Analysis of the initial nerve connections in the embryonic vertebrate brain

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Abstract

The complex organisation of the vertebrate brain starts off as a simple arrangement of axon tracts in the early embryo termed the early axon scaffold. These initial axon tracts were important for the correct guidance of later, follower axons. Yet, little is known about the temporal and spatial control of neuronal differentiation, or the control of axon guidance for the early axon scaffold. The aim of this study was to provide a detailed anatomical description of the early axon scaffold as a basis for functional experiments, to identify candidate genes involved in the differentiation of the early neurones, and to gain insight into the axon guidance for a particular early tract.

The anatomical formation of the early axon scaffold in the chick embryonic brain has been analysed in detail using immunohistochemistry and axon tracing. The early tracts in the chick were directly compared with cat shark, *Xenopus*, zebra finch and mouse. These results highlight the conservation of early axon scaffold development. The medial longitudinal fascicle (MLF) was shown to be the most conserved tract forming first in all vertebrates, apart from mouse where it forms later. Since the genes involved in specification of neurones to an MLF fate are unknown, microarray analysis was used to identify candidate genes with a possible role in MLF neurone specification. CRABPI was shown by *in situ* hybridisation to be specifically expressed by the MLF neurones.

Another highly conserved early tract is the tract of the posterior commissure (TPC). Its neurones were shown to be located in the ventral diencephalon of the chick embryonic brain. While the TPC neurones were intermingled with the MLF neurones, their axons project along very separate paths, suggesting that their outgrowth is directed by different guidance cues present. Netrin1 and Netrin2 were identified as candidate genes for repelling the TPC axons along their correct path. Gain-of-function experiments led to reduction or loss of the TPC, suggesting that Netrins act as repellents on the TPC axons in the chick embryonic brain.

Declaration

Whilst registered as a candidate for the above degree, I have not been registered for any other research award. The results and conclusions embodied in this thesis are the work of the named candidate and have not been submitted for any other academic award.

Acknowledgments

Firstly I would like to thank my supervisor, Dr Frank Schubert for putting up with me for the last 6 years. I am indebted to him for sharing his knowledge, always being there to answer my '100 and one' questions and providing the guidance and encouragement I needed to complete my PhD. The list is endless but in particular thank-you for getting me through the *in situ* hybridisation nightmare and I should also mention Dr Suzanne Dietrich for lots of suggestions.

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Abbreviations

AC	Anterior Commissure
ANR	Anterior Neural Ridge
AP	Anterior-posterior
bHLH	Basic helix-loop-helix
CNS	Central Nervous System
di	Diencephalon
DLL	Dorsoventral longitudinal fascicle
drc	Dorso-rostral cluster
DTmesV	Descending tract of the mesencephalic nucleus of the trigeminal nerve
DV	Dorsoventral
DVDT	Dorsoventral diencephalic tract
E	Embryonic day
ep	Epiphysis
FR	Fasciculus retroflexus
GFP	Green Fluorescent Protein
HH	Hamburger and Hamilton stage
hpf	Hours post fertilisation
IsO	Isthmic Organiser
LLF	Lateral longitudinal fascicle
Mes	Mesencephalon
MFB	Midbrain-Forebrain Boundary
MHB	Midbrain-Hindbrain Boundary
MLF	Medial Longitudinal Fascicle
MTT	Mammillotegmental tract
p1-p3	Prosomere 1-Prosomere 3
PBS	Phosphate Buffered Solution
PFA	Paraformaldehyde
POC	Postoptic Commissure
PNS	Peripheral Nervous System
Pro	Prosencephalon
rho	Rhombencephalon
SM	Stria Medullaris
SOT	Supraoptic Tract
tel	Telencephalon
THC	Tract of the Habenular Commissure
TPC	Tract of the Posterior Commissure
TPOC	Tract of the Postoptic Commissure
VC	Ventral Commissure
vcc	Ventral-caudal cluster
VLT	Ventral Longitudinal Tract
vrc	Ventral-rostral cluster
ZLI	zona limitans intrathalamica

Chapter 1

Introduction

All vertebrates consist of a neural tube that divides the central nervous system (CNS) into the brain (rostral) and the spinal cord (caudal). The formation of the CNS needs to be tightly regulated to ensure the correct connections are made. This study investigates the development of the very first connections set up in the vertebrate embryonic CNS.

1.1 The nervous system

The nervous system has evolved from a simple nerve net in organisms such as sea anemone to a highly complex system of millions of nerve connections found in humans. The nervous system is made up of neurones and glial cells that form in either the central nervous system (CNS) or the peripheral nervous system (PNS). The PNS is involved in the communication between the CNS and the rest of the body. The CNS evolved during the divergence of bilateral organisms, to facilitate many basic functions such as movement and sight.

The developing embryo is formed from three germ layers, the mesoderm, ectoderm and endoderm. Neural induction specifies ectoderm into a neural fate involving signals from a primary signalling organiser forming the neural plate. The primary signalling organisers are Hensen's node in amniotes and Spemann's Organiser in *Xenopus*. Experiments have been done to show when tissue from these organisers were transplanted into non-neural tissue a second neural axis was induced (Spemann, 1938; Waddington, 1933). The signals released

from the organisers include Bone Morphogenetic Protein (BMP) antagonists such as Chordin, Noggin and Follistatin and Wingless (Wnt) antagonists such as Cerberus and Dickkopf. These antagonists inhibit BMP and Wnt signalling to enable the formation of the rostral neural plate while BMPs and Wnts pattern the caudal neural plate. The neural plate that arises from the ectoderm layer that rolls up to form the neural tube that will eventually form the CNS. The neural tube consists of the floor plate that extends along the entire ventral midline, the basal plate, the alar plate and the roof plate that extends along the dorsal midline. The floor plate and roof plate structures are involved in patterning of the dorsoventral axis.

1.1.1 The peripheral nervous system (PNS)

The PNS arises from neural crest cells that migrate from the neural/epithelial ectoderm interface located above the neural tube and epidermal placodes. The PNS consists of autonomic and sensory ganglia that consist of many cell bodies forming nerves that project as a collection of axons and the Schwann cells. The PNS is organised into ganglia and nerves whereas the CNS is organised into nuclei and tracts.

1.2 Patterning of the neural tube

Most of the caudal neural tube will give rise to the spinal cord and the brain forms in the rostral region of the neural tube. The brain is divided into the prosencephalon (forebrain), mesencephalon (midbrain) and rhombencephalon (hindbrain). As the neural tube closes patterning along the anterior-posterior (AP) axis and the dorsoventral (DV) axis is critical for development and controlling the formation of neurones at specific locations.

1.2.1 Signalling pathways and Transcription factors

Signalling between cells is essential for communication and involves the release of a signalling molecule from one cell that binds to a receptor on another cell and mediates a signalling cascade within the cell causing changes in gene expression. Signalling pathways in

neural patterning involves activation from sonic hedgehog (Shh), Bone Morphogenetic Proteins (BMPs), Fibroblast Growth Factors (FGFs), wingless (Wnts), and Notch. Gene expression needs to be tightly regulated to allow different cell types to differentiate. The resulting differential gene expression is a critical factor in cell fate determination and differentiation. Transcription factors are proteins, required by RNA polymerase, and activated downstream of the signalling pathway. Transcription factors are involved in the control of transcription and regulate many biological processes. Transcription factors can act as activators or repressors of transcription. The specificity of a transcription factor to a DNA sequence is determined by the DNA-binding domain present. Transcription factors are involved in the regulation of neural tube patterning setting up domains that will allow specific neurones to differentiate.

1.2.2 Neurogenesis

Neurogenesis is the process in which neurones are generated from progenitor cells. At least in arthropods and vertebrates, this is regulated by lateral inhibition and the Delta-Notch signalling pathway, suggesting the molecular mechanisms of neurogenesis are conserved. Lateral inhibition regulates commitment to a neuronal fate by preventing neighbouring cells from adopting the same fate. In *Drosophila,* the CNS develops from neuroblasts that express proneural transcription factors to give them their neuronal identity (Reviewed by Bertrand et al., 2002; Chitnis, 1999; Kageyama and Nakanishi, 1997; Lee, 1997; Lewis, 1996). Proneural genes achaete-scute (AS-C), atonal and amos regulate the expression of the neurogenic gene Delta, which in turn activates Notch or Jagged. Binding of ligands Notch and Jagged leads to repression of proneural genes by the expression of anti-neural genes Hairy and Enhancer of split E (Spl). Proneural basic helix-loop-helix (bHLH) transcription factors act downstream of the Delta-Notch signalling pathway and promote differentiation. The progenitor cells activated by proneural genes become neuroblasts. These bHLH proteins can function as

homodimers or heterodimers and bind to a common DNA sequence called the E-box (motif: CANNTG). The basic domain makes contact with the DNA and the HLH domain are involved in dimerisation (Murre et al., 1989). Proneural genes are key regulators of neurogenesis and promote switching from the growth phase to the differentiation phase (Kageyama and Nakanishi, 1997). In vertebrates, the neural cells at different stages of differentiation are present in different layers with undifferentiated progenitor cells being present in the ventricular zone of the neuroepithelium (Bertrand et al., 2002). The postmitotic neurones then migrate radially to form the mantle zone. All cells in the ventricular zone express Notch1 and by the time these cells move to the mantle zone the Delta-Notch pathway has been switched off. Mash-1 and Mash-2 (mammalian achaete-scute homologues), Math, NeuroD and neurogenin (Ngn) (mammalian atonal homologues) and the NSCL (neurological stem cell leukaemia) family function in determining neuronal fate. NeuroD, NSCL-1 and NSCL-2 function as differentiation factors. Hes (vertebrate Hairy homologue) and Id (vertebrate E (Spl) homologue) act as anti-neural factors. Hes proteins bind to the N-box (motif: CACNAG) which allows it to act as a repressor.

During neurogenesis, in order for the CNS to establish a fully functioning network of connections, these neuronal cells must also adopt a specific identity. The identity of a neuronal cell is then determined by patterning genes.
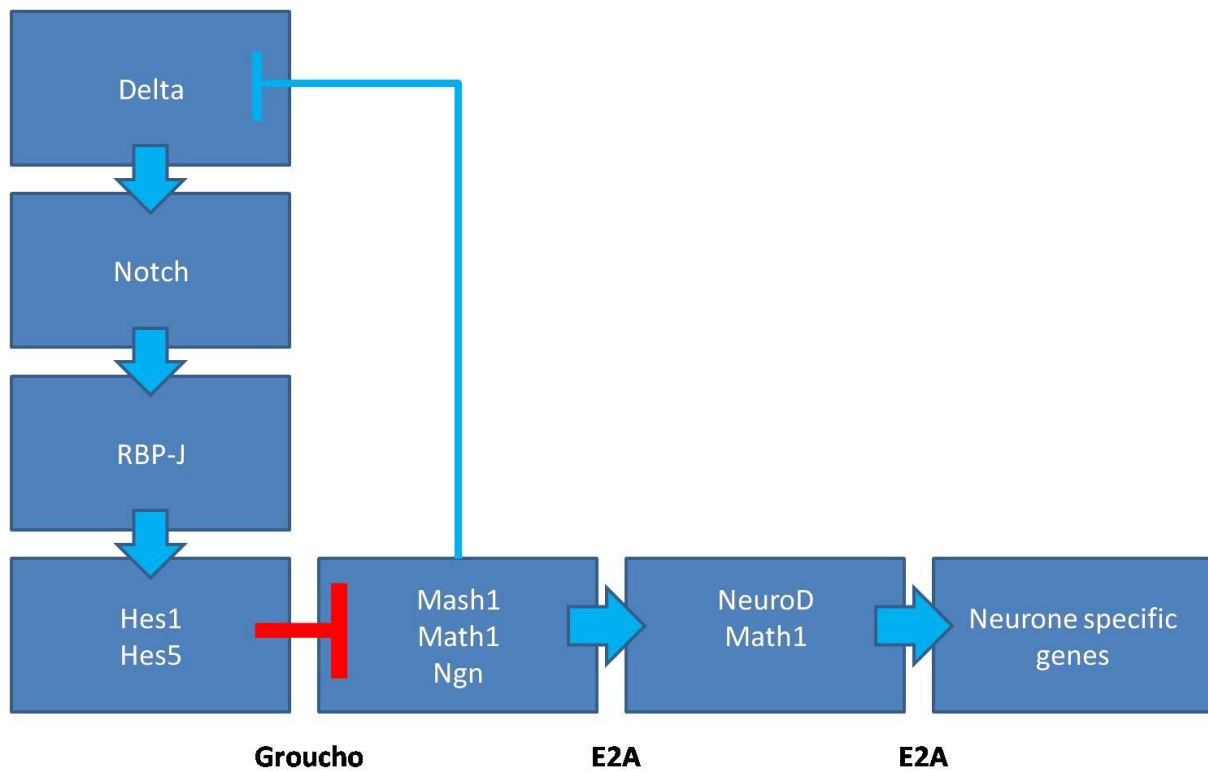
Figure 1.1 Neurogenesis in vertebrate precursor cells

