite stage. The expression is found throughout the neural tube. (D, E) 9 somite stage. The expression in the neural tube is not restricted to segments. (F, G) HH stage 11. The expression in the caudal neural tube from the MHB is weakened. Strong expression remains, particularly in the MB (bracket). (H, I) Transverse section (H) section of MB at HH stage 11. c*RFng* is expressed strongly on the dorsal side of MB (arrow head). (I) HB at HH stage 11. The expression in HB is weak but even dorsoventrally. Arrows indicate the morphological MHB. Bracket shows the strong expression. i:MHB.

Figure 3.4(1) *cLFng* expression is excluded from the MHB from HH stage 9 to HH stage 12.

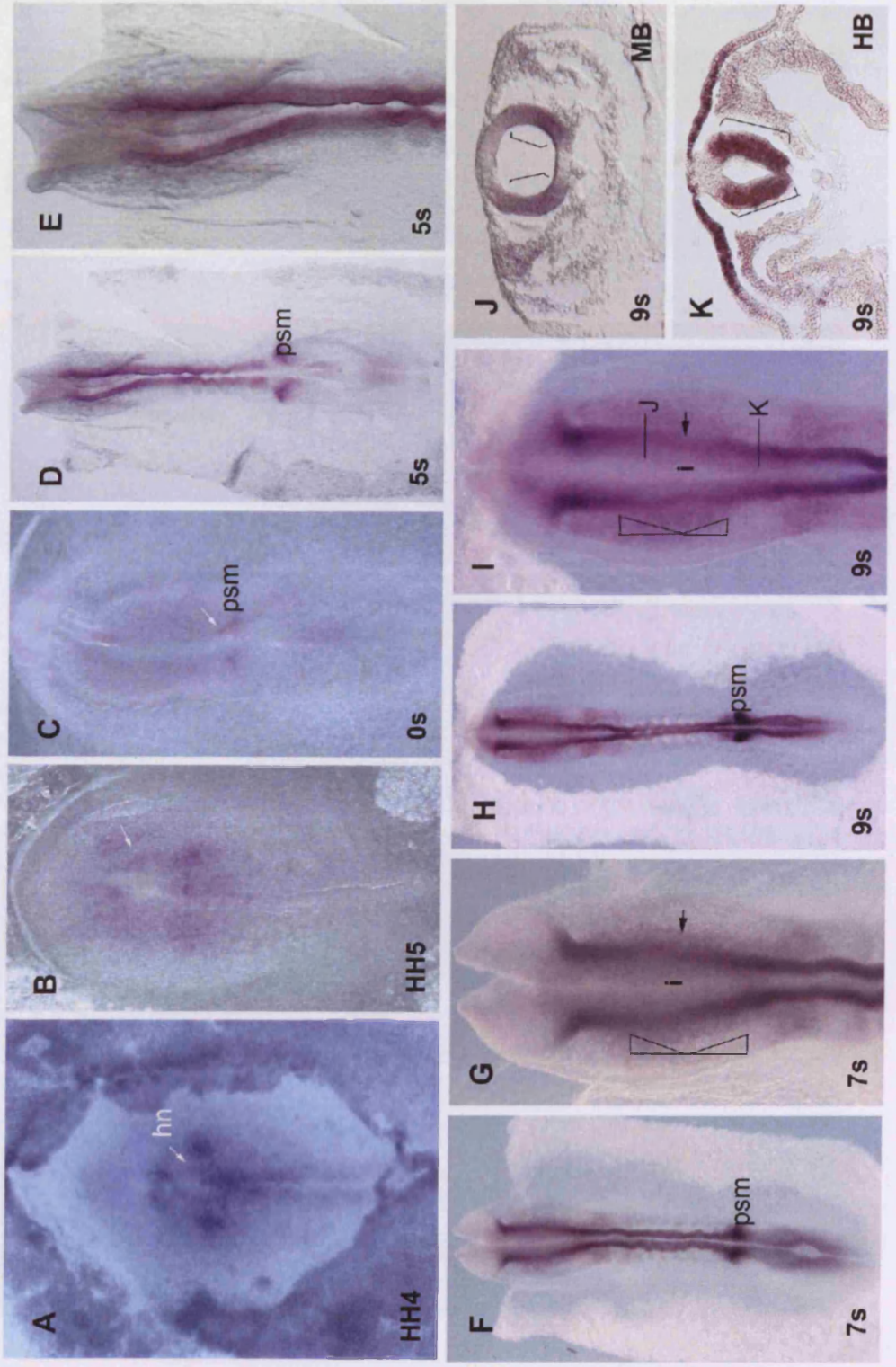


Figure 3.4(2) c*LFng* expression is excluded from the MHB from HH stage 9 to HH stage 12.

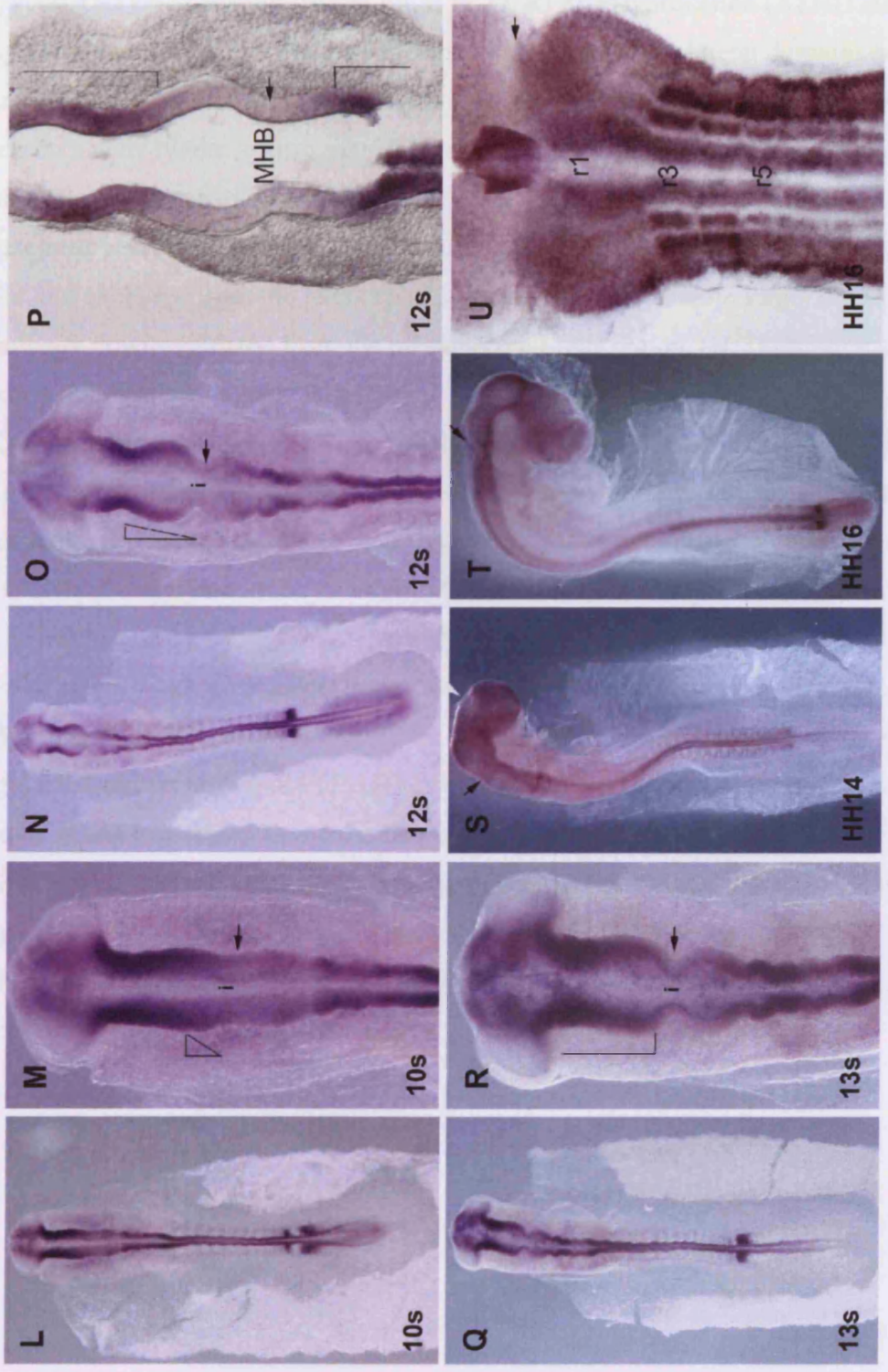


Figure 3.4(1-2) c*LFng* expression is excluded from the MHB from HH stage 9 to HH stage 12

in situ hybridisation of c*LFng* (A-I, L-O, Q,R) dorsal view, (S, T) lateral view: (A-D, F, H, L, N, Q-T) 3.2x magnification, (G, I, M, O, R) 8x magnification (A) HH stage 4. The expression appears in the mid-streak region and some in two lateral domains of adjacent mesoderm. Note two distinct expression domains are apparent anterior and posterior to Hensen's node (white arrow). (B) HH stage 5. Two stripes of expression can be seen anteriorly and posteriorly from Hensen's node. (C) 0 somite stage. Expression in presomitic mesoderm. (D, E) 5 somite stage. High levels of expression in the neural tube, but excluded from the telencephalic region. (F, G) 7 somite stage. Expression is gradually down-regulated from the presumptive MHB region. (H, I) 9 somite stage. Expression is weak at the MB, MHB, r1 and r2. Transverse sections of MB (J) and HB (K) show that expression of c*LFng* is excluded from both floor plate and roof plate. (L, M) 10 somite stage. The posterior limit of MB expression remains anterior to the morphological MHB. (N, O) 12 somite stage. Expression appears to be excluded from the MHB domain, r1 and the anterior half of r2. (P) Longitudinal section of ventral part of neural tube at 12 somite stage. No c*LFng* expression in the MHB domain. (Q, R) 13 somite stage. Weak expression at the MHB. Strong expression in the diencephalon and MB remains (Bracket). (S) HH stage 14. Expression in the ZLI appears to be down-regulated (white arrowhead). (T, U) HH stage 16. The flat mount of neural tube (U) shows strong expression in the rhombomeres. Black arrows indicate the morphological MHB. Brackets show areas of strong expression. Triangles indicate gradients of mRNA expression. White arrows point to Hensen's node. White arrowhead shows the ZLI. i: MHB, hn: Hensen's node, psm: presomitic mesoderm, r1: rhomobomere 1, r3: rhomobomere 3, r5: rhomobomere 5.

Figure 3.5(1) c*Delta1* expression and the morphological MHB in chick CNS development.

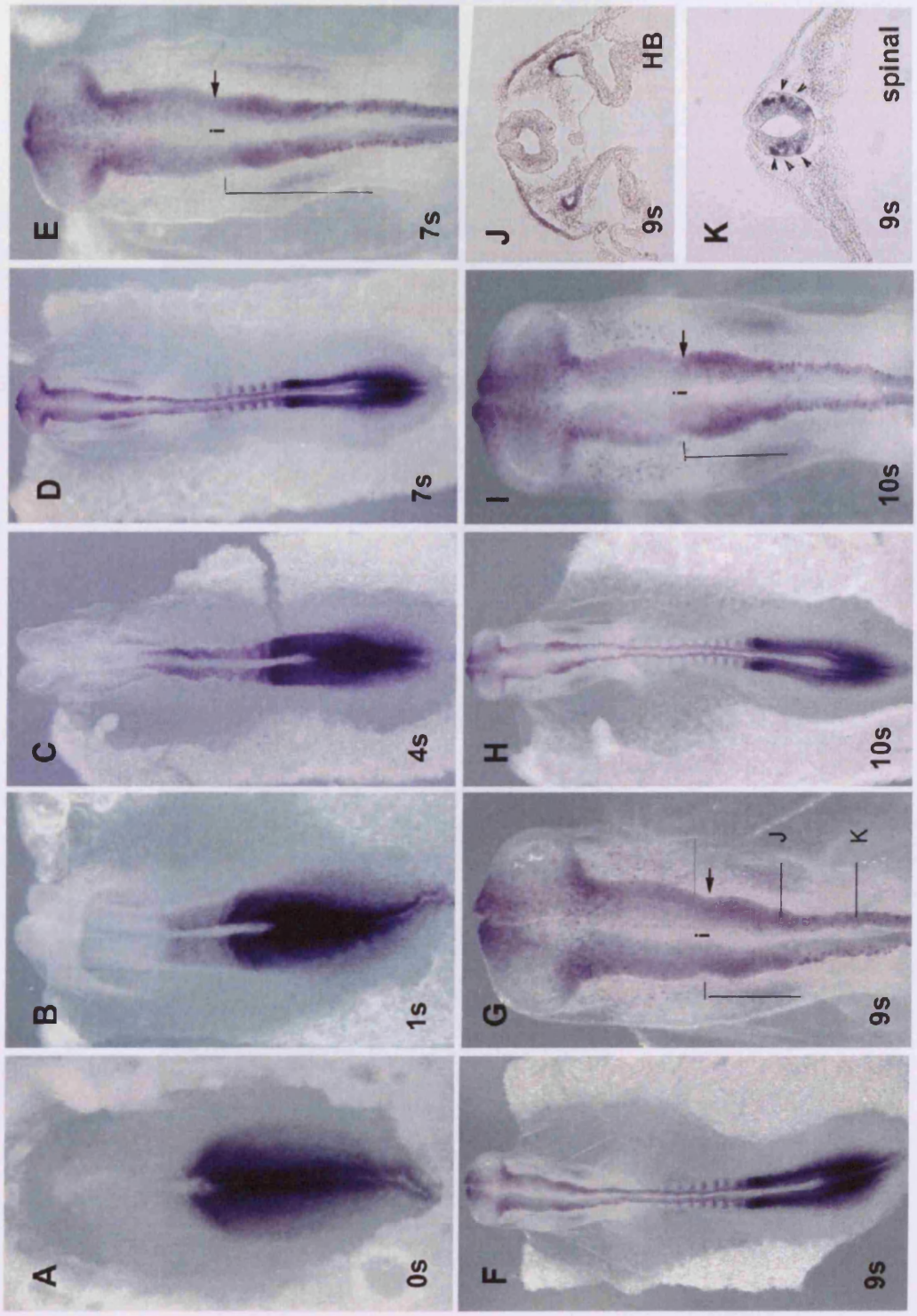


Figure 3.5(2) c*Delta1* expression and the morphological MHB in chick CNS development.

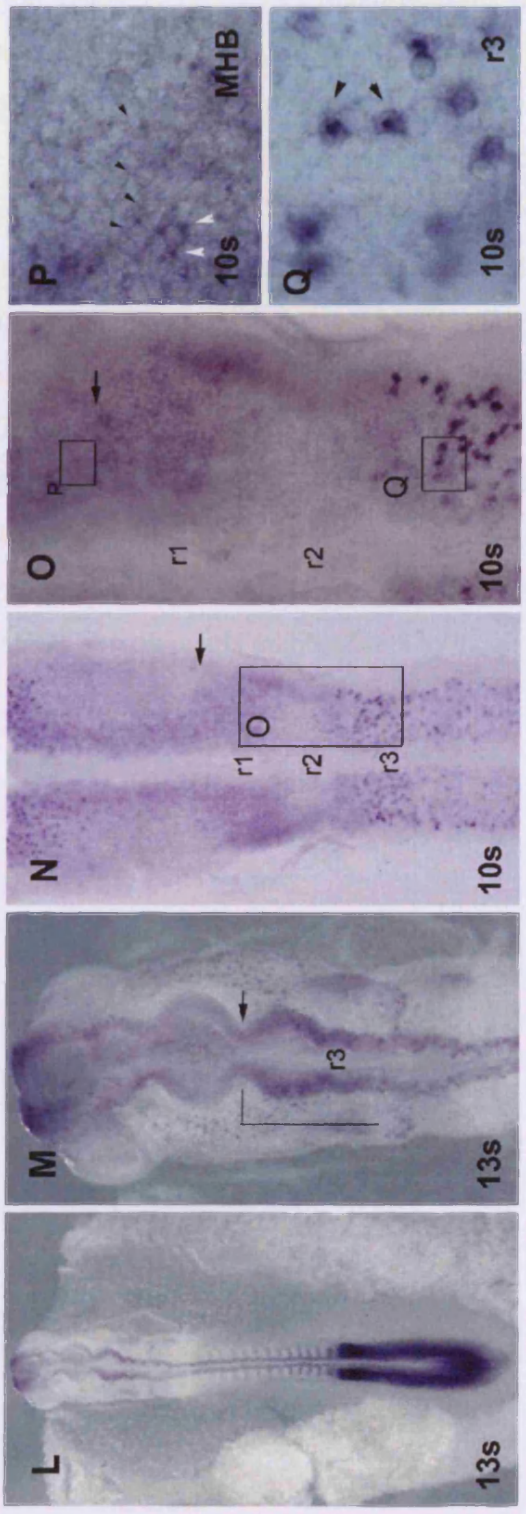


Figure 3.5(1-2) c*Delta1* expression and the morphological MHB in chick CNS development.

Whole mount *in situ* hybridisation of c*Dll*. (A-I, L, M) dorsal views of embryos (J, K) Transverse section. (A-D, F, H, L) 3.2x magnification. (E, G, I, M) 8x magnification. (A) 0 somite stage. (B) 1 somite stage. Strong mesodermal expression is observed. (C) 4 somite stage. Expression of c*Dll* appears strongly only in the posterior half of embryos. (D, E) 7 somite stage. Expression in telencephalon and diencephalon (F, G) 9 somite stage. Strong expression in the HB. Transverse sections reveal c*Dll* mRNA is localised intracellularly in hindbrain compartments and spinal cord (J, K: Arrowheads). (H, I) 10 somite stage. Posterior to the morphological MHB, c*Dll* is strongly expressed. (L, M) 13 somite stage. Expression in the HB remains strong. (N-Q) Flat mount of 10 somite stage embryo: (N) low magnification. Strong expression of c*Dll* mRNA appears in r1. (O) Middle magnification. Expression is excluded from r2. (P,Q) 17.6x magnification. (P) MHB. mRNA is localised to the cell membrane (White arrow heads). (Q) r3. mRNA is localised within the nucleus in some cells (Black arrowheads). Arrows show the morphological MHB. Arrowheads and brackets indicate distinct expression. i:MHB

Figure 3.6(1) *cSerrate1* expression.

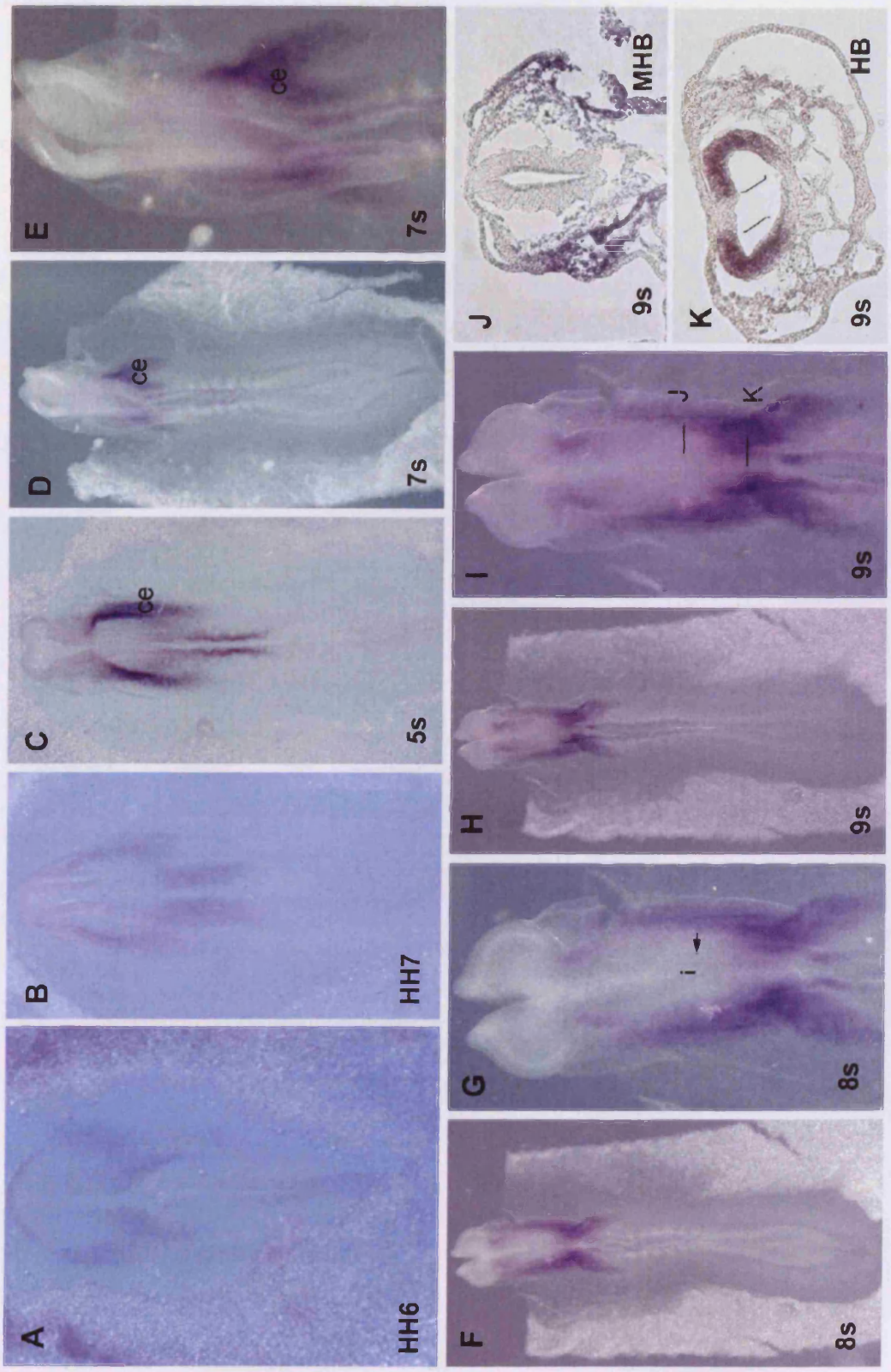


Figure 3.6(2) c*Serrate1* expression.

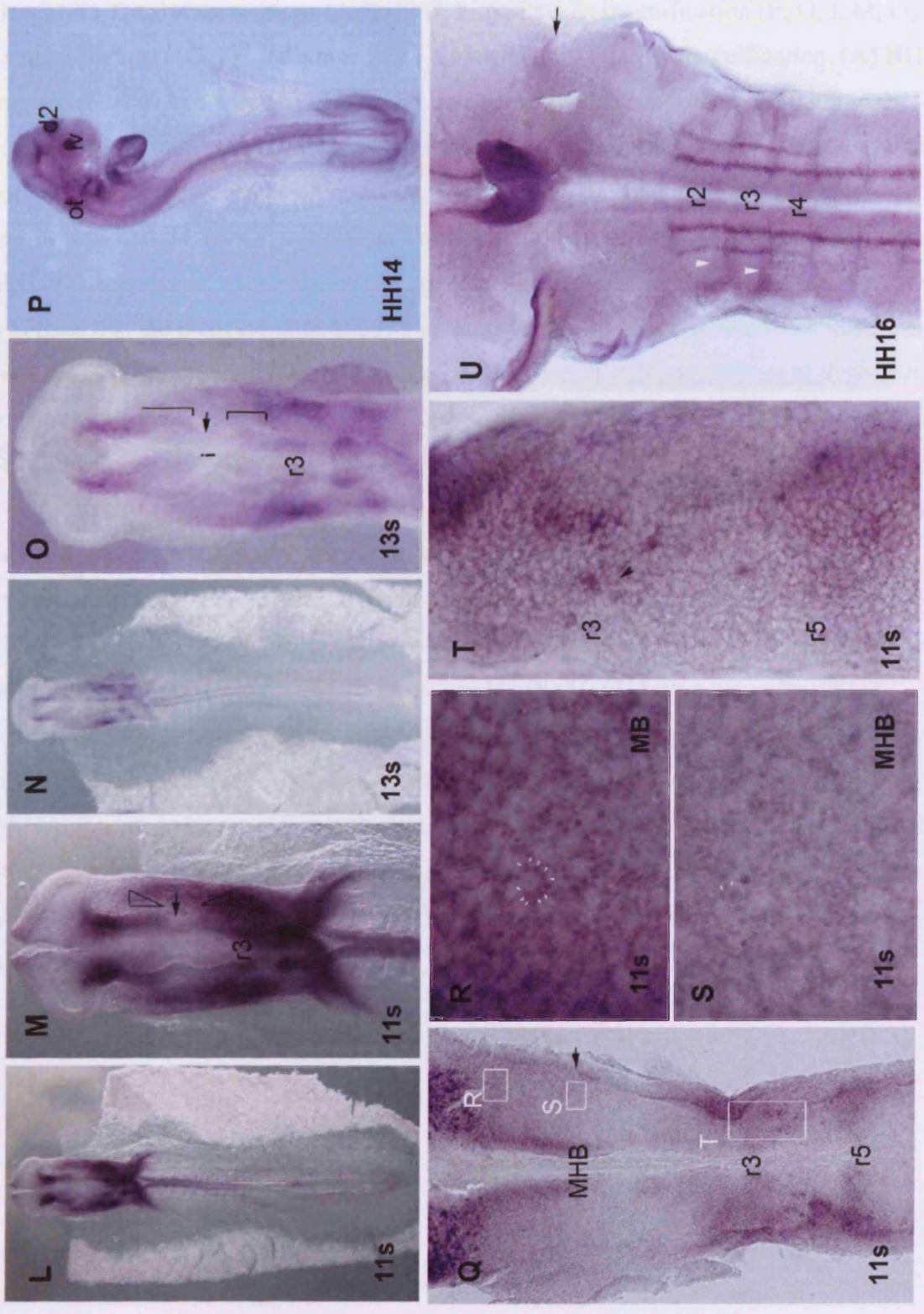


Figure 3.6(1-2) c*Serrate1* expression.

Whole mount *in situ* hybridisation of c*Ser1*. (A-I, L-O) dorsal view. (P) dorsolateral view (J,K) Transverse sections (A-D, F, H, L, N, P) 3.2x magnification (E, G, I, M, O) 8x magnification (Q-T) Flat mount of 11 somite stage. (Q) Low magnification. (A) HH stage 6. c*Ser1* is expressed in neurogenic ectoderm. (B) HH stage 7. Expression in the presumptive cranial ectoderm starts to appear. (C) 5 somite stage. c*Ser1* mRNA spreads out within the cranial ectoderm intensely. Expression also appears at the posterior neuroectoderm. (D, E) 7 somite stage. Transcripts remain strong in the cranial ectoderm. (F,G) 8 somite stage. c*Ser1* expression appears within r3 and r5. Some weak expression is apparent in the diencephalon. (H, I) 9 somite stage. Expression is observed within the diencephalic domain, and HB. Transverse sections reveal weak expression at the MHB and strong expression in the HB (J, K). The expression in the HB is excluded from roof plate and floor plate. (L, M) 11 somite stage. Expression is first seen in the MB and r1,2. c*Ser1* mRNA is excluded from the MHB.(N, O) 13 somite stage. Expression in the MB and r1,2 appears weakly. (P) HH stage 14. Expression in the MB is strongest in the ventral most tissue. High levels of expression are seen in r3 and r5. c*Ser1* transcripts are excluded from the MHB. (R, S) 17.6x magnification. (R) MB. mRNA is localised to the cell membrane in some cells (white arrow heads). (S) MHB. Few cells with cell membrane localisation appear in the MHB. (T) Middle magnification. Some cells show nuclear localisation of c*Ser1* (Black arrow heads). (U) Flat mount of HH stage 16. c*Ser1* mRNA appears to be restricted to the hindbrain boundaries (white arrow heads). Arrows show the morphological MHB. Brackets show the distinct expression. Triangles indicate gradients of mRNA expression. i:MHB, ce: cranial ectoderm, d2: neuromere D2, ot: otic vesicle, lv: lens vesicle

Figure 3.7(1) c*Serrate2* expression.

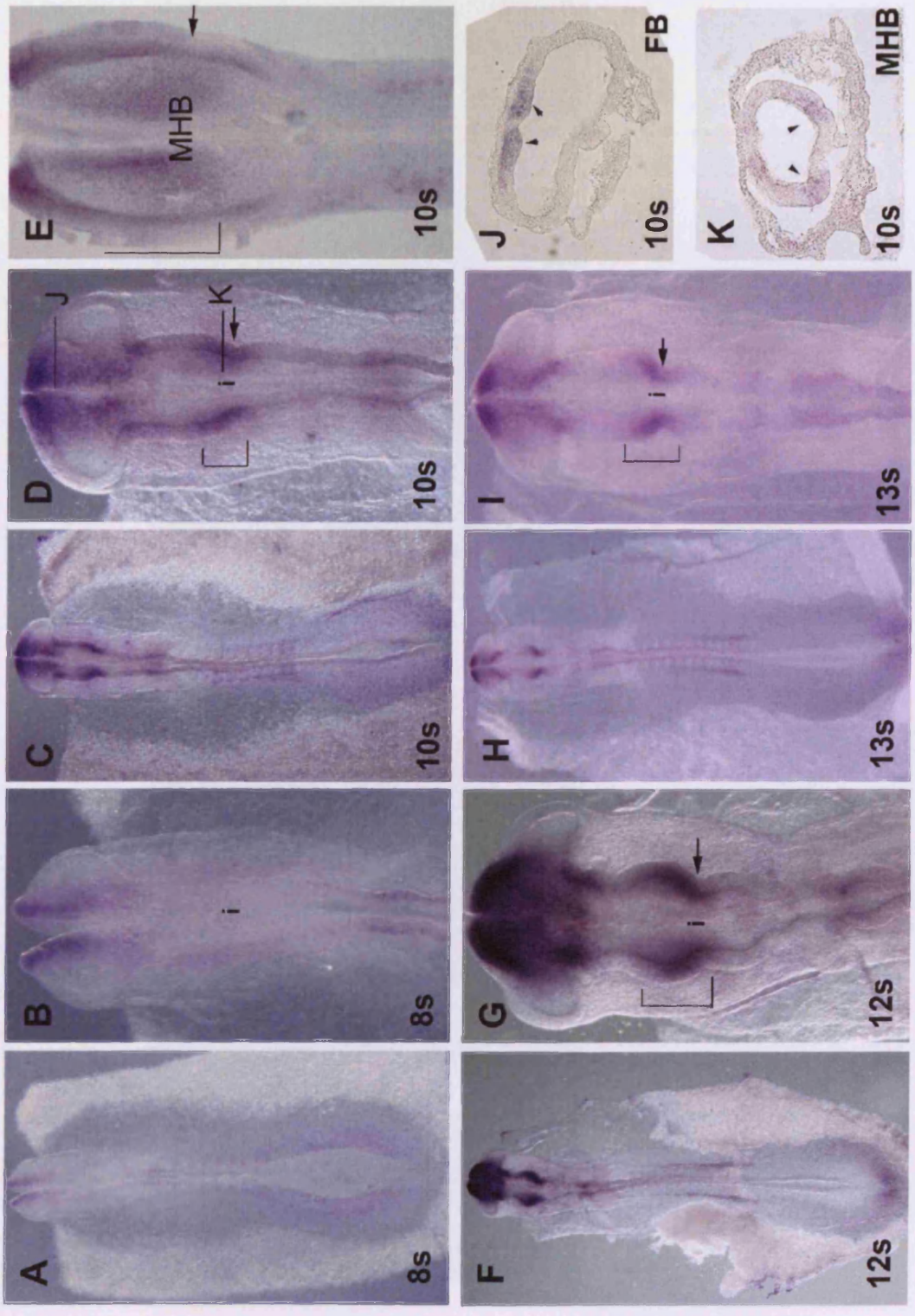


Figure 3.7(2) c*Serrate2* expression

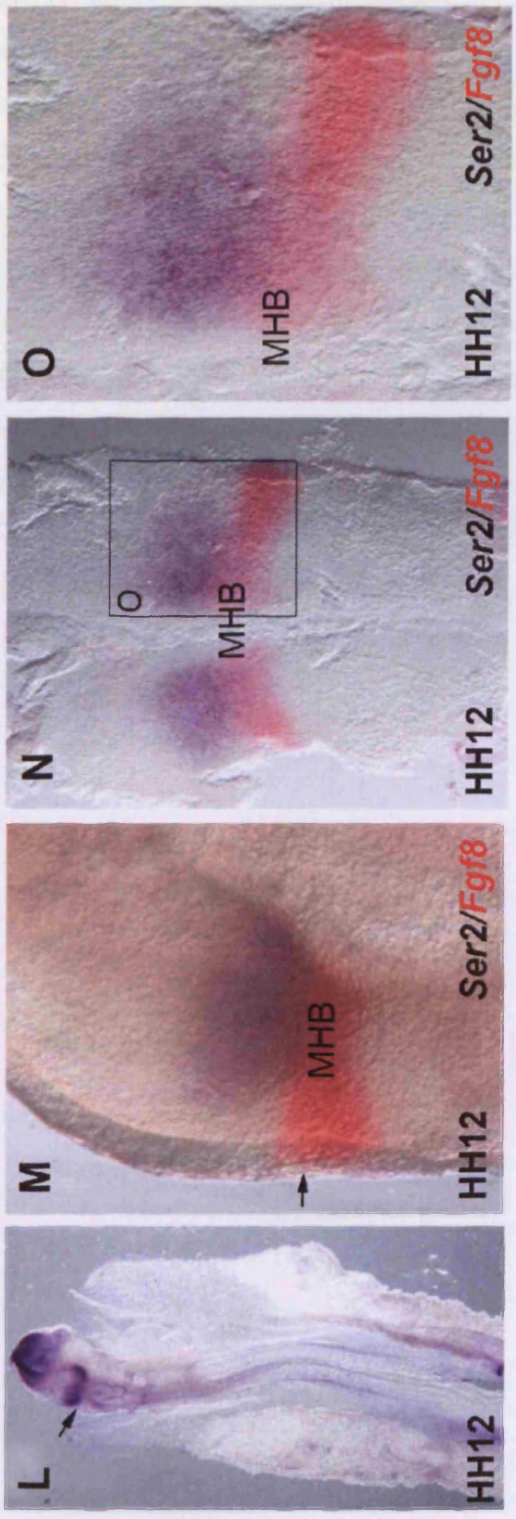


Figure 3.7(1-2) c*Serrate2* expression

c*Ser2* whole mount *in situ* hybridisation. (A-D, F-I) dorsal view. (L) dorsolateral view. (M) Lateral view. (J, K) Transverse sections. (A-C, F, H, L) 3.2x magnification (D, G, I) 8x magnification. (M) 17.6x magnification. (A,B) 8 somite stage. c*Ser2* transcripts appear in the telencephalon. (C-E) 10 somite stage. Expression is strongly restricted within the MHB and telencephalon. Transverse section of FB shows the expression appears only in the dorsal tissue (J), while the expression in the MHB is strong in ventral (K). (F, G) 12 somite stage. The expression in the MHB is expanded to the MB side. (H, I) 13 somite stage. (L-O) HH stage 12. Expression at the MHB appears to be anteriorised within the MB. (M-O) double *in situ* hybridisation. c*Ser2* in blue. c*Fgf8* in red. (M) c*Ser2* expression is adjacent to the anterior most *Fgf8* expression. (N,O) flat mount. c*Ser2* expression in the MB abuts *Fgf8* expression in the posterior MHB (anterior HB). c*Ser2* transcripts are observed in the ventral half of the neural tube. Arrows show the morphological MHB. Brackets and arrowheads indicate strong expression. i:MHB

Figure 3.8(1) c*Notch1* is broadly expressed in the CNS.

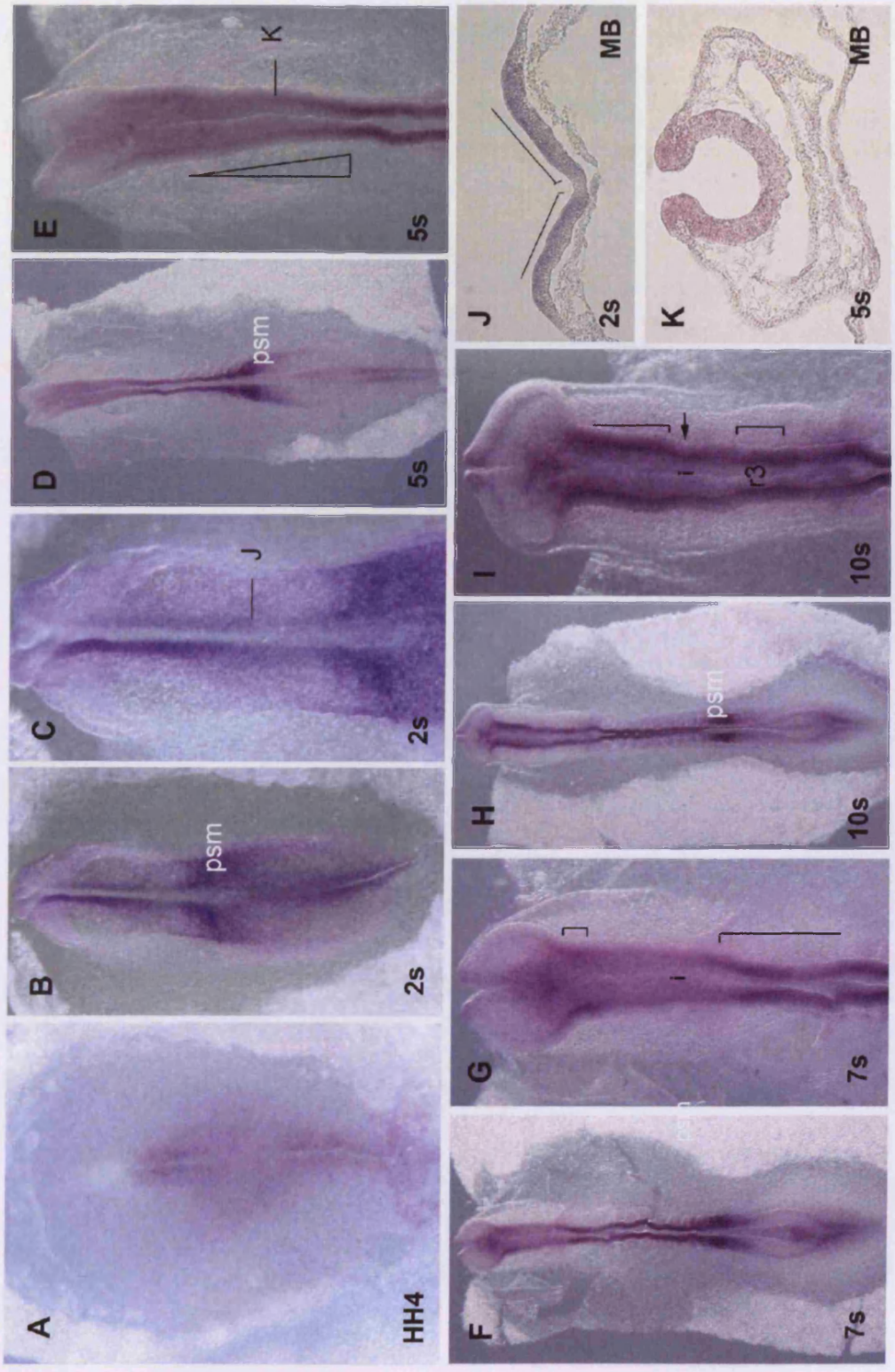


Figure 3.8(2) c*Notch1* is broadly expressed in the CNS.

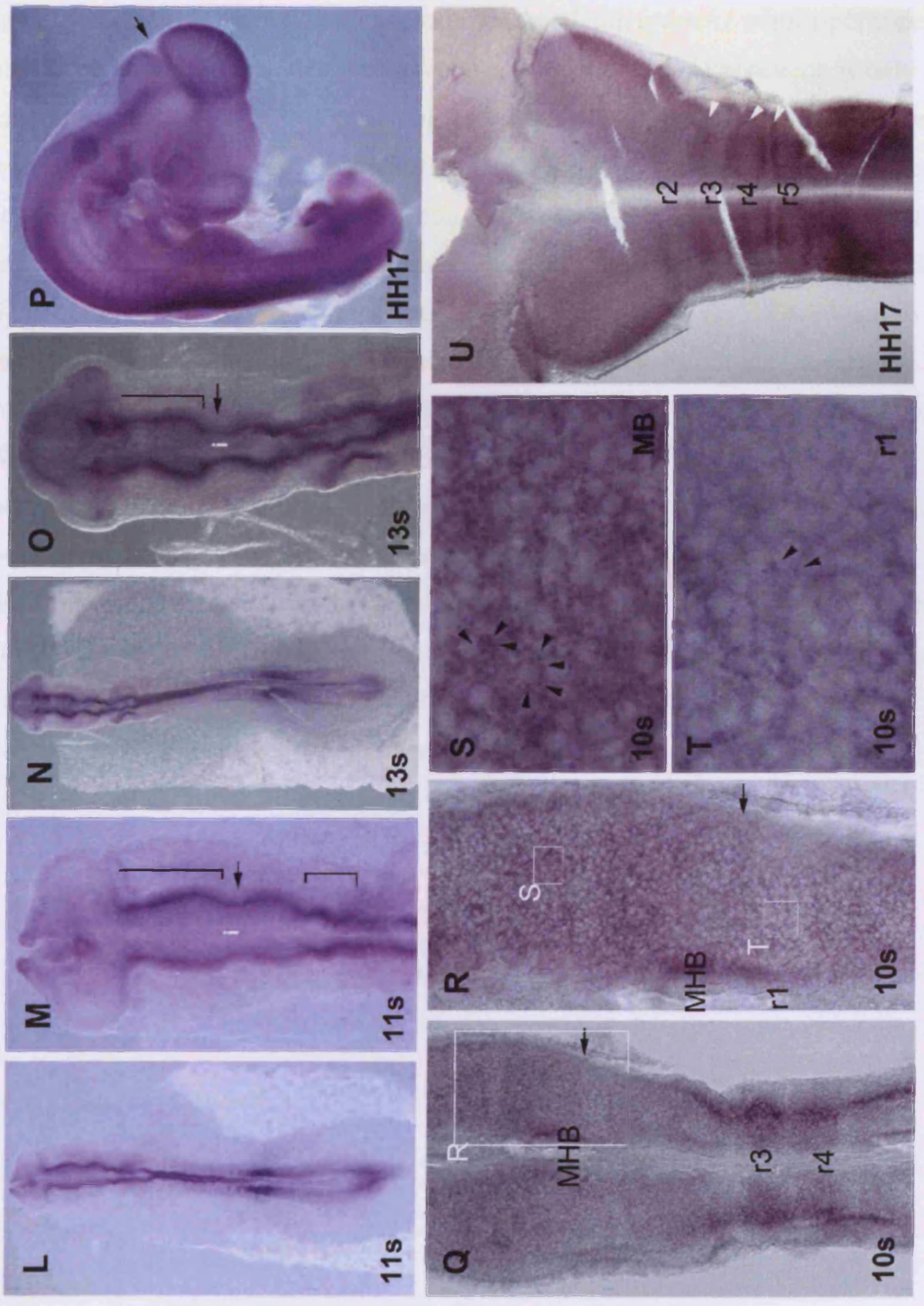


Figure 3.8(1-2) c*Notch1* is broadly expressed in the CNS.

(A, B, D, F, H, L, N and P) 3.2x magnification, (C, E, G, I, M and O) 8x magnification. (A) HH stage 4. Expression appears around the primitive streak. (B,C) 2 somite stage. Strong expression is observed in the presomitic mesoderm; c*Notch1* transcripts appear within the neural fold. Transverse section (J) shows that c*Notch1* expression is only in the ectodermal tissue. (D, E) 5 somite stage. Expression within the neural folds appears to be weakened gradually towards the future forebrain. Transverse section (K) indicates that transcripts do not show any localisation, ventrally or dorsally. (F, G) 7 somite stage. Strong expression is observed in the presumptive diencephalon and hindbrain (brackets). (H, I) 10 somite stage. c*Notch1* transcripts appear strongly in the FB, MB, r3 and r4 (brackets). Expression at the MHB, r1 and r2 is weak. (Q-T) Flat mount view of the neural tube. (Q) 3.2x magnification. (R) 8x magnification. (S, T) 17.6x magnification. (S) Midbrain. Strongly localised transcripts appear on the edge of cells (Black arrowheads). (T) rhomobomere 1. Few localised transcripts appear (Black arrowheads) (L, M) 11 somite stage. The expression at the MHB, r1 and r2 is weak. (N, O) 13 somite stage. Strong restriction of expression at the HB is lost. (P) HH stage 17. Flat mount (U) shows c*Notch1* transcripts localised to both sides of the hindbrain boundaries (White arrowheads). Arrows show the morphological MHB. Triangles indicate gradients of mRNA expression. psm: presomitic mesoderm

Figure 3.9(1) *cHairy1* expression is restricted to the MHB at 10 somite stage.

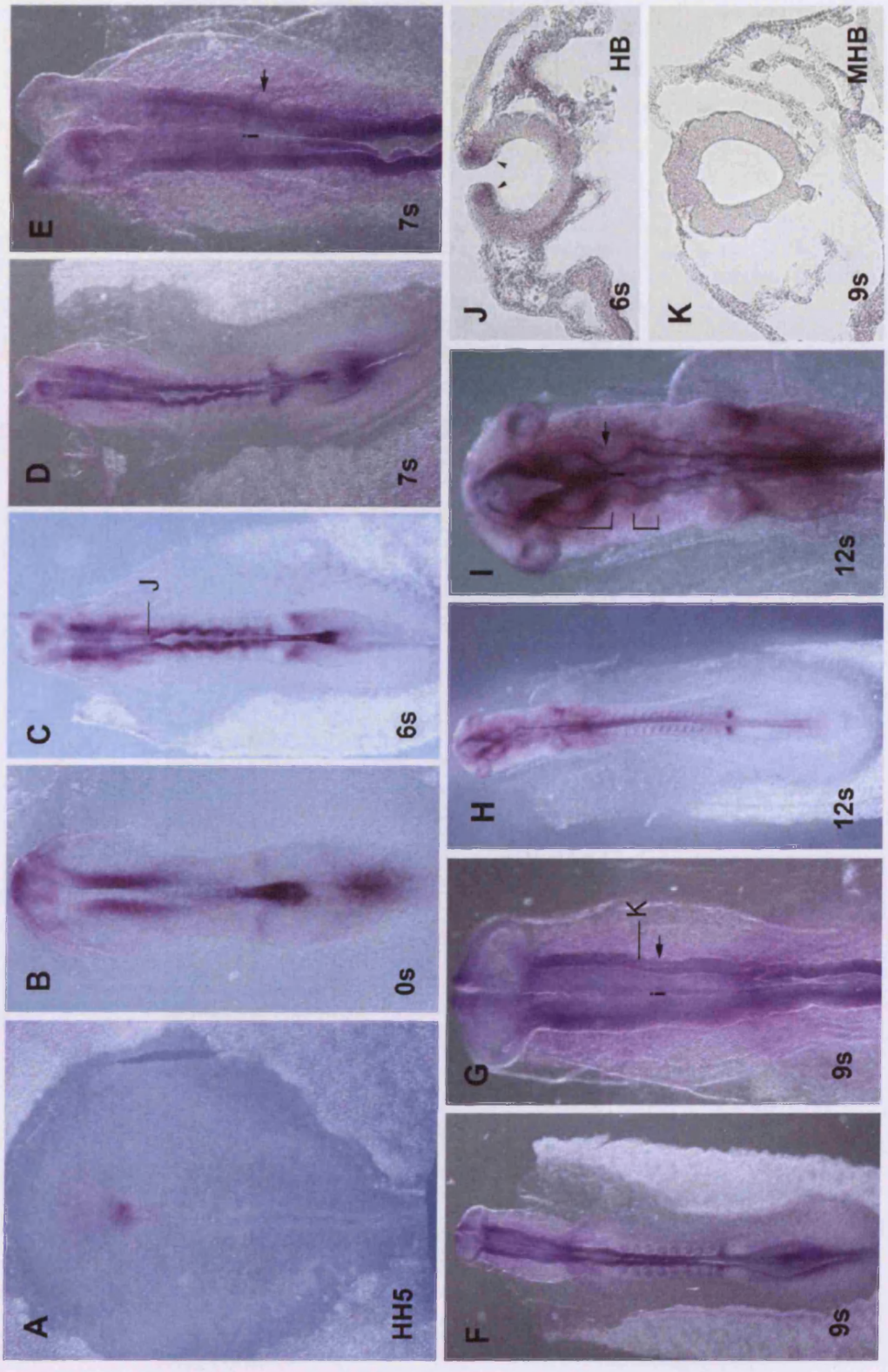


Figure 3.9(2) c*Hairy1* expression is restricted to the MHB at 10 somite stage.

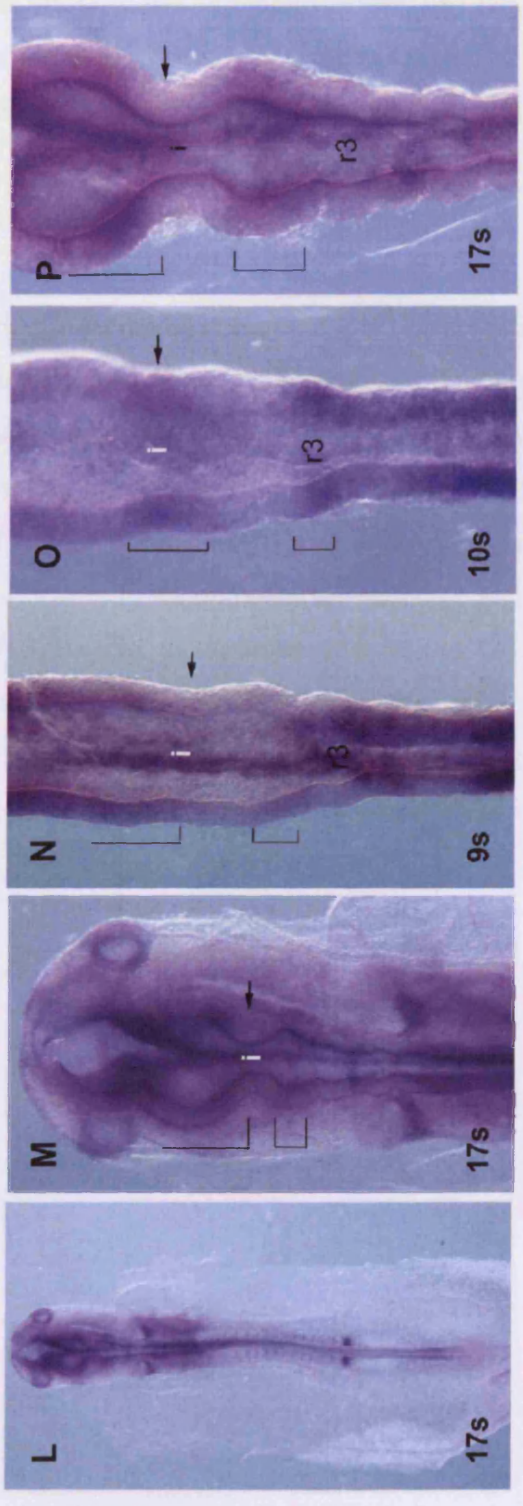


Figure 3.9(1-2) c*Hairy1* expression is restricted to the MHB at 10 somite stage.

Whole mount *in situ* hybridisation of c*Hairy1*. (A-I, L-P) dorsal view (A-D, F, H, L) 3.2x magnification. (E, G, I, M) 8x magnification. (A) HH stage 5. c*Hairy1* transcripts only appear on the Hensen's node. (B) 0 somite stage. The expression in the node remained strong. Strong expression appears in the neural folds. (C) 6 somite stage. Expression is seen throughout the neural tube. Transverse section (J) shows the expression is strongly restricted within the dorsal lip (Arrow heads). (D, E) 7 somite stage. Expression continues throughout the neural tube. (F, G) 9 somite stage. The expression, posterior from the morphological MHB is weakened. Transverse section at the MHB shows c*Hairy1* is expressed ubiquitously within the neural tube (K). (H, I) 12 somite stage. c*Hairy1* expression is excluded from the morphological MHB (bracket). (L, M) 17 somite stage. Exclusion of c*Hairy1* from the morphological MHB remains. Strong expression appears within the MB and r1, 2 (Brackets). (N-P) Dorsal views of the neural tube: (N) 9 somite stage. Posterior boundary of the strong expression is at the morphological MHB. Expression appears within r2. (O) 10 somite stage. c*Hairy1* mRNA is detected at the MHB and r3. (P) 17 somite stage. Posterior boundary of expression appears to be more anterior than the morphological MHB. Arrows show the morphological MHB. Arrowheads and brackets indicate distinct expression. i: MHB, r3: rhombomere 3.

Figure 3.10(1) c*Hairy2* is expressed in the MHB at HH stage 10.

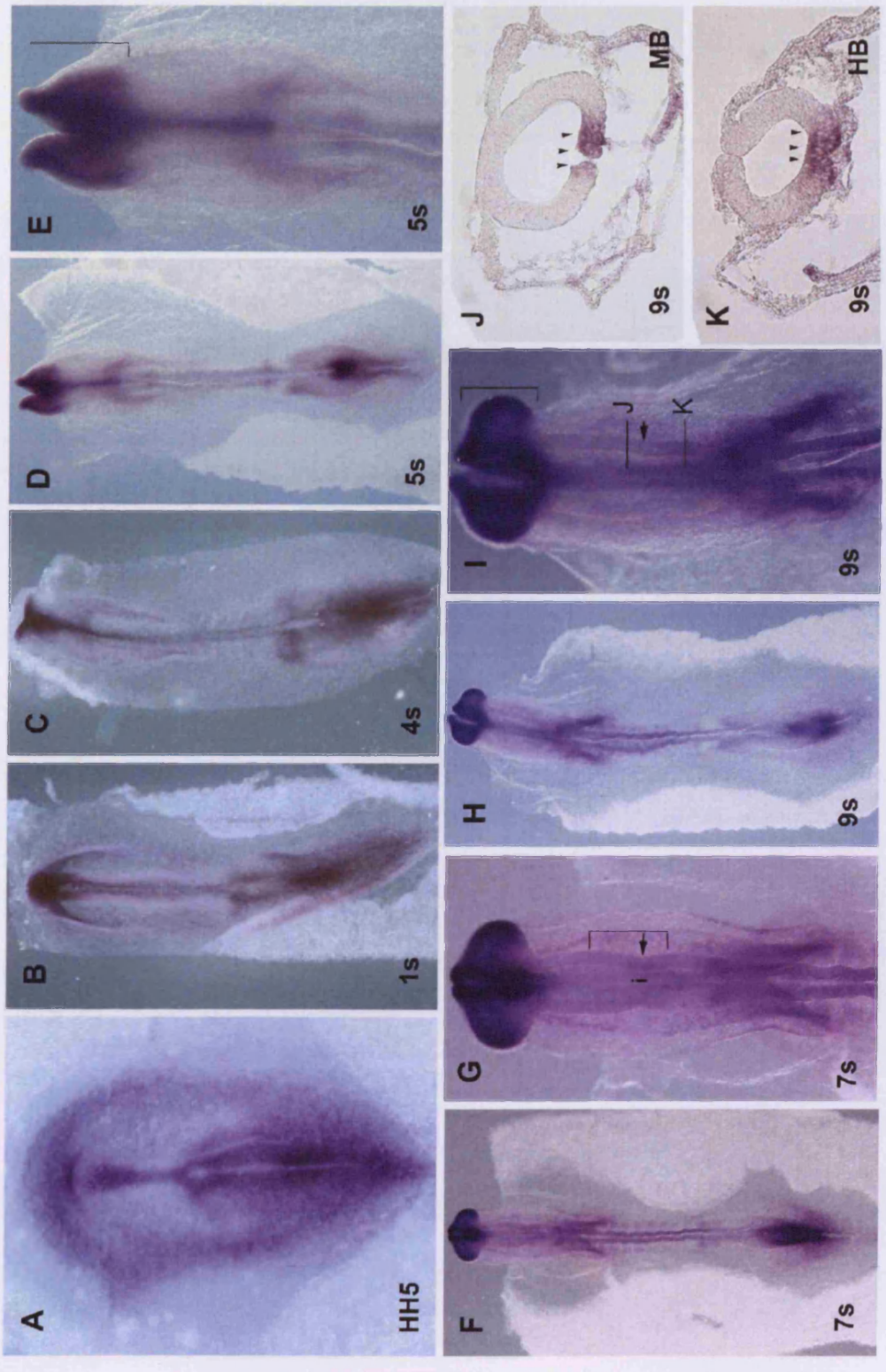


Figure 3.10(2) c*Hairy2* is expressed in the MHB at HH stage 10

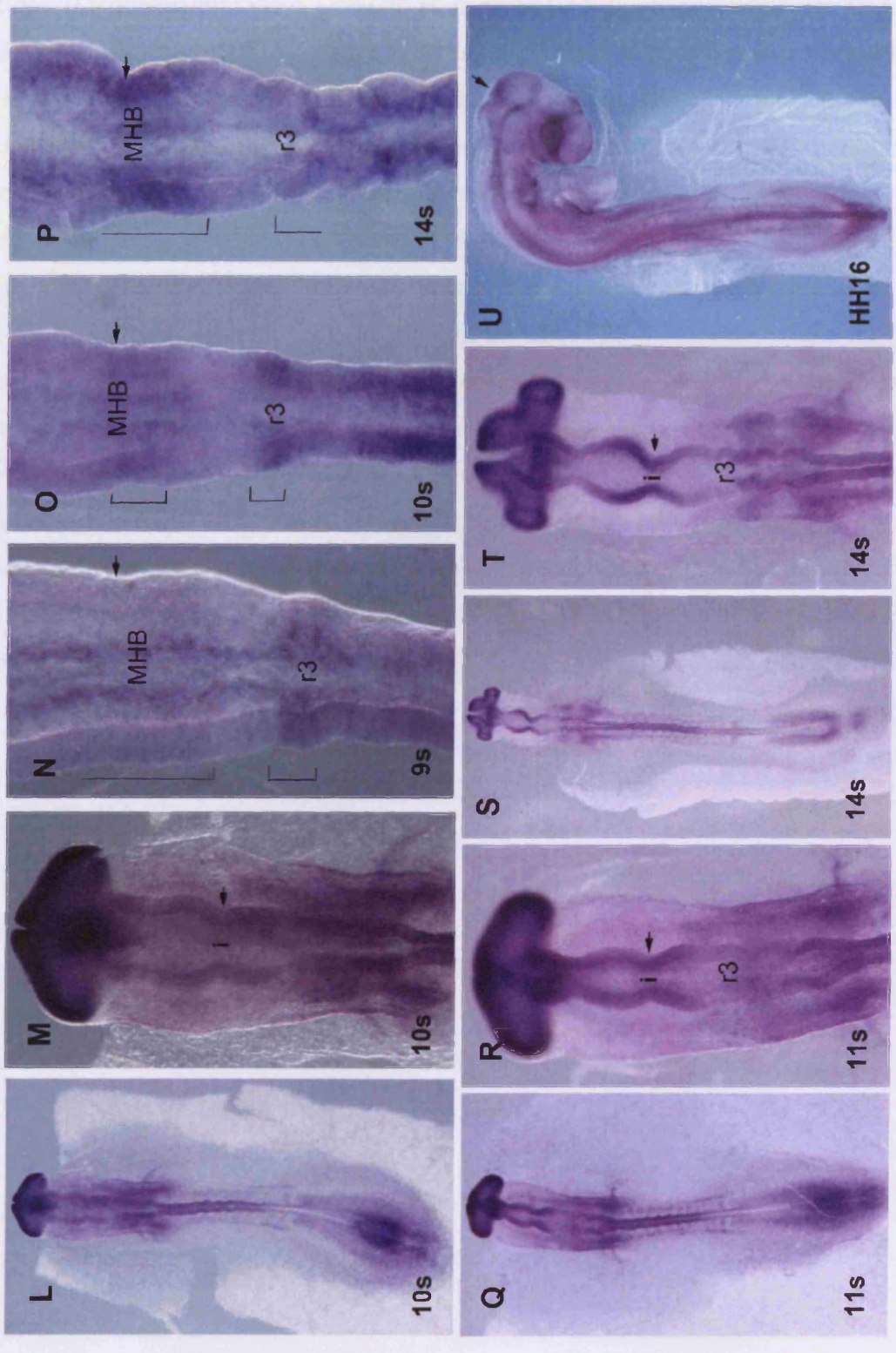


Figure 3.10(1-2) c*Hairy2* is expressed in the MHB at HH stage 10

c*Hairy2* whole mount *in situ* hybridisation. (A-I, L-T) dorsal view, (J, K) Transverse sections. (U) dorsolateral view. (A-D, F, H, L, Q, S, U) 3.2x magnification. (E, G, I, M, R, T) 8x magnification. (A) HH stage 5. Strong expression is observed at the primitive streak. (B) 1 somite stage. (C) 4 somite stage. c*Hairy2* is expressed strongly in the most anterior part of the neural fold and ventral midline. (D, E) 5 somite stage. Expression is restricted to the future forebrain and ventral midline. (F, G) 7 somite stage. Strong expression in telencephalon (bracket). (H, I) 9 somite stage. Strong expression is visible in the telencephalon and presumptive optic cups. Little expression is apparent in the MB and HB domains. Transverse sections show restriction of the expression within the floor plate (J, K). (L, M) 10 somite stage. c*Hairy2* mRNA appears within the MHB. Strong expression is observed in telencephalon. (Q, R) 11 somite stage. The expression fields in MB and telencephalon meet each other. The MHB expression extends to r1. (S,T) 14 somite stage. c*Hairy2* is restricted strongly in the MB, MHB and telencephalon, including the optic cups. (U) HH stage 16. (N-P) Dorsal views of neural tube: (N) 9 somite stage. c*Hairy2* mRNA appears in the MB and MHB, but not in r2.(O) 10 somite stage. Expression is restricted to the MHB domain. (P) 14 somite stage. The restriction of the expression is maintained strongly in the MHB. c*Hairy2* expression appears in the MB. Arrows show the morphological MHB. Arrowheads and brackets indicate distinct expression. i:MHB, r3: rhombomere 3.

Figure 3.11(1) c*Lrrn1* expression is excluded from the MHB domain in a similar time period to c*LFng*.

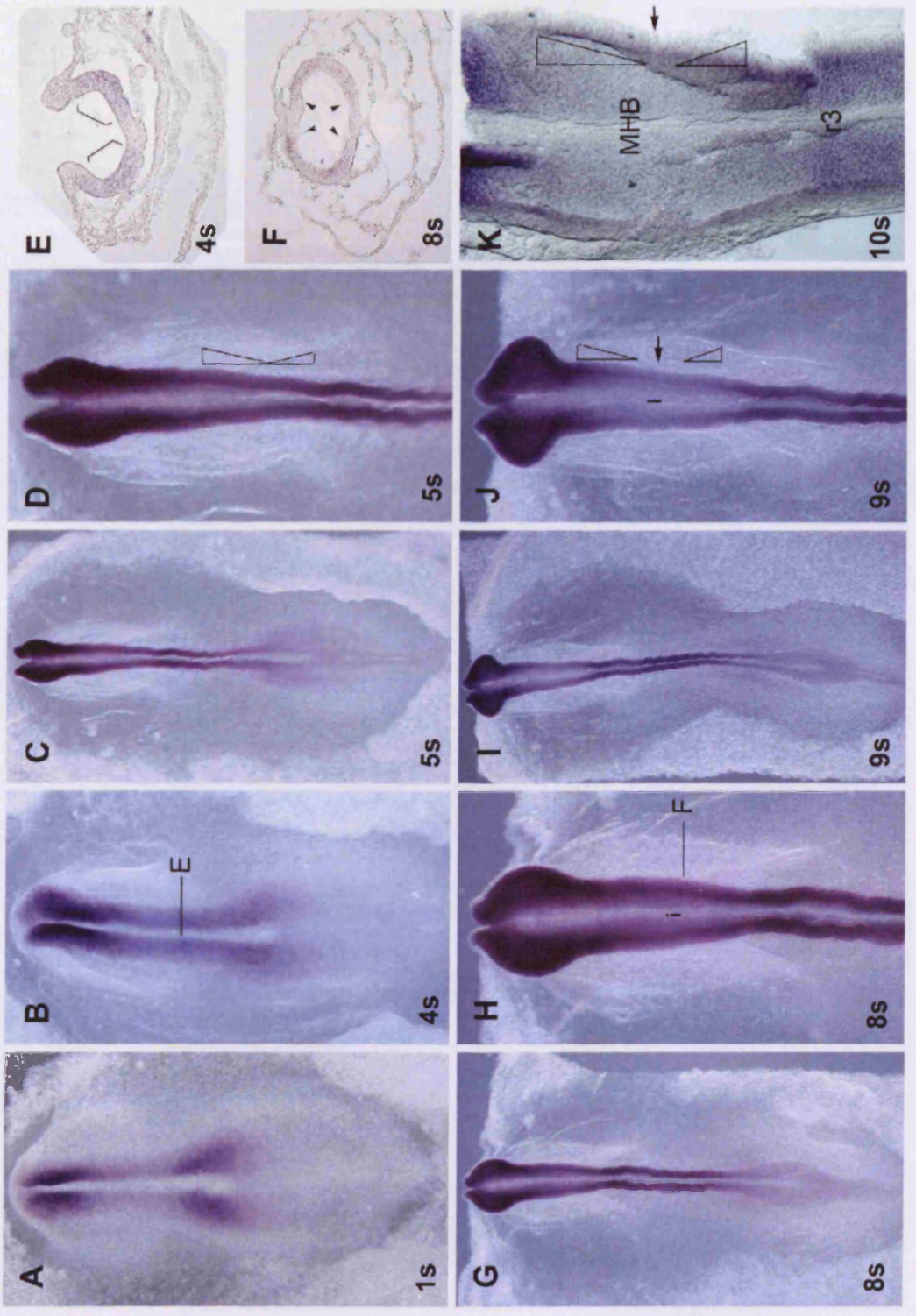


Figure 3.11(2) c*Lrrn1* expression is excluded from the MHB domain in a similar time period to c*LFng*.