When Notch is activated, it causes inhibition of proneural bHLH genes such as Mash1, Math1 and Ngn by Hes genes. When the anti-neural bHLH genes (Hes1 and Hes5) are inactivated, this switches on transcription of neurone specific genes allowing the cell to adopt a neural fate (adapted from Kageyama and Nakanishi, 1997).

1.2.3 Dorsoventral (DV) patterning

Throughout the neural tube, the notochord and floor plate express the secreted signalling molecule sonic hedgehog (Shh). Shh is expressed in a narrow stripe in the floor plate throughout the spinal cord and rhombencephalon; however in the prosencephalon and mesencephalon expression is more diffuse in the basal plate. Shh produces a graded morphogen effect to pattern the ventral neural tube by regulating the expression of specific transcription factors (Briscoe et al., 2000; Ericson et al., 1997). As a morphogen, the concentration of Shh expression decreases dorsally towards the roof plate where BMPs and Wnts pattern the dorsal spinal cord. Correct expression of these signalling molecules is required to ensure the correct formation of different types of neurones along the neural tube. In the ventral spinal cord, Shh induces the expression of class I (Dbx1, Dbx2, Irx3 and Pax6) and class II (Nkx6.2, Nkx6.1, Olig2 and Nkx2.2) transcription factors, which mutually repel each other to set up boundaries in which specific progenitor domains are generated (Fig 1.2). Each domain gives rise to a distinct neuronal type. There are five classes of ventral neurones set up: VO, V1, V2, MN and V3. When Shh is lacking in the embryonic mouse, ventral cell types within the neural tube were missing (Chiang et al., 1996). An example of cross repulsion involves the expression of Pax6 and Nkx2.2 at the pMN/p3 boundary (Briscoe et al., 2000 Fig 1.2). Misexpression of Pax6 causes repression of Nkx2.2 expression in the cells within the p3 domain that would normally express Nkx2.2. When Nkx2.2 was misexpressed, cells repressed Pax6 expression. This led to the conclusion that Pax6 and Nkx2.2 have cross-repulsive activity. Loss of Nkx2.2 results in the loss of V3 neurones and ectopic generation of MNs within the p3 domain (Briscoe et al., 1999).
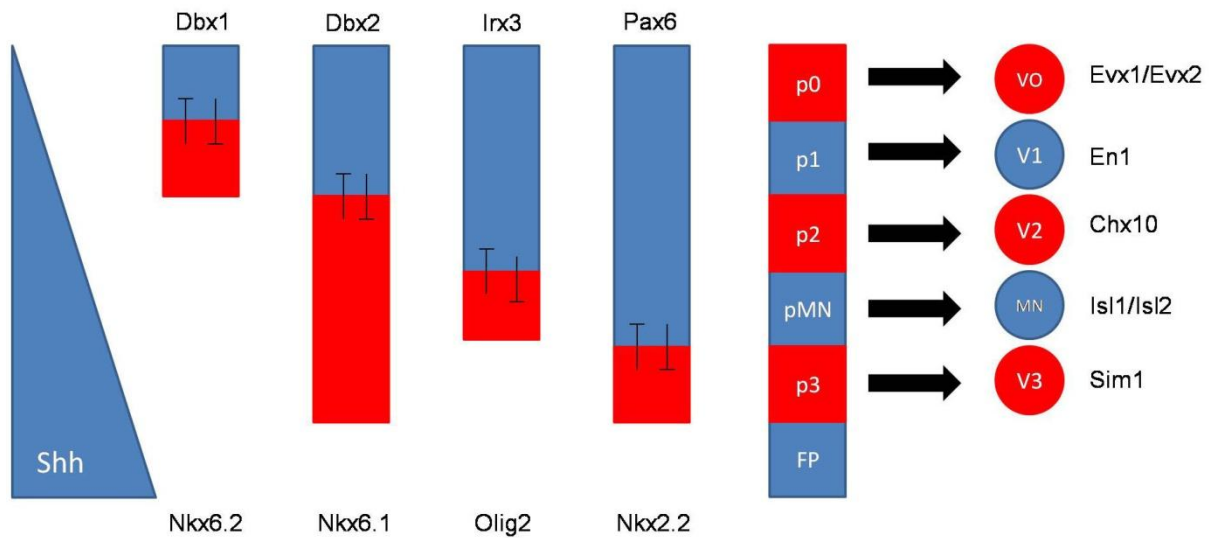
Figure 1.2 Regulation of transcription factors in the spinal cords in response to Shh

Shh is expressed as a gradient from the floor plate (FP) in the spinal cord. This induces cross repulsion of class I genes (Dbx1, Dbx2, Irx3 and Pax6) with Class II genes (Nkx6.2, Nkx6.1, Olig2 and Nkx2.2). Class I genes are repressed by Shh and Class II genes are activated by Shh. The cross repulsion of these genes sets up the patterning of different cell types in the ventral spinal cord and determines which interneurones (VO, V1, V2 and V3) and motor neurones (MN) are generated. (Adapted from Dessaud et al., 2008)

Like in the spinal cord, many of the neurones are formed in highly organised clusters at specific regions within the brain. The notochord expresses Shh leading to induction of Shh signalling in the floor plate. The notochord underlies the floor plate up to the p2/p3 boundary of the diencephalon (see 1.2.5) and the prechordal plate that forms from the mesendoderm underlies the secondary telencephalon. This would suggest DV patterning of the brain is similar to that shown in the spinal cord (Briscoe et al., 2000; Ericson et al., 1997). A similar mechanism occurs in the ventral mesencephalon, in which Shh is required for neuronal and molecular identity. The ventral mesencephalon is highly organised into an array of reiterative arcuate territories arranged bilaterally along the floor plate formed from neuronal cells (Sanders et al., 2002). Within these arcs of neuronal cells, specific transcription factors are expressed like in the spinal cord. These arcs also appear to extend into the diencephalon up to the p2/p3 boundary although the number of arcs is reduced (Sanders et al., 2002). The ventral mesencephalon is organised into five distinct arcs that express specific transcription factors: arc1 (Phox2A and Isl1), arc2 (GATA2 and Fox2A), Pax6 stripe, arc3 (GATA2) and EVX1 stripe (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Sanders et al., 2002). When Shh was ectopically expressed in the embryonic chick brain, it produced a mirror image of the midbrain arcs (Agarwala et al., 2001). Similarly when Shh was knocked down in mouse, the formation of midbrain arcs were severely disrupted and dorsal markers were also upregulated (Fogel et al., 2008). The transcription factors Emx2, Sax1 and Six3 have overlapping expression in arcs 2 and 3, with Pax6 expression separating the arcs (Schubert and Lumsden, 2005). Otx2 is involved in the specification of identity and fate of neuronal progenitors in the ventral mesencephalon (Puelles et al., 2004). Like in the spinal cord, neuronal populations have been shown to be specified by the transcription factor expression of the arcs. The oculomotor and red nucleus neurone development in the ventral mesencephalon are regulated by Shh (Agarwala and Ragsdale, 2002).

1.2.4 Anterior-posterior (AP) patterning of the brain

The prosencephalon and mesencephalon are patterned along the AP axis by signalling molecules located in specific regions of the brain. Main secondary organisers in the brain involved in AP patterning are the anterior neural ridge (ANR), the zona limitans intrathalamica (ZLI) and the isthmic organiser (IsO).

The ANR is located in the rostral most part of the brain and expression of the signalling molecule FGF8 is involved in the patterning of the telencephalon. Transplantation of ANR cells from either zebrafish or chick embryos to a more caudal position induces the expression of Nkx2.1, Emx and Dlx genes that are typically expressed in the telencephalon (reviewed by Echevarría et al., 2003; Houart et al., 1998). If the ANR cells were ablated, the anterior structures in the zebrafish brain were lost, including neuronal differentiation (Houart et al., 1998).

The ZLI forms at the border of p2 and p3 in the diencephalon (Kiecker and Lumsden, 2004 see 1.2.5). Expression of Shh from the ZLI causes Gbx2 and Irx3 to be expressed caudally and Dlx2 and Six3 to be expressed rostrally (Fig 1.3). Mutual repression between Six3 and Irx3 define the ZLI border (Kobayashi et al., 2002).

The formation of the IsO at the midbrain-hindbrain boundary (MHB) controls mesencephalon and rostral rhombencephalon development. Placing FGF8 soaked beads ectopically in the diencephalon induces ectopic expression of mesencephalic marker genes (Crossley et al., 1996; Liu and Joyner, 2001). Otx2 expression is throughout the prosencephalon and mesencephalon, while Gbx2 expression is throughout the rostral rhombencephalon. These homeodomain transcription factors are expressed early in development and set up the positioning of the MHB by mutual repression (Fig 1.3). Otx2 and Gbx2 gain-of-function experiments show a shift in the position of the IsO (Broccoli et al., 1999; Katahira et al.,

2000; Millet et al., 1999). Otx2 and Gbx2 are important for the positioning of FGF8, but Pax2 and En1/En2 are required for induction of FGF8 (Ye et al., 2001). En1 and En2 are expressed as a gradient that is highest at the IsO, rostrally and caudally away from the MHB. In chick there are two FGF8 isoforms: FGF8a and FGF8b expressed at the IsO (Sato et al., 2001). FGF8b was the isoform involved in patterning from the IsO, misexpression caused Otx2 expression to be repressed and Gbx2 and Irx3 to be upregulated. FGF18 and FGF17 are also expressed in the isthmic region and are involved in the organisation of the mesencephalon (reviewed by Sato et al., 2004).