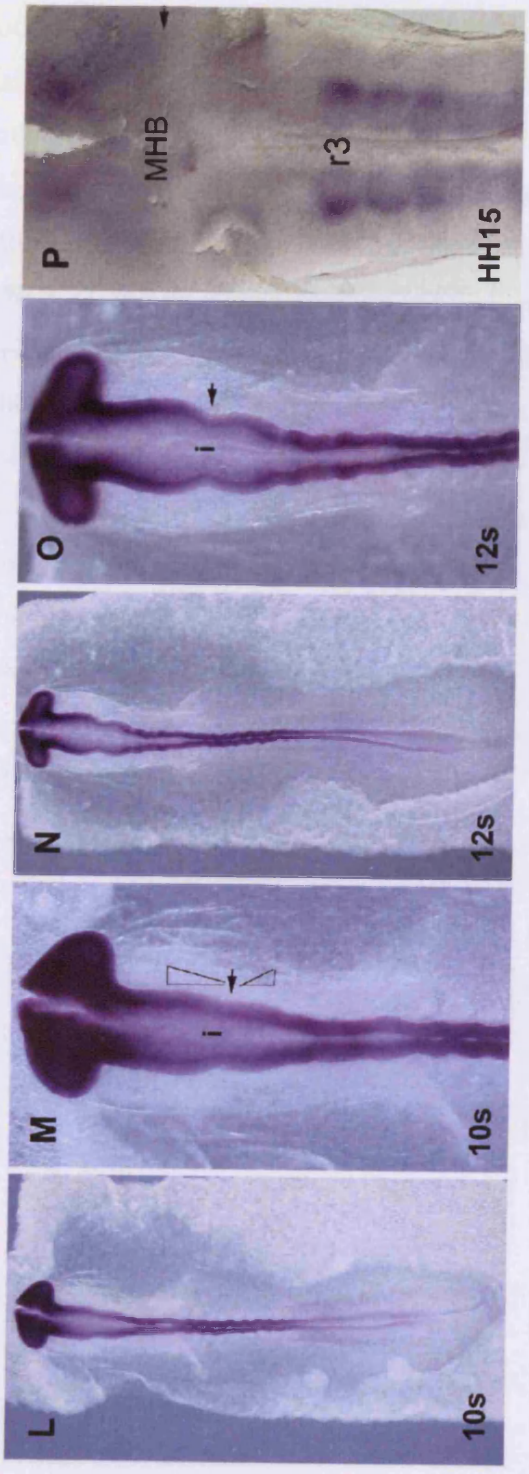


Figure 3.11(1-2) c*Lrrn1* expression is excluded from the MHB domain in a similar time period to c*LFng*.

(A-C, G, I, L and N) 3.2x magnification. (D, H, J, M and O) 8x magnification (A) 1 somite stage. c*Lrrn1* mRNA appears strongly in the presumptive telencephalon and presomitic mesoderm. (B) 4 somite stage. Strong expression is seen in the neural plate of future forebrain. (C, D) 5 somite stage. The expression in the presumptive MHB appears to be down-regulated gradually. (G, H) 8 somite stage. Strong expression remains in the forebrain and hindbrain. (I, J) 9 somite stage. Expression is downregulated from MB to r2. (L, M) 10 somite stage. Expression is still excluded from posterior MB, MHB, r1 and r2. The flat mount view of neural tube (K) indicates that the down-regulation around the MHB region is graded. (N,O) 12 somite stage. c*Lrrn1* is excluded from the MHB in a more restricted manner than c*LFng*. Gradients are less visible. (P) HH stage 15. Flat mount view. c*Lrrn1* is only expressed in the ventral half of the hindbrain, posterior from r3. Weak expression appears in the MB. (E, F) Transverse sections of 4 somite (E) and 8 somite (F) embryo. Expression at the neural tube appears to be in the neural folds, and this is excluded from roof plate and floor plate. Brackets show distinct expressions. Arrowheads indicate the border of expression. Arrows show the morphological MHB. Triangles indicate gradients of mRNA expression. i:MHB, r3: rhomobomere 3

Figure 3.12(1) Comparison of gene expression patterns at the MHB at HH stage 10.

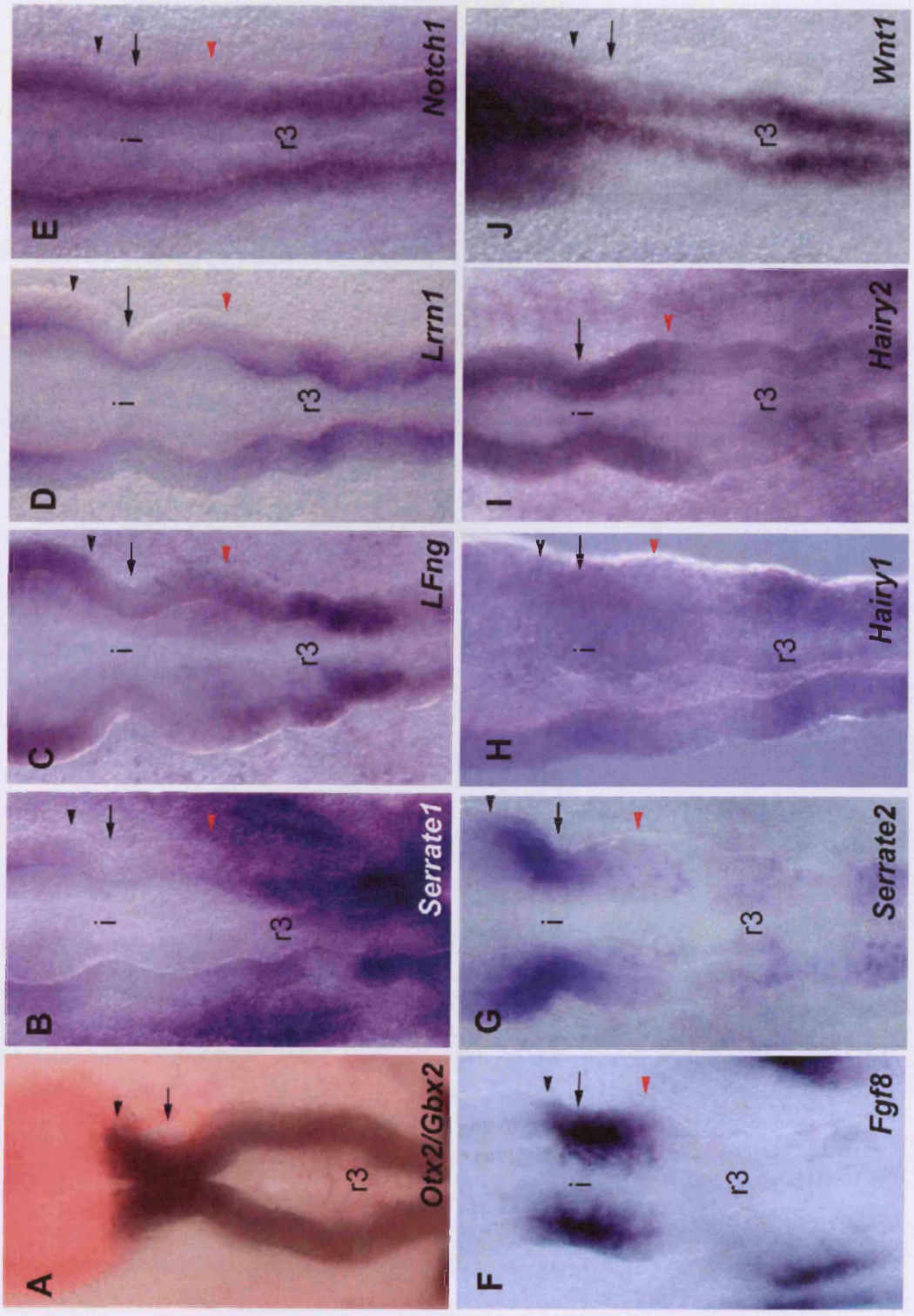


Figure 3.12(2) Comparison of gene expression patterns at the MHB at HH stage 10.

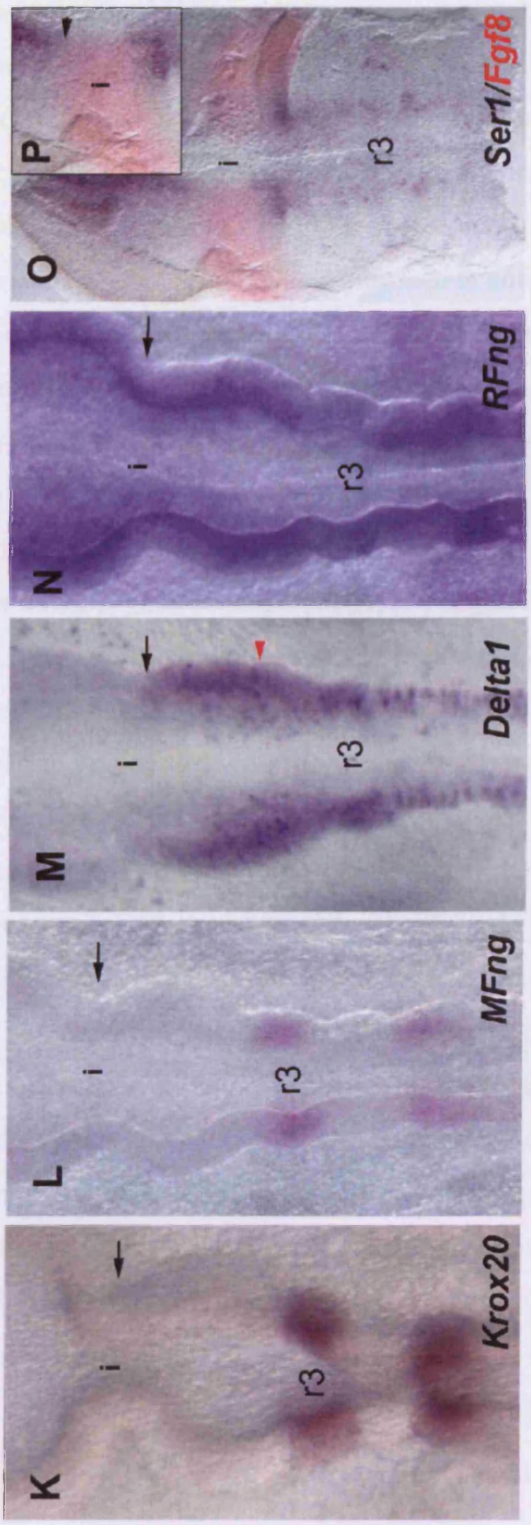


Figure 3.12(1-2) Comparison of gene expression patterns at the MHB at HH stage 10.

Gene expression of the MHB marker genes and Notch related genes at HH stage 10. (A-N) Dorsal views, 17.6x magnification around the MHB. Black arrow heads indicate the interface of *Otx2/Gbx2* (molecular MHB markers), and black arrows show the morphological MHB constriction. Red arrow heads indicate the r1/r2 border which coincides with the posterior most *Fgf8* expression of this stage. (A) *Otx2* in red, *Gbx2* in blue. At this stage, the *Otx2* and *Gbx2* interface is anterior to the morphological MHB. (B) c*Ser1* is excluded from the MHB. The posterior border of the MB expression coincides with the *Otx2/Gbx2* interface. (C) c*LFng* is not expressed in the MHB, and the posterior border of expression in the MB coincides with the *Otx2/Gbx2* interface. The posterior expression appears from r2. (D) c*Lrrn1* expression is down-regulated at the MHB, and both anterior and posterior borders are the same as c*LFng*.(E) c*Notch1* expression is down-regulated at the MHB. (F) c*Fgf8* is expressed in the posterior MHB. The anterior limit of this expression sits at the Otx2/Gbx2 interface, and posterior most limit is at the r1/r2 border. (G) c*Ser2* is expressed within the MHB. The anterior most edge of expression coincides with the Otx2/Gbx2 border. The posterior most expression reaches the r1/r2 border, although expression is weak here. (H) c*Hairy1* expression appears in the MHB. The anterior border of this expression is at the Otx2/Gbx2 border, and posterior most is at the r1/r2 border. (I) c*Hairy2* expression is also observed within the MHB. While this gene is expressed in the MB, the posterior border is at the r1/r2 boundary. (J) c*Wnt1* expression in the MB is restricted at the *Otx2/Gbx2* interface. (K) c*Krox20* is expressed in r3 and r5. (L) c*MFng* is not expressed in the MHB. Expression is observed only in r3 and r5. (M) c*Dll* is excluded from the MB. The anterior most limit of strong c*Dll* expression in the HB is at the morphological MHB. (N) c*RFng* is expressed ubiquitously at this stage. (O,P) double staining, c*Ser1* in blue and c*Fgf8* in red. In the high magnification view (P), c*Ser1* expression is complementary to the c*Fgf8* expression, and clearly abuts on both sides

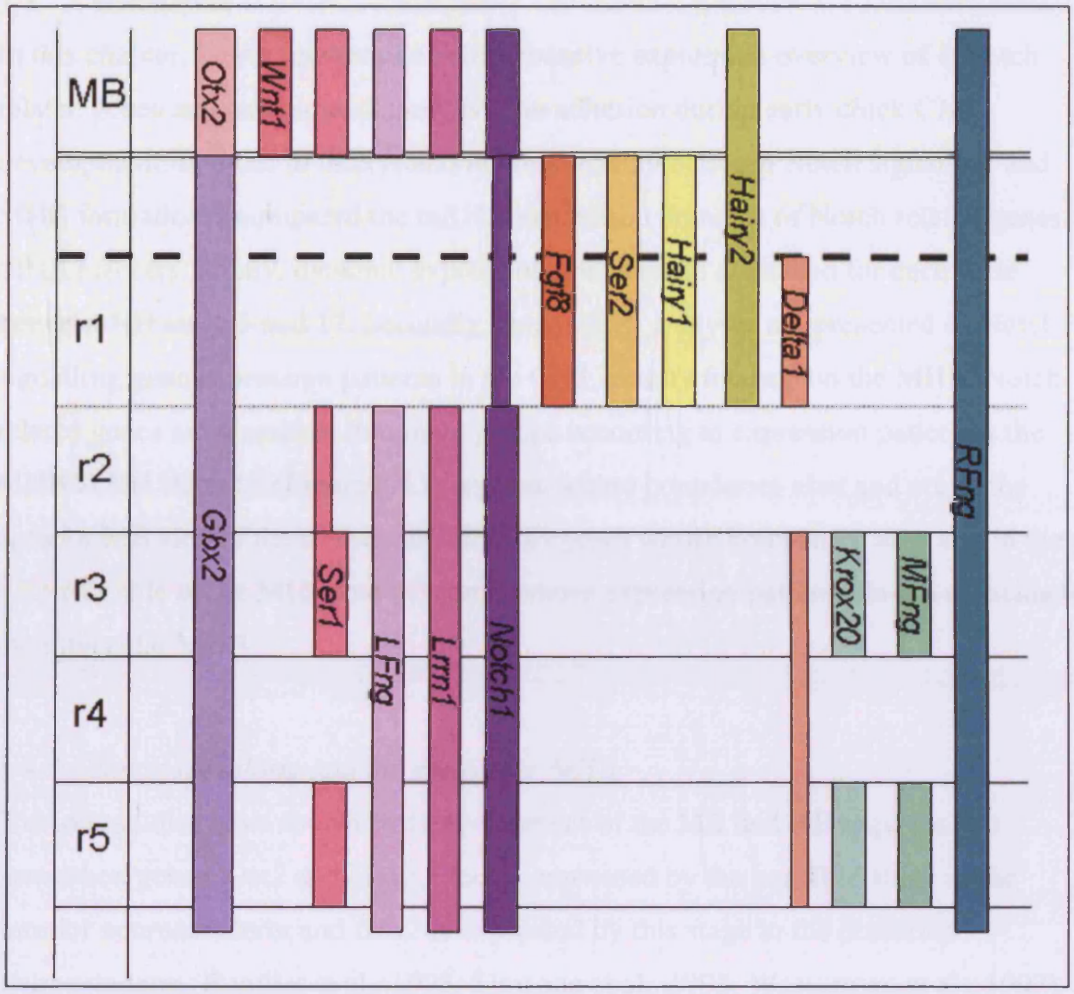


Figure 3.13 Comparison of the expression pattern of Notch related genes and MHB markers at the MHB.

Notch related gene expression compared to that of the MHB markers' expression. Double line indicates the molecular MHB. Dotted line shows the morphological MHB (constriction).

Otx2 rostrally and *Gbx2* caudally abut anterior to the morphological MHB, setting a molecular MHB. *Wnt1* is restricted to the *Otx2* positive region and its expression boundary follows this molecular MHB. The posterior expression boundary of c*Ser1*, c*LFng* and c*Lrrn1* also coincides with the molecular MHB border. c*Notch1* is downregulated from this same domain.

Fgf8 is expressed within the MHB, with its anterior limit at the molecular MHB. c*Ser2* and c*Hairy1* appear to be expressed in an almost identical manner to *Fgf8* at 10 somite stage. The posterior limit of c*Hairy2* matches the posterior limit of *Fgf8* expression.

3-4 Discussion

In this chapter, I have presented a comprehensive expression overview of 8 Notch related genes and one molecule involved in adhesion during early chick CNS development. In order to understand the relationship between Notch signalling and MHB formation, I compared the mRNA expression domains of Notch related genes and MHB markers. Firstly, dynamic expression domains are described for each gene between HH stage 5 and 17. Secondly, comparison analyses are presented of Notch signalling gene expression patterns in the CNS, mainly focused on the MHB. Notch related genes are classified into three groups according to expression pattern at the MHB at HH stage 10 (Figure 3.13) i) genes whose boundaries abut and are in the anterior MB side of the molecular MHB, ii) genes whose boundaries abut and in the posterior side of the MHB and iii) genes whose expression patterns do not coincide to the molecular MHB.

3-4-1 Notch signalling and the molecular MHB

Previous studies have shown that development of the MB and HB requires two homeobox genes, *Otx2* and *Gbx2*. *Otx2* is expressed by the headfold stage in the anterior neuroectoderm and *Gbx2* is expressed by this stage in the posterior neuroectoderm (Bouillet et al., 1995; Simeone et al., 1993; Wassarman et al., 1997). It is known that these two genes share a border of expression and that this interface demarcates the presumptive MHB. Studies using genetically modified mouse embryos have demonstrated that a lack of *Otx2* caused clear defects of caudal neural structures up to r3, and embryos lacking *Gbx2* failed to develop anterior HB structures including r1-3 (Matsuo et al., 1995; Wassarman et al., 1997). This strongly suggests that the *Otx2/Gbx2* interface is significant for the positioning of the MHB organiser that will go on to regulate MB and cerebellum development. Expression of *Fgf8* and other MHB organiser genes, in particular *Wnt1*, shares this domain and contribute to the further formation and maintenance of the MHB.

The gene expression analysis showed that there was a strong correlation between the expression patterns of Notch related genes and the *Otx2/Gbx2* interface at the molecular MHB (Figure 3.13). It is clear that *cSer1*, *cLFng* and *cLrrn1* are all downregulated on the posterior side of the molecular MHB (in *Gbx2* positive cells) at HH stage 10.

Comparatively, *cSer2* and *cHairy1* are downregulated at the anterior side of the molecular MHB (in *Otx2* positive cells) at this stage.

During *Drosophila* wing disc DV boundary formation, *Fng*, *Serrate* and *tartan* are only expressed in dorsal cells, while *Delta* is expressed in ventral cells. Restricted high Notch activation between a DV boundary then leads to *wg* expression at the boundary. Compared to the *Drosophila* wing disc model, gene expression patterns of Notch related genes were more complex in chick MHB. Similar to *Drosophila*, *cLFng* and *cLrrn1* are only expressed at the MB side of the molecular MHB. As *Fng* does in *Drosophila*, *LFng* may have a role for creating a boundary at the MHB by giving preference for ligands for Notch activation. In contrast with these similarities, however, the anterior most boundary of *cDll1* expression is not related to the molecular MHB. Rather, expression of *cDll1* appears to be found in the HB segments where neurogenesis takes place. Instead of *cDll1*, *cSer1* and *cSer2* are at the either side of molecular MHB, and may create the necessary ligand bias. Thus, it is clear that Notch related genes may play a significant function for the formation or maintenance of the MHB. The DV boundary of the *Drosophila* wing disc shares many similarities with the boundary between midbrain and hindbrain, and may provide a basic model upon which a more complex model of MHB boundary formation may be built.

3-4-2 Notch ligands and the molecular MHB

Following flat mount analysis, I observed greater cytoplasmic localisation of *cNotch1* transcripts on the anterior side of the molecular MHB which probably suggests the existence of higher level of cNotch1 protein due to active translation. *cSer1* also behaved in this manner. More interestingly, expression of *cSer1* is clearly excluded from the domain where *cFgf8* is expressed, and these two expressions abut both anteriorly and posteriorly. This suggests that *cSer1* is also correlated with the molecular MHB and this ligand localisation may have a significant role in Notch activity at the molecular MHB. By contrast, *cSer2* expression was observed at the posterior side of molecular MHB at HH stage 10, overlapping with *cFgf8* expression (data not shown). This contrasting expression pattern at the molecular MHB reveals that c*Ser1* and c*Ser2* may have antagonistic or cooperative function for the formation or maintenance of molecular MHB. Meanwhile, another Notch ligand, *cDll1* displayed its anterior most expression boundary at the morphological MHB. *cDll1* is generally required during

neurogenesis where it leads *Dll* expressing cells to be neurons. At the CNS boundaries, neurogenesis is delayed compared with the segmental regions (Raible and Eisen, 1995; Trevarrow et al., 1990). Thus, *cDll* may be required only for the neurogenesis around the MHB, and may be different from the function of *cSer1* and *cSer2*.

3-4-3 On/off expression of Notch related genes in the chick CNS

Several Notch related genes, such as vertebrate *hairy* homologues, *cHairy1*, *cHairy2*, *HES1*, *HES7* or *her1* (Jouve et al., 2000; McGrew et al., 1998; Palmeirim et al., 1997), *LFng* (Aulehla and Johnson, 1999), and *DeltaC* (Jiang et al., 2000) are known to be cyclic genes. A dynamic wave of these genes appears to cross the entire presomitic mesoderm (PSM) once during each somite formation, and waves of Notch activation generates the somite boundaries (Pourquie, 1999). Jouve and her colleagues reported that oscillations in the expression patterns of these cyclic genes are observed as early as primitive streak stages (Jouve et al., 2000). However, the expression of cyclic vertebrate genes reported to be restricted to particular boundaries or rhombomeres did not show oscillations in the CNS (Baek et al., 2006; Johnston et al., 1997; Qiu et al., 2004). Here, I have shown that *cHairy1* and *cMFng* display on/off expression between HH stage 9 to HH stage 11 at the MHB (Figure 2K-M, 7N, O). While *cHairy1* was expressed at the MHB at HH stage 10, *cMFng* expression was downregulated at the same period. It suggests that *cHairy1* expression is required at the MHB at HH stage 10, but this signalling would not require modification of Notch by cMFng. *cHairy1* expression is reported to be more similar to *HES1* than *cHairy2* in chick (Jouve et al., 2000). In mouse, both mRNA and protein of HES1 were demonstrated to be expressed at the MHB in a restricted manner, reducing cell proliferation and maintaining undifferentiated cells (Baek et al., 2006). In the absence of *HES1* and its related gene *HES3*, MHB cells differentiated into neurons (Hirata et al., 2001). It is possible that temporal upregulation of *cHairy1* follows a similar role to the mouse *HES1* and is acting to maintain a neuron-free zone which is important for the organiser activity of MHB.

3-4-4 Probability of Notch signalling function in the MHB formation and maintenance

The gene expression pattern analysis in this chapter raises two questions about Notch signalling function at the MHB. The first is whether Notch signalling is required for the formation of the local signalling centre domain rather than a boundary. The posterior most boundary of both *cHairy1* and *cHairy2* expression at the MHB sits at r1/r2, where the HB expression of *cSer1*, *LFng* and *Lrrn1* starts. This mirror image-like expression pattern of *cSer1*, *LFng* and *Lrrn1* is similar to that found at the ZLI and zebrafish HB. *LFng* expression is strongly excluded from the ZLI (Zeltser et al., 2001). Zebrafish *delta* genes are selectively absent from the HB boundaries (Cheng et al., 2004; Qiu et al., 2004). However, counter to this comparison, the MHB has a major difference from either ZLI or HB – neither *Shh* nor *Wnt1* are expressed across the MHB. More significantly, *Wnt1* expression at the MHB is only restricted to the rostral to the *Otx2/Gbx2* interface. This gives rise to a second question: is Notch signalling required for the cell lineage restriction at the MHB? In the *Drosophila* wing disc, Notch signalling is also required for the formation of a morphological wall which prevents cells from intermingling. In chick CNS, *cSer1*, *cLFng* and *cLrrn1* selectively create the positive/negative expression domains, and Notch could be highly activated at the boundaries: the molecular MHB and r1/r2 boundary. In the ZLI, cells misexpressing *LFng* are selectively excluded from the *LFng* domain (Zeltser et al., 2001). In addition, cells expressing ectopically activated Notch (following introduction of NICD) showed selective segregation to the HB boundary (Cheng et al., 2004). It is important to understand how these Notch-related genes are functioning and influencing the formation of the MHB.

Chapter 4 Notch signalling and MHB boundary and organiser formation

4-1 Introduction

Notch related genes are noteworthy for their expression at the molecular MHB in HH stage 10 chick embryos. Notch signalling has been implicated in the establishment of crucial signalling centres in the vertebrate CNS. In the zebrafish HB, *rfng* is expressed within the boundary cells, while *deltaA* and *deltaD* are expressed adjacent to boundaries (Cheng et al., 2004; Qiu et al., 2004). Similar to the Notch function in *Drosophila* wing disc, wnt1 is induced by rfng mediated modulation of Notch activity at the HB boundaries (Amoyel et al., 2005; Cheng et al., 2004). Lack of *rfng* downregulates *wnt1* expression at the HB boundaries which is normally expressed in HB boundary cells and roof plate cells (Cheng et al., 2004). *delta* genes in rhombomeres segments are upregulated by a proneural gene, *as-c/ash*, whom expression is regulated by Wnt signalling from both boundary cells and roof plate cells (Amoyel et al., 2005). These HB boundaries attract both NICD expressing cells and dominant active Su(H) expressing cells, while dominant negative Su(H) expressing cells remains in rhombomeres segments and stay away from the HB boundaries (Cheng et al., 2004). Thus, in the zebrafish HB, Notch signalling is required for boundary cell segregation and for correct neurogenesis via lateral inhibition which is regulated by *wnt1* (Amoyel et al., 2005; Cheng et al., 2004). In the chick CNS, Notch signalling has not to date been demonstrated in boundary formation or maintenance, but cells expressing ectopic *LFng* selectively move out from the zona limitans intrathalamica (ZLI), a boundary and compartment which lies between dorsal and ventral thalamus (Zeltser et al., 2001). *LFng* is normally expressed in both the dorsal and ventral thalamus, but not in the ZLI, thus demarcating this region as a discrete compartment. *Delta* and *Serrate* follow the same expression pattern. It is not clear whether this selectivity of *LFng* occurs through the Notch signalling pathway or by another mechanism. However, the simplest explanation is that the presence of *LFng* affects the affinity difference between cells in the thalamus and the ZLI.

Notch activation occurs when NICD is cleaved from the transmembrane Notch receptor; the NICD fragment is then able to act directly as a transcriptional activator of its target genes. NICD is a fragment which comprises of one RAM23 domain and 6

CDC10/Ankyrin repeat domains which are crucial for the binding of Su(H)/CSL transcription factor proteins (Kato et al., 1997; Tamura et al., 1995). Despite the complexity of the Notch signalling pathway, it has been reported that NICD alone is sufficient to activate Notch signalling within cells both *in vitro* and *in vivo* (Schroeter et al., 1998; Takke and Campos-Ortega, 1999).

Here I show that misexpression of active Notch via *in ovo* electroporation of NICD fragments leads to two crucial phenotypes; cell sorting and cell fate changes. In cases where the first phenotype is apparent I reveal that cells expressing ectopically active Notch are selectively excluded from the metencephalon (r1 and r2), a region where expression of other Notch related genes is also excluded. In examples of the second phenotype, cells misexpressing active Notch undergo a cell fate change from r1 to MB fate. Cell population analysis shows that the presence or absence of a community effect may be the factor that decides between these two phenotypes. I propose a model where Notch activation at the MHB regulates MHB organiser gene expression, including *Otx2* and *Gbx2*, genes which position the MHB.

4-2 Materials and methods

4-2-1 in ovo electroporation of DNA

A construct containing a Notch1 intracellular domain (NICD) linked with green fluorescent protein by an internal ribosomal entry site (IRES-GFP) at the upstream of IRES was used for *in ovo* electroporation. This plasmid was constructed and kindly given by O. Voiculescu. pCAB-IRES-eGFP plasmid was used as a control. DNA injection and *in ovo* electroporation was performed as described (Chapter 2). DNA was injected at HH stage 8 and 9, and electroporated by placing electrodes either side of the neural tube. Embryos were left to develop and processed for the whole mount *in situ* hybridisation or whole mount double *in situ* hybridisation as described in Chapter 2. Immunohistological analysis was also carried out as described in Chapter 2. Detailed description of the RNA probes used can be found in the appendices (Appendix A).

4-2-2 GFP-RFP in ovo co-electroporation

GFP and red fluorescent protein (RFP) co-electroporation was perfomed following the same method as single *in ovo* electroporation. pCAB-IRES-mRFP1-grip was used as a

control (this cDNA was a gift from J. Gilthorpe., King's College, London). The RFP plasmid was mixed with GFP constructs prior to injection.

4-2-3 Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling (TUNEL) analysis

The whole mount TUNEL analysis followed the manufacturer's protocol with revision by A. Gibson (Terminal transferase recombinant (Roche: 333574)). Immunohistochemical analysis of GFP was done after TUNEL analysis using anti-eGFP (Sigma) primary antibody. Alexa Fluor 568 fragment of goat anti-rabbit (Invitrogen) was used as a secondary antibody.

4-2-4 EC culture and real-time fluorescent analysis

EC culture was performed as described by S. C. Chapman (Chapman et al., 2001). HH stage 8-9 embryos were electroporated *in ovo* and incubated for 2 hours to allow them to recover. The embryos were then moved to EC culture beds and incubated for 24 hours. Real-time fluorescent analysis was carried out every 30 mins from 6 hours post-electroporation using a Nikon SMZ1500 microscope. Fluorescent photographs were taken individually with a Nikon digital camera (DXM1200F) attached to a Nikon SMZ1500 microscope. Any embryos showing malformation of the heart or damage to the area opaca were excluded during the experiments.

4-3 Results

4-3-1 Ectopic Notch activation causes cells to move out of the metencephalon (r1/r2)

In order to introduce active Notch domains in a mosaic fashion, I performed electroporation of NICD-GFP throughout the MHB domain at HH stages 8 and 9. DNA vectors were electroporated unilaterally in the neural tube, thus, ectopic expression of these genes was localized to only one side of the neural tube. Misexpression of constitutively active Notch expressing cells across the MHB showed two distinctive phenotypes. In the first, activated Notch cells are excluded from r1 and r2 (n=30/55). In some of these embryos regional gene expression is perturbed. In the second phenotype, activated Notch cells are not excluded from r1 and r2 territory, but regional gene expression is perturbed (n=25/55).

Here, I describe the phenotype which showed selective cell exclusion from r1 and r2. Electroporation technique was controlled for by viewing embryos electroporated with a control GFP construct under UV light to check that misexpression of this construct appeared in a constant manner throughout the targeted domain within the neural tube (Figure 4.1A, n=0/21). However, cells ectopically expressing constitutively active Notch were not found in longitudinal strips along the length of the neural tube like the controls. Instead they appeared in patches excluded from r1 and r2 (Figure 4.1B, n= 30/55). To understand the position of the restricted constitutively active Notch expressing cells, immunohistochemistry analysis was applied after whole mount *in situ* hybridisation using neural compartment marker genes (Figure 4.1C-H). *Hoxa2* marks all rhombomeres up to but excluding r1 and *Fgf8* marks the MHB. Thus *Fgf8/ Hoxa2* double whole mount *in situ* hybridisation was used to demarcate r1. Ectopically introduced constitutively active Notch expressing cells were absent from the r1 and r2 domain and thus were restricted either anteriorly within the MHB or posterior to r2/r3 boundary (Figure 4.1C). For further analysis, mesenchymal tissues and notochord were removed from samples and the neural tube was flatmounted. Constitutively active Notch expressing cells were seen located within the MB, MHB and r3-5, but not in r1 and r2 (Figure 4.1E). Under high magnification analysis, it is clear that cells expressing active Notch were strongly localised toward the MHB and posterior from r2/r3 boundary.