Cross-repulsion between the prosencephalic marker Pax6 and mesencephalic markers En1/En2 sets up the midbrain-forebrain boundary (MFB) (Fig 1.4) (Araki and Nakamura, 1999; Matsunaga et al., 2000). Pax6 mutants (small eye) in mice have an effect on the positioning of the p1/mes boundary, by shifting p1 into a mesencephalic identity further suggesting Pax6 is involved in setting up the MFB (Mastick et al., 1997). The rhombencephalon is formed of repeated segments called rhombomeres. Expression of retinoic acid within the posterior region of the neural tube leads to expression of Hox genes. Hox genes are expressed caudally from r2 and are involved in patterning of the rhombencephalon and spinal cord (Marshall et al., 1992).
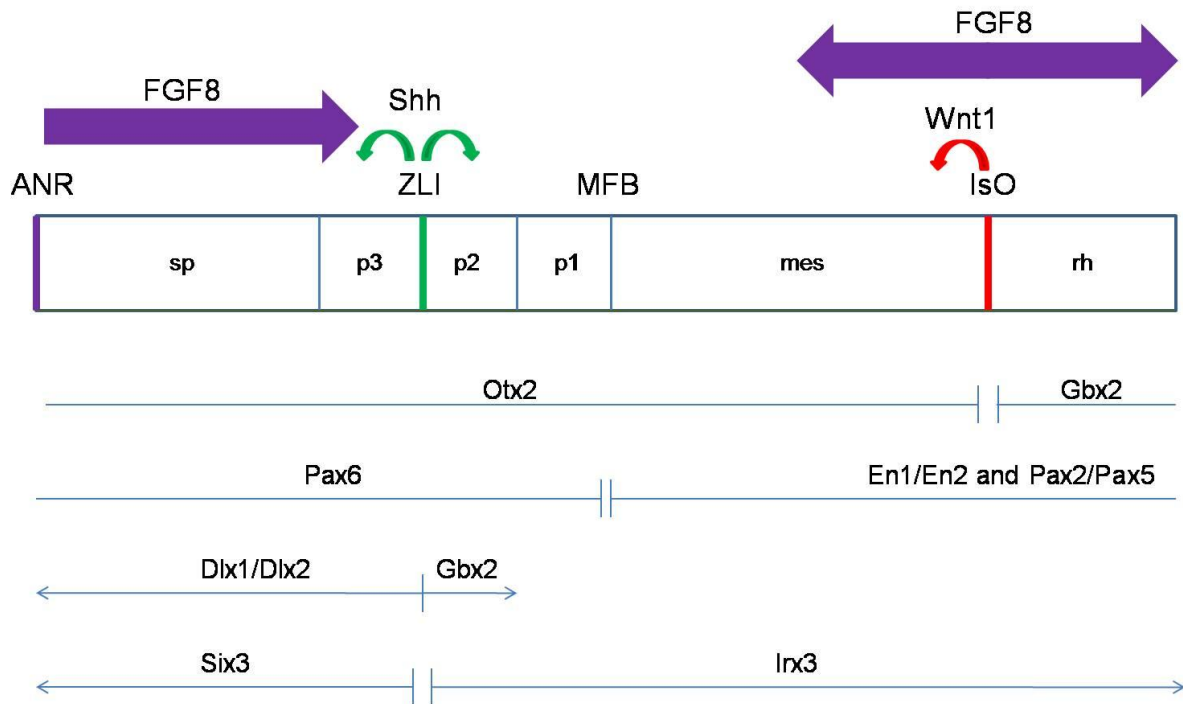
Figure 1.3 Patterning of the rostral neural tube in the embryonic vertebrate brain

There are three signalling centres located in the brain that are involved in patterning. At the rostral most region is the ANR (purple), between p3 and p2 the ZLI (green) and at the MHB the IsO (red). FGF8 (purple), Wnt1 (red) and Shh (green) are the main signalling molecules involved in the patterning from these signalling centres. There are numerous transcription factors involved in patterning of the brain, expressed in response to the signalling centres.

ANR, anterior neural ridge; IsO, isthmic organiser; mes, mesencephalon; MFB, midbrain-forebrain boundary; p1, prosomere1; p2, prosomere2; p3, prosomere3; rh, rhombencephalon; sp, secondary prosencephalon; ZLI, zona limitans intrathalamica

1.2.5 The Prosomeric model

The prosencephalon is located in the most rostral part of the neural tube and is further subdivided into transversal neuromeres along the AP axis into the telencephalon and diencephalon. The mesencephalon remains undivided transversally (Fig 1.4). Puelles and Rubenstein (1993) showed that expression patterns of homeobox and regulatory genes are regional markers that subdivide the prosencephalon further transversally into prosomeres. The model was initially suggested to consist of p1-p4 in the diencephalon and the telencephalon consisted of p5 and p6 (Puelles, 2001; Puelles and Rubenstein, 1993). However, subsequent studies found little evidence to support the p4-p6 subdivision so the model has now been simplified showing p1 (or pretectum), p2 (or thalamus) and p3 (or prethalamus) make up the caudal diencephalon. The rostral diencephalon and telencephalon are non-segmented rostral to p3 making up the secondary prosencephalon (Puelles and Rubenstein, 2003). The alar and basal plate divides the entire AP axis longitudinally. In the brain, this boundary is marked by the expression of Nkx2.2 (Shimamura et al., 1995).
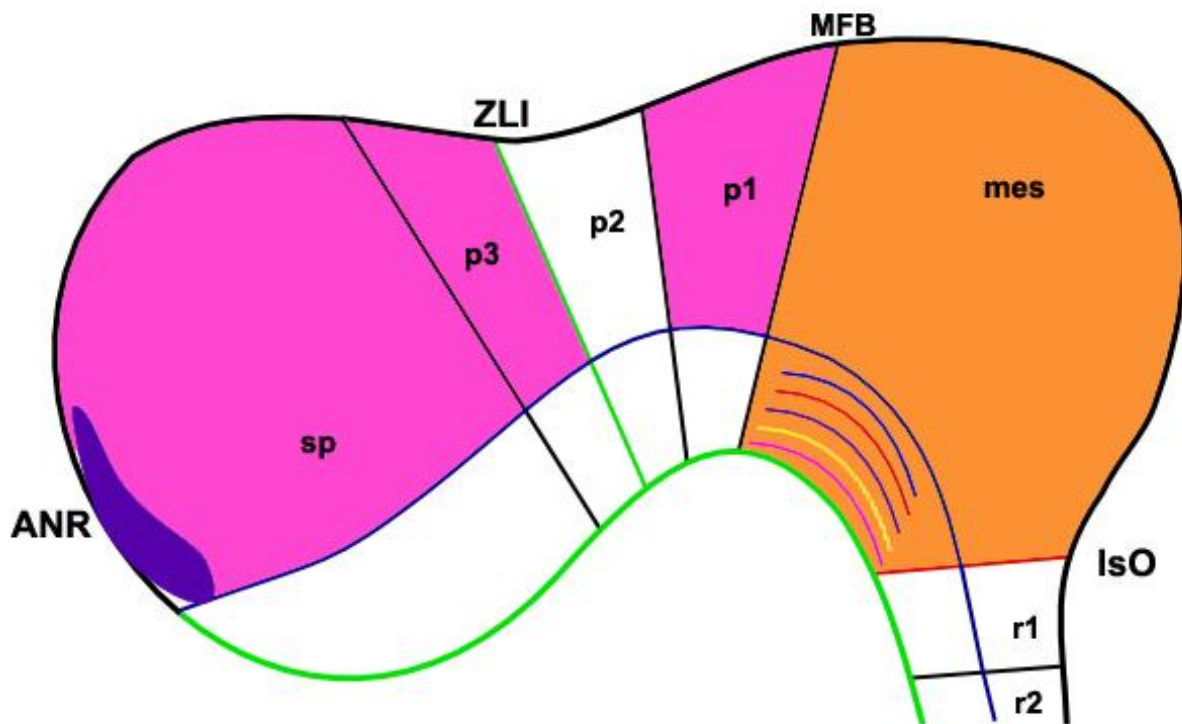
Figure 1.4 Overview of brain patterning and development of neuromeres

The brain is subdivided into the telencephalon (tel), diencephalon (p1-p3), mesencephalon (mes) and rhombencephalon (r1-r7). In specific regions of the brain, signalling centres are involved in AP patterning of the brain (ANR, ZLI and IsO). Shh (green) is expressed by the floor plate and the ZLI. Shh is responsible for inducing midbrain arcs; arc1 (pink), arc2 (yellow), Pax6 stripe (purple), arc3 (red) and EVX1 (blue). The brain is also divided by the alar and basal plate, which is marked by the expression of Nkx2.2 (Blue longitudinal line). The MFB is marked by Pax6 (pink) rostrally and En1 (orange) caudally. Pax6 is not present in the basal plate throughout the prosencephalon or the alar plate of p2 at later stages,. In earlier stages Pax6 is expressed throughout the entire alar diencephalon (Ferran et al., 2007).

ANR, anterior neural ridge; IsO, isthmic organiser; mes, mesencephalon; p1, prosomere1; p2, prosomere2; p3, prosomere3; r1, rhombomere1; r2, rhombomere2; sp, secondary prosencephalon; ZLI, zona limitans intrathalamica

1.3 The early axon scaffold

The complex organisation of the adult vertebrate brain is pioneered during early embryonic development by a small number of neurones and associated axon tracts. The basic array of early longitudinal tracts, transversal tracts and commissures was first described in the zebrafish embryonic brain and was termed the early axon scaffold (Chitnis and Kuwada, 1990; Wilson et al., 1990 and Fig 1.5). Subsequent studies in zebrafish have demonstrated that the neurones of the early axon scaffold have an important function in pioneering the major axon pathways in the brain allowing more complex connections to form (Chitnis and Kuwada, 1990).

The early axon scaffold has since been studied in various anamniotes such as *Xenopus* (Hartenstein, 1993; Key and Anderson, 1999), turbot (Doldan et al., 2000), medaka (Ishikawa et al., 2004), and sea lamprey (Barreiro-Iglesias et al., 2008). In contrast, among amniotes only the mouse brain has been studied in detail (Easter et al., 1993; Mastick and Easter, 1996) and early tracts have been briefly described in chick (Chédotal et al., 1995; Lyser, 1966) and alligator (Pritz, 2010). The basic tract system is remarkably well conserved during evolution. As the early axon scaffold is present in both non-jawed vertebrates (Barreiro-Iglesias et al., 2008; Kuratani et al., 1998a) and jawed vertebrates this would suggest the structure appeared before the divergence of the vertebrates (Fig 1.6). A common feature of all vertebrates analysed is the ventral longitudinal tract (VLT) system, formed by the medial longitudinal fascicle (MLF) and the tract of the postoptic commissure (TPOC). The MLF originates from a cluster of neurones located at the midbrain-forebrain boundary (MFB), while the TPOC neurones are located in the rostral basal hypothalamus. Prominent commissures are the postoptic commissure (POC) and anterior commissure (AC) in the rostral telencephalon, the posterior commissure (TPC) in the caudal diencephalon and the ventral commissure (VC) crossing at the ventral MFB. The TPC is a well-conserved transversal tract that aligns the

MFB. An additional transversal tract, the dorsoventral diencephalic tract (DVDT) is only clearly distinguished in anamniotes. On the other hand, in the mouse and chick neurones in the dorsal mesencephalon form the prominent dorsal tract of the mesencephalic nucleus of the trigeminus (DTmesV), this tract has no obvious counterpart in the early anamniote brain.

1.3.1 Formation of the medial longitudinal fascicle (MLF)

The MLF has been described as the first axon tract to form in the embryonic vertebrate brain of all the vertebrates studied, excepted in mouse where it appears slightly later (Easter et al., 1993). The MLF originates from neurones located around the MFB. In zebrafish this population of neurones has been termed the ventral-caudal cluster (vcc) (Ross et al., 1992) and in other vertebrates the nMLF (nucleus of the MLF). The MLF axons project caudally along the floor plate as a highly fasciculated tract into the rhombencephalon, crossing the MHB at around 18hpf in zebrafish (Metcalfe et al., 1990).

In zebrafish larvae, the MLF consists of different populations of neurones (Sankrithi and O'Malley, 2010), which has also been suggested in the chick embryonic brain (Ahsan et al., 2007). The zebrafish MLF neuronal populations are termed MeM (medial-lateral) and MeL (medial-lateral). The MeL population is further subdivided into MeLr (rostral), MeLc (caudal) and MeLm (medial) (Sankrithi and O'Malley, 2010). Ablation studies of two of the MLF neurone populations (MeLr and MeLc) that project axons into the spinal cord shows involvement in visually guided prey capture in zebrafish larvae (Gahtan et al., 2005). These MLF neurones act downstream of signals received from the tectum. MLF neurones have also been shown to be involved in escape and swimming behaviour (Gahtan and O'Malley, 2003; O'Malley et al., 2004; Sankrithi and O'Malley, 2010).
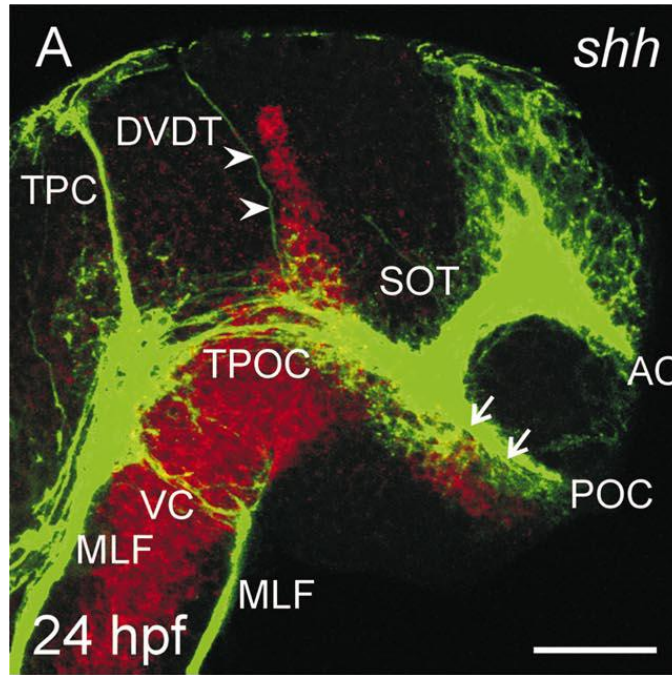
Figure 1.5 Early axon scaffold in the zebrafish (*Danio Rerio*) embryonic brain (green) with expression of Shh (red) at 24hpf (Copied from Hjorth and Key, 2001)

The early axon scaffold well established at 24hpf and is formed of longitudinal tracts: TPOC and MLF, transversal tracts: DVDT, TPC and SOT and commissures: AC, POC, VC. Shh is expressed throughout the floor plate and ZLI (arrowheads).

AC, anterior commissure; POC, postoptic commissure; SOT, supraoptic tract; MLF, medial longitudinal tract; VC, ventral commissure; TPOC, tract of the postoptic commissure; TPC, tract of the posterior commissure; DVDT, dorsoventral diencephalic tract
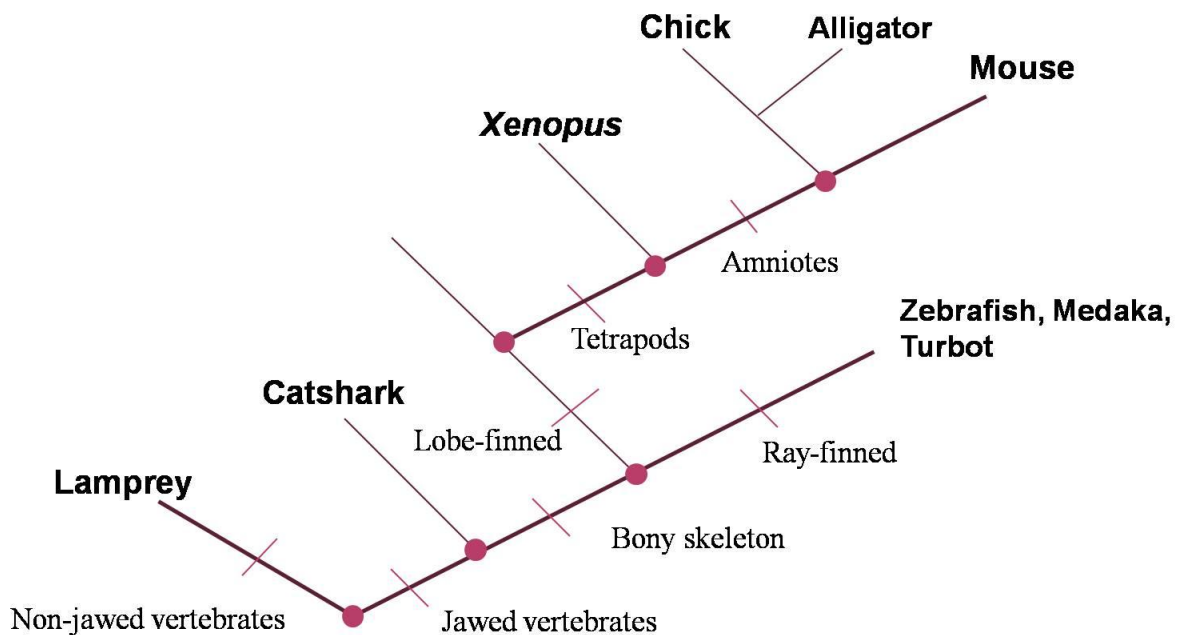
Figure 1.6 Evolutionary tree of vertebrates already analysed

1.3.2 Formation of the tract of the postoptic commissure (TPOC)

The TPOC originates from neurones located in the rostral basal hypothalamus in all vertebrates studied. In zebrafish this population of neurones has been termed the ventral-rostral cluster (vrc) (Ross et al., 1992) and in other vertebrates the nTPOC (nucleus of the TPOC). The TPOC projects caudally through the basal plate, ventral to the optic stalk, towards the MFB. Once the TPOC axons reach the MFB they will form the VLT along with the MLF, connecting the prosencephalon and mesencephalon. In amniotes, the mammillotegmental tract (MTT) projects along with TPOC. The TPOC also uses the VC as well as the POC to connect the TPOC on both sides of the brain (Anderson and Key, 1999). The supraoptic tract (SOT), DVDT and TPC (see 1.3.4) use the TPOC axon tract for guidance once their axons reach the basal plate in zebrafish (Chitnis and Kuwada, 1990; Chitnis and Kuwada, 1991) and *Xenopus* (Anderson and Key, 1999). The SOT projects axons ventrally from neurones located in the dorsal telencephalon (dorsal-rostral cluster; drc) (Ross et al., 1992) and turns caudally when its axons encounter the TPOC. The DVDT axons project ventrally, initially as a single tract from the epiphysis and turn rostrally when its axon encounters the TPOC. In addition to the caudal projection, the vrc also projects axons rostrally to form the postoptic commissure (POC). The POC connects the TPOC on both sides of the brain (Anderson and Key, 1999). In *Xenopus* and zebrafish, the POC is used by optic axons from the retinal ganglion cells to enter the brain (Easter and Taylor, 1989; Wilson et al., 1990) and project alongside the TPOC to the tectum (Burrill and Easter, 1995).

1.3.3 Formation of the descending tract of the mesencephalic nucleus of the trigeminal nerve (DTmesV)

In mouse, the DTmesV is the first tract to form at E8.5, which is different to other vertebrates studied where the DTmesV forms later. In some vertebrates the DTmesV forms only after the early axon scaffold has been set up (Kimmel et al., 1985; Kollros and Thiesse, 1985).

The DTmesV neurones appear along the dorsal midline of the mesencephalon and project axons ventrally in the alar plate before turning caudally when they reach the sulcus limitans (at the alar/basal boundary). The DTmesV axons pioneer the lateral longitudinal fascicle (LLF) that projects axons into the rhombencephalon. In the embryonic chick brain, the DTmesV axons exit the CNS at around HH20 (Chédotal et al., 1995), which is when the production of DTmesV neurones stops (Hunter et al., 2001). In the mouse, the DTmesV neurones leave the CNS at E15.5 (Mastick and Easter, 1996). The DTmesV axons exit at r2 in both chick and mouse, pass along the trigeminal (V) nerve and form connections at the mandibular arch innervating jaw-closing muscles. The DTmesV neurones act as proprioceptive receptors that convey information to help determine positions of the upper and lower jaw and to coordinate biting and mastication (Hunter et al., 2001).

The formation of the DTmesV is closely linked with the evolution of the jaws in jawed vertebrates and is not present in non-jawed vertebrates such as lamprey (Hunter et al., 2001; Kuratani et al., 1998b; Murakami and Kuratani, 2008).

1.3.4 Formation of the tract of the posterior commissure (TPC)

The TPC is a highly conserved transversal tract that marks the MFB. In anamniotes, the TPC has been described to form from two populations of neurones, one located dorsally and the other located ventrally. The neurones located dorsally project axons ventrally and when they encounter the TPOC, they turn and project caudally (Chitnis and Kuwada, 1991). The ventrally located neurones project axons dorsally towards the midline where they will cross connecting the two halves of the brain. In mouse, the TPC neurones have been shown to be located ventrally and project axons towards the dorsal midline (Mastick and Easter, 1996). The organisation of the TPC in chick is unclear however, it has been suggested that the TPC neurones are also located ventrally (Schubert and Lumsden, 2005).

1.3.5 Axon tracts as pioneers

The early axon tracts play an important role as pioneers; setting up a scaffold for follower axons and several examples highlight this function. In the zebrafish embryonic brain the TPC axons project from the dorsally located neurones, ventrally towards the TPOC. When the TPC axons encounter the TPOC they turn caudally and project with the TPOC. Ablation of the TPOC, by making a transverse cut in the diencephalon, caused the TPC axons to extend along aberrant pathways (Chitnis and Kuwada, 1991; Chitnis et al., 1992). The initial projection of the TPC axons ventrally was normal. However, as the axons reached the VLT, most turned caudally along the correct pathway, however using the MLF instead of the TPOC. The SOT was another transversal axon tract that projects ventrally from the drc in the dorsal telencephalon and turns caudally when it encounters the TPOC. When the TPOC was ablated in the *Xenopus* embryonic brain (by lesioning the nucleus of the TPOC), some of the SOT axons continued projecting ventrally instead of turning caudally, however some SOT axons did project caudally along the correct path (Anderson and Key, 1999). These results suggest that while the TPC and SOT axons use the TPOC for guidance there are also other axon guidance cues present in the brain to ensure these axons project along the correct path. *Cyclops* mutant zebrafish where embryos are missing the floor plate confirm the importance of the TPOC for guiding other axon tracts (Hatta, 1992; Patel et al., 1994). As the growth of the TPOC and MLF were affected in these mutants, the DVDT axons projecting from the epiphysis make aberrant projections once they reached the TPOC and the TPC axons projecting from the dorsal neurones made errors (Patel et al., 1994). Cell adhesion molecules (CAMs) are likely to be present on these early axons to allow the follower axons to recognise the CAM and project along the correct pioneer axons. NOC-2 is an example of a cell surface molecule present on selected early axon scaffold tracts in the embryonic *Xenopus* brain (Anderson and Key, 1999).