Figure 4.1 Misexpressed constitutively active Notch expressing cells are excluded from the metencephalon (r1 and r2).

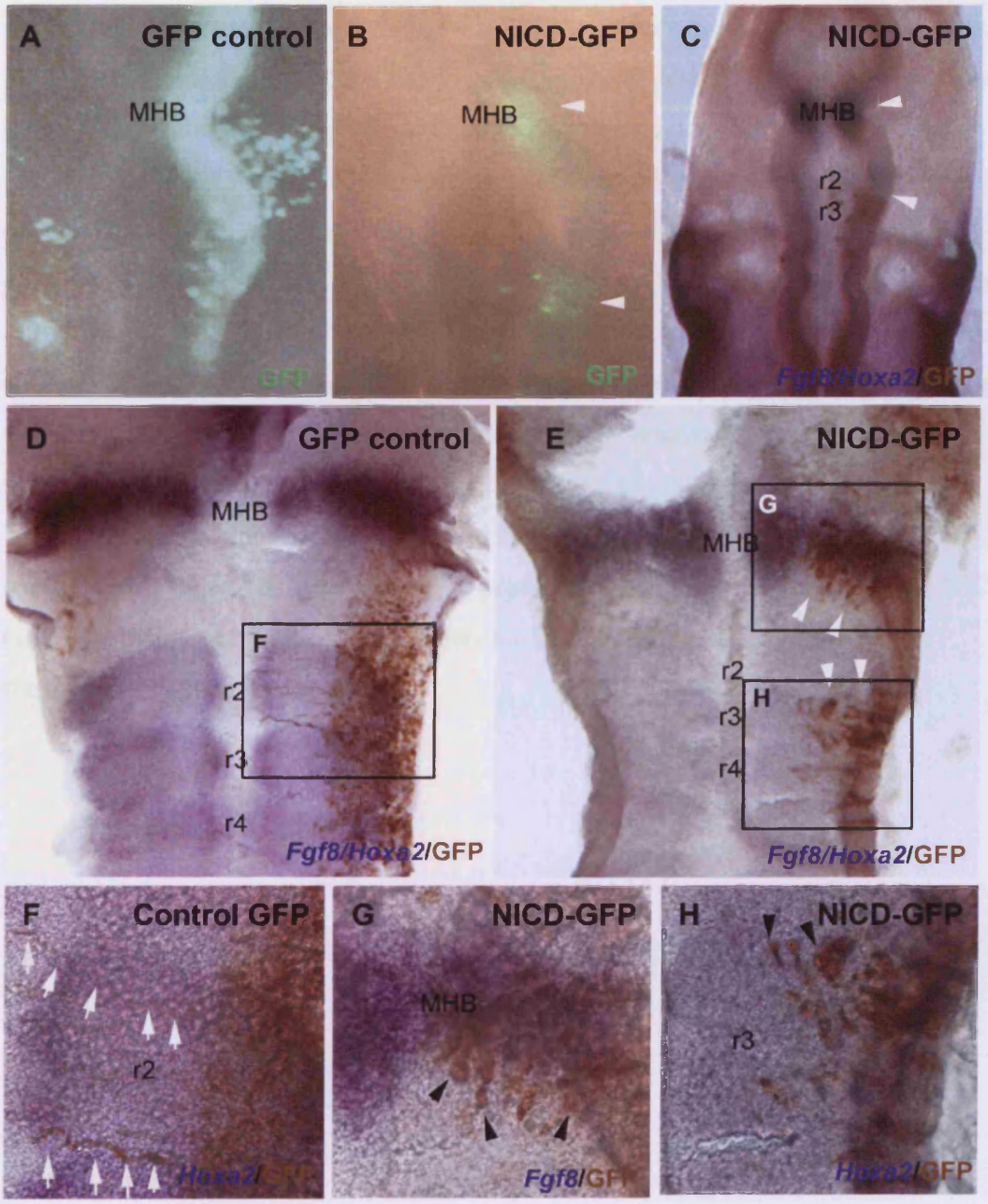


Figure 4.1 Misexpressed constitutively active Notch expressing cells are excluded from the metencephalon (r1 and r2)

All *in ovo* electroporation was carried out at HH stage 8 and 9. Electroporation was unilateral and covered a broad anterioposterior domain from midbrain to hindbrain. (Garcia-Calero et al.) High magnification dorsal view (D-H) The neural tube from MHB to hindbrain was flatmounted; dorsal = lateral, anterior = top. Whole mount *in situ* hybridisation marked the MHB with *Fgf8* (blue), and posterior rhombomeres from r2 with *Hoxa2* (blue). GFP protein was stained immunohistochemically (brown).
(A, D) Control GFP expressing cells appeared in a continuous line within the neural tube 24 hours post- *in ovo* electroporation. Control GFP expressing cells were observed consistently across the MHB and hindbrain. (B, C, E) NICD-GFP expressing cells were only found in the MHB and posterior from r2/r3 boundary. (F) High magnification view shows that a number of control GFP expressing cells produce processes toward the ventral midline. (G, H) NICD-GFP expressing cells are round and have short processes toward the dorsal roof plates in both the MHB and hindbrain.
White arrow heads indicate the localised NICD-GFP expressing cells. White arrows show the processes which contain GFP proteins. Black arrow heads show the NICD GFP expressing cells. MHB: mid-hindbrain boundary; r2: rhombomere 2; r3: rhombomere3; r4: rhombomere 4

4-3-2 The absence of constitutively active Notch expressing cells in r1/r2 is not due to selective cell death

There are a number of possible reasons why I did not observe constitutively active Notch expressing cells in r1 and 2. Firstly, precise levels of ectopically introduced cDNAs are difficult to obtain by the *in ovo* electroporation method, despite a consistent amount of DNA injected into each neural tube. Hence, this region of the neural tube may have failed to incorporate the NICD-GFP DNA. This seems unlikely however, as this phenotype was found in a high percentage of cases compared to embryos which were electroporated with control GFP. Secondly, it is possible that either the ectopically manipulated Notch signalling level or physical electronic current caused r1 and 2 domain cells to die. Thirdly, cells that contained high levels of constitutively active Notch may be excluded from this domain due to a change in cell surface properties, and may even actively move out of this domain to incorporate into regions of the neural tube with more similar cell surface properties.

To test these hypotheses, I performed Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labelling (TUNEL) analysis on embryos which were electroporated with the NICD-GFP construct at HH stage 8 to 9. TUNEL analysis marks apoptotic cells by binding the nicked DNA within the cells. Dorsally localised apoptotic cells were found in both non-treated embryos (data not shown) and control GFP embryos (Figure 4.2A, B, n=65/65). In both of the cases, TUNEL stained cells were detected throughout the r1/2 region at a moderate level. However, I could not detect any increase in apoptotic cells in r1 and r2 where constitutively active Notch expressing cells were excluded (Figure 4.2A-D, n=53/64). In fact, there were fewer apoptotic cells as compared to control GFP embryos (compare Figure 4.2B and 4.2D). Ectopically introduced constitutively active Notch expressing cells did not cause selective cell death in either a cell autonomous or non- cell autonomous manner (Figure 4.2E-J). Similar results were observed in embryos grown for 6 hrs and 12 hrs post-eletroporation (data not shown). This suggests that the selective exclusion of constitutively active Notch expressing cells at r1 and r2 is not due to selective cell death.

4-3-3 Constitutively active Notch expressing cells are selectively excluded from a morphological compartment

To test whether the restriction of constitutively active Notch expressing cells was due to cell movement, IRES-RFP was co-electroporated with each GFP construct. Since synthesised RFP proteins from the control construct are identified using a red fluorescence filter, co-electroporation of this construct along with GFP constructs marked an electroporated domain and allowed visualisation of the specific cell movements of GFP-expressing cells. cDNAs which were introduced ectopically via *in ovo* electroporation require a certain length of time for translation within the cells. GFP proteins expressed from a chicken β-actin promoter were observed 3hrs post-electroporation in the chick neural tube. However, I could only observe GFP proteins expressed by NICD-IRES-GFP clearly 6 hrs post-electoporation in the chick neural tube, possibly due to comparatively inefficient translation with the IRES sequence in the construct. Here, both RFP and GFP expression were harvested 6-24 hrs apost-electroporation. Each time points of 6, 12, 24 hrs showed the same phenotypes. Cells containing both RFP and GFP (observed as yellow cells from dual channel immunoflourescence) were compared with those that only contained either RFP (red cells) or NICD-GFP (green cells). Control RFP proteins were observed to share the same domain as control GFP proteins (Figure 4.3A, B, n=6/6). However, NICD-GFP proteins showed clear exclusion from the morphological compartment (Figure 4.3D-F, n=5/7), and the cells containing both RFP and GFP (yellow cells) were found only in the morphological boundaries (Figure 4.3F, n=5/7). In contrast, only RFP protein-expressing cells were found in the morphological compartment. This shows that exclusion of constitutively active Notch expressing cells from the r1 and r2 domain occurred selectively to NICD-GFP cells. Interestingly, the expression pattern and levels of RFP when electroporated with control GFP, as compared with RFP together with NICD-GFP, showed higher levels of RFP expression at the morphological boundary in NICD embryos (Compare Figure 4.3B and 4.3E). One explanation for this would be as a result of selective cell sorting of NICD-GFP cells. It appears that NICD-GFP cells have actively localised to the morphological boundaries.

Figure 4.2 Absence of NICD-GFP cells in r1 and r2 is not due to selective cell death.

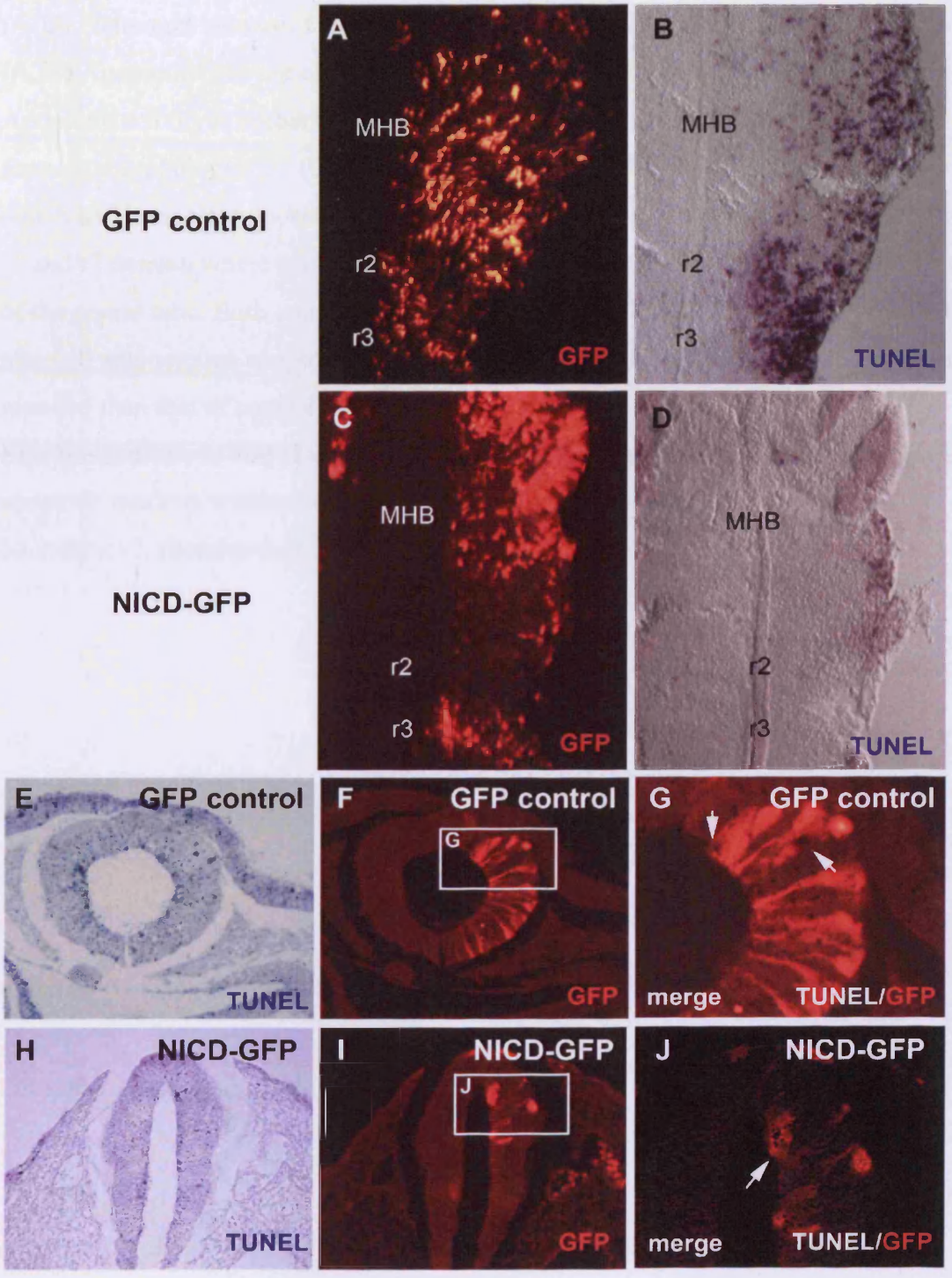


Figure 4.2 Absence of NICD-GFP cells in r1 and r2 is not due to selective cell death

Apoptotic cells are observed at 12 and 24 hours post electroporation. Apoptotic cells were stained by TUNEL (blue), GFP protein was stained immunohistochemically (red). (A-D) Flatmount views of GFP control embryo and NICD-GFP embryo.
(A, B) Apoptotic cells are observed mainly in the dorsal side of the neural tube. Apoptotic activity is higher around r2/r3 boundary at this stage where the boundary is forming morphologically. (C, D) Few apoptotic cells were found in the neural tube which had been electroporated with NICD-GFP. GFP proteins (red) are excluded from r1 and r2 domain where apoptotic cells are also absent. (E-J) Transverse section views of the neural tube. Both control GFP cells (E-G) and NICD-GFP cells (H-J) showed no non-cell autonomous apoptosis. (J) The shape of NICD-GFP expressing cells was more rounded than that of control cells.
Brackets indicate r1 and r2 domains. White arrows show GFP expressing cells in which apoptotic markers within the nucleus were also observed. MHB; mid-hindbrain boundary; r2; rhombomere 2; r3; rhombomere 3

Figure 4.3 Cells expressing constitutively active Notch only co-localised with RFP at the morphological boundaries.

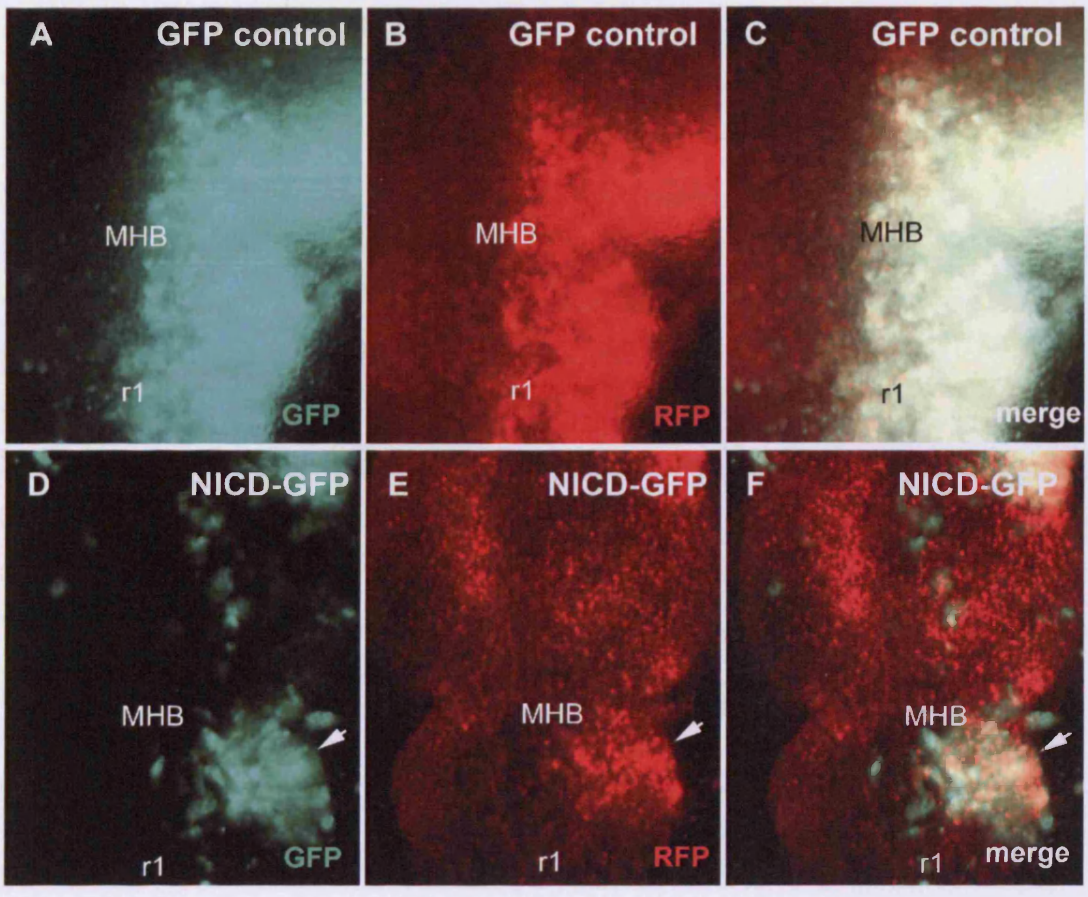


Figure 4.3 Cells expressing constitutively active Notch only co-localised with RFP at the morphological boundaries.

GFP constructs and control RFP construct were co-electroporated at HH stage 7-8. (A-C) 24hrs after co-electroporation of GFP control and RFP control. Photos were taken dorsally. (A) GFP proteins were observed in a consistent manner across the MHB. (B) RFP proteins were observed in a consistent manner across the MHB laterally. (C) Merged view of GFP and RFP. Cells containing both GFP and RFP appear yellow/white in colour. There are no gaps between GFP and RFP expression. (D-F) 24 hrs after co-electroporation of NICD-GFP construct and RFP control. (D) GFP proteins were excluded from the morphological compartment, and only found around the morphological boundaries. The localisation at the MHB is posterior to the morphological MHB, on the r1 side of the constriction. (E) RFP expression. Significantly, some RFP cells are localised to the same domain as the NICD-GFP cells. (H) Merged view of GFP and RFP. Cells containing both GFP and RFP appeared only at the morphological boundaries.
White arrows indicates the position where RFP positive cells are highly localised.
MHB; mid-hindbrain boundary

4-3-4 Ectopically activated Notch expression causes cell fate changes; repressing r1 and MHB markers, while inducing MB and MHB markers

Another phenotype of NICD misexpression was a change of regional gene expression at the MHB (n=30/55). To investigate any effect on the viability of the MHB after ectopic activation of Notch signalling, I performed whole mount *in situ* hybridisation of MHB molecular markers following electroporation of NICD-GFP. I used *Fgf8* as a marker of MHB organiser activity since *Fgf8* is the only protein to have been demonstrated to mimic MHB organiser activity when implanted on heparin beads into the neural tube (Crossley and Martin, 1995; Irving and Mason, 2000). In the presence of ectopic active Notch expressing cells across the MHB, *Fgf8* was downregulated (Figure 4.5A, B and I, control; n=1/48, NICD; n=41/60). *Gbx2* was used as a molecular marker of anterior hindbrain, and together with *Otx2,* is an early determinant of MHB position (Hidalgo-Sanchez et al., 2000; Hidalgo-Sanchez et al., 2005). Following ectopically activated Notch expression, *Gbx2* expression in the hindbrain, posterior to the MHB, was dramatically downregulated (Figure 4.5E, F, control; n=0/10, NICD; n=11/16).

Following misexpression of active Notch expressing cells, the anteriormost boundary of *Hoxa2* was shifted rostrally into r1 co-incident with the observed repression of *Fgf8* (Figure 4.4A, B, I, control; n=0/29, NICD; n=20/34). *Hoxa2* is expressed in the hindbrain with an anterior limit at the r1/r2 boundary. It has previously been reported that *Hoxa2* expression is repressed in r2 following FGF bead implantation into the r1/r2 boundary, while the implantation of a bead soaked in an anti-FGF8 blocking antibody in r1 expands *Hoxa2* expression into r1 (Irving and Mason, 2000). Therefore, this observed shift in *Hoxa2* is most likely due to the lack of *Fgf8* which normally acts to repress *Hoxa2* and position the r1/2 boundary. *Wnt1* is normally expressed on the anterior side of the MHB only. After ectopic Notch activation, cells expressing *Wnt1* were detected in the hindbrain. *Wnt1* expression in the MB and MHB was expanded into r1 dorsally (Figure 4.4G, H, L, control; n=0/32, NICD; n=15/21). I also looked at *Fgf3* expression as it is expressed from HH stage 12 on the midbrain side of the MHB after MHB formation, and is also expressed in rhombomere boundaries after they become morphologically apparent (Mahmood et al., 1995). Therefore I used it here as a marker of boundary cells. Interestingly, in the presence of ectopic active Notch expressing cells, *Fgf3* expression at the MHB was widely upregulated (Figure 4.4C, D, L, control; n=0/10, NICD; n=9/11). Strikingly, the downregulation of *Fgf8* and ectopic

expression of *Wnt1* both occurred in a non-cell autonomous manner (Figure 4.4L). Taken together, these data indicate that ectopically activating the Notch signalling pathway across the region that will give rise to the MHB organiser, disrupts the molecular identity of the MHB, both at the level of *Otx2* and *Gbx2* which position the MHB, and at the level of the organiser itself (as analysed by *Fgf8* expression). Furthermore, activating Notch signalling within cells appeared to promote a boundary cell fate, as identified by *Fgf3* expression.

Figure 4.4 Constitutive activation of Notch in cells across the MHB causes changes in expression of MHB marker genes.

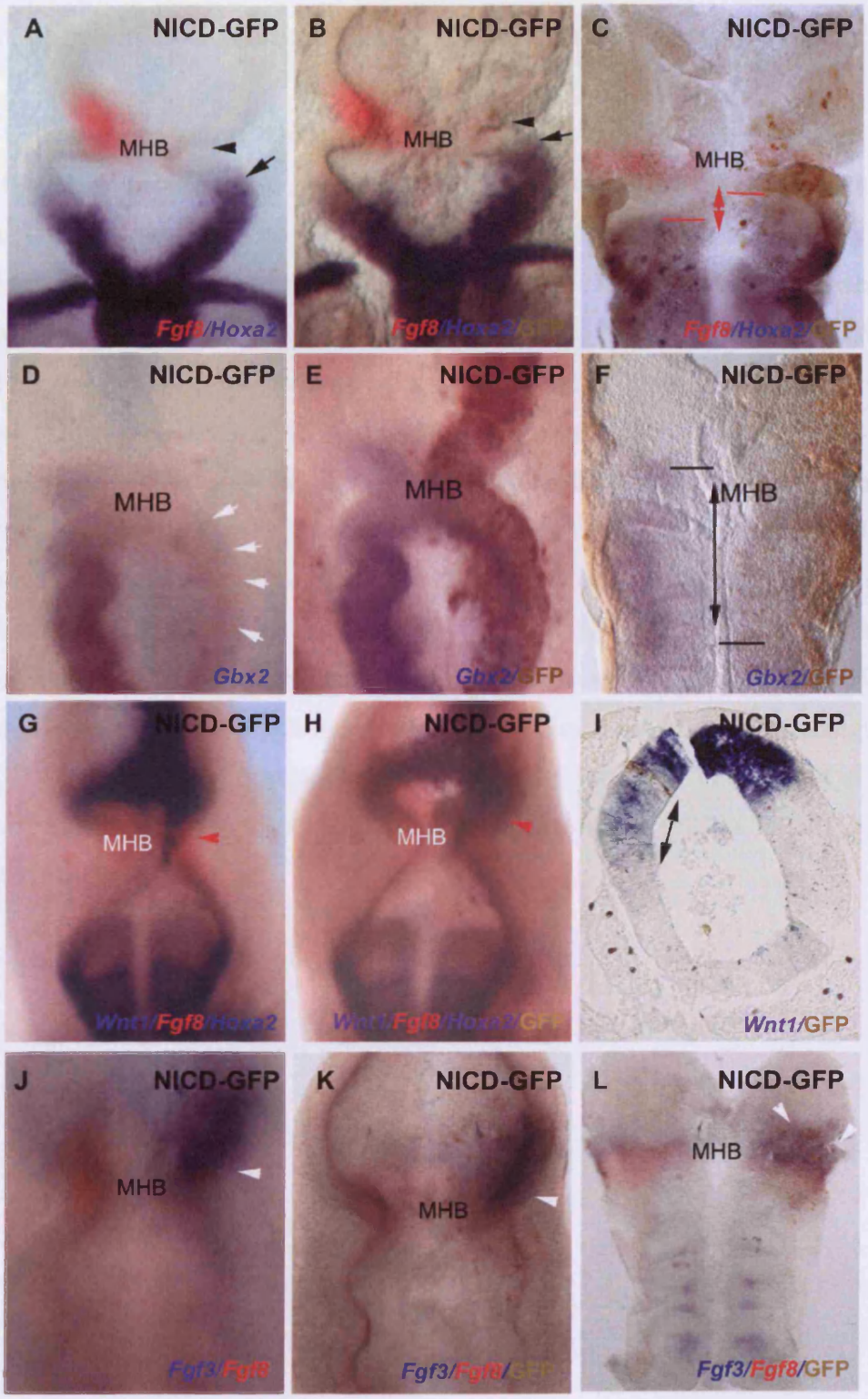


Figure 4.4 Constitutive activation of Notch in cells across the MHB causes changes in expression of MHB marker genes.

Regional gene expression was examined 24 hrs after the NICD-GFP *in ovo* electroporation. Immunohistochemistry shows GFP in brown (A-L).

(A-C) In the presence of NICD-GFP cells, *Fgf8* expression is downregulated at the MHB (Black arrow heads). (A) Double *in situ* hybridisation with *Fgf8* (red) and *Hoxa2* (blue) reveals that the anterior border of *Hoxa2* expression is shifted rostrally due to a lack of *Fgf8* (black arrow). (B) GFP positive cells are present at the MHB where *Fgf8* is downregulated. (C) The flatmount view shows a clear shift of *Hoxa2* expression limit (Red arrow head). (D-F) Intensive misexpression of NICD-GFP downregulates rostral *Gbx2* expression (D, E). (F) A flatmount view shows a clear shift of the *Gbx2* rostral limit (Black double arrow head). (G-I) After NICD-GFP electroporation, ectopic *Wnt1* expression (blue) is seen in the dorsal MHB (red arrow heads). (G) Ectopic *Wnt1* expression is seen dorsally at the MHB. (H) NICD-GFP positive cells are detected across the MHB. (I) Transverse sections of the midbrain side of the MHB show non-cell autonomous ectopic *Wnt1* expression. (J-L) *Fgf3* expression (blue) at the MHB is highly upregulated (white arrow heads). (J) *Fgf8* expression at the MHB is downregulated, (K) NICD-GFP expressing cells are coincident with the domain (L) Flatmount view reveals clearly that *Fgf8* and *Fgf3* expressions are perturbed within the NICD-GFP domain. MHB; mid-hindbrain

4-3-5 Ectopic Notch activation causes both cell fate changes and cell sorting, depending on the number and density of ectopic cells

Of the embryos expressing ectopically activated Notch, 54% showed a selective cell exclusion from r1 and r2, while the others underwent a cell fate change without any relocation. To investigate the reason for these different phenotypes, NICD embryos that showed cell sorting and those that showed cell fate changes were compared (Table 4.1). Out of 55 experimental embryos, 15 showed only cell sorting, 25 showed only cell fate changes, and 15 showed both cell sorting and cell fate changes. These results led to the conclusion that cell sorting does not necessarily correspond to cell fate changes at the MHB. However, these differences could be due to the absolute numbers of NICD expressing cells. It is possible that a critical number of NICD cells were necessary to cause cell fate changes, and below this number, cells would move to a "like "environment. To investigate if this was the case, I counted the total number of cells per rhombomeric segment expressing constitutively active Notch. I performed whole mount *in situ* hybridisation to confirm the molecular phenotype of the cells expressing activated Notch, and the host environment. Within the embryos which showed neither cell sorting nor fate changes, the total number of activated Notch expressing cells in any segment did not exceed 30 and usually contained less than 10. However, all segments where more than 30 activated Notch expressing cells were counted displayed either a cell sorting or a cell fate change phenotype. Higher numbers of activated Notch expressing cells were observed in embryos that had cell fate changes (Table 4.2). It is possible that the tendency toward cell sorting and fate changes may due to the community effect of cells. The community effect is an effect in which cells to respond to many nearby cells, as a result of which these cells activate tissue-specific genes and differentiate co-ordinately as a uniform population (Gurdon, 1988). A sandwich experiment with X*enopus* muscle cells demonstrated that a large number of cells are required for the community effect (Gurdon et al., 1993). In the case of NICD misexpression, when the number of ectopically activated Notch expressing cells is small and their distribution mosaic i.e. distributed with a low density within the tissue, these cells may relocate to the morphological boundary i.e. their preferred environment. On the other hand, when greater numbers of ectopically activated Notch expressing cells are present and they are closely packed, a community effect may mean that a new environment is created by the cells themselves, such that they have no need to relocate.

This ectopic population of active Notch cells would become a new source of signals, causing the cell fate changes that I have observed.

	Control	*NICD*
Cell sorting	N=0/21 (0%)	**N=30/55 (54.5%)**
Cell fate changes	N=0/21 (0%)	**N=40/55 (72.7%)**
Cell sorting + cell fate changes	**N=0/21 (0%)**	**N=15/55 (27.2%)**

Table 4.1 Frequency of cell sorting and cell fate changes.

Misexpression of constitutively active Notch expressing cells across the MHB showed two different phenotypes. One is the exclusion of the NICD-GFP expressing cells from r1 and r2 (cell sorting). Another is the perturbation of regional gene expression at the MHB (cell fate change). The frequency of each phenotype was examined. In 6 out of 21 embryos, control GFP expressing cells showed random exclusion from r1 and r2, while 30 out of 55 NICD-GFP embryos showed strong exclusion from r1 and r2. None of the control GFP treated group showed cell fate changes, while 40 out of 55 NICD-GFP embryos showed perturbed regional gene expression at the MHB. 15 embryos of NICD-GFP showed both phenotypes.

Number of cells	*Cell sorting*	*Cell fate changes*
3	X	**X**
5	X	**X**
10	√	√
30	√	√
52	√	√
76	X	√
114	**X**	√

Table 4.2 Frequency of cell fate changes and total cell numbers in the MHB.

GFP-expressing cell numbers in each compartment (MHB, r1, r2, r3) were counted, and compared to the phenotype. In total, 52 embryos were examined. Cell sorting was found when each compartment contained more than 10 NICD-GFP positive cells, but less than 70. Cell fate changes were found when each compartment contained more than 10 GFP positive cells. When the cell number of NICD-GFP positive cells exceeded 100, only the cell fate change phenotype was observed.

4-4 Discussion

I have demonstrated in this chapter that Notch activation can perturb MHB formation. Using *in ovo* electroporation techniques, molecular biology and immunohistochemistry I have established that ectopically activated Notch expressing cells are selectively excluded from the r1 and r2 (metencephalon) domain during CNS development. The domains to which the cells move coincide with areas where several Notch related genes are expressed at HH stage 10. Furthermore, the existence of ectopically activated Notch expressing cells across the MHB caused downregulation of *Gbx2* and *Fgf8*. Consistent with this, *Wnt1* expression was ectopically induced dorsally in r1. These cell fate changes were observed in a non-cell autonomous manner. Therefore, I propose a model where the level of Notch activation at the MHB is responsible for the positioning, formation and maintenance of the MHB.

4-4-1 Active Notch expressing cells are undifferentiated during neurogenesis

Notch activation within the neuroepithelium cell keeps cell fate undifferentiated by an inhibition of proneuronal gene expression (de la Pompa et al., 1997; Henrique et al., 1995; Williams et al., 1995). During neurogenesis, the number of cells which undergo neuronal differentiation is tightly controlled by lateral inhibition through a Notch-*Delta* interaction (Chapter1). High levels of *Delta* expression lead to Notch activation in neighbouring cells (Jennings et al., 1994). As *Hairy/E(spl)* family members repress the proneuronal gene *AS-C/MASH* cell-autonomously, activated Notch cells repress cell differentiation, and cells remain in a primary fate (Heitzler et al., 1996; Schrons et al., 1992). Indeed, in various populations of neuronal progenitor cells, misexpression of NICD is reported to show the increase number of undifferentiated cells causing Notch target genes to remain its expression (Austin et al., 1995; Chitnis et al., 1995; Henrique et al., 1997; Wettstein et al., 1997). Here, after *in ovo* electroporation, some control GFP cells oriented their projections, probably axons, towards the ventral midline (Figure 4.1D, F: white arrows). In contrast, the active Notch expressing cells often appeared to be round and their projections were short, wide and projected dorsally (Figure 4.1E, G and H: black arrow heads). This suggests that the cells are prevented from differentiating by the presence of activated Notch. In these cells, therefore, proneural genes such as *Mash1*, *neurogenin1 (ngn1)* and *neurogenin2 (ngn2)* may be repressed in a cell-autonomous manner.

4-4-2 Active Notch expressing cells sort to "like" domains

I found that ectopically active Notch expressing cells are excluded from r1 and r2. TUNEL analysis revealed that this absence of active Notch expressing cells was not due to selective cell death. Furthermore, GFP/RFP co-electroporation showed that this cell exclusion happened selectively to active Notch expressing cells. There are two possible explanations for why the ectopically active Notch expressing cells are excluded from the r1 and r2 domain. Firstly, these cells "dislike" the r1/2 domain as they carry cell surface properties that prevent them from mixing with cells within r1 and r2. Secondly, the domains surrounding r1 and r2, i.e. the MHB and r2/r3 boundary, create a "like" environment since their cell surface properties are similar to the ectopically active Notch expressing cells.

During zebrafish HB boundary formation, ectopic active Notch expressing cells were seen to sort into boundaries where one of the Notch modulator genes, *rfng*, was specifically expressed (Cheng et al., 2004). Cheng et al proposed that the cell sorting seen in the ectopic active Notch expressing cells in zebrafish HB was due to an affinity balance between cells. Interestingly, whole mount *in situ* hybridisation analysis of the Notch related genes showed that several genes were specifically absent from r1 and r2 at HH stage 10, but were expressed in the adjacent segments (MB and r3), hence forming a molecular boundary (as described in Chapter 3). These genes included the Notch modulator, *LFng* and *Lrrn1* which is known to have a role in adhesion in *Drosophila* (Milan et al., 2001). Hence, the sorting of ectopic active Notch expressing cells from r1 and r2 to the adjacent domains may be due to the existence of "like" environments where the modulator and affinity molecule are expressed (Figure 4.5 A, B).

Figure 4.5 Domains of constitutively active Notch expressing cells correlate with *LFng* and *Lrrn1* expressing domains.

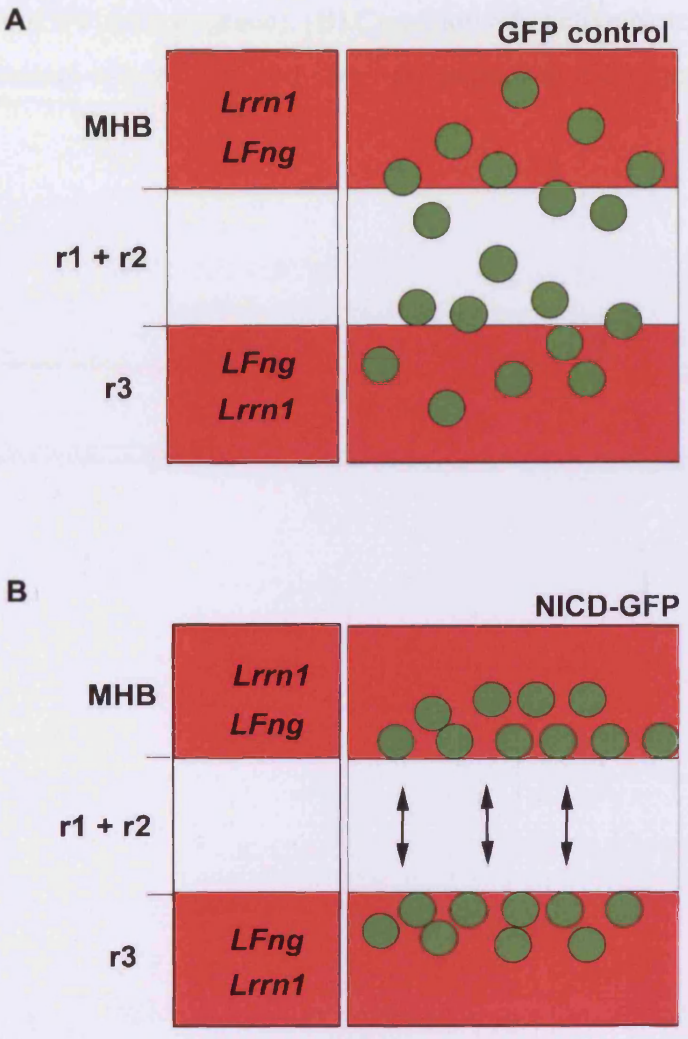


Figure 4.5 Domains of constitutively active Notch expressing cells correlate with *LFng* and *Lrrn1* expressing domains.

(A) A hypothetical model of cell sorting. *LFng* and *Lrrn1* expressing domains are shown in red. A. In control embryos, GFP spreads out equally across the hindbrain boundaries (GFP cells in green). (B) Constitutively active Notch expressing cells sort into the domain where *LFng* and *Lrrn1* are expressed (Black arrow heads).

4-4-3 Morphological boundaries expressing endogenous active Notch form a 'like' domain for cells ectopically expressing active Notch

LFng is known to modify the sensitivity of the Notch-ligand interaction by its glycosyltranferase activity in the same cells that express Notch receptor (Bruckner et al., 2000; Panin et al., 1997). *Drosophila Fng* modulates the Notch receptor to be sensitive to *Delta*, and insensitive to *Serrate* at the DV boundary of the wing disc (de Celis et al., 1996; Micchelli and Blair, 1999). Therefore, *Fng* acts as a key factor in making two different cell identities in dorsoventral boundary formation. Under the hypothesis I propose, ectopically active Notch expressing cells would prefer the environment of the *LFng* expressing domains and move towards these. As *LFng* expression is restricted to the midbrain/ anterior side of the molecular MHB, it would follow that there is a high level of Notch activity at/around the molecular MHB. It is important to know whether Notch signalling is active at the MHB. Whole mount *in situ* hybridisation analysis showed that *Notch1* is expressed on both sides of the molecular MHB at HH stage 10. In zebrafish HB, it is reported that the restrictive expression of *rfng* within a *delta* negative area is required for making a clear border (Amoyel et al., 2005; Cheng et al., 2004). Surprisingly, chick *Dl1* did not show any complementary expression patterns to the chick *fng*s at the molecular MHB and r2/r3 boundary at HH stage 10 (Chapter 3). In fact, another Notch ligand *Ser1* is expressed at the midbrain/anterior side of the molecular MHB, and *Ser 2* is posterior to this boundary in a complementary fashion. In *Drosophila* wing discs, Notch is active in the cells immediately adjacent to the dorsoventral boundary on both sides (Micchelli and Blair, 1999). It could be that the same is true during the formation of the molecular MHB and r2/r3 boundary, with active Notch present in the cells immediately adjacent to these boundaries and ectopic active Notch expressing cells being attracted to these domains. Interestingly, real-time time lapse analysis (data not shown) and GFP/RFP co-electroporation analysis revealed that the cell sorting is synchronised with the formation of morphological boundaries. Thereby, it suggests that Notch signalling is active at the MHB and r2/3 boundaries while these boundaries are morphologically forming.

To counter this hypothesis, however, *Hairy/E(spl)* family members, *cHairy1* and *cHairy2* expression were expressed across the MHB (Chapter3). *Hairy/E(spl)* family genes originally identified as Notch target genes (Delidakis and Artavanis-Tsakonas, 1992; Sasai et al., 1992). Therefore their expression here could indicate that Notch is

active throughout the MHB. However, recent reports have demonstrated that some *Hairy/E(spl)* family genes are expressed in Notch independent manner (Bae et al., 2005; Geling et al., 2003; Hans et al., 2004; Pasini et al., 2001). *Hes1* is expressed at the MHB in mice (Hirata et al., 2001). The mouse homologue of both chick *Hairy1* and *Hairy2*, *Hes1*, is expressed in a *Mash1*-independent manner at the olfactory placodal domain, suggesting this is Notch independent (Cau et al., 2000). In zebrafish, *her5* and *him* (*her11*) are expressed at the MHB, and the function and expression of these genes are independent of the Notch signalling pathway (Bally-Cuif et al., 2000; Geling et al., 2003). Ectopically activating Notch represses expression of these genes at the MHB (Geling et al., 2003). Thus, it is not yet clear that whether Notch signalling is activating *Hairy* at the MHB. For future studies it will be important to optimise antibodies that can detect just the intracellular fragment of Notch, and hence reveal domains of active Notch in the chick embryo as have been demonstrated in mouse, as these tools are not currently available for chicken (Schroeter et al., 1998).

4-4-4 Notch activation levels regulate the establishment of r1 and MHB cell fates

Misexpression of active Notch cells across the MHB *in ovo* showed that the correct level of the Notch activation is required for *Fgf8* expression at the MHB. Ectopic expression of *Wnt1* on the r1 side of the MHB due to irregular levels of Notch activation, coincides with a complete lack of *Fgf8,* suggesting that the adoption of MB MHB cell fate occurred in the r1 cells. When considered with the elevated changes seen in *Hoxa2* expression which is normally repressed by *Fgf8* at the r1/2 boundary, it is clear that a high level of Notch activation leads to a lack of *Fgf8* signalling during MHB formation. More significantly, when ectopic active Notch expressing cells were observed across the MHB, one of the MHB positioning markers, *Gbx2*, was also down-regulated in a mosaic manner in caudally. The expression domains of *Otx2* and *Gbx2* are initially established independently of each other at the early headfold stage, but by HH stage 10, they are complementarily and antagonistically expressed (Li and Joyner, 2001). *Gbx2* appears to regulate *Wnt1* negatively and *Fgf8* positively (Katahira et al., 2000; Liu and Joyner, 2001b; Millet et al., 1999). This strongly supports the hypothesis that a high level of Notch activation causes disruption of the correct positioning of the MHB through down regulation of *Gbx2*, and allows r1 MHB cells to adopt the MB MHB cell fate.

As discussed, Amoyel and his colleagues suggested that a clear expression border of the *delta* and *rfng* is necessary to create fine definition for the segregation of boundary cells in zebrafish HB (Amoyel et al., 2005). The molecular phenotype which I have shown here strongly implies that the activity of Notch signalling is required not only for the segregation but also the positioning and formation of the MHB. Unlike in the zebrafish, it appears that the ligands *Ser 1* and *Ser2* play the key roles in signalling at the MHB since the expression patterns in the chick are more restricted to the molecular MHB than *Dl1* is.

4-4-5 Cell number in the ectopic activated Notch cell population dictates the switch between cell sorting and cell fate changes

Active Notch expression causes either cell sorting or cell fate changes within the MHB region of the developing neural tube. Cell population analysis showed that a critical number of active Notch expressing cells are necessary in order to effect either cell fate changes or cell sorting. This may be due to a "community effect" operating between neighbouring cells in the neural tube (Figure 4.6A). A community effect model would account for the fact that sometimes I observed cell sorting and sometimes cell fate changes. Since the Notch signalling pathway is a cell-cell communication signalling pathway, it is possible that in cases where an extremely high population of Notch activated cells are present, there is no requirement to sort; instead they create their own 'like' environment, causing cell fate changes at their 'domain' boundary. Indeed, Gurdon and his colleagues demonstrated that large number of cells are required for the community effect (Gurdon et al., 1993). Once dissociated cells reaggregate and adopt new fates only when there is certain number of grouped cells nearby (Gurdon et al., 1993). In the case of misexpression of active Notch cells in my experiments, cell fate changes were only observed when more than 10 NICD-GFP positive cells were present in each compartment. While cell sorting required minimum and maximum numbers of NICD-GFP positive cells, cell fate changes did not display a maximum limit in cell numbers.

Alternatively, the concentration level of active Notch could also account for these two phenotypes. In various systems, the dosage of Notch activity is crucial in determining Notch signalling outcomes (Louvi and Artavanis-Tsakonas, 2006). It is possible that a cluster of ectopic active Notch cells establishes high dosage of Notch activity in a cell-

autonomous manner; and this causes the cell fate changes at the MHB. It is important to test this point; however, the technique I used here is incapable of controlling the quantity of DNA which is electroporated, and hence analysing dosage is beyond the limits of these experiments. In order to address this I would carry out quantitative RT-PCR on the MHB tissue after electroporation of the active Notch.

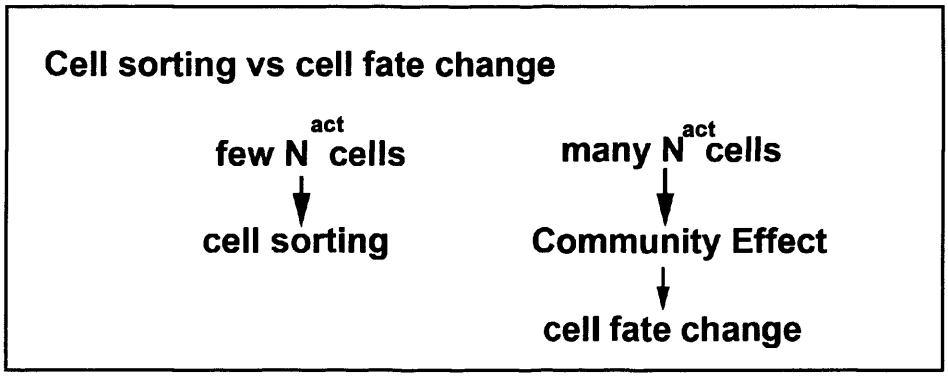


Figure 4.6 Cell sorting versus cell fate changes.

A model to describe the two different phenotypes following NICD GFP electroporation in the neural tube: cell sorting versus cell fate changes. When few constitutively active Notch expressing cells exist in segments, these cells move to a "like" environment. However, when many active Notch expressing cells exist in same area, a community effect-like interaction between active Notch expressing cells occurs. This causes the cell fate changes observed.

Chapter 5 The restricted expression of both *LFng* and *Lrrn1* is required for the formation and maintenance of the molecular MHB

5-1 Introduction

In Chapter 4, I demonstrated that ectopically expressed constitutively active Notch expressing cells are preferentially excluded from r1 and r2. Since *LFng* and *Lrrn1* were both found to be expressed anterior from the molecular MHB and posterior from r2/r3, and were not expressed in the r1/2 domain, I hypothesised that ectopic constitutively active Notch expressing cells preferentially sorted into these two domains.

At the DV boundary of the *Drosophila* wing disc, *Fng* is expressed only in the dorsal cells. *Fng* acts as a glycosyltransferase to modulate the Notch receptor in the dorsal compartment (Micchelli and Blair, 1999; Rauskolb et al., 1999). *Fng* activity makes dorsal cells more sensitive to *Delta* in ventral cells, and more insensitive to *Serrate* in dorsal cells (Fleming et al., 1997; Panin et al., 1997). Together with the restriction of ligand activity to the cells adjacent to the boundary, high-level Notch activity is limited to a narrow band of the cells along the boundary. In *Drosophila*, it has been shown that modulation of *Fng* activity allows cells to move across the boundary (Rauskolb et al., 1999). This cell behaviour is influenced by *Fng* activity through Notch signalling (Milan et al., 2001). In the chick ZLI, misexpression of *LFng* within the ZLI allows cells to move across the boundary to domains outside of the ZLI, where *LFng* is expressed (Zeltser et al., 2001). It is not yet known whether *LFng* activity in this instance is also acting through the Notch signalling pathway. However, it is likely that cell movement across CNS boundaries is regulated via Notch signalling as several Notch related genes, including Notch ligands, are known to be expressed in a restricted manner at the ZLI boundary. In Chapter 3, I showed that *LFng* is expressed only on the anterior side of the molecular MHB at HH stage 10. I hypothesised in Chapter 4 that the molecular boundary of *LFng* is crucial for midbrain MHB cells to maintain their position.

Tartan (*Trn*) is reported as an adhesion molecule which is also expressed only in the dorsal cells at the *Drosophila* wing disc (Milan et al., 2001). *Trn* is a cell surface protein

which mediates cell interactions via its leucine rich repeats domains (Shishido et al., 1998). Misexpression of *Trn* across the DV boundary induces the formation of cellular processes that project from ventral cells toward dorsal cells (Milan et al., 2001). Thus, *Trn* supports boundary formation by controlling cell behaviour in short-range cell interactions, and this does not require Notch signalling (Milan et al., 2002). LRR family genes, homologues of *Trn*, have been reported in several species in vertebrates (Bormann et al., 1999; Haines et al., 2005; Hayata et al., 1998). One member, *Lrrn1,* was cloned in chick due to its expression pattern at the MHB – specifically absent from r1 and r2 at HH stage 10, but expressed in adjacent segments MB and r3 (Chapter3, (Andreae et al., 2007; Garcia-Calero et al., 2006)). Although both *Fng* and *Trn* are expressed in dorsal cells of *Drosophila* wing disc during DV boundary formation, their functions are different. *Fng* modifies dorsal signalling properties without affecting DV affinities, while *Trn* supports boundary formation without affecting dorsal signalling properties. Milan and his colleagues suggested that *Trn* with another adhesion protein, *Capricious* (*Cap*), may lead to a segregation of two cell populations, and maintain the cell numbers within the dorsal compartment, then *Fng* will maintain the boundary (Milan et al., 2002; Milan et al., 2001). However, the expression of *Fng* is observed as early as the first instar in *Drosophila* wing disc, while *Trn* and *Cap* expression are observed from second instar in the dorsal cells (Milan et al., 2001; Panin et al., 1997). Little is known about the molecular interaction between *Fng* and *Trn* during boundary formation but they act in independent pathways. Gene expression analysis showed that both *LFng* and *Lrrn1* are expressed only anterior to the molecular MHB at HH stage 10 in the chick CNS (as shown in Chapter 3). The movement of ectopically introduced constitutively active Notch expressing cells suggested that there is a high level of Notch activation at the molecular MHB.

In this chapter, I show that the perturbation of the Notch modulator, *LFng* at the border of the MHB causes disruption of MHB formation. DiI label analysis reveals that ectopically expressed *LFng* allows cells to cross the MHB. I also show that *Lrrn1* has a similar function to that of *LFng* in MHB formation. Cross-analysis reveals that *Lrrn1* can selectively induce *LFng* expression. Here I propose a model stating that the demarcation of *LFng* and *Lrrn1* at the molecular MHB plays a significant role in the formation and maintenance of the MHB.

5-2 Materials and Methods

5-2-1 DNA in ovo electroporation

Complete coding sequence of Mouse *Lunatic Fringe* (*LFng*) is linked with green fluorescent protein by an internal ribosomal entry site (IRES-GFP). This plasmid was constructed and kindly given by Dr. O. Cinquin. Coding sequence of chick *Lrrn1* was linked with IRES-GFP. This plasmid was constructed and kindly given by J. Gilthorpe. For constitutively active Notch, NICD which is linked with IRES-GFP was used (as described in Chapter 4). pCAB-IRES-eGFP plasmid was used as a control GFP. DNA injection and *in ovo* electroporation was performed as described (Chapter 2). DNA was injected at HH stage 8 and 9, and electroporated laterally. Embryos were left to develop and processed for whole mount *in situ* hybridisation or whole mount double *in situ* hybridisation, Immunohistochemical analysis was carried out as described in Chapter 2. The detailed description of RNA probes can be found in the appendix.

5-2-2 Iontophoresis DiI labels

Small deposits of DiI (Molecular Probes; D-282) were applied *in vivo* to the molecular MHB by iontophoresis (Nittenberg et al., 1997). *In ovo* electroporation was carried out with appropriate GFP constructs at HH stage 8 and 9, and then DiI was immediately applied to the cells at the molecular MHB. The position of the dye was confirmed using an epifluorescence microscope, and imaged with Nikon digital camera (DXM1200F).

5-3 Results

5-3-1 Active Notch expressing cells are attracted to LFng and Lrrn1 positive domains

Ectopically active Notch cells showed selective localisation to the MHB and r2/r3 domain (Figure 5.1A). In Chapter 4, I hypothesised that constitutively active Notch cells may sort to a "like" domain, the MHB, and posterior to the r2/3 boundary. *LFng* and *Lrrn1* expression is absent or severely reduced in the MHB and r1 domain at HH stage 10 (Figure 5.1A, B), and the exclusion of these genes from this domain continues during CNS development. To determine whether *LFng* or *Lrrn1* expression is coincident with the re-localised NICD-GFP cells, I carried out whole mount *in situ* hybridisation with *LFng* and *Lrrn1* after *in ovo* electroporation of NICD-GFP. Following electroporation, NICD-GFP cells were excluded from r1 and r2, and these cells were located in the *LFng*

and *Lrrn1* expressing domains (Figure 5.1E, F). This supports the hypothesis that active Notch cells moved into the domains where Notch is hyperactive, as *Fng* has previously been reported to maintain a high level of Notch activation (Wu and Rao, 1999).

Figure 5.1 Sorted domains of constitutively active Notch expressing cells correlate with *LFng* and *Lrrn1* expressing domains.

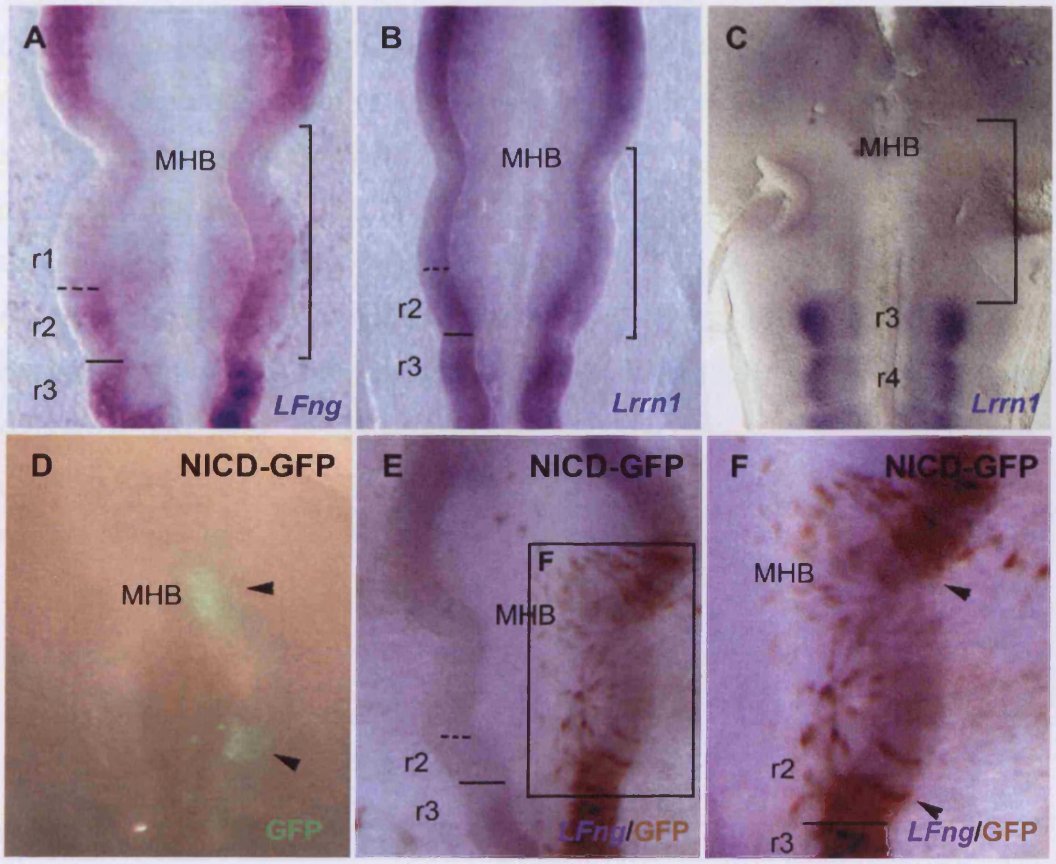


Figure 5.1 Sorted domains of constitutively active Notch expressing cells correlate with *LFng* and *Lrrn1* expressing domains.

Whole mount *in situ* hybridisation reveals *LFng* in blue (A, E, F), *Lrrn1* in blue (B, C).Immunohistochemistry analysis shows GFP in brown (E, F).
(A) *LFng* expression is absent from the domain posterior to the molecular MHB to r2/r3 at HH stage 10 (bracket). (B) *Lrrn1* expression is downregulated in the domain posterior to the molecular MHB to r2.r3 at HH stage 10 (bracket). (C) At HH stage 15, the restriction of *Lrrn1* is maintained (bracket). (D) NICD-GFP cells are excluded from r1 and r2, and observed in the MHB and posterior from r2/r3 boundary (black arrow heads). (E) *LFng* expression following electroporation of NICD-GFP shows that NICD cells are located in the *LFng* domain.
(F) High magnification view shows that NICD-GFP cell exclusion is coincident with the domain in which *LFng* expression is downregulated.

5-3-2 Perturbing the LFng boundary into r1 disrupts the expression of the MHB organiser genes

To determine the requirement for the *LFng* expression border in the formation or maintenance of the MHB, I performed *in ovo* electroporation of mouse *LFng*-IRES-GFP into HH stage 8 and 9 chick embryos. In order to perturb the expression border, ectopic *LFng* was introduced across the molecular MHB and posterior to the molecular MHB (Figure 5.2A). mRNA expression of MHB organiser genes was then analysed by whole mount *in situ* hybridisation. The position of *LFng* positive cells was detected by co-expression of GFP detected via immunohistochemistry.

Expression of the MHB organiser molecule *Fgf8* is restricted at the MHB, from the posterior side of the molecular MHB to r1/r2 (Hidalgo-Sanchez et al., 1999a; Shamim et al., 1999). Misexpression of *LFng* on the r1 side of the MHB caused severe downregulation of *Fgf8* at the MHB (Figure 5.2B, C, control; n=0/30, LFng; n=5/7). Interestingly, several *Fgf8* expressing cells were detected spreading dorsally in r1 which indicating a loss of *Fgf8* restriction to the MHB (Figure 5.2C; black arrows). In addition to these findings, it appeared that the MHB had lost its morphological constriction on the manipulated side (Figure 5.2C). *Wnt1* expression also spread and lost its restriction to the anterior MHB following *LFng* electroporation (Figure 5.2D-F, control; n=0/7, LFng; n=4/5). Following flatmounting of the neural tube it was clear that the MHB morphological boundary was absent on the experimental side as compared to the control side. *Wnt1* and *Fgf8* positive cells were intermingling at the interface of midbrain and hindbrain on the experimental side (Figure 5.2F,J), whereas on the control side *Wnt1* and *Fgf8* were detected clearly in domains separated by the MHB.

To test whether *LFng* electroporation also caused disruption of the positioning of the MHB, I carried out the whole mount *in situ* hybridisation of the MHB positioning markers, *Otx2* and *Gbx2*. The results showed that *Otx2* and *Gbx2* expressing cells were no longer tightly restricted with a sharp boundary between them; instead they also appeared to be intermingled (Figure 5.2G-J, control; n=0/8, LFng; n=5/6). Remarkably, all *LFng*-treated embryos showed a clear loss of constriction at the MHB (Figure 5.2F, I; double heads arrows). Together, these data suggest that the MHB fails to form correctly in the absence of a molecular *LFng* expression boundary.

Figure 5.2 Perturbation of *LFng* expression into r1 causes disruption of the MHB.

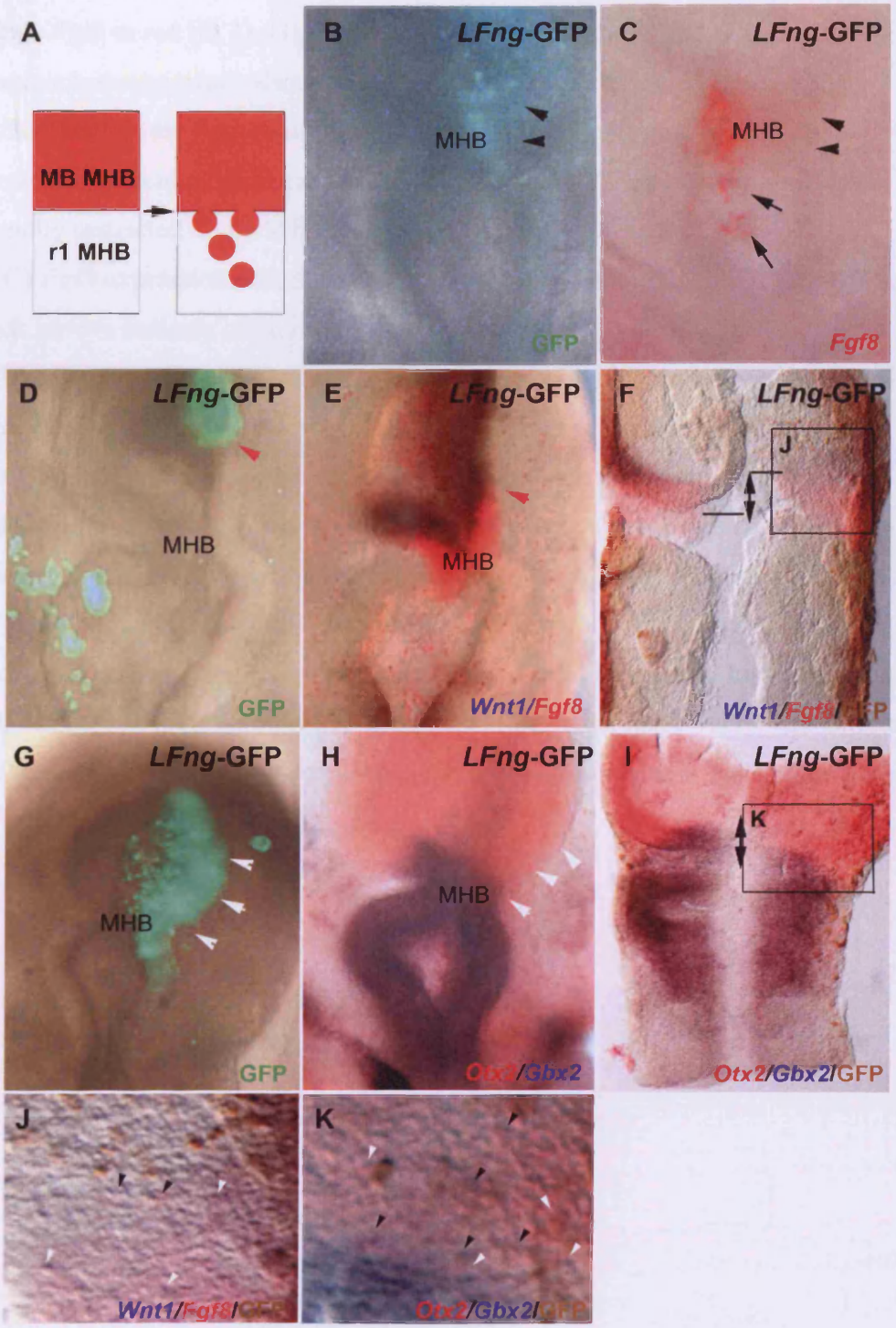


Figure 5.2 Perturbation of *LFng* expression into r1 causes disruption of the MHB.

MHB and hindbrains are flatmounted in G, H. Whole mount *in situ* hybridisation reveals *Fgf8* in red (B, D, G), *Wnt1* in blue (D), *Otx2* in red and *Gbx2* in blue (F, H). Immunochistochemistry shows GFP in brown (F, I, J, K).

(A) A model of the perturbation of *LFng* restricted expression. Misexpression of *LFng* across the molecular MHB causes the perturbation of *LFng* expression which is normally restricted in the MB and MHB.

(B, C) *Fgf8* expression was downregulated in *LFng*-GFP cells (black arrow heads). Black arrows indicate scattered ectopic *Fgf8* expression dorsally in r1.

(D-F) *LFng* electoporation causes a loss of morphological constriction at the MHB (red arrow heads). Inset (J) shows that *Wnt1* and *Fgf8* gene expression patterns are intermingled (white arrow heads: blue cells, black arrow heads: red cells).

(G-I) Misexpression of *LFng* across the MHB (white arrow heads) caused ectopic *Otx2* expression in r1 and ectopic *Gbx2* expression in the MB. (I) The *Otx2*/*Gbx2* interface on the experimental side is more posterior than control side and not clearly defined (double heads arrow). The experimental side (right hand side) has clearly lost its morphological MHB constriction as compared to the control (left) side. Inset (K) reveals intermixing of *Otx2* and *Gbx2* cells (white arrow heads: red cells, black arrow heads: blue cells).