In order for the initial nerve connections in the embryonic brain to be set up correctly progenitor cells within the neural tube must undergo patterning by signalling molecules that will activate neuronal differentiation by neurogenesis. The specific neuronal type of the differentiating cell also has to be specified. Once the neuronal fate has been determined, the cell body starts to project its axon. The correct pathway of the axons is determined by axon guidance cues. The axons must then be able to recognise their targets and form synapses.

1.4 Molecular mechanisms of early axon scaffold formation

The formation of the early axon scaffold has to be tightly regulated to ensure correct positioning, differentiation and specification of the neurones. It is also important that the neurones differentiate at the correct time in development. Sanders et al., (2002) have shown homeobox genes lie in arcs organising the ventral mesencephalon (see 1.2.3). A homeobox is a highly conserved gene sequence that codes for the DNA-binding domain of homeodomain proteins that act as transcriptional regulators throughout development making them interesting to research (Vollmer and Clerc, 1998). These homeobox genes are expressed in the pretectum (p1) and the mesencephalon in longitudinal striped patterns (see 1.2.3), which indicates DV patterning similar to that which occurs in the spinal cord (Briscoe et al., 2000; Ericson et al., 1997).

Sax1, Six3, Emx2 and Nkx2.2 are all homeobox genes that are expressed within the midbrain arcs and in close proximity to the MLF and TPC neurones, suggesting they could be involved in MLF neuronal specification. Schubert and Lumsden, (2005) studied these homeobox genes and show expression begins appearing in the mesencephalon of the chick embryonic brain at HH15. The involvement of Emx2 and Sax1 in the formation of the MLF is discussed further in chapter 6. Pax6 expression begins early in development (Matsunaga et al., 2000) within the prosencephalon, however later in development Pax6 is also expressed as a single longitudinal

stripe in the ventral mesencephalon and forms part of the midbrain arcs (Agarwala and Ragsdale, 2002).

Transcription factors have been shown to play a role in the formation of axon tracts in other vertebrates. In the embryonic zebrafish brain, Six3 is expressed in the ventral mesencephalon from 18 hours post fertilisation (hpf) and expression overlaps with the TPOC neurones while being specifically expressed by the MLF and TPC neurones. Emx3 is required for the differentiation of drc neurones in the zebrafish dorsal telencephalon (Viktorin et al., 2009). Pax6 expression throughout the prosencephalon is highly conserved marking the MFB in cat shark (Derobert et al., 2002), zebrafish (Hjorth and Key, 2001), *Xenopus* (Bachy et al., 2002), chick (Ferran et al., 2007) and mouse (Mastick et al., 1997). Pax6 (small eye) mutants cause the p1/mes boundary to be affected (see 1.2.4) and as a result, the TPC axons fail to cross the midline (Mastick et al., 1997; Matsunaga et al., 2000). The TPOC axons in these mutants were misrouted into the alar plate and also failed to cross the p2/p3 boundary (Mastick et al., 1997).

In Cyclops zebrafish mutants, the floor plate is missing and therefore Shh signalling is missing, the ventral early axon scaffold tracts are also disrupted or missing (Patel et al., 1994). This is also true in chick, where the MLF is affected by ectopic expression of Shh (Ahsan et al., 2007). Like in the spinal cord, these experiments provide evidence that Shh is required for patterning of the ventral brain and regulating the formation of axon tracts.

1.5 Axon guidance of axon tracts

1.5.1 The growth cone

After a neurone differentiates, it sends out an axon that often projects over long distances towards its target where it will form a synapse. Axons grow along a defined route by following the path of existing axons. However, the initial axons in the rostral neural tube

project into an environment where no other axons are present. Therefore, in order for them to reach their target they require specific guidance cues. At the end of the axon is a structure called the growth cone, which was discovered by Ramon y Cajal (Cajal, 1890). The growth cone features protrusions called filopodia, which guide the direction of the axon by responding to external factors in the surrounding environment. New axonal growth occurs just behind the growth cone. New components are synthesised in the cell body, transported down the axons, and then incorporated into the membrane. Receptors present on the growth cone are responsible for binding axon guidance molecules and directing the axon in the correct direction by modulating the cytoskeleton. Raman y Cajal suggested that axonal growth was directed by chemotropic cues (Cajal, 1892) proposing that commissural axons reached the floor plate in response to a gradient of factors secreted by the floor plate (Cajal, 1899).

1.5.2 Axon guidance molecules

Axon guidance by a chemical gradient was suggested by Roger Sperry (Sperry, 1963) based on the model of axons projecting from the retinal ganglion cells to the tectum in amphibians (reviewed by Guan and Rao, 2003). Since then, axon guidance has been studied extensively and a number of axon guidance molecules have been identified. Axon guidance molecules either repel or attract the growth of an axon and can act at short-range, by contact-mediated mechanisms or by long-range secreted mechanisms. Axon guidance molecules are highly conserved ligands that bind to receptors located within the plasma membrane of the growth cone activating intracellular signalling pathways. Most axon guidance molecules work via modulating the activity of specific G proteins within the axon. RhoA is downregulated and Rac1 and Cdc42 are upregulated to promote attraction while RhoA is upregulated to promote repulsion (Guan and Rao, 2003). This leads to changes in the actin cytoskeleton, influencing the projection of the growth cone. The distance an axon may have to project can be up to

several millimetres therefore the pathway may be broken down into 'choice points' aiding the accuracy of an axon reaching its target. There are four well characterised families of axon guidance molecules, these are Ephrins, Semaphorins, Netrins and Slits (Chilton, 2006).

1.5.3 Ephrins

Eph receptors are the largest group of receptor tyrosine kinases (RTKs) and are activated by interaction with their ligands, the Ephrins. There are two classes of Ephrin ligands: EphrinAs that are attached to the cell membrane via a GPI anchor and EphrinBs that are actual transmembrane proteins. The corresponding Eph receptors are also divided into two classes: EphAs and EphBs. Ephrins and their receptors function in many processes throughout development such as gastrulation, segmentation of the somites and rhombencephalon and neural pathfinding. Since Ephrins are membrane associated, they mediate short-range, contact-dependent interactions. Ephrins can act as bidirectional axon guidance cues. They are expressed throughout the developing embryo, in the primitive streak, somites, vascular system and brain (Baker and Antin, 2003). In the embryonic chick brain, EphA4 and EphB1 are expressed in rhombomeres r3 and r5 and EphrinB1 is expressed in r4 the expression of these Eph receptors and their ligands, is essential to mediate cell contact repulsion to establish boundaries between the rhombomeres. This prevents neuronal growth cones projecting into the wrong territory (O'Leary and Wilkinson, 1999). Many of these Ephrins and Ephs are expressed throughout the prosencephalon and mesencephalon, suggesting they are involved in segmentation of the rostral brain as well as the rhombencephalon. EphrinA5 is involved in guidance of retinal axons as knockout mice have defects in the projection of these axons (Frisen et al., 1998).

1.5.4 Netrins

Netrins are bifunctional axon guidance cues, involved in the attraction and repulsion of axons. The receptors DCC (Keino-Masu et al., 1996) and neogenin (Meyerhardt et al., 1997)

mediate attractive effects of Netrin while the Unc5 receptor mediates the repellent effects of Netrin (Leonardo et al., 1997). Netrin1 and Netrin2 were first isolated from the embryonic chick brain and shown to be involved in the attraction of commissural axons in the embryonic chick spinal cord (Kennedy et al., 1994; Serafini et al., 1994). In the embryonic chick spinal cord, Netrin1 is expressed by the floor plate and Netrin2 is expressed at lower levels in the ventral two thirds of the spinal cord. In the chick embryonic brain, Netrin1 is expressed along the floor plate and dorsally into the basal plate. Netrin2 has a more complex expression pattern in the rostral mesencephalon and diencephalon (see 7.2.1). Expression of Netrin2 in these regions expands into the alar plate. The receptor neogenin is expressed throughout the embryonic brain and Unc5H4 is expressed at the ventral MFB (Riley et al., 2009). The expression of Netrin1, Netrin2 and Unc5H4 are described further in chapter 7. Neogenin interacts with another family of axon guidance cues, the repulsive guidance molecules (RGM) that was identified as a repulsive cue for mapping temporal retinal axons onto the caudal region of the chick tectum (reviewed by De Vries and Cooper, 2008; Monnier et al., 2002). RGMa interacts with neogenin to repel the SOT axons away from the dorsal telencephalon ventrally towards the TPOC while Netrin1 attracts the SOT axons also through the interaction with neogenin in the embryonic *Xenopus* brain (Wilson and Key, 2006).

1.5.5 Semaphorins

Semaphorins (Semas) are a large family of signalling proteins that can be secreted or membrane bound. These axon guidance molecules mostly mediate repulsive activity. The family is divided into 8 classes with the secreted class 3 Semas being the most studied. This class is known for its growth cone collapsing properties through a receptor complex of Neuropilin and PlexinA. Neuropilin binds to the Semaphorin ligand, while PlexinA is responsible for the signal transduction. Apart from Sema3E, the other Sema3s are expressed within the mesencephalon of the chick embryonic brain. Sema3A is expressed rostral to the

nMLF. Neuropilin1 and PlexinA4 expression has been shown to be overlapping with the MLF neurones (Riley et al., 2009). Neuropilin1 interacts with Sema3A to repel MLF axons away from the rostral diencephalon. This ensures the MLF axons project along a caudal route into the mesencephalon and rhombencephalon (Riley, 2008).

1.5.6 Slits

The Roundabout (Robo) receptor was first identified in *Drosophila* and Robo mutants cause commissural axons to cross and re-cross the midline several times (Seeger et al., 1993). Robo homologues have been identified in other vertebrates (Kidd et al., 1998). Slit ligands are expressed at the midline and interact with Robo receptors to repel axons from the midline in both *Drosophila* and vertebrates. On commissure axons Robo gets upregulated once the axons have crossed the midline to prevent them re-crossing (Kidd et al., 1998). In the chick embryonic brain, Slit1 and Slit2 are expressed along the ventral midline. The receptors, Robo1 is expressed throughout the mesencephalon and Robo2 is initially expressed caudally between the MLF and LLF and overlapping the oculomotor nucleus (Riley et al., 2009). Robo1 is expressed by the MLF neurones in mouse and prevents the MLF axons crossing the midline by Slit repulsion (Farmer et al., 2008). Robo2 is expressed by the LLF axons. In Robo1/Robo2 double knockouts, longitudinal axons are able to enter the floor plate, suggesting a complete loss of midline signalling. Rig1 is another vertebrate Slit receptor, in knockout mice the commissural axons fail to cross in the midline (Sabatier et al., 2004).

1.5.7 Draxin

Draxin was recently identified as a repulsive axon guidance molecule expressed in the chick spinal cord and brain (Islam et al., 2009). In knockout mice, spinal cord and forebrain commissural axons are misrouted. Draxin is expressed along the dorsal midline in the chick embryonic brain and has a role in guiding DTmesV axon by repelling then away from the midline (Naser et al., 2009).

Axon guidance molecule	Receptor	Function
Slit1-3	Robo/Rig	Repulsion
Netrin	DCC/neogenin	Attraction
Netrin	Unc5/DCC	Repulsion
RGM	Neogenin	Repulsion
Sema3A-3F	Plexin/neuropilin	Repulsion
Ephrins	Ephs	Bidirectional
Draxin		Repulsion

Table 1.1 Summary of axon guidance molecules and the receptors they bind

As well as the axon guidance molecules described above, morphogens such as Wnts, BMPs and Shh have been suggested to play a role in axon guidance. Shh is involved in the patterning of the ventral spinal cord, but also plays a role in attracting commissural axons to the ventral midline. BMPs are expressed by the roof plate and as well as patterning the dorsal spinal cord, they play a role in repelling the commissural axons away from the dorsal midline (Augsburger et al., 1999).