5-3-3 Disrupting the molecular LFng boundary allows cells to move across the MHB boundary

The lateral MHB cells at HH stage 9-10 maintain their position within the MHB during development, the dorsal-central MHB cells however, spread out along the roof plate (Alexandre and Wassef, 2003; Louvi et al., 2003). By HH stage 17-18, the cell movements of the dorsal-central MHB cells cease as the MHB territory has become more fixed. There are two possibilities explaining the intermingling phenotype observed for *Otx2* and *Gbx2* cells. Firstly, misexpressing *LFng* across the MHB caused a cell fate change, and *Otx2* and *Gbx2* expression were induced ectopically. Secondly, ectopic *LFng* caused a loss of restriction at the MHB, allowing cells to move and mingle across the MHB and MB-HB interface.

To determine which of these possibilities was occuring, I analysed cell movements at the MHB following *LFng* electroporation. After *in ovo* electroporation of *LFng* across the MHB at HH stage 8 and 9, cells anterior to the molecular MHB (midbrain, *Otx2* positive) were marked with DiI (Figure 5.3A). Embryos were then cultured for 24 hrs. The cells labelled with DiI were observed within the anterior MHB of both non-treated embryos and those treated with control-GFP (Figure 5.3D-F, control; n=6/6). In contrast, in the presence of ectopic *LFng* across the MHB, DiI labelled cells were seen to spread out from the MB into r1 (Figure 5.3G-I, n=6/8). Overlay of DiI and GFP expression revealed that DiI labelled cells did not co-express GFP, but were neighbouring cells (Figure 5.3G, H; white arrows). The distance travelled by DiI labelled cells was remarkably long along the anteroposterior axis, but within the domain of ectopic *LFng*. Taken together, these results show that disruption of the molecular *LFng* boundary causes cells to lose their restriction to either the MB or HB and move between these domains. Therefore, the presence of *Otx2* positive (MB marker) cells in anterior hindbrain and *Gbx2* positive (HB marker) cells in midbrain is likely due to this loss of restriction rather than ectopic induction of these markers.

Figure 5.3 Disrupting the molecular *LFng* boundary allow cells to move across the MHB boundary.

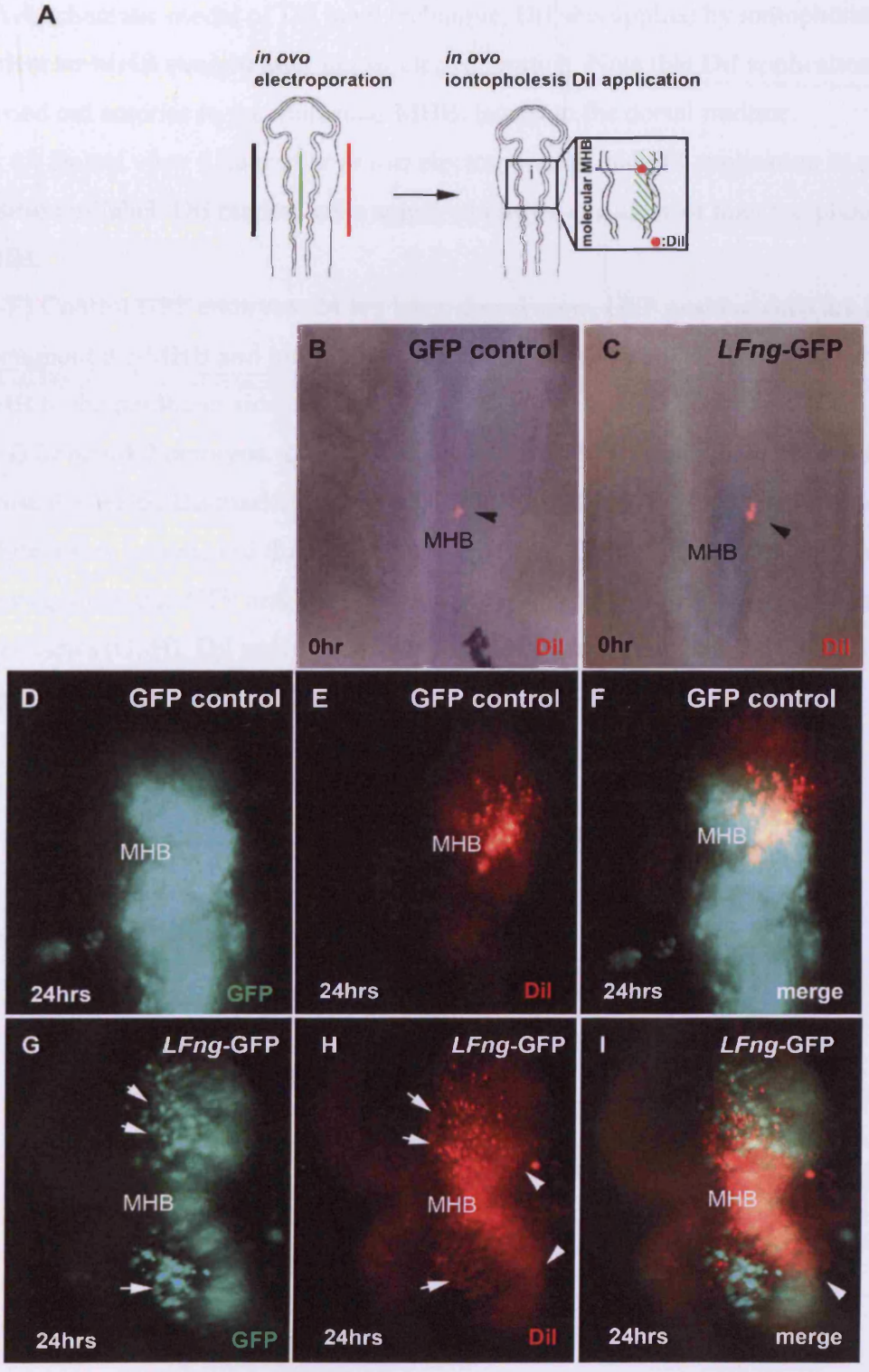


Figure 5.3 Disrupting the molecular *LFng* boundary allow cells to move across the MHB boundary.

Cell movement was observed by labelling cells in the anterior MHB with DiI, after *in ovo* electroporation.

(A) A schematic model of DiI label technique. DiI was applied by iontophoresis at the molecular MHB straight after *in ovo* electroporation. Note that DiI application was carried out anterior to the molecular MHB, lateral to the dorsal midline.

(B, C) Dorsal view 0 hour after *in ovo* electroporation and DiI application to confirm position of label. DiI marked cells appear slightly more anterior than morphological MHB.

(D-F) Control GFP embryos. 24 hrs later, dorsal view. GFP positive cells are found throughout the MHB and hindbrain. DiI marked cells spread out, but do not cross the MHB to the hindbrain side.

(G-I) *LFng* GFP embryos. 24 hrs later, dorsal view. GFP expressing cells are found across the MHB. DiI marked cells are widely visible from the midbrain to hindbrain (white arrow heads), and this area is adjacent to the GFP positive domains. Merged view (I) show that GFP and DiI labels do not overlap. However, together with single filter views (G, H), DiI positive cells are adjacent to the neighbouring *LFng* GFP expressing cells (white arrows).

5-3-4 Ectopic active Notch expressing cells induce ectopic LFng expression across the MHB

Misexpression of active Notch expressing cells across the MHB showed that Notch signalling is necessary for the formation and maintenance of the MHB (Chapter 4). *Fng* is known to modulate the Notch-ligand interaction within the cells where Notch receptors are expressed (Panin et al., 1997). However little is known about the precise relationship between *Fng* and its ability to control ligand choice and Notch activity levels. To determine the function of *LFng* during MHB formation and maintenance, *LFng* mRNA expression at the MHB following misexpression of active Notch expressing cells was examined.

Interestingly, in the presence of high levels of ectopic active Notch expression across the MHB, ectopic *LFng* mRNA was detected (Figure 5.4A, B, control; n=0/17, NICD; n=16/16). This result showed that Notch activation can lead to *LFng* expression in r1 and r2 domain where *LFng* expression is normally excluded at HH stage 10. Since one function of *LFng* is to modify the Notch-ligand interaction, there must be a positive regulation loop on the MB side of the molecular MHB. However, as *LFng* is an enzyme, there must be an additional factor to initiate the *LFng* expression at the MB side of the molecular MHB (Figure 5.4C).

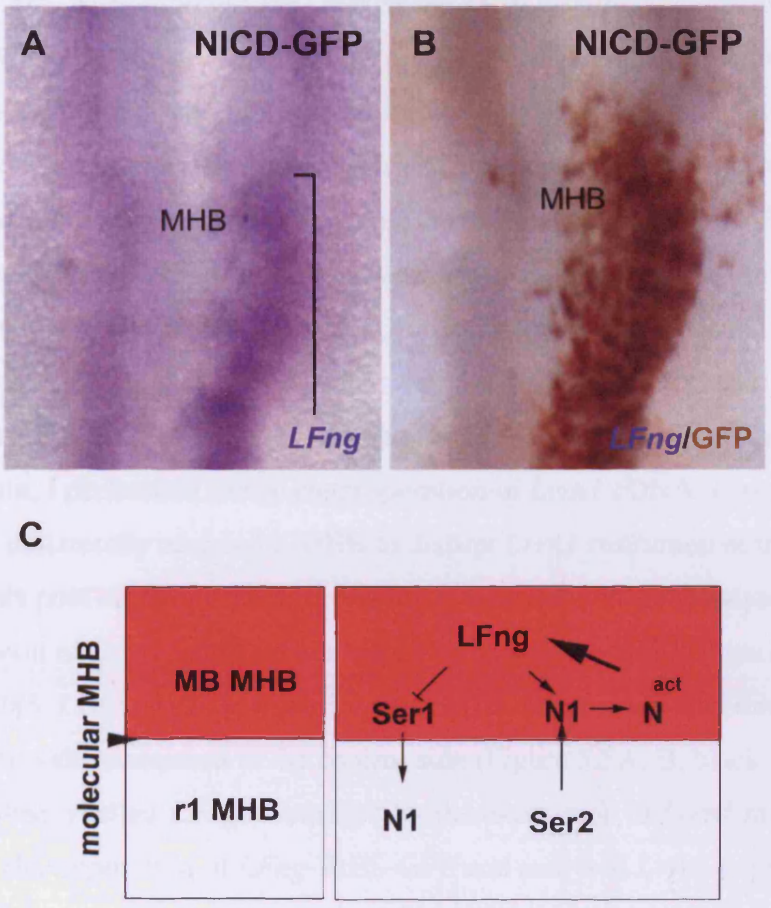


Figure 5.4 Notch activation can induce *LFng* expression.

in ovo electroporation of constitutively active Notch was carried out at HH stage 8 and 9. Whole mount *in situ* hybridisation reveals *LFng* in blue (A, B). Immunohistochemistry shows GFP in brown (B). (A, B) *in-situ* hybridisation (A) and post-GFP antibody staining (B) *LFng* is overexpressed where GFP expressing cells exist (bracket). (C) A hypothetical model of molecular MHB formation in chick CNS. Notch activation, modified by *LFng*, is required for the formation and maintenance of the MHB (Chapter 4). However, this Notch activation regulates *LFng* expression, possibly to maintain the level of Notch activation.

5-3-5 Ectopic expression of Lrrn1 across the MHB disrupts the expression of LFng

In *Drosophila*, *Fng* is regulated by *Apterous* (*Ap*) for the signalling between CV compartments. Indirectly to this regulation, *Tartan* (*Trn*) is also regulated by *Ap* and mediates cell-cell interactions, causing *Trn*-expressing cells to stay grouped in the same domain, and helping to refine DV boundaries (Milan et al., 2001). In Chapter 3, I showed that one of the chick orthologues of *Trn*, *Lrrn1*, is expressed on the anterior side of the molecular MHB at HH stage 10 in chick CNS and its expression domain is coincide to *LFng*. To investigate whether *Lrrn1* is responsible for restriction of cells to the MB side of the molecular MHB so that it maintains *LFng* expressing cells in the same domain, I performed *in ovo* electroporation of *Lrrn1* cDNA. *Lrrn1*-IRES-GFP was introduced unilaterally across the MHB to disrupt *Lrrn1* restriction at the molecular MHB. 24 hrs post-electroporation, *LFng* mRNA expression was analysed. Misexpression of *Lrrn1* led to upregulate *LFng* across the MHB (Figure 5.5A, B, control; n=0/5, Lrrn1; n=2/3). Significantly, MHB morphology was lost on the experimental side, compared to the control side (Figure 5.5A, B; black arrow heads; n=2/3). To test whether *LFng* is required for the expression of *Lrrn1* mRNA, I performed electroporation of *LFng*-IRES-GFP and analysed *Lrrn1* expression. As for control GFP embryos (data not shown), *LFng* GFP electroporation did not generate any major changes in *Lrrn1* expression (Figure 5.5C, D, control; n=9/9, Lrrn1; n=0/9). These results revealed that *Lrrn1* is able to induce *LFng* expression ectopically, but *LFng* does not have the same function towards *Lrrn1*.

5-3-6 Perturbation of the Lrrn1 expression boundary at the MHB causes intermingling of midbrain and hindbrain cells

If *Lrrn1* is responsible for the restricted *LFng* expression, then restricted *Lrrn1* expression would also be essential for MHB formation. LRR molecules are reported to mediate cell-cell interactions by short-range signalling (Milan et al., 2001). Misexpression of *Lrrn1* downregulates the MHB organiser signalling molecules (Figure 5.6A, B, control; n=0/11, Lrrn1; n=4/5), and there is obvious deformation of the MHB morphologically (Figure 5.6A, B). In flat mount analyses, *Wnt1* and *Fgf8* expressing cells showed an intermingling within the MHB region following *Lrrn1* electroporation (Figure 5.6B, C; double arrow heads). In addition to these molecular phenotypes, after

electroporation of *Lrrn1* the morphological constriction at the MHB was clearly lost on the experimental side (Figure 5.6B).

To further test the effect of misexpression of *Lrrn1* across the MHB, I investigated any change in the MHB positioning molecules, *Otx2* and *Gbx2*. Following ectopic *Lrrn1* expression, the *Otx2*/*Gbx2* gene expression interface was absent from the expected position (Figure 5.6D, E, control; n=0/7, Lrrn1; n=5/5). Flat mount analysis revealed that misexpression of *Lrrn1* caused a loss of the normally clear *Otx2*/*Gbx2* interface (Figure 5.6E, F). Instead, *Otx2* and *Gbx2* were observed intermingling in the region of ectopic *Lrrn1*-GFP cells. Taken together, these results show that restricted *Lrrn1* expression is necessary for the restriction of midbrain and hindbrain markers, *Otx2* and *Gbx2*, and subsequently for restricted expression of MHB organiser genes *Wnt1* and *Fgf8*.

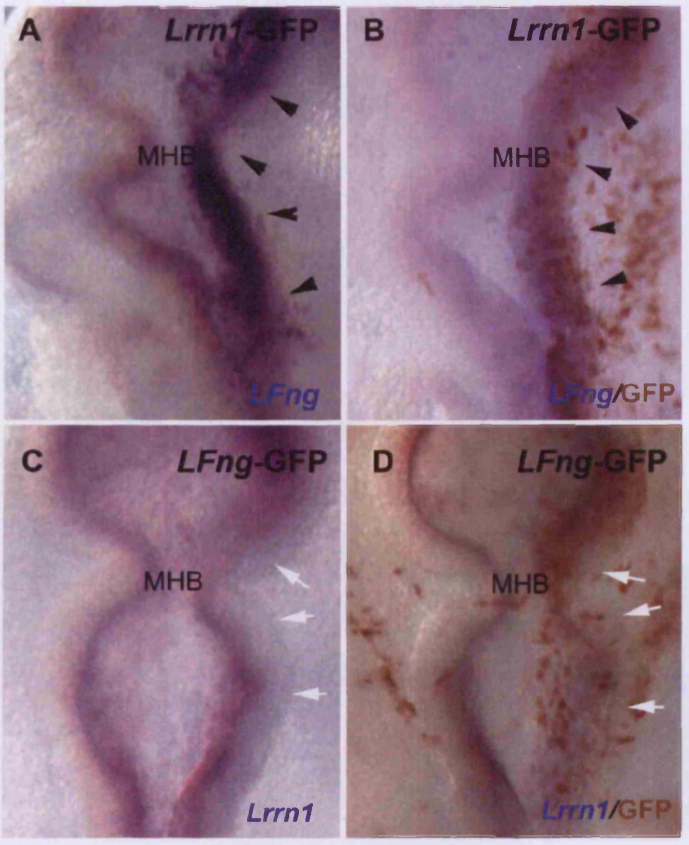


Figure 5.5 Misexpression of *Lrrn1* causes upregulation *LFng* mRNA expression.

In ovo electroporation of *Lrrn1* and *LFng* cDNA was carried out at HH stage 8 and 9, across the MHB. Whole mount *in situ* hybridisation reveals L*Fng* in blue (A, B) and *Lrrn1* in blue (C, D). Immunohistochemistry shows GFP in brown (B, D).
(A, B) Misexpression of *Lrrn1* leads to dramatic upregulation of *LFng* (black arrow heads). (C, D) Misexpression of *LFng* did not show any change in *Lrrn1* mRNA expression (white arrows).

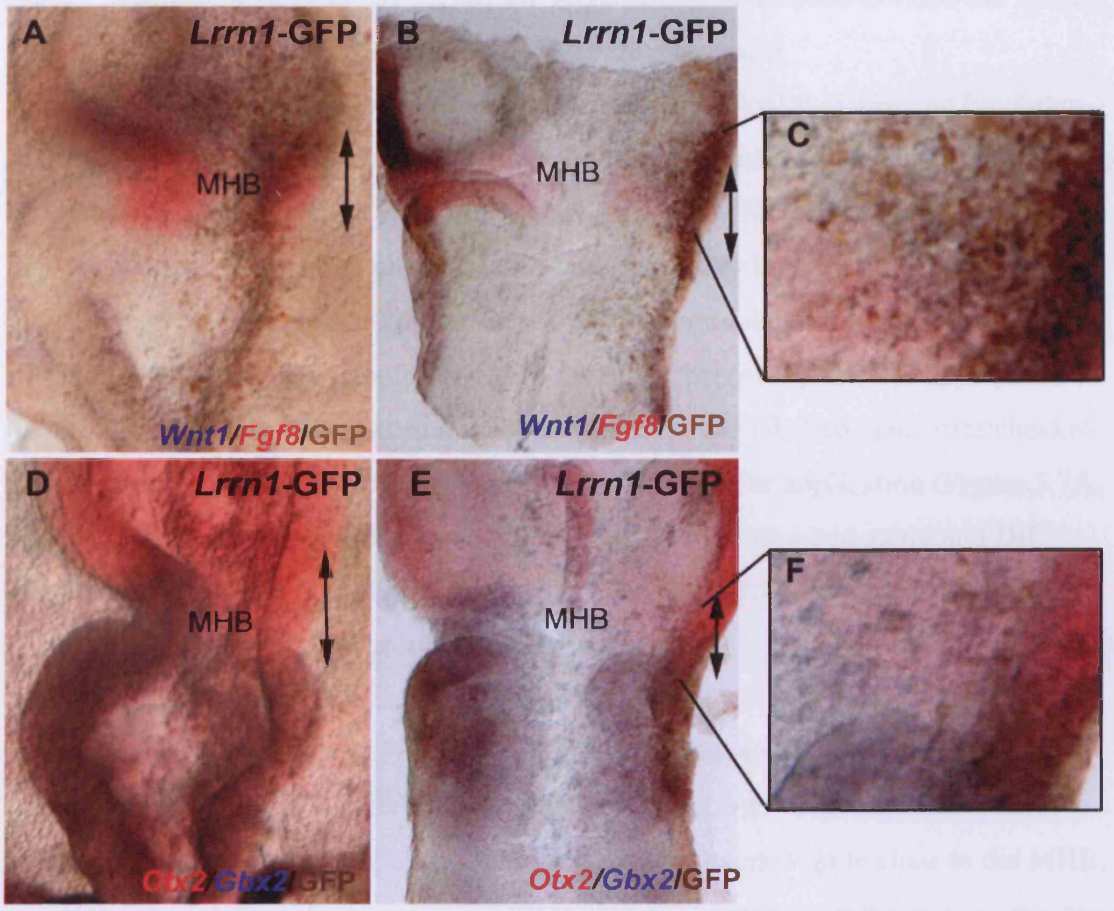


Figure 5.6 Misexpression of *Lrrn1* across the MHB causes loss of the constriction of the MHB molecularly and morphologically.

in ovo electroporation of *Lrrn1*-GFP across the MHB was carried out at HH stage 8 and 9. Whole mount *in situ* hybridisation reveals *Wnt1* in blue and *Fgf8* in red (A-C), *Otx2* in red and *Gbx2* in blue (D-F). Immunohistochemistry shows GFP in brown (A-D). MHB and hindbrains are flatmounted in B and D.

(A, B) Misexpression of *Lrrn1*-GFP causes intermingling of *Wnt1* and *Fgf8* positive cells in the region of MHB (double arrowheads). Note that the physical constriction is lost. Inset (C) shows intermingling of *Wnt1* and *Fgf8* cells. (D, E) *Otx2* and *Gbx2* expressing cells are also seen mixing into adjacent domains. The physical constriction is lost. Inset (F) shows intermingling of *Otx2* and *Gbx2* cells. (D) Ectopic morphological MHB is found dorsally where *Otx2* and *Gbx2* expressions abut. Double headed arrows show the loss of morphological MHB. i: isthmus (MHB)

5-3-7 Misexpression of Lrrn1 across the MHB allows MHB cells to cross the MHB, but does not allow further movement towards the hindbrain.

There are two possibilities explaining why cells are ectopically expressing hindbrain markers (*Gbx2* and *Fgf8*) in the midbrain, and midbrain markers (*Otx2* and *Wnt1*) in the hindbrain. Firstly, cells may have lost restriction at the MHB and moved into the adjacent domains due to the perturbation of this boundary by *Lrrn1*. Secondly, cells may have ectopically induced midbrain and hindbrain markers due to abnormal cell signalling caused by the ectopic *Lrrn1*. To test these two possibilities, I applied DiI to the molecular MHB cells after *in ovo* electroporation. DiI labelled cells were checked and observed to be at the molecular MHB immediately after application (Figure 5.7A, B). After 24 hours incubation, control GFP embryos were checked again and DiI labelled cells were found only within the MHB, and not outside of the MHB region (Figure 5.7C-E, n=0/5). In contrast, in embryos where *Lrrn1* was ectopically expressed across the MHB, DiI labelled cells were found in the places where the *Lrrn1*-GFP positive cells were (Figure 5.7F-H, n=6/6). Merged analysis of the two expressions revealed that DiI labelled cells were co-localised with *Lrrn1*-GFP expressing cells (Figure 5.7H). However, this cell movement always remained quite close to the MHB, not venturing far away from the *Lrrn1* expressing cells into the midbrain or r1 territories. This result reveals that perturbation of the MHB by ectopic *Lrrn1* causes cells to lose their restriction and move within the ectopic domain. Cells appear to move between midbrain and hindbrain, and this is likely to account for the intermingling of *Otx2* and *Gbx2*, *Wnt1* and *Fgf8* expressing cells observed.

5-3-8 Misexpression of constitutively active Notch expressing cells across the MHB does not change the Lrrn1 expression

Constitutively active Notch expressing cells led to ectopic expression of *LFng* across the MHB. The misexpression of *Lrrn1*, however, induced ectopic *LFng* expression. To investigate the requirement of restriction of *Lrrn1* mRNA expression to the midbrain/anterior side of the molecular MHB, constitutively active Notch expressing cells were introduced across the MHB. 24 hours post-electroporation, *Lrrn1* mRNA was observed by whole mount *in situ* hybridisation. Ectopic active Notch expressing cells did not lead to expression of *Lrrn1* in the MHB and r1 (Figure 5.8A, B, control; n=6/6, NICD; n=15/15). Taking these data together, I propose a model where *Lrrn1* is a key

adhesion molecule which maintains restriction of cells to the midbrain/anterior side of the molecular MHB via regulation of *LFng* expression, and is upstream of and independent from Notch signalling (Figure 5.8C).

Figure 5.7 Misexpression of *Lrrn1* allows cells to cross the MHB, but not spread further than the ectopic *Lrrn1* region.

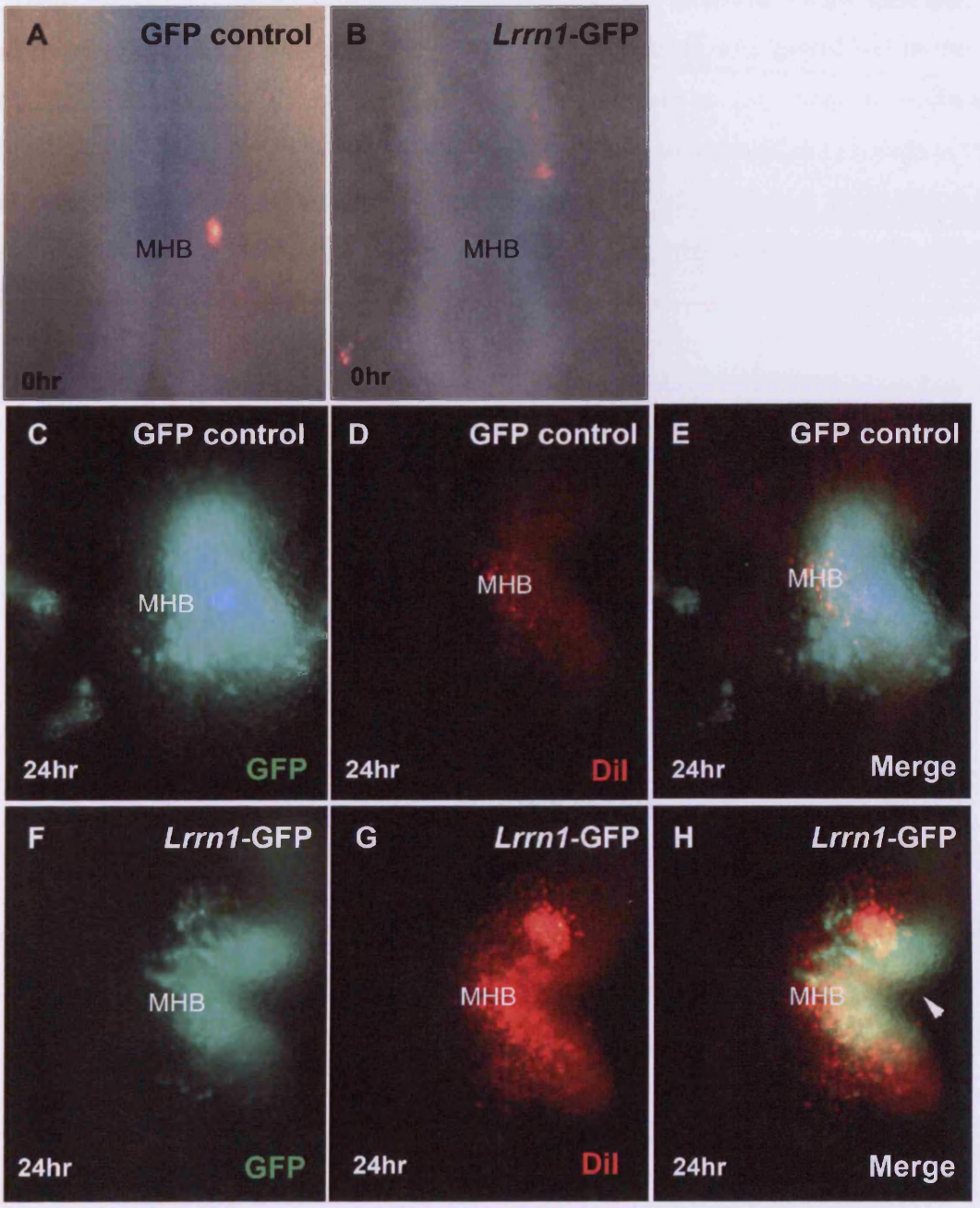


Figure 5.7 Misexpression of *Lrrn1* allows cells to cross the MHB, but not spread further than the ectopic *Lrrn1* region.

Cell movement was observed with DiI application to the molecular MHB cells after *in ovo* electroporation of *Lrrn1*-GFP. (A, B) control GFP and *Lrrn1*-GFP embryos at 0hr. Size of DiI label is similar and small. (C-E) control GFP embryos. 24 hrs after the eletcroporation and iontophoresis of DiI. Some DiI labelled cells spread within the MHB but do not correspond to the GFP-positive cells, and do not cross into midbrain or hindbrain. (F-H) *Lrrn1*-GFP embryos. DiI labelled cells are spread out as wide as the ectopic *Lrrn1*-GFP cells, but never further than around the MHB area. Note that there is strong co-localisation of *Lrrn1*-GFP expressing cells and DiI labelled cells (H; Shown in yellow colour, white arrow heads).

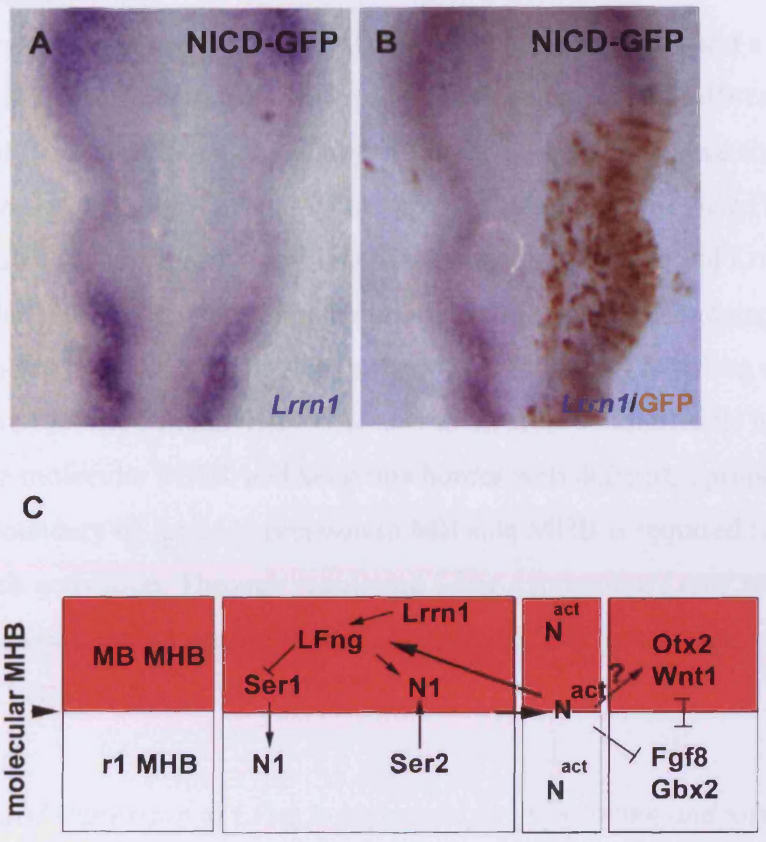


Figure 5.8 Misexpression of constitutively active Notch expressing cells does not affect *Lrrn1* mRNA expression.

(A, B) *in ovo* electroporation of NICD-GFP followed by whole mount *in situ* hybridisation of *Lrrn1* in blue. Immunohistochemistry analysis reveals GFP in brown. *Lrrn1* expression does not change after the electroporation of constitutively active Notch expressing cells. (C) A hypothetical model of the molecular MHB. Both *Lrrn1* and *LFng* are expressed on the midbrain side of the molecular MHB. *Lrrn1* regulates *LFng* and restricts cells to the midbrain side of the molecular MHB. Notch activation at the midbrain side regulates midbrain MHB genes, *Otx2* and *Wnt1*. This Notch activation also maintains the level of *LFng* expression at the midbrain side of the molecular MHB. i: isthmus (MHB)

5-4 Discussion

In this chapter, I have looked at the role of a Notch modulator *LFng* and a LRR protein *Lrrn1*, during MHB formation. Misexpression of *LFng* across the MHB causes deformation of the MHB both molecularly and morphologically. I have shown that this deformation was as a result of irregular cell movement which was caused by misexpressed *LFng*. I further revealed that perturbing the restriction of *Lrrn1* expression at the molecular MHB causes not only the intermingling of MHB organiser genes, but also the ectopic expression of *LFng* across the MHB. Using DiI labelling assays and misexpression of *Lrrn1*, I found that *Lrrn1* acts as an adhesion molecule to maintain the MB side of the molecular MHB, and keep this border well defined. I proposed a model whereby the boundary of *Lrrn1* expression in MB side MHB is required for the restricted Notch activation. Through regulating *LFng* expression, *Lrrn1* modulates affinity differences between compartments at the MHB, and thus assists boundary formation and maintenance of the MHB.

5-4-1 Restricted expression of LFng is important for positioning and formation of the MHB, but is not sufficient.

In the *Drosophila* wing disc, a distinctive stripe of high level Notch activation is required for dorsoventral boundary formation (Rauskolb et al., 1999). Rauskolb and his colleagues demonstrated that misexpression of Notch ligands or mutation of the Notch receptor can disrupt boundary cells on both sides of the boundary. However, activation of Notch itself does not confer dorsal or ventral cell identity. Misexpression of *Fng* across the boundary showed that the cell separation at the dorsoventral boundary is *Fng* dependent (Rauskolb et al., 1999). Therefore, it is clear that the restriction of *Fng* plays a significant role in boundary formation.

In Chapter 3, I showed that *LFng* is expressed only on the MB side of the molecular MHB at HH stage 10. At the MHB, perturbation of the *LFng* expression boundary resulted in dramatic disruption of *Fgf8* and *Wnt1* expression. Analysis of MHB positioning gene expressions, *Otx2* and *Gbx2*, revealed that perturbation of the *LFng* expression boundary at the MHB causes disruption of the positioning of the anterior and posterior cells. Together with the remarkable lack of morphological constriction at the MHB observed under these conditions, I propose that the ectopic *Otx2* cells seen in the hindbrain would also express *Wnt1* and correspond to those seen in Figure 1C, D, and G.

Likewise, ectopic *Gbx2* cells in the midbrain would also express *Fgf8* and correspond to those seen in Figure 1B. This molecular phenotype is supported by the results of DiI labelling analysis. Alexandre and her colleagues demonstrated that the lateral MHB cells keep their movement within the MHB region at HH stage 9-10, as those cells are maintained at the domain (Alexandre and Wassef, 2003). Following electroporation of control GFP, DiI labelled cells at the molecular MHB did not move significantly. In contrast, misexpressed *LFng* allowed DiI labelled cells to spread out from the MHB domain. In both invertebrate and vertebrate models, it has been reported that *Fng*s act to restrict cells to boundaries (Rauskolb et al., 1999; Zeltser et al., 2001). However in these experimental situations their role was identified by misexpression, which shows cells move and cross boundaries. Here, DiI labelling analysis clearly showed that ectopic *LFng* across the MHB has the ability to let cells cross the MHB. Furthermore, this suggests that perturbation of the *LFng* expression boundary by the misexpression of *LFng* into the surrounding area leads MHB cells to cross the MHB. As a result of this cell movement, cell restriction at the MHB is lost and MHB cells are intermixed. In other words, restriction of *LFng* expression at the midbrain side of the molecular MHB is significant for correct MHB formation.

It was previously thought that *Otx2* and *Gbx2* imparted properties that caused restriction of cells across the MHB (Joyner et al., 2000). Here I have shown that *LFng* activity sits upstream of this event. According to the phenotypes I obtained, restriction of *LFng* expression to the midbrain domain of the molecular MHB is important for the cells to maintain their positions. However, *LFng* is upregulated by the electroporation of constitutively active Notch across the MHB. This suggests that *LFng* itself is not enough to maintain the Notch activated domain, and requires another factor "X" to support it. Indeed, it has been reported that restoring *Fng* in Notch mutant flies is not sufficient to restore a normal DV boundary at the wing disc (Milan et al., 2002; Milan et al., 2001).

5-4-2 The LRR protein Lrrn1 restricts MHB cells to their original domain

In the *Drosophila* wing disc, cell adhesion molecules act to restrict cells within compartments, and help to refine the boundary. *Tartan* (*Trn*) is one of these adhesion molecules, known for its activity in the dorsal compartment during dorsoventral

boundary formation in the *Drosophila* wing disc. In addition, it has been shown that the activity of this molecule in maintenance of the dorsal compartment is independent of *Fng (Milan et al., 2001). Fng* is expressed in the dorsal compartment of the *Drosophila* wing disc and within this region, *Trn* acts independently to retain dorsal-specified cells in the dorsal domain, preventing them from crossing into the ventral. The perturbation of *Lrrn1*, a homologue of *Trn*, showed that the *Lrrn1* expression border plays a significant role in normal MHB formation both morphologically and molecularly. *Lrrn1* is a transmembrane protein which has a PDZ-domain binding site at its cytoplasmic C-terminal (Andreae et al., 2007). PDZ domain containing proteins are reported to regulate the subcellular trafficking of signalling groups (Fanning and Anderson, 1999; Gomperts, 1996; Ponting et al., 1997). This suggests the ability of *Lrrn1* to integrate cell signals at the boundary, which in this case would be the integration of cell adhesion and Notch signalling. At the MHB, DiI labelled cells showed selective cell movement within the *Lrrn1* positive territory. Under the misexpression of *Lrrn1* across the MHB, DiI labelled cells coincided with the *Lrrn1* positively expressing cells. Taken together, these data suggest that *Lrrn1* may function by forming an affinity difference between the MB and HB compartments. *Lrrn1* prevents cells from intermixing between MB and HB at the molecular MHB, enabling a boundary to form at their interface. Indeed, preliminary data strongly support this observation. Loss of function of *Lrrn1* through dominant negative *Lrrn1* and *Lrrn1* morpholino oligonucleotides led to the loss of expression of MHB organiser markers, *Fgf8* and *Wnt1* (C. Irving, data not shown). Furthermore, analysis of molecular MHB markers *Otx2* and *Gbx2* showed that no boundary formed. Significantly, *Lrrn1* expression was not affected under the ectopic Notch activation, while *LFng* was upregulated across the MHB by ectopically activated Notch. Thus, *Lrrn1* regulates cell affinity difference between MB and HB compartments in a Notch activation independent manner, probably through unknown signalling.

Strikingly, ectopic expression of *Lrrn1* upregulated *LFng* expression at the MHB, where both *Lrrn1* and *LFng* are normally absent. In contrast, *LFng* ectopic expression did not affect *Lrrn1* expression, and *Lrrn1* maintained its normal expression, a strict absence from r1 and r2. The normal expression of *LFng* only in midbrain cells could be due to the restricted expression of *Lrrn1* in MB cells. *Lrrn1* may regulate *LFng* expression

here normally, so that a high level of Notch activation is restricted to the molecular MHB. It is not yet clear how *Lrrn1* regulates *LFng*; neither of the LRR proteins, *Cap* and *Trn*, are reported to regulate *fng* in the *Drosophila* wing disc. Since *Lrrn1* is not a direct transcriptional activator, it could regulate *LFng* indirectly through an as yet unidentified signalling pathway. Both *Trn* and *Fng* are regulated by LIM homeodomain protein, *Ap* in the *Drosophila* wing disc (Blair et al., 1994; Milan et al., 2002; Milan et al., 2001). In this way, both the cell affinity and signalling components of boundary formation are coupled and controlled by *Ap*, the selector gene, the function of genes that specify cell, tissue, organ, as well as regional identity in animals. Interestingly, the LIM family member, *Lmx1b* is expressed at the MHB and is capable of inducing *Wnt1* (Matsunaga et al., 2002). Following *in ovo* electroporation, misexpressed *Lmx1b* also induces *Fgf8* non-cell autonomously, and represses *Fgf8* cell autonomously (Matsunaga et al., 2002). Thus, it is possible that both *LFng* and *Lrrn1* are regulated through *Lmx1b* in a similar mechanism to *Drosphila* and it would be interesting to address this in future work. However, here I have found that the affinity regulator, *Lrrn1*, can also regulate signalling through regulation of *LFng*. Coupling cell signalling and cell adhesion in this way may ensure integration of these two important facets of boundary formation at the MHB.

Chapter 6 Notch signalling is both necessary and sufficient for boundary formation in the mid-hindbrain and hindbrain

6-1 Introduction

In Chapters 3-5, I have demonstrated that Notch signalling activity can affect the formation and maintenance of the MHB in the developing chick CNS. Here I show that Notch signalling is required for MHB formation. I have used a number of strategies to perform loss of function analyses for this purpose. One strategy is to block Notch signalling using a chemical inhibitor, DAPT; a γ-secretase inhibitor. γ- secretase activity is important in the activation of Notch as it is responsible for the cleavage of NICD from the transmembrane receptor. After ligand binding, the intracellular domain of Notch receptor (NICD) is released from its extracellular domain by three stages of cleavage. A ligand binding to the extracellular domain of Notch receptor causes the proteolytic release of its cytoplasmic domain via ligand interaction with *Neuralized* (Greenwald, 1998). Ligand endocytosis then triggers a conformational change of the Notch receptor, and this leads to cleavage of the Notch receptor at a second site (S2). After this, the extracellular domain of the Notch receptor is trans-endocytosed to the ligand-expressing cell (Parks et al., 2000). This is then followed by another cleavage of the Notch receptor at a third site (S3), and this cleavage releases NICD to be translocated to the nucleus (Mumm et al., 2000). In order to release the Notch receptor extracellular domain at S2, γ- secretase activates intramembranous proteolysis (Mumm et al., 2000). Therefore, inhibition of γ- secretase activity prevents the Notch receptor from finishing S2 cleavage, and without this enzymatic activity, active Notch signalling cannot take place in the nucleus.

However, γ- secretase mediates intramembranous cleavage of other transmembrane proteins. For example, γ- secretase activity is required for the release of Amyloid-β peptide from β-amyloid precursor protein which is responsible for Alzheimer's disease (De Strooper et al., 1999). As γ- secretase activity is not entirely restricted to the Notch pathway, another more specific strategy is to use dominant negative ligands to block signalling. In Chapter 3, I have shown that there are a number of ligands present in the MHB region of the neural tube. It has previously been demonstrated that dominant

negative ligands act in *cis* to block Notch signalling in the same signal receiving cell. Therefore, all dominant negative ligands act indiscriminately to block all Notch signalling in the cell in which they are expressed (Kramer, 2001; le Roux et al., 2003). Meanwhile, in cells expressing both Notch and ligand, ligand interferes with the process of inter-translocation of NICD to the nucleus. It has been thought that this interference may be relieved by the neuralized protein, which targets ligands for degradation (Kramer, 2001). Here I have utilised the dominant negative effect of the ligand by misexpressing its extracellular domain, thereby blocking the Notch signalling pathway.

6-2 Materials and Methods

6-2-1 in ovo electroporation of DNA

The viral vector, Rous Sarcoma Virus, containing human Serrate1 (1-1222 a.a.) linked with green fluorescent protein by an internal ribosomal entry site (IRES-GFP) was used for *in ovo* electroporation. For *in ovo* electroporation of the dominant negative Serrate1, the pseudotype viral vector containing truncated human Serrate1 (1-1102) lacking most of the intracellular domain, linked with IRES-GFP was used. Both vectors were a kind gift from Dr. J. Lewis (le Roux et al., 2003).

6-2-2 DAPT soaked Affi-Gel Blue bead implantation

Affi-Gel Blue beads (150-200um, BioRad) were rinsed once, and washed 3 times in 1xPBS. γ-secretase inhibitor DAPT was added to the PBS with beads at 1:1000 ratios per bead. Beads were incubated for 1 hr at 37 °C before implantation.

HH stage 9 embryos were prepared for implantation. 1x ink solution was injected with a 25 gauge needle underneath the embryo to visualise the embryos before implantation. One drop of sterile room temperature PBS was added on top of the embryos to moisten them. Using forceps one Affi-Gel Blue bead was placed onto the embryo close to the site of implantation and a nick was made though the dorsal-most part of the neural tube, adjacent to the MHB, with a tungsten needle. The bead was then pushed into the neural tube through the nick, and another drop of sterile room temperature PBS containing antibiotic was added on top of the embryo. Embryos were incubated for a further 6hrs, 12 hrs and 24 hrs.

6-3 Results

6-3-1 Notch signalling is required for Fgf8 expression at the MHB;

6-3-1-1 Blocking Notch signalling through a chemical inhibitor disrupts *Fgf8* expression at the MHB

In Chapters 3-5, my results suggest that Notch signalling activity may be significant for the formation and maintenance of the MHB in developing chick CNS.

After ligand binding, the intracellular domain of Notch receptor (NICD) is cleaved from its extracellular domain through three stages of cleavage. For release of the Notch receptor extracellular domain at S2, γ- secretase activates intramembranous proteolysis (Mumm et al., 2000). Inhibition of γ-secretase activity prevents the Notch receptor from completing S2 cleavage and Notch signalling is blocked. In order to understand the importance of Notch signalling activity in the formation and maintenance of MHB, endogenous Notch signalling activity was blocked in this way using a chemical γ-secretase inhibitor (DAPT). Affi-Gel Blue beads were soaked in DAPT as described in the Materials and Methods and implanted into the region of the neural tube fated to give rise to the MHB at HH stage 7-8 (Figure 6.1A). After 6, 12 and 24 hour post-implantation, molecular changes at the MHB organiser were analysed by whole mount *in situ* hybridisation. Following control PBS-soaked bead implantation, *Fgf8* mRNA expression was found in the MHB (Figure 6.1B). In contrast, only 6 hours after DAPT soaked beads were implanted, *Fgf8* expression was visibly downregulated (data not shown, n=9/11). After 24 hours, *Fgf8* expression was lost entirely from the MHB in embryos treated with DAPT beads (Figure 6.1C, n=2/2). Significantly, the morphological MHB also failed to form. Interestingly, this *Fgf8* downregulation occurred when the DAPT beads were implanted either on the MB side or on the HB side of the MHB. These results suggest that Notch signalling at the MHB is necessary for the formation of the molecular and morphological MHB.

Figure 6.1 Implantation of DAPT soaked beads in the MHB causes the downregulation of *Fgf8*.

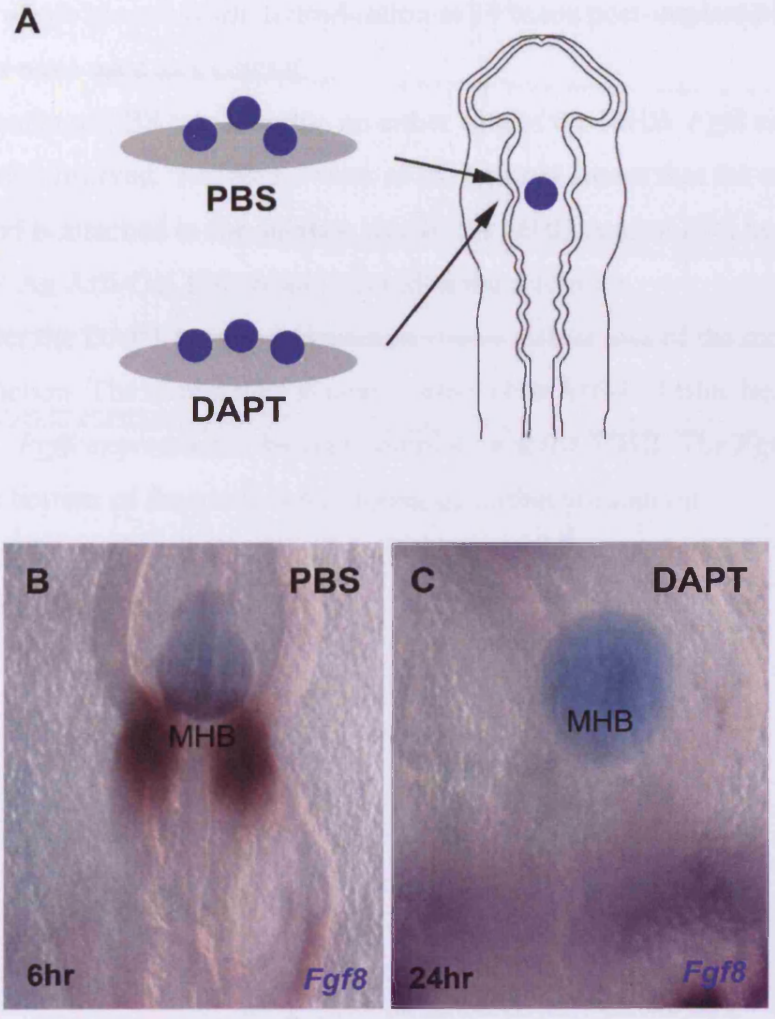


Figure 6.1 Implantation of DAPT soaked beads in the MHB causes the downregulation of *Fgf8*.

(A) A model of the bead implantation into HH stage 9 embryos. DAPT soaked Affi-Gel Blue beads were implanted into the MHB. Molecular changes at the MHB were analysed by whole mount *in situ* hybridisation at 24 hours post-implantation. PBS soaked beads were used as a control.
(B) After 6 hours of PBS implantation on either side of the MHB, *Fgf8* expression (in blue) is clearly observed. The dorsal view of the embryo shows that the edge of an Affi-Gel Blue bead is attached to the anterior side of the MHB control PBS bead implantation. An Affi-Gel Blue bead is found in the midbrain.
(C) 24hrs after the DAPT beads implantation shows a clear loss of the morphological MHB constriction. The neural tube is neatly intact after Affi-Gel Blue bead implantation. *Fgf8* expression is lacking completely at the MHB. The *Fgf8* expression visible at the bottom of the panel is that found in cardiac mesoderm.

MHB; mid-hindbrain boundary

6-3-1-2 Blocking Notch signalling through *Ser1* at the MHB causes the deformation of the MHB

Using DAPT applied on a bead to block Notch signalling provided a crude approach that indicated that cleavage via a γ-secretase enzyme was necessary for *Fgf8* expression at the MHB. In order to confirm that it was in fact the Notch pathway that was disrupted by this inhibition, and that Notch signalling is required for the establishment of the molecular and morphological MHB, I then chose to block the Notch signalling pathway specifically, by disrupting the ligand-receptor interaction.

Gene expression pattern analysis in Chapter 3 showed that a number of Notch ligands are expressed at the MHB, and the expression of one of the Notch ligands *Ser1* is demarcated at the molecular MHB in HH stage 10 chick embryos. By contrast, *Ser2* has a complementary expression pattern to *Ser1*, and *Dll1* is expressed in a more punctuated manner throughout r1.

In order to perturb Notch activation through *Ser1*, I performed *in ovo* electroporation of a truncated *Ser1* (dn*Ser1*) linked to IRES-GFP. This cDNA lacks most of the intracellular domain of *Ser1* which is fundamental for ligand activity. Truncated ligands are able to bind to but not activate the Notch receptor, and hence block endogenous signalling in a dominant negative fashion (Kramer, 2001; le Roux et al., 2003). dn*Ser1* *in ovo* electroporation was carried out on HH stage 8 and 9 embryos (prior to MHB constriction formation), and molecular changes were analysed by whole mount *in situ* hybridisation at 24 hour post-electroporation. When dn*Ser1*-GFP was expressed throughout the midbrain and anterior hindbrain, *Fgf8* mRNA expression was dramatically downregulated on the electroporated side compared to the control contra-lateral side (Figure 6.2A-C: black arrow heads: control; n=0/37, dnSer1; n=17/17). By contrast, the level of *Wnt1* expression did not visibly change, although its sharp boundary of expression was lost (Figure 6.2E: control; n=0/15, dnSer1; n=6/6). Sectioning and examination under higher magnification allowed further analysis. In transverse sections of the MB side of the MHB, *Wnt1* was observed throughout the neural tube from dorsal to ventral on the control side. In contrast, ventral expression of *Wnt1* was clearly downregulated on the experimental side (Figure 6.2F). Moreover, *Wnt1* was observed to be downregulated in cells containing GFP. Together, these results

showed that Notch signalling through *Ser1* is required for the formation of the MHB. To test the genes involved in initial positioning of the MHB, the expression of *Otx2* and *Gbx2* were analysed. Blocking Notch-*Ser1* activity across the MHB caused the interface between *Otx2* and *Gbx2* to become unclear and shift in a posterior direction. Upon flatmounting, it became clear that this might be due to intermingling of *Otx2* positive and *Gbx2* positive cells in the region of the MHB and that the apparent shift in the interface was due to the relative intensity of staining of *Otx2* and *Gbx2* (Figure 6.2G-I: control; n=0/10, dnSer1; n=4/5). Furthermore, in all cases where dn*Ser1*-GFP was expressed across the MHB, the boundary appeared to be lost and the morphological constriction was absent; the electroporated side of the embryo was markedly straight as compared to the control contra-lateral side (Figure 6.2A-C, G, H). Previously, in chick hindbrain and spinal cord, dominant negative *Dl1* and *Ser1* have been demonstrated to elicit a weak block of Notch activity (le Roux et al., 2003). Thus, these results revealed that Notch signalling is required for the correct positioning and formation of the MHB in developing chick CNS, but this may be occurring through any of the expressed ligands *in vivo*. Indeed, I obtained similar results using a dominant negative *Dl1* to block Notch signalling (data not shown).

6-3-2 Disruption of Notch signalling by ectopic expression of the ligand Ser1 leads to perturbation of the MHB

Ser1 expression is restricted to the anterior/midbrain side of the molecular MHB at HH stage 10 (Chapter 3). Blocking of Notch signalling through *Ser1* across the MHB caused deformation of the MHB both molecularly and morphologically.

To investigate whether the restriction of *Ser1* expression is required for MHB establishment, I performed *in ovo* electoporation with cDNA which encodes a coding sequence of *Ser1*. Ectopic *Ser1*-GFP was introduced across the MHB at HH stage 8 and 9, and expression of the MHB positioning molecules *Otx2* and *Gbx2* were tested by whole mount *in situ* hybridisation after 24 hour post-electroporation. In the embryos which were electroporated with control GFP, the interface between *Otx2* and *Gbx2* was clear and a perfect morphological MHB formed. In the presence of *Ser1*, however, the anteriormost boundary of *Gbx2* became indistinct (Figure 6.3A: control; n=0/14, Ser1; n=6/6). Surprisingly, *Otx2* expression was observed in r1, and expression of these two

homeobox genes, *Otx2* and *Gbx2,* were mixed where *Ser1*-GFP expressing cells were present (bracket, Figure 6.3B). Furthermore, flatmount analysis of these embryos revealed that misexpression of *Ser1* across the MHB causes intermingling of *Otx2* and *Gbx2* expressing cells (Figure 6.3C). Interestingly, misexpression of *Ser1*-GFP led to two morphological boundaries. The formation of the morphological MHB was visible at the same level as the MHB on the non-manipulated side (white arrow heads, Figure 6.3A, B). However, another morphological boundary was observed in the hindbrain (black arrow heads, Figure 6.3A-C: control; n=0/42, Ser1; n=15/15). The level of the second morphological MHB was identical to the posteriormost edge of the ectopic *Otx2* expression (Figure 6.3B). Flatmounting showed that interference in the positioning of the *Otx2/Gbx2* interface caused the formation of an ectopic morphological constriction (Figure 6.3C). In order to investigate the link between changes in MHB positioning and MHB organiser genes, whole mount *in situ* hybridisation of *Fgf8* and *Wnt1* was performed. Interestingly, expression of both *Fgf8* and *Wnt1* was maintained at the MHB (Figure 6.3D-F), however, their expression appeared in wider domains than control embryos (white arrows: control; n=0/54, Ser1; n=26/26). Together, these experiments revealed that *Ser1*-GFP misexpression at the MHB caused the MHB positioning genes, *Otx2* and *Gbx2*, to alter their expression domains and lose their restriction at the MHB, and this might allow a second MHB constriction to form at the new interface of their expression. However, *Ser1* has little or no effect on the MHB organiser gene expressions, *Wnt1* and *Fgf8*.

Figure 6.2 Blocking Notch signalling using a dominant negative ligand disrupts MHB organiser genes.

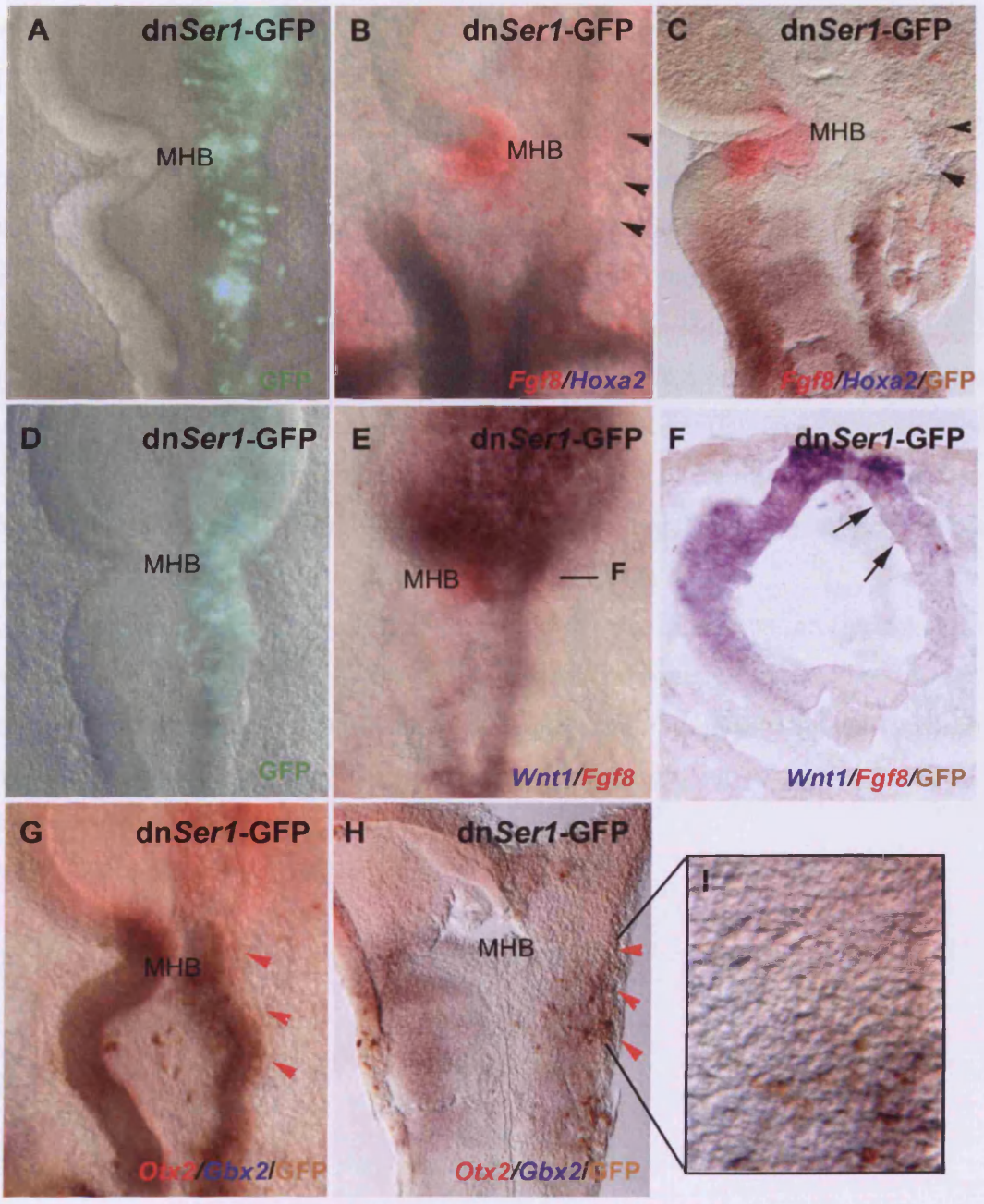


Figure 6.2 Blocking Notch signalling using a dominant negative ligand disrupts MHB organiser genes.

In ovo electroporation of dn*Ser1*-GFP across the MHB was performed on HH stage 8 and 9 chick embryos. Whole mount *in situ* hybridisation and immunohistochemistry analysis were carried out after 24 hrs culture.

(A-C) Misexpression of dn*Ser1*-GFP shows downregulation of *Fgf8*. (A) GFP fluorescent view shows consistent expression of dn*Ser1*-GFP construct. dn*Ser1*-GFP positive domain lacks morphological compartment. (B) *in situ* hybridisation reveals complete loss of *Fgf8* (red: black arrow heads), and the anterior limit of *Hoxa2* expression (blue) is not clear. (C) Flat mounting reveals the loss of morphological compartments in the domain where dn*Ser1*-GFP is expressed.

(D-F) Misexpression of dn*Ser1*-GFP perturbs *Wnt1* expression. (D) GFP fluorescent view shows consistent expression of dn*Ser1*-GFP construct. (E) *Wnt1* expression on the electroporated side is disrupted. (F) Transverse sections of the midbrain side of the MHB reveals that *Wnt1* expression is massively downregulated non-cell autonomously. GFP positive cells (black arrows, brown) do not express *Wnt1*.

(G-I) The boundary between *Otx2* and *Gbx2* domains is diffuse on the electroporated side (red arrow heads). (G) *Otx2* and *Gbx2* expression domains appear to be overlapping as compared to the control contralateral side of the embryo. (H) Flatmount view shows that the interface of *Otx2* and *Gbx2* is diffuse. (I) Intermingling of *Otx2* and *Gbx2* positive cells can be seen.

MHB; mid-hindbrain boundary

Figure 6.3 Misexpression of *Ser1* leads to intermingling of MHB gene expression and formation of ectopic morphological boundaries.

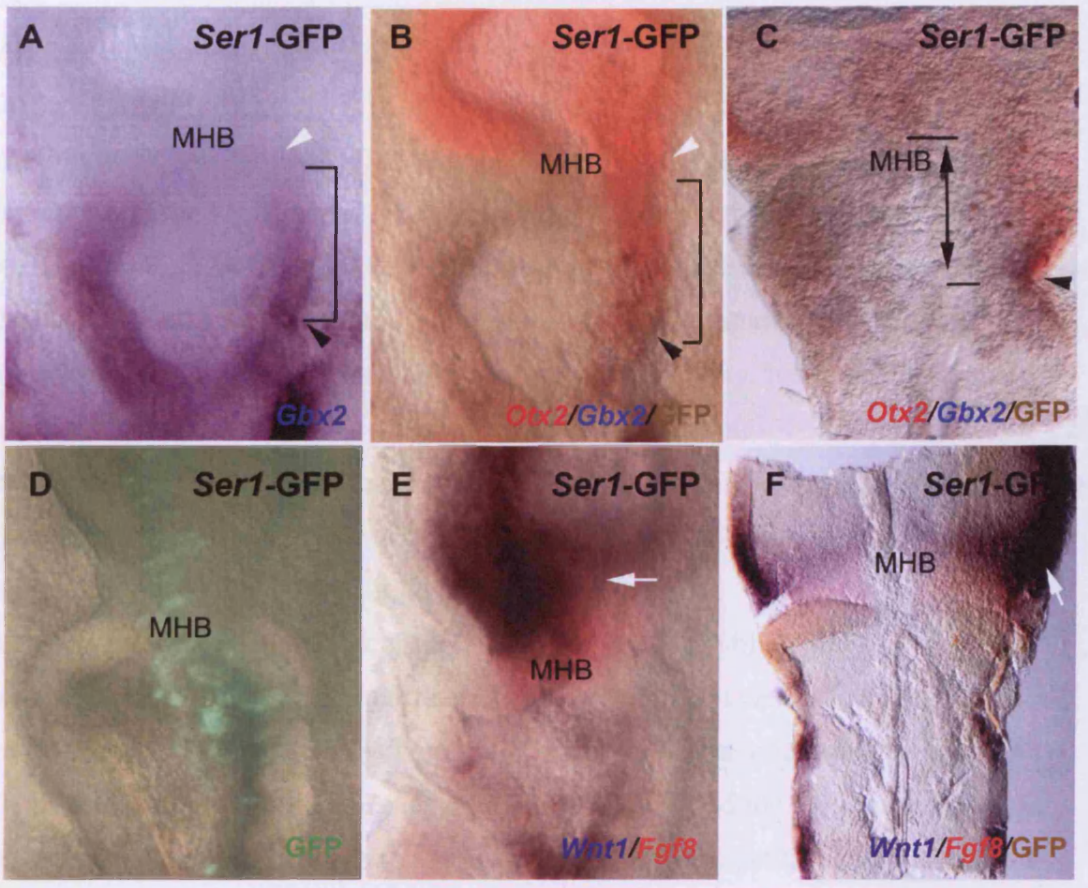


Figure 6.3 Misexpression of *Ser1* leads to intermingling of MHB gene expression and formation of ectopic morphological boundaries.

In ovo electroporation of *Ser1*-GFP across the MHB was performed at HH stage 8 and 9. Whole mount *in situ* hybridisation and immunohistochemistry were carried out after 24 hrs.

(A-C) Misexpression of *Ser1*-GFP across the MHB shows overlapping expression of *Otx2* and *Gbx2* (brackets). (A) The rostral limit of *Gbx2* expression (in blue) at the MHB is weak and diffuse. (B) *Otx2* expression (in red) is overlapping with *Gbx2* expression in the MHB and r1. GFP cells (in brown) are coincident with this overlapping domain. (C) The flatmount view reveals that the MHB domain has lost the clear *Otx2/Gbx2* interface on the experimental side as compared to the control contralateral side, and these two cell populations are mixing. Note that there is another morphological boundary in the hindbrain where posteriormost boundary of ectopic *Otx2* expression lies (Black arrow heads). (D-F) Misexpression of *Ser1*-GFP does not change the expression of the MHB organiser markers, *Fgf8* and *Wnt1*. (D) GFP fluorescence expression shows the *Ser1*-GFP positive domain.