1.6 Aims for the project

As the early axon scaffold sets up the first neuronal pathways in the brain, understanding the anatomy and development of these tracts is essential for investigating the formation of the more complex connections that form. While the initial axon tracts have been studied extensively in many anamniotes and amniotes, chick is a major developmental model organism, yet the development of the early axon scaffold has been poorly characterised. A direct comparison of the early axon scaffold is also missing between the major model organisms.

The aim of this thesis was to map the early axon scaffold in various vertebrates including cat shark (*Scyliorhinus canicula*), *Xenopus laevis,* chick (*Gallus Gallus*), zebra finch (*Taeniopygia guttata*) and mouse (*Mus musculus)* investigating the formation of the axon tracts and their development through evolution (Chapter 5). An antibody was identified in chapter 4 that labels all the axons and neurones in the embryonic vertebrate brain and can be used as a comparative antibody. As chick is used as a model organism, particularly for in vivo experiments, therefore the anatomy of the early axon scaffold needed to be characterised in more detail (Chapter 3). The comparative analysis in chapter 5 will highlight the most conserved tracts as a first step to understanding the molecular mechanisms involved in the formation of the basic vertebrate axon scaffold. The formation of the MLF is already known to be highly conserved as well as being the first axon tract to arise in the embryonic vertebrate brain makes it a particularly interesting tract to investigate. Homeobox genes have been suggested to have a role in MLF formation however; Emx2 and Sax1 already identified to have a role in MLF formation but are not involved in specifying the MLF neurones due to

their late expression. In the chick embryonic brain, genes need to be identified that are involved in specifying neuronal cells into an MLF fate (Chapter 6). As the early axon scaffold is set up from pioneering axons, guidance of these tracts is essential to ensure correct formation. The TPC is a highly conserved transversal tract in which the position of the neurones needs to be confirmed in the chick embryonic brain. This will then allow candidate axon guidance molecules to be investigated in chapter 7 and their role will be identified using overexpression experiments.

Chapter 2

Materials and methods

2.1 Stock solutions

Distilled water (dH$_2$O, Fisher)

PBS (Phosphate Buffered Solution)
      1 part 10X PBS (Fisher)
      9 parts dH$_2$O

PBT
      1X PBS
      0.1% Tween-20 (Sigma)

2M Tris-HCl
      121g Tris Base (Sigma) per 500ml dH$_2$O
      5M HCl was added for the required pH.

10X MBS (Modified Bart's Saline)
      88mM NaCl
      1mM KCl
      2.4mM HaHCO$_3$
      0.82mM MgSO$_4$.7H$_2$O
      0.33mM Ca (NO3)$_2$.2H$_2$O
      10mM HERPES
      For pH7.6, solution was adjusted with 5M NaOH

0.5M EDTA
      37.2g EDTA per 200ml dH$_2$O
      For pH8.0, solution was adjusted with 5M NaOH (Autoclaved)

0.5M EGTA
      38.04g EGTA per 200ml dH$_2$O
      For pH8.0, solution was adjusted with 5M NaOH (Autoclaved)

0.5M MOPS (4-morpholinopropanesulfonic acid)
      41.85g MOPS (Sigma)
      6.8g sodium acetate
      For pH7.0, solution was adjusted with 5M NaOH (Autoclaved)

1M MgCl$_2$
	40.7g MgCl$_2$ per 200ml dH$_2$O (Autoclaved)

0.5 M MgSO$_4$
	24.7g MgSO$_4$ per 200ml dH$_2$O (Autoclaved)

5M NaCl
	58.4g NaCl in 200ml dH$_2$O (Autoclaved)

20X SSC (Saline Sodium Citrate)
	3M NaCl
	0.3M Sodium Citrate
	For pH4.5 solution was adjusted with citric acid

2.1.1 Fixatives

4% PFA/PBS
	8g of Paraformaldehyde (Fisher) per 200ml H$_2$O, dissolved with a drop of 5M NaOH
	and heated to 60°C. Once dissolved, 1 PBS tablet (Sigma) was added per 200ml.

MEMFA
	0.1M MOPS
	3.7% formaldehyde (Sigma)
	2mM EGTA
	1mM MgSO$_4$

Dent's
	80% MeOH (methanol)
	20% DMSO (Dimethyl Sulfoxide, Sigma)

Mirsky's
	1ml 10X Buffer (National Diagnostics)
	1ml 10X Concentrate (National Diagnostics)
	8ml dH$_2$0

2.1.2 Immunohistochemistry solutions

Triton X-100
	5ml Triton X-100 (Sigma)
	45ml 1X PBS

DAB stain
	10mg DAB tablet (Fluka/Fisher) dissolved in 15ml 0.1M Tris-HCl pH7.5 and filtered
	(0.22μm)

2.1.3 In situ hybridisation solutions

10% BBR (Blocking reagent)
      10g BBR (Roche) per 100ml 5X MAB

Detergent Mix
      1% Igepal
      1% SDS
      0.5% Deoxycholate
      50mM Tris-HCl pH8.0
      1mM EDTA
      150mM NaCl (Fisher)

5X MAB
      0.5M Maleic acid
      0.75M NaCl
      For pH7.5 solution was adjusted with 5M NaCl

1X MABT
      1X MAB
      0.1% Tween-20

NTMT
      100mM NaCl
      100mM Tris-HCl pH9.5
      50mM $MgCl_2$
      1% Tween-20
      2mM Levamisole (Fisher)

Pre-hybridisation mix
      50% Formamide
      5X SSC pH4.5
      2% BBR
      250µg/ml yeast RNA
      100µg/ml Heparin

Solution X
      50% Formamide
      2X SSC pH4.5
      1% SDS

2.1.4 Electrophoresis solutions

50X TAE running buffer
        242g Tris Base
        57.1ml glacial acetic acid (Fisher)
        100ml 0.5M EDTA pH8
        Made up to 1l with dH$_2$O

10 mg/ml ethidium bromide (EtBr) (Sigma)

2.1.5 Microbiological solutions

Carbenicillin
        1g Ampicillin (Fisher) was added per 10ml dH$_2$O

Kanamycin
        1g Kanamycin (Gibco) was added per 10ml dH$_2$O

LB agar
        20g LB agar mix (Fisher) per 1l H$_2$O and autoclaved
        Once cooled add 100µl Kanamycin or Carbenicillin
        Pour into petri dishes and leave to set

LB broth
        4g LB broth mix (Fisher) per 100ml H$_2$O and autoclaved

2.2 Harvesting embryos

2.2.1 Chick embryos

Fertilised chicken eggs were obtained from Henry Stewart and Co (Peterborough, UK). They were incubated at 38°C for the required stage (4.5 hours per stage). The embryo was harvested by cracking the egg open into a glass dish. The embryo was removed from the yolk by cutting into the yolk sac and putting the embryo into PBS using a spatula to wash. The vitelline membrane was removed and for embryos HH14 onwards the amnion was removed over the top of the rostral region. Embryos were then put into fixative.

For embryos HH9 to around HH14, the embryos were staged by counting somites. For older stages embryos staged according to the morphological features described by Hamburger and Hamilton (Hamburger and Hamilton, 1951).

2.2.2 Zebra finch embryos

Zebra finch embryos were removed from the egg in the same way as the chick embryos (2.2.1). As the developmental stages of zebra finch have not yet been described, the zebra finch embryo was compared to the chick embryo at equivalent stages of development.

2.2.3 Xenopus embryos

2% Cysteine
 4g cysteine (Sigma)
 150ml 0.1M MBS
 10 pellets of concentrated NaOH.
 5M NaOH was added to bring the solution to pH8.0

Bleaching solution
 5% Formamide (Fluka)
 0.5X SSC pH4.5
 10% H_2O_2 (VWR International)

Xenopus laevis embryos were de-jellied in 2% cysteine to separate the embryos, and were carefully washed in 1X MBS to remove the cysteine. The embryos were then transferred into 0.1X MBS and no more than 50 embryos were placed into a large Petri dish. The embryos were incubated at 14°C, 18°C or 23°C for the required stage. Once the embryos reached the required stage they were then into fixative. If the embryos were not hatched (younger than stage 25), the membrane was removed before fixing. Embryos were staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1994).

2.2.4 Cat shark embryos

The cat shark eggs were incubated in seawater at around 17°C for the required stage. As the eggs were transparent, depending on the stage, the embryo could be seen moving on the top of the yolk. A window was cut into the egg to expose the embryo. For younger stages, the embryos were cut out of the yolk. For older stages, embryos were only attached to the yolk at one point close to the heart. The embryos were removed using a spatula and washed in PBS

before fixing with 4% PFA/PBS or MEMFA. Staging was done according to Ballard (Ballard et al., 1993).

2.2.5 Mouse embryos

Timed mating of outbred mice was set up. Pregnant mice were killed, by asphyxiation and cervical dislocation according to regulations issued by the Home Office of the United Kingdom under the Animals (Scientific Procedures) Act, 1986. The uterus was removed into ice cold PBS and embryos were dissected out of the decidua and fixed in ice cold 4% PFA/PBS or MEMFA.

2.2.6 Fixing embryos

Embryos fixed in 4% PFA/PBS or Mirsky's were left overnight and then stored long term in PBS. Embryos were fixed in MEMFA for 30-40 minutes, followed by two 30 minute methanol washes and then stored long term in methanol. Embryos fixed in Dent's were left overnight then stored long term in methanol. All embryos were stored at 4°C.

2.3 Immunohistochemistry

2.3.1 Preparation of embryos

Embryos fixed in 4% PFA/PBS and Mirsky's were already stored in PBS so were ready to use. As embryos fixed in MEMFA and Dent's were stored in methanol, they needed to be rehydrated from 75% MeOH to 25% MeOH and then placed in PBS. Cat shark, zebra finch, chick and mouse embryos were prepared by opening up the hindbrain roof plate and making a cut between the telencephalon vesicles, to improve penetration of the antibody into the tissue. *Xenopus* embryos were prepared by cutting the embryo in half and removing the skin from the rostral end of the embryo. The yolk, somites and notochord were all removed to expose the neural tube.

2.3.2 Addition of primary antibody

Embryos were placed into individual wells in a 24-well dish, where all washes took place and the dish was left in the cold room at 4°C on a shaking platform for all washes. The pigmentation of the *Xenopus* embryos was removed in an additional bleaching step. Bleaching solution was added to the embryos and placed on a light box for approximately 20 minutes until the embryos were white. The bleaching solution was removed by washing three times with PBS. Three 1 hour washes with PBS removed any excess fixative or bleaching solution, followed by PBS/5% serum/1% Triton X-100/0.1% H_2O_2 that was left overnight at 4°C. Goat serum (Sigma) was used to block non-specific protein binding. Triton X-100 was used as it is a non-ionic detergent and allows cell permeabilisation and PBS (Phosphate Buffered Saline) was used as it ensures the pH of the solutions remain constant.

The H_2O_2 solution was washed off with three 1 hour washes of PBS/5% serum/1% Triton X-100. The primary antibody was then added at the relevant concentration (table 2.1) to the following solution: PBS/10% serum/1% Triton X-100/0.02% Na-azide. This solution was left on the embryos for 3-4 nights at 4°C to allow the antibody to diffuse throughout the embryo and bind to its appropriate antigen. The addition of Na-azide allows the primary antibody solution to be preserved and used again.