(E) The morphological MHB constriction is lost. *Wnt1* (in blue) and *Fgf8* (in red) expression is visible, but the interface of these genes is not clear. (F) Flatmount views reveal that there is slight upregulation of *Wnt1* in the dorsal midbrain (white arrows). There is intermingling of *Wnt1* and *Fgf8* positive cells, and the MHB organiser gene expressing domain in total is expanded as compared to the control contralateral side. White arrow heads; the MHB morphological boundary. MHB; mid-hindbrain boundary

6-3-3 Misexpression of Ser1 leads to ectopic Fgf3 expression in r1 and r2

Blocking of Notch signalling through *Ser1* inhibits the folding of the neural tube normally evident at the MHB. In contrast, misexpression of *Ser1* leads to ectopic morphological boundary formation in the hindbrain. To investigate whether the Notch ligand *Ser1* is responsible for constriction formation in the CNS, I ectopically expressed it across the MHB towards the anterior hindbrain and analysed *Fgf3* expression, the earliest marker of rhombomere boundaries (Mumm et al., 2000). Interestingly, after *in ovo* electroporation of *Ser1*-GFP, an ectopic stripe-like expression of *Fgf3* was observed (Figure 6.4A-D: control; n=0/28, Ser1; n=9/9). On the non-manipulated side of the neural tube, *Fgf3* expression was observed in the MHB and posterior from r3, and it was completely absent from r1 and r2. Following ectopic *Ser1*-GFP expression, at least 4 extra stripes of *Fgf3* were observed clearly in the r1 and r2 domain on the manipulated side (black arrow heads; Figure 6.4A, B). In order to examine this phenotype further, these embryos were flatmounted (Figure 6.4C, D). High magnification analysis showed co-localisation of GFP cells with ectopic *Fgf3* expression (Figure 6.4D). Interestingly, the *Fgf3* expression at the MHB was also up-regulated (red arrow heads). These results revealed that ectopic *Ser1* expression is sufficient to create ectopic boundaries in r1, r2 - the metencephalon. Furthermore, these ectopic stripes exhibited a periodicity similar to rhombomere boundaries.

Figure 6.4 Ectopic *Ser1* expression leads to formation of ectopic boundaries.

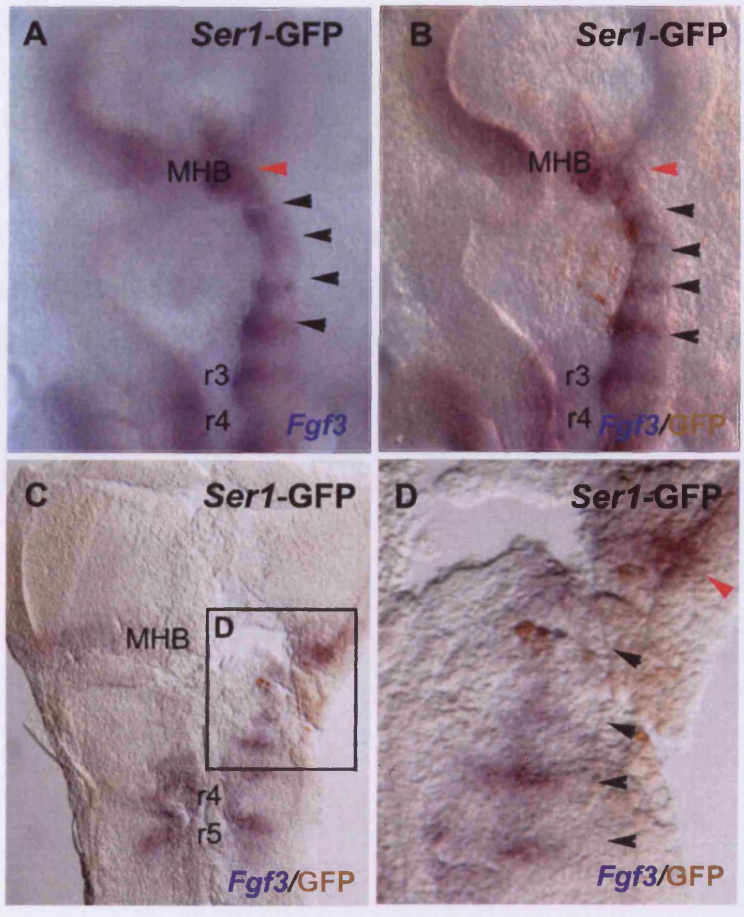


Figure 6.4 Ectopic *Ser1* expression leads to formation of ectopic boundaries.

In ovo electroporation of *Ser1*-GFP was carried out in HH stage 8-9 chick embryos. Whole mount *in situ* hybridisation and immunohistochemistry analysis were performed 24 hrs after electroporation.

(A-D) Ectopic expression of *Ser1*-GFP leads to the formation of ectopic boundaries in r1 and r2 (black arrow heads). (A) Stripe-like ectopic expression of *Fgf3* (in blue) in r1 and r2 is observed after *Ser1*-GFP electroporation. (B) GFP positive cells (brown) are coincident with the ectopic *Fgf3* expression. (C) Flatmounts of the MHB and hindbrain reveal that the ectopic *Fgf3* expression is arranged in a boundary-like manner. At the MHB, *Fgf3* is upregulated (red arrow heads). (D) A high magnification view of r1 and r2 reveals that ectopic boundaries form where *Ser1*-GFP positive cells are present. MHB; mid-hindbrain. r3; rhombomere 3. r4; rhombomere 4

6-4 Discussion

Previous studies have implicated Notch signalling in segmentation and boundary formation in the vertebrate CNS. Cheng et al described the role of Notch in segregation and differentiation of rhombomere boundary cells in Zebrafish, and Zeltser et al have implicated Notch signalling (or at least *Lunatic Fringe* activity) in the formation of the ZLI (Cheng et al., 2004; Zeltser et al., 2001). In neither of these cases was it demonstrated that Notch signalling is required for boundary formation. Indeed, in Cheng's study it was shown that Notch was not sufficient for boundary formation; ectopically activated Notch signalling through the use of NICD did not generate ectopic boundaries.

In this chapter I have sought to understand whether Notch signalling is both necessary and sufficient for MHB boundary formation. Firstly, I have shown that by blocking Notch signalling using a fairly broad spectrum chemical inhibitor, DAPT, the MHB was disrupted as evidenced by looking at *Fgf8* expression. By then taking a dominant negative ligand strategy I was able to confirm that Notch signalling is required for boundary formation. When Notch signalling was blocked using dn*Ser1*-GFP, the positioning of *Otx2* and *Gbx2* at the MHB were disrupted.

6-4-1 Notch-Ser1 signalling is required for the Otx2/Gbx2 interface formation

In the absence of Notch signalling, the early markers of the MHB, *Otx2* and *Gbx2*, do not form a sharp boundary. Previous studies have shown that the MHB forms at the interface of these two genes (Hidalgo-Sanchez et al., 1999b; Joyner et al., 2000; Katahira et al., 2000). In the absence of such a sharp boundary, *Fgf8* and *Wnt1* are also disrupted. It appears that midbrain (*Otx2*) and hindbrain (*Gbx2*) cells are able to mix, indicating that lineage restriction may be lost across the MHB in the absence of Notch signalling, although this was not tested in this case. As well as a molecular disruption to the boundary, the morphological constriction itself appeared to be lost in the absence of Notch signalling. The neural tube became flat and thin, losing the characteristic pinching at the interface of midbrain and hindbrain. This could be a direct consequence of loss of the MHB, but it is also possible that blocking Notch signalling interfered with another role of Notch – that of neurogenesis. Notch is required for the maintenance of proliferating neural precursors (de la Pompa et al., 1997; Huppert et al., 2000; Ishibashi

et al., 1995; le Roux et al., 2003). In the absence of Notch signalling, cells may be driven to differentiate prematurely resulting in a thinner neural tube. Preliminary data using the dn*Ser1*-GFP construct to block Notch signalling, followed by an analysis of the neurogenesis markers, *NeuroM* and *NeuroD*, suggests that this is not the case, as there was no change of these genes observed (data not shown).

When Notch signalling was ectopically activated at the MHB using *Ser1*-GFP, a similar intermingling of midbrain and hindbrain cells was seen, but this time an additional boundary seemed to form, as a second pinching or constriction was observed. Cells appeared of mixed origin between the two MHB constrictions. This may be due to a partial shift in the MHB due to a shift in *Ser1* boundary. As electroporation is only able to target a subset of cells in a mosaic fashion, the levels of *Ser1* may not be sufficient to shift the MHB entirely, and hence result in the formation of one MHB boundary at the original *Ser1* boundary and one MHB boundary at the ectopic *Ser1* boundary. This ectopic constriction did not express *Fgf8* or *Wnt1* and consequently was not a functional ectopic MHB organiser. Again, this may be due to insufficient levels of ectopic *Ser1* to form a precise boundary.

During experiments in which the boundaries of *Otx2* or *Gbx2* were shifted using transgenic mice, *Fgf8* and *Wnt1* expression domains both expanded widely (Broccoli et al., 1999; Millet et al., 1999). In *Otx2* and *Gbx2* null mice, *Fgf8* and *Wnt1* are still expressed at the MHB, but lose the restriction of their expression and are expressed over a greater area (Millet et al., 1999). These results confirm that the induction of MHB organiser marker genes is not regulated by *Otx2* or *Gbx2*, but correct positioning of *Otx2* and *Gbx2* is required for their restriction to appropriate domains. Following misexpression of *Ser1*-GFP, the interface of *Fgf8* and *Wnt1* expression was no longer clear at the MHB, and the entire domain appeared abnormally wide. Supported by these results in mice, the alteration of the organiser gene expression at the MHB is due to the loss of positioning of the *Otx2/Gbx2* interface, and may not be due to a direct impact of the *Ser1*-GFP misexpression.

6-4-2 Notch-Ser1 signalling is required for the boundary formation in the developing CNS

Notch signalling is required for the formation of hindbrain boundaries, as in the absence of Notch signalling this region of the neural tube becomes flattened, losing the characteristic pinching at boundaries and expression of *Krox20*, a gene that demarcates specific compartments (data not shown). A clear boundary constriction may be necessary for the proper segmental identity of rhombomeres. Furthermore, I have shown that Notch signalling is sufficient for boundary formation in the hindbrain using *Ser1* ligand to activate Notch ectopically. Under these conditions, ectopic stripes of *Fgf3* were observed in r1 and r2. In one case, a domain of ectopic *Krox20* expression was observed within r1, indicative of a new compartment formed within the ectopic boundaries and with the molecular properties of rhombomeres. More commonly, small blebs were observed extruding from the neural tube in r1, which might indicate that cells of a different character (e.g. *Krox20* expressing rhombomere) were induced but then excluded from the neural tube. Future experiments are necessary to investigate this phenotype fully. Interestingly, I was only able to generate ectopic boundaries in the r1/r2 region of the hindbrain, probably due to an underlying competence of the neural tube in this region. Intriguingly, these ectopic boundaries exhibited a periodicity similar to the other rhombomere boundaries more posterior in the hindbrain. This study differs from that of Cheng et al, in that I used the ligand *Ser1* to activate Notch in the hindbrain, rather than introduce the active nuclear fragment of NICD. This may account for the differences – activating the receptor at the cell surface may activate the associated signalling pathways more fully than directly targeting the nucleus with an activated Notch fragment. This study has been limited to the analysis of *Fgf3*, the earliest known boundary marker in hindbrain. Future work is necessary to investigate more fully the properties of these boundaries. Do they express other molecular markers and subsequently exhibit morphological properties of boundaries? Do they represent a true compartment i.e. are they cell lineage restricted? Ongoing work is trying to investigate these questions and repeat the experiment to analyse *Krox20* expression and that of other specific rhombomere markers, to see if ectopic rhomobomeres with specific characteristics are generated within the boundaries. It would also be interesting to investigate how and why these boundaries form with a defined periodicity.

Chapter 7 General discussion

7-1 Notch signalling and MHB development

I have shown that Notch signalling is important for the formation and maintenance of the MHB boundary and organiser. Genes involved in the Notch signalling pathway are expressed in restricted patterns that coincide with the MHB boundary. Both gain and loss of function analyses of Notch signalling molecules have revealed that Notch signalling is necessary for boundary formation in this region of the CNS.

7-1-1 A Notch-Serrate1 interface is sufficient for boundary formation

At the MHB, perturbation of *Ser1* through both gain and loss of function analyses strongly suggested that restriction of *Ser1* on the midbrain side of the molecular MHB is crucial for positioning of *Otx2* and *Gbx2,* genes which position the MHB during CNS development. Strikingly, a second *Otx2/Gbx2* interface was created following ectopic *Ser1* expression in the r1/2 domain, and a constriction was visible at this interface suggesting a second morphological boundary had also formed. Moreover, loss of function analysis of Notch signalling through *Ser1* showed malformation of both the MHB and hindbrain boundaries - boundaries were lost both molecularly and morphologically. Hence, I propose that Notch-*Ser1* interaction at the molecular MHB is required for the correct positioning of *Otx2* and *Gbx2* expressing domains, and assists them in forming an interface at the MHB. This interface is required for correct expression of *Fgf8* and *Wnt1*. Ectopic expression of *Ser1* did not affect the expression of these MHB organiser genes, but did affect their restriction to the boundary. This is probably as a secondary consequence of the lack of *Otx2/Gbx2* boundary, as other manipulations which perturb the *Otx2/Gbx2* boundary also result in lack of restriction of these genes at the MHB (Broccoli et al., 1999).

Ectopic expression of *Ser1* was also sufficient for the induction of *Fgf3*, an early boundary marker, in stripes within the r1/2 domain. Furthermore, these stripes appeared to have a periodicity indicative of hindbrain boundaries. Thus it seems that activating

Notch signalling here is sufficient for the formation of ectopic boundaries. Indeed, I was unable to induce ectopic *Fgf3* expression (as a marker of boundaries) through introduction of NICD, however, by creating a new Notch-ligand interface through ectopic expression of *Ser1* I was able to induce *Fgf3* in transverse stripes.
Ongoing and future work will investigate further the properties of these potential boundaries, and the properties of the r1/2 domain that provide competence for boundary induction.

7-1-2 The role of Serrate at the MHB

Notch signalling through *Delta* has been widely reported in relation to its role in lateral inhibition and neurogenesis (Dorsky et al., 1997; Henrique et al., 1997; le Roux et al., 2003). In chick CNS, misexpression of *Dll* blocks differentiation of neural progenitor cells, while dn*Dll* promotes differentiation of progenitor cells. Lateral inhibition through Notch-Delta signalling regulates the population balance of progenitor cells and neuronal cells. In contrast, little is known about the function of the Notch-Serrate interaction. Le Roux and her colleagues suggested that *Ser1* might have a function other than lateral inhibition in the neural tube. When *Ser1* or dn*Ser1* was misexpressed in the developing chick CNS, no irregular proliferation or differentiation in the hindbrain and spinal cord was seen (le Roux et al., 2003). Indeed, my own preliminary investigations also showed that misexpression of either *Ser1* or dn*Ser1* did not cause any changes in neurogenesis marker gene expression across the MHB region.

This finding links to an idea that *Ser1* may play more than one role around the MHB. *Ser1* C-terminal encodes a putative PDZ domain which is well conserved amongst species (Ascano et al., 2003; Hock et al., 1998). Mutation of the PDZ domain does not disrupt the ability of *human Ser1*(*hSer1*) to initiate Notch signalling in neighbour cells *in vitro*, however, the PDZ domain is required for changes in the expression of *hSer1* target genes and transcriptional activation of luciferase reporter construct (Ascano et al., 2003). At the MB side of the molecular MHB, *Ser1* shares its expression domain with *LFng* (Chapter3). In *Drosophila*, *Fng* modulates Notch to decrease its affinity for *Serrate* and increase its affinity to *Delta* (Bruckner et al., 2000; Hicks et al., 2000; Moloney et al., 2000; Panin et al., 1997). It is possible that *Ser1*-Notch interaction at the *LFng* positive domain in MB side of the MHB is weak, and instead, *Ser1* mediates

PDZ-dependent signals in the *Ser1* expressing cells. Interestingly, many PDZ domain-containing proteins are known to interact with cytoskeletal elements (Gomperts, 1996; Ponting et al., 1997). Further analysis over the *Ser1* mediated PDZ-dependent signals may be a key to understanding the Ser1-Notch signalling function at the MB.

The *Ser2* expression is strikingly restricted at the MHB at HH stage 10, in a complementary manner to *Ser1*. Unlike *Ser1*, *Ser2* is reported to lack a PDZ domain at its cytoplasmic C-terminal (Ascano et al., 2003). This finding predicts that *Ser2* only functions in Notch activation. In fact, this view is supported by a recent report in which only *Jagged2*, a zebrafish homologue of *Ser2*, and not *Jagged1*, is shown to play a significant role in the maintenance of proliferating progenitor cells and differentiation of secondary neurons in ventral spinal cord (Yeo and Chitnis, 2007). Thus, *Ser2* may play different role from *Ser1* at the MHB. Preliminary experiments showed that knockdown of *Ser2* does not significantly affect MHB formation or maintenance (date not shown). There is no evidence to show that *Ser1* and *Ser2* regulate each other, however, as the complementary expression pattern brings speculation about which *Ser2* may assist the formation of the clear border of *Ser1* expression at the MHB.

7-1-3 Contradictory views on the role of Notch at the MHB

Gene expression analysis in HH stage 10 chick embryos revealed that Notch related genes could be separated into three categories; genes which are significantly downregulated, or lacked expression from the MHB, genes which are expressed at the MHB and genes which are not expressed at the MHB. Investigating potential Notch target genes at the MHB was outside the scope of this thesis. However, it is interesting to note that amongst the Notch related genes analysed at the MHB, *Hairy2* was specifically expressed at the MHB at HH stage 10. Both *Hairy1* and *Hairy2* are chick homologues of the *Hairy/ E(spl)* family which is firstly shown as a family of Notch target genes (Jouve et al., 2000; Palmeirim et al., 1997). In zebrafish, *her5*, a zebrafish *hairy* orthologue, is reported to be expressed at the MHB throughout CNS development (Lun and Brand, 1998). Previously, Geling demonstrated that *her5* plays a key role in the inhibition of neurogenesis at the MHB in zebrafish (Geling et al., 2004). Loss of function analysis of *her5* through morpholino injection causes overexpression of neurogenesis markers, *ngn1* and *coe2,* across the MB. Similarly, *Hes1* and *Hes3* are

expressed at the MHB in mouse (Hirata et al., 2001; Lobe, 1997). In the absence of both *Hes1* and *Hes3*, MHB cells are prematurely differentiated into neurons resulting in the loss of midbrain and anterior hindbrain structures, and suggesting that the function of these genes at the MHB is to maintain the MHB organiser activity by preventing differentiation of these cells (Hirata et al., 2001). It is not known whether these genes are activated by Notch signalling in mouse, but in zebrafish Geling's further analysis suggested that the activity of *her5* is not directly regulated by Notch signalling (Geling et al., 2004). *her5* expression was severely downregulated following ectopic expression of NICD. Thus, the activity of the some Hairy/E(*spl*) family members at the MHB could be independent of Notch signalling. Further analysis in chick using NICD-GFP to investigate any effect on *Hairy2* would be interesting.

At HH stage 10, the expression boundary of *cDll1* coincided with the morphological MHB, and did not align with the molecular MHB. Furthermore, *cDll1* expression appeared mosaic, particularly within r3, suggestive of ongoing lateral inhibition and neurogenesis. It is possible that Notch-Delta signalling plays a significant role in neurogenesis in the neural tube, while Notch-Serrate1 signalling positions the *Otx2*:*Gbx2* interface correctly and induces the boundary marker, *Fgf3*, therefore assisting MHB boundary formation. However this contradicts data obtained in zebrafish by Cheng, who found that although Notch signalling is necessary for the segregation and differentiation of boundary cells in the hindbrain, Notch activation itself was not sufficient for the specification of boundary cells (Cheng et al., 2004). This apparent discrepancy could be due to the different molecular tools used to activate Notch signalling. In zebrafish, Notch activation in non-boundary regions by using dominant active Su(H) was tested, however it did not induce any ectopic *rfng* expression in the domain (Cheng et al., 2004). Here I used *Ser1* to activate Notch in the neighbouring cells. It is possible that the specification of boundary cells requires more than direct nuclear activation of Notch targets through Su(H). Directly activating Notch using *Ser1* may activate a more complete signalling response than just activating Su(H) targets in the nucleus and may circumvent the need for restricted *fng* expression, to activate downstream boundary cell markers.

There is also a possibility that this difference is due to the organism used and differences in experimental methods. Unlike zebrafish, none of chick *fng* genes were expressed in boundary restricted fashion (Chapter3). Furthermore, in the zebrafish, *Jagged1a*, a homologue of mammalian *Ser1* is reported to be expressed in a very similar manner to *deltaA* and *deltaD* at the HB, adjacent to the HB boundaries (Cheng et al., 2004; Zecchin et al., 2005). Although Notch related genes are highly conserved amongst species, these expression differences add more complexity to the understanding of the role of Notch signalling during development.

7-2 Fgf3 and Fgf8 expression at the MHB

Interestingly, misexpression of the constitutively active Notch led not only to the downregulation of *Fgf8* expression, but also the upregulation of *Fgf3* at the MHB (Chapter 4). Whereas NICD was not sufficient to generate new boundaries, as analysed by *Fgf3*, ectopic expression of *Fgf3* was seen in an expanded domain at the MHB. One explanation is that NICD may be sufficient to enlarge a population of boundary cells that normally express *Fgf3* from HH stage 15 (Mahmood et al., 1995). The onset of normal *Fgf3* expression is much later than that of *Fgf8*, and little is known about the molecular interaction of *Fgf8* and *Fgf3* at the MHB. However, members of the FGF family have been reported to be able to compensate for one another (Liu et al., 2003), and at the MHB a number of FGFs are expressed. *Fgf8* maintains *Fgf17* and *Fgf18* expression in zebrafish (Reifers et al., 2000). Therefore, another explanation of this phenotype is that *Fgf3* is upregulated in response to the downregulation of *Fgf8* by NICD. To distinguish between these probabilities, other boundary markers (e.g. follistatin) could be tested.

7-3 Cell lineage restriction at the MHB

For the segregation of the CNS into distinct regional compartments, cells need to be free to mix within a given compartment, but not across the boundary into the neighbouring compartment (Pasini and Wilkinson, 2002). This lineage restriction is known to play a fundamental role during the formation of the hindbrain compartments in the developing CNS (Fraser et al., 1990; Mellitzer et al., 1999). Despite this, it has been proposed that cells around the MHB are not cell lineage restricted and can cross the MHB, readjusting their gene expression to that of their new environment (Jungbluth et al., 2001). In

contrast, other studies showed that there is restriction of cell movement across the MHB in chick (Alexandre and Wassef, 2003; Louvi et al., 2003). Recently, Langenberg and his colleagues demonstrated that lineage restriction has already been established by the end of gastrulation at the MHB in zebrafish (Langenberg and Brand, 2005). A single marked cell cannot cross the MHB, and maintains its position within the developing midbrain. In this study, I have shown that DiI labelled cells on the midbrain side of the MHB cannot cross the boundary (Chapter 5: Figure 5.3F). This experiment was carried out at HH stage 8-9, before a morphological constriction is visible. Therefore, as in the zebrafish, it appears that lineage restriction at the MHB is established before morphological MHB formation. Discrepancies in results obtained by myself and others could be due to the methods used, the number of cells labelled and the position of label.

Strikingly, perturbation of *LFng* expression on the midbrain side of the MHB resulted in a clear disturbance of cell lineage restriction. Ectopic expression of *LFng* across the boundary resulted in cells which were initially on the midbrain side of the molecular MHB moving across the MHB, sometimes as far as posterior r1. However, these cells were always within the electroporated *LFng* domain. The ZLI is another cell lineage restricted boundary in the CNS (Figdor and Stern, 1993; Larsen et al., 2001). In the ZLI, misexpressed *LFng* cells are reported to sort to the *LFng* positive domains, either side of the ZLI compartment (Zeltser et al., 2001). It was possible that I would also see cells electroporated with *LFng* moving to the *LFng* positive domain at the MHB boundary. However, although LFng-positive cells were occasionally seen in two separate domains 24hrs after electroporation, this was a rare event and not statistically relevant. Interestingly, ectopically introduced *fng* clones in the ventral side of the *Drosophila* wing disc, where *fng* is normally absent, do not move (Micchelli and Blair, 1999; Milan et al., 2001). Furthermore, Andreae obtained a conflicting result at the ZLI, where misexpressed *LFng* cells did not move out of the *LFng* negative area into dorsal and ventral thalamus (Andreae, 2004). It is more likely that the ectopic *fng/LFng* cells maintain a group of cells which have the same status, in this case, *fng/LFng* positive cells (Micchelli and Blair, 1999; Rauskolb et al., 1999). Therefore, I conclude that sorting of *LFng*-positive cells does not take place at the MHB and that the cell movements observed are due to the disruption of the boundary, and therefore disruption of cell lineage restriction at the MHB.

7-4 Real time analysis of constitutively active Notch cell exclusion from r1/2

Ectopic expression of constitutively active Notch expressing cells showed the selective exclusion from r1 and r2 in 54.5% of embryos. Further analysis in Chapter 4 showed that this was not due to cell death, and was more likely due to cell exclusion specifically from this domain. This exclusion was also observed to coincide with morphological boundary formation. To understand the mechanisms of this cell exclusion, real-time cell movement analysis is an ideal technique to use. Preliminary data was obtained using real-time fluorescent analysis, carried out every 30 mins from 6 hours post-electroporation using a Nikon SMZ1500 microscope. Fluorescent photographs were taken individually with a Nikon digital camera (DXM1200F) (data not shown). Although NICD expressing cells showed restriction within the neural tube which appeared to co-localise with boundaries, it was not possible to capture the precise manner of cell migration. A number of reasons made it difficult to visualise the NICD cells in real time. Firstly, electroporation had to be carried out 6hrs prior to analysis in order for sufficient levels of GFP to be observed - at HH stage 7-8. Embryos then had to be cultured *in vitro* during the analysis. Standard New culture techniques require the embryo to be placed ventral side uppermost on an albumin bed, and this was not possible in order for the neural tube to be observed. Therefore, a modified EC culture method was used (Chapman et al., 2001). Finally, a compound microscope enclosed in a heated chamber is necessary to provide a good resolution and correct incubation temperature. Future work would include the optimisation of these techniques to image the NICD cells in real time under high power magnification, in order to determine the exact mechanism of their exclusion from the r1/2 domain.

7-5 Notch activation and cell affinity/adhesion

In order to prove the hypothesis that Notch activation is changing cell affinity properties, which leads to exclusion of activated Notch cells from r1/2, it would be interesting to perform *in vitro* cell adhesion assays. Cells from different regions of the neural tube could be dissociated and labelled, before mixing together to assay sorting behaviour, in the presence or absence of Notch signalling. In this thesis I have identified a candidate adhesion molecule, *Lrrn1*, that is required to maintain compartments in *Drosophila*. In chick, this gene is specifically expressed and demarcates the domain into which cells sort. Misexpression of *Lrrn1* across the MHB domain disrupts the boundary and cell

lineage restriction there. It would be interesting to see whether activated Notch cells sort in the presence of ectopic *Lrrn1* in r1/2, and also, whether cells mix in cell adhesion assays in the presence or absence of *Lrrn1*. Currently, further work is underway using antisense morpholino oligonucleotides to block translation of *Lrrn1* transcripts and hence provide a knockdown of gene function for a loss of function analysis in order to test the requirement for *Lrrn1* in boundary formation.

7-6 The role of Notch signalling in MHB formation

Understanding where Notch is active at/around the MHB is significant for further consideration of the function of Notch signalling. Gain of function experiments suggested that Notch might activate *Wnt1*. Ectopically induced active Notch cells led to ectopic *Wnt1* expression. In *Drosophila* wing disc, the *Wnt1* homologue, *wg* is induced at the DV boundary where Notch is highly active (Rauskolb et al., 1999). Unlike wg, *Wnt1* is expressed only on the midbrain side of the molecular MHB. However, numbers of ectopic boundaries were induced under the Notch activation through *Ser1*. Thus, Notch signalling seems to promote boundary cell fate in chick developing CNS. In zebrafish hindbrain, Notch activation is required for the segmentation and differentiation of the boundary cells where *Wnt1* is expressed (Amoyel et al., 2005; Cheng et al., 2004). Thus, Notch is likely to be active in a narrow strip of cells at the boundary itself, which presumably lies between midbrain and hindbrain, although *Wnt1* expression appears at the midbrain side of the molecular MHB. To address this question, I performed an immunohistochemistry analysis of active Notch expressing cells using Val1744, an antibody of a cleaved-form of Notch1 (at the Val1744 site). I also used a lacZ reporter assay to detect where Notch is active in the neural tube. *In ovo* electroporation of a lacZ construct which is fused with multiple duplications of *Su(H)* binding site of *Notch1* demonstrates where Notch is active in the CNS, post-Xgal staining. However, neither study showed conclusive results (data not shown). Evidence from Zebrafish suggests that Notch is active in MB and posterior HB but not in r1/2 however, as a *Her4* transgenic reporter line that reveals where Notch is active in the CNS, shows clear GFP reporter expression in MB and posterior HB, but no staining in r1/2 (Fig.3B from Yeo et al, 2007). This would correlate with the hypothesis that ectopically activated Notch cells sort out of the r1/2 domain to domains either side where Notch is normally active.

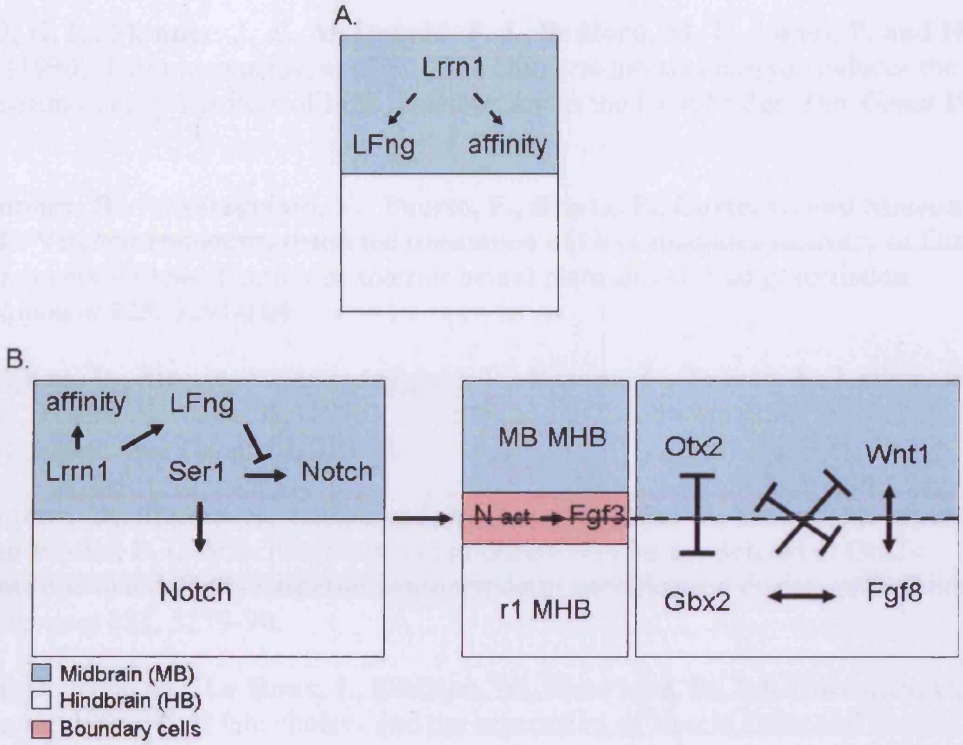


Figure 7.1 A model of the role of Notch signalling in MHB positioning.

(A) *Lrrn1* regulates *LFng* and an affinity balance, and two distinctive compartments (MB and HB) are formed. (B) At the MB side, Ser1-Notch interaction is negatively modulated by *LFng*. Thus, *Ser1* only activates Notch in the boundary. Notch activation at the Ser1-Notch interface leads to the specification of boundary cells, marked by *Fgf3*, and the fine boundary of MHB is formed.

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Appendix A: Description of the RNA probes

cDNA	Restriction enzyme site	RNA polymerase
c-Delta1	EcoRI	T3
c-Fgf3	NotI	T3
c-Fgf8	BamHI	T7
c-Gbx2	BglII	T3
c-Hairy1	HindIII	T7
c-Hairy2	HindIII	T7
c-Hoxa2	XhoI	T7
c-Krox20	EcoRI	T3
c-LFng	ClaI	T3
c-Lrrn1	SalI	T3
c-MFng	NotI	T3
c-Notch1	HindIII	T7
c-Otx2	XhoI	T3
c-RFng	ClaI	T3
c-Serrate1	XhoI	T7
c-Serrate2	BamHI	T3
c-Wnt1	EcoRI	T7