2.3.3 Addition of secondary antibody

Any unbound primary antibody was washed off with three 1 hour washes of PBS/1% serum/1% Triton X-100, followed by the addition of the secondary antibody at the relevant concentration (table 2.2) with the following solution: PBS/5% serum/1% Triton X-100. The secondary antibody solution was left on overnight at 4°C. If a fluorescent antibody was used the embryos were kept in the dark. Any unbound secondary antibody was then washed off with three 1 hour washes of PBS/1% serum/1% Triton X-100.

2.3.4 Fluorescent labelling

For fluorescently labelled antibodies, the embryos were washed with PBS for 30 minutes followed by fixing with 4% PFA/PBS for 1 hour. If embryos were originally fixed with MEMFA, 4% PFA/PBS was left on overnight to harden the embryos further for easier preparation. The embryos were then put into long term storage with 80% glycerol.

2.3.5 DAB peroxidise labelling

Embryos were washed twice with 100mM Tris-HCl pH7.5 for 30 minutes and incubated with inactive DAB solution (filtered) for 3 hours. Active DAB stain (3µl H_2O_2 per 1ml DAB) was added to the embryos for up to 35 minutes (no longer than 1 hour) until the axon tracts were clearly labelled. To stop the reaction the embryos were rinsed with dH_2O three times, followed by three 30 minute PBS washes. The embryos were fixed with 4% PFA/PBS overnight and then put into 80% glycerol for long term storage.

2.3.6 Double-labelling immunohistochemistry

The basic protocol (2.3.2 and 2.3.3) was followed but two different primary antibodies that were raised in different species (rabbit and mouse) were used. Both the primary and secondary antibodies were added to the solutions at the same time. The secondary antibodies used were different colours so they excite at different wavelengths.

Primary antibody	Raised in	Concentration used for chick, mouse and cat shark	Concentration used for *Xenopus*	Supplier	Antigen
RMO-270	Mouse	1:2000	1:2000	Zymed	Neurofilament-M (neuronal intermediate filament protein)
Zn-12	Mouse	1:100	1:100	DSHB	Neuronal call surface marker
Tuj1	Mouse	1:1000	1:1000	Abcam	Neurone-specific βIII tubulin
Tuj1	Mouse	1:1000		R&D systems	Neurone-specific βIII tubulin
Tuj1	Rabbit	1:1000		Abcam	Neurone-specific βIII tubulin
SV2	Mouse	1:100	1:100	DSHB	Synaptic vesicles (SV2)
HNK-1	Mouse (IgM)	1:100	1:100	Sigma	Associated surface glycoprotein CD57/HNK-1
6-11B-1	Mouse	1:20	1:100	Sigma	Acetylated tubulin
HuC/D	Mouse	1:500	1:500	Molecular Probes	Neuronal proteins HuC and HuD
CYN-1	Mouse	1:100		DSHB	Neurones, cytoplasmic
4H6	Mouse	1:100		DSDB	Neurofilament
40E-C	Mouse	1:100		DSDB	Radial cells (Vimentin)
GAD-6	Mouse	1:100		DSDB	Glutamic acid decarboxylase
αTH	Mouse	1:100		DSDB	Tyrosine hydroxylase
GABA	Rabbit	1:2000		Abcam	γ-amino butyric acid (GABA)
BEN	Mouse	1:100		DSDB	Neuronal, motor marker. Cell surface glycoprotein
23.4.5	Mouse	1:100		DSDB	TAG-1 neuronal marker
Pax6	Mouse	1:100		DSDB	Transcription factor Pax6
Pax7	Mouse	1:100		DSDB	Transcription factor Pax7
GFP	Rabbit	1:500		Invitrogen	Green Fluorescent Protein
Unc5d	Mouse	1:500		Abcam	Unc5d Receptor (Unc5H4)

Table 2.1 Concentration of primary antibodies used for immunohistochemistry and a description of what antigen they label

DSHB, Developmental Studies Hybridoma Bank

Secondary antibody	Concentration	Company
Alexa 488 goat anti-mouse IgG	1:100-1:500	Invitrogen
Alexa 543 goat anti-mouse IgG	1:100-1:500	Invitrogen
Alexa 488 goat anti-rabbit IgG	1:100	Invitrogen
Cy2 goat anti-mouse IgM	1:100	Jackson ImmunoResearch
Peroxidase-conjugated goat anti-mouse IgM + IgG	1:100	Jackson ImmunoResearch

Table 2.2 Concentration of secondary antibodies used for immunohistochemistry

2.4 Lipophilic tracing

Carbocyanine dyes: DiI (1'-dioctadecyl-3, 3, 3', 3'-tetramethylindocarbocyanine perchlorate, Molecular Probes, D282) and DiO (3, 3'-dioctadecyloxacarbocyanine perchlorate, Molecular Probes, D275) were used to trace specific axon tracts. These dyes were highly lipophilic and move through the lipid membranes of axons by lateral diffusion. These dyes have the advantage of being non-toxic and can be used on fixed tissue. As DiI (549) and DiO (484) have different wavelengths they can be used to trace different tracts within the same embryo. The dyes were dissolved in 100% ethanol and injected into the chick embryonic brain using fine glass needles (World Precision Instruments). For fixed embryos, the diffusion of the dye worked only in embryos that have previously been fixed with 4% PFA/PBS not with MEMFA. Once injected the embryos were left in PBS at room temperature in the dark, for 3-4 days, for older embryos (E4 and above) this was increased to 1 week. The embryos were put into 80% glycerol for long term storage.

2.4.1 Lipophilic tracing with immunohistochemistry

DiI labelling can be combined with immunohistochemistry. Embryos were first injected with DiI and left for 3-4 days to allow the dye to diffuse through the axon tracts. The immunohistochemistry protocol (2.3.2-2.3.4) was followed however, 100μg/ml digitonin (Fisher) was used instead of Triton X-100. Digitonin is a cholesterol specific detergent that does not solubilise all lipid in the cell membranes like Triton X-100 and leads to more efficient penetration of the antibody (Matsubayashi et al., 2008).

2.4.2 Photo-conversion of DiI

Chick embryos that had previously been injected with DiI were pre-soaked in 0.05M Tris-HCl pH8.5 for one hour, then incubated in the DAB solution (one DAB tablet was dissolved in 10ml 0.05M Tris-HCl pH8.5) on ice for 30 minutes. The embryo was placed onto a welled microscope slide and covered with a glass cover slip. The slide was placed under a microscope (Nikon Eclipse E800) using the 20X objective and GFP (R-LP) wavelength to allow photo-oxidation of DAB by the fluorescence emitted from the excited DiI. The embryos were left at room temperature in PBS overnight, put into 80% glycerol for long-term storage and flat mounted.

2.4.3 Whole-mount preparation of embryos

All embryos were re-fixed with 4% PFA/PBS then put into 80% glycerol for long term storage. For cat shark, chick, and mouse the neural tube was prepared by removing the eyes and mesenchyme. Much of the spinal cord, notochord and heart were removed. The brain was opened by cutting in half along the dorsal and ventral midlines and placed onto a microscope slide. The brain tissue was covered with a glass cover slip with silicone feet and sealed using nail varnish. As the *Xenopus* embryo neural tubes were already prepared, they were flat-mounted straight onto a microscope slide.

2.5 Microscopy

A Zeiss Stereo Lumar V12 fluorescent stereomicroscope was used to obtain low magnification images of the embryos. For more detailed images, Zeiss LSM 510 and LSM 710 confocal microscopes were used.

All embryos with blue substrate (*in situ* hybridisation) or brown substrate (DAB immunohistochemistry) were visualised using the compound microscope (Nikon Eclipse E800).

2.5.1 Image processing

Images were processed using ImageJ and Photoshop. ImageJ was used to combine z-stack images taken by the confocal microscope into single images.

2.6 *In situ* hybridisation

2.6.1 Template synthesis

The template for the genes required for analysis by *in situ* hybridisation was amplified using PCR (see 2.7.3).

The following reagents were mixed in a 0.2ml PCR tube: 21.5µl dH$_2$O, 25µl 2X BioMix Red (Bioline), 2µl DMSO, 0.5µl M13 (100µm) F-primer, 0.5µl M13 (100µm) R-primer, 0.5µl Plasmid.

The programme used was: 1 minute at 95°C, 5X (95°C for 15 seconds, 65°C for 15 seconds, 72°C for 2 minutes), 25X (95°C for 15 seconds, 50°C for 15 seconds, 72°C for 4 minutes) and a final step 72°C for 8 minutes.

2.6.2 RNA probe synthesis

A reaction mixture containing 37.5µl dH$_2$O, 5µl 10X transcription buffer (Roche), 2µl RNA labelling mix (DIG or Fluorescein, Roche), 0.5µl RNase inhibitor (Roche), 3µl PCR template (see 2.6.1) and 2µl RNA polymerase (T3, T7 or SP6, depending on the template, Roche) was put into a 1.5ml reaction tube. The reaction was incubated for 2 hours at 37°C and 2µl of the reaction was run on a gel to check the probe has been synthesised correctly. 2µl RNase-free DNaseI (Roche) was added to the reaction and incubated for 15 minutes at 37°C. This was then purified with post reaction clean up columns (Sigma) and the probe was stored at -20°C.

2.6.3 Preparation of embryos

Cat shark and chick embryos fixed in 4% PFA/PBS were prepared by cutting between the telencephalic vesicles and opening the hindbrain roof. The embryos were dehydrated then stored in methanol at -20°C for about 1 week. This allows the membranes to become permeable and enhance staining.

2.6.4 Hybridisation of the probe

Embryos were placed into individual wells in a 24-well dish, rehydrated and bleached with 6% H$_2$O$_2$/MeOH, followed by two 5 minute PBT washes, to remove any remaining methanol. The embryos were washed three times in detergent mix for 20 minutes, washed in MEMFA for 20 minutes and again followed by PBT washes. The embryos were then put into 0.1M triethanolamine with 5µl acetic anhydride per 10ml for 20 minutes followed by PBT washes. The pre-hybridisation mix was pre-warmed to 65°C and pre-incubated with the embryos for 1 hour. The hybridisation mix, which contains 5µl of probe per 1ml pre-hybridisation mix was added to the embryos and incubated overnight at 65°C.

2.6.5 Addition of AP-conjugated antibody

Solution X was pre-warmed to 65°C and used to wash the embryos four times for 30 minutes each removing the hybridisation mix and any unbound probe. MABT was then used to remove the Solution X with two 30 minute washes at room temperature. MABT/2%BBR/20%serum bleaching solution was added to the embryos for 1 hour. AP-conjugated anti-digoxigenin antibody (1:2000, Roche) was added to the MABT/2%BBR/20%serum solution and incubated with the embryos for 3 nights at 4°C. The antibody was then washed off with seven 1 hour MABT washes and then one overnight wash at 4°C.

2.6.6 Staining of the AP- conjugated antibody

To stain the embryos they were first equilibrated with NTMT (pH9.5) for 10 minutes. The substrate solution was made with 10-20µl of NBT/BCIP (Roche) per 10ml NTMT. The substrate solution was removed from the embryos and washed with NTMT for 5 minutes and replaced with fresh substrate solution every 1-3 hours. The substrate solution was left on the embryos for hours or days to allow staining. Once staining was finished, the embryos were washed in NTMT and then PBT for 10 minutes each. The embryos were finally fixed in 4% PFA/PBS overnight and then put either into 80% glycerol for long-term storage or PBS for double-labelling with immunohistochemistry.

2.6.7 In situ hybridisation followed by immunohistochemistry

The same *in situ* hybridisation protocol (2.6.3-2.6.6) was followed, however after fixing with 4% PFA/PBS at the end of the protocol, this was washed off with three 1 hour PBS washes and three 1 hour washes with PBS/5%serum/1%Triton X-100. The primary antibody was then added and the remainder of the immunohistochemistry protocol was followed (2.3.3 and 2.3.5).

Gene	Source
cWnt2b	S. Dietrich
cSatb1	This study
cCx40	Zheng-Zheng Bao
cCRABPI	Richard Wingate
cASCL1/Mash1	Salvador Martinez
cTac1	Qinfu Ma
cZic1	Luis Puelles/Jose Ferran
cEphA7	A. Hunter
cWnt5a	Anthony Graham/Loretta Tumiotto
cMab21L2	This Study
cTFAP2α	Roseline Godbout
cNeuroD	N. Adams
cNrg1	Jo Begbie
cHes5	Domingos Henrique
cDlx5	A. Streit/R. Koxher
cFli1	Marianne Bronner
cFGF3	Gary Schoenwolf/Christian Paxton
cJag2	S. Lowell
cFGF18	Gary Schoenwolf/Christian Paxton
cPRTG	Yuji Watanabe
cTcf4	Luis Puelles/Jose Ferran
cCldn5	This Study
cSRGAP1	This Study
cPlxDC2	This Study
cGbx2	Frank Schubert
cCHRDL1	J. Collignon
cSlit2	A. Chedotal via R. Wingate
cSRGAP3	This Study
cEmx2	E. Bell/A. Lumsden
cNkx2.2	Frank Schubert
cSax1	Abraham Fainsod/Yossi Gruenbaum
cSix3	Frank Schubert
cNetrin 1	M. Tessier-Lavigne Cell 78, 409
cNetrin 2	M. Tessier-Lavigne Cell 78, 409
Cat shark Netrin1	Frank Schubert
cUnc5H4	chEST741P10

Table 2.3 *In situ* hybridisation probes used

The probes made in this study were amplified from chick cDNA by PCR (see 2.7.3).

Gene	Forward	Reverse	Forward (gateway)	Reverse (gateway)
cCLDN5	CGGGTTTCCGAAGAGCAG	AGTCTCAAAGGCGCACAGAT	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGGCTTCGGCGGCGGTG	GGGGACCACTTTGTACAAGAAAGCTGGGTG'GACGTAGTTCTTCTTGTC
cPLXDC2	TTGTTTTCCCCAGCAGTGAT	TGAGCCCAATTTCATTGTGA	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGGCGAGGCTGCGGAGA	GGGGACCACTTTGTACAAGAAAGCTGGGTG'GCATTGCTCTGATACAAT
cMAB21L2	GCATTGGATCCCTCAACGTA	CTGAAAGAGGTCGAGGTTGG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGATCGCCGCCCAGGCC	GGGGACCACTTTGTACAAGAAAGCTGGGTG'CCACGTCGCGGTAGCTGC
cSatb1	TCTTTAGAGGCGGGACTGAG	TTTGACAGAACGCAAGATGG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGGATCATTTGAACGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTG'GTCTTTCAATTCAGCATT
cSRGAP1	AGAAGAGGGAGAAGCGGAAG	GTGGCGAGAGGAGTTTCTTG	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGTCAACCCCGAGCAGA	GGGGACCACTTTGTACAAGAAAGCTGGGTG'CATTGTGCAAGATTTGTC
cSRGAP3	CCCGCGCCCTCTCGAA	TCAGCAATCCACATGAACAGA	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGTCCTCGCAGGGCAAG	GGGGACCACTTTGTACAAGAAAGCTGGGTG'CCGGCCCATGACTCCGCC
cNetrin1	TTCTCCGCGGGGTTTGGCCG	CACCCGGCAGCGCTCAGTCC	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGCCGCGGAGGGGCGCG	GGGGACCACTTTGTACAAGAAAGCTGGGTG'CGCCTTCCTACACTTCCC
cNetrin2	CGGTGCGGCAACGCGTGAAG	GTGTGCAGTGGGGCCGGGAC	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGGAGGCCCCTCAGCTC	GGGGACCACTTTGTACAAGAAAGCTGGGTG'GGGCTTCACACACTTCCC
xNetrin2	AAGTCCTTTCCCCAAACCAT	ACAGGGTTCCCAATGTCTTT	GGGGACAAGTTTGTACAAAAAAGCAGGCTCC'ACCATGTTTTACCTTCGGGAG	GGGGACCACTTTGTACAAGAAAGCTGGGTG'GGGTTTGAGACATTTGCC

Table 2.4 Primers used for cloning (MWG Biotech)

Flanking primers designed using Primer3

2.7 Molecular cloning

2.7.1 Electrophoresis

A 1% agarose gel was made by heating 0.5g agarose (Bioline) with 50ml 2X TAE buffer until dissolved. When the solution had cooled 0.5µl ethidium bromide was added and the gel poured. Gels were run at 40V for approximately 30 minutes to separate the DNA. As a size marker, HyperLadder 1 (Bioline) was used.

2.7.2 Production of chick cDNA

To make cDNA from animal tissue, RNA was first extracted from an E5 chick brain, cat shark head, mouse embryo and *Xenopus* embryo by homogenising the tissue first using a pipette then, a fine needle. This was followed by the RNeasy mini protocol (Qiagen). cDNA was then made from the RNA using SuperScript III First-strand synthesis kit (Invitrogen).

2.7.3 Polymerase Chain Reaction (PCR)

PCR utilised thermostable DNA polymerases to produce rapid, multiple copies of a DNA molecule. It involves cycles of denaturing the double stranded DNA to break the H-bond at 90-95°C, annealed primers to the target sequence at 40-65°C and elongation of the primers at 72°C.

For PCR (first reaction) with flanking primers the following reagents were mixed in a 0.2ml PCR reaction tube: 21µl dH$_2$O, 25µl 2X Pwo polymerase mix (Roche), 1µl (50mM) MgCl$_2$ (Bioline), 1µl (100µm) F-primer, 1µl (100µm) R-primer, 1µl cDNA.

The programme used was: 2 minutes at 95°C, 30X (95°C for 30 seconds, 58°C for 30 seconds, 72°C for 5 minutes), and a final step 72°C for 10 minutes.

For PCR (second reaction) with gateway nested primers the following reagents were mixed in a 0.2ml PCR reaction tube: 21µl dH$_2$O, 25µl PWO (Roche), 1µl (50mM) MgCl$_2$ (Bioline), 1µl (100µm) F-primer, 1µl (100µm) R-primer, 1µl 1st PCR reaction.

The programme used was: 1 minute at 95°C, 5X (95°C for 30 seconds, 40°C for 30 seconds, 72°C for 5 minutes), 25X (95°C for 30 seconds, 58°C for 30 seconds, 72°C for 5 minutes) and a final step 72°C for 15 minutes.

For some primers the annealing temperature was changed for optimum binding of the primers (52°C-62°C).

2µl of the PCR reaction was run on a 1% agarose gel to check the gene for the correct size.

2.7.4 Construction of entry vector

The expression constructs were made using gateway cloning technology, which uses recombinases that were more efficient than using restriction enzymes as it was easier to clone multiple fragments.

All of the second PCR reaction was run on a gel and the DNA was removed from the gel using UV light to visualise the bands and a clean, sharp scalpel to remove the bands. The DNA was isolated from the gel using QIAquick Gel Extraction (Qiagen). 2µl of the purified DNA was run on a gel to check the DNA was correctly removed from the gel.

The entry vector was then made using Gateway BP clonase II enzyme mix (Invitrogen). The following reagents were mixed together in a 1.5ml reaction tube: 3µl purified DNA, 0.5µl donor vector p221 (Invitrogen) and 1µl BP clonase II.

The reaction was vortexed and centrifuged briefly then incubated for 1 hour at 25°C. 0.5μl Proteinase K was added to terminate the reaction and vortexed briefly. The reaction was incubated for 10 minutes at 37°C.

2.7.5 Construction of expression vector

The expression vector was made using Gateway LR clonase II enzyme mix (Invitrogen). The following reagents are mixed together in a 1.5ml reaction tube: 1μl purified middle entry vector, 1μl 5' entry chick β-actin, 1μl 3' entry IRES-GFP PolyA, 1μl pDEST Tol2TR and 2μl LR clonase II.

The reaction was vortexed, centrifuged briefly and incubated for 1 hour at 25°C. 0.5μl Proteinase K was added and vortexed briefly. The reaction was incubated for 10 minutes at 37°C.

2.7.6 Transformation

The BP/LR reaction was added to ~70μl of competent cells (Silver efficiency, Bioline) and incubated on ice for 15 minutes. The cells were heat shocked at 42°C for 30 seconds to allow the vector to enter the *E.Coli* cells then placed back onto ice. 200μl LB medium was added and incubated for 1 hour at 37°C with shaking. All of the reaction was spread evenly onto kanamycin (BP reaction) or carbenicillin (LR reaction) LB agar petri dishes and incubated overnight at 37°C.

2.7.7 Plasmid purification

To set up liquid cultures individual colonies were taken from each plate and added to universal tubes containing 2ml of LB with kanamycin or carbenicillin. The tubes were left overnight at 37°C with shaking. Approximately 1.5ml of liquid culture was put into a 1.5ml reaction tube and centrifuged for 30 seconds to pellet the bacterial cells. The supernatant was

discarded, removing as much liquid as possible. The plasmid was purified using Nucleospin plasmid QuickPure protocol (Macherey-Nagel).

To replicate larger quantities of the plasmids, liquid cultures were set up in conical flask containing 50ml LB medium, 50µl AMP and an individual colony from the plate. This was incubated at 37°C overnight with shaking. For large quantities of plasmid, these were purified using HiPure Plasmid Midi prep kit (Invitrogen).
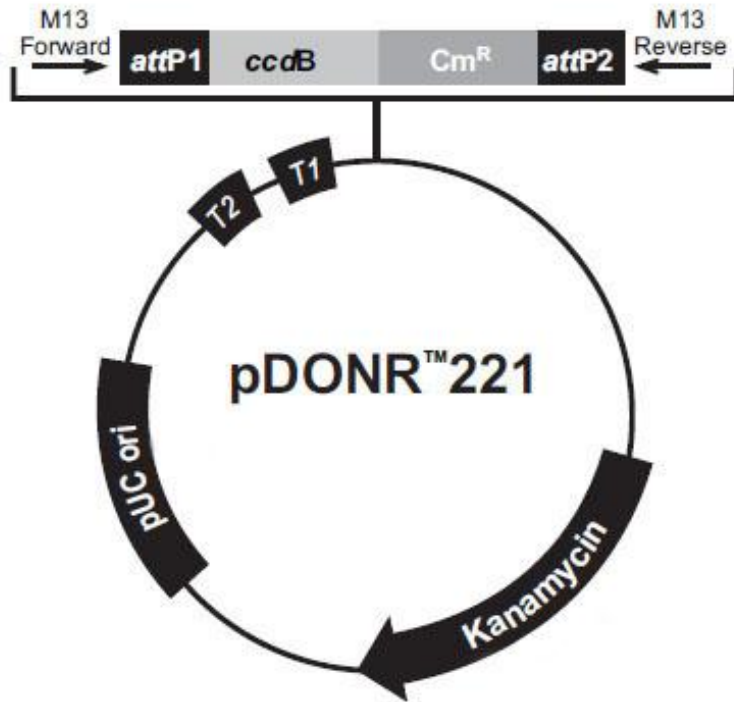
Figure 2.1 Vector map for pDONR 221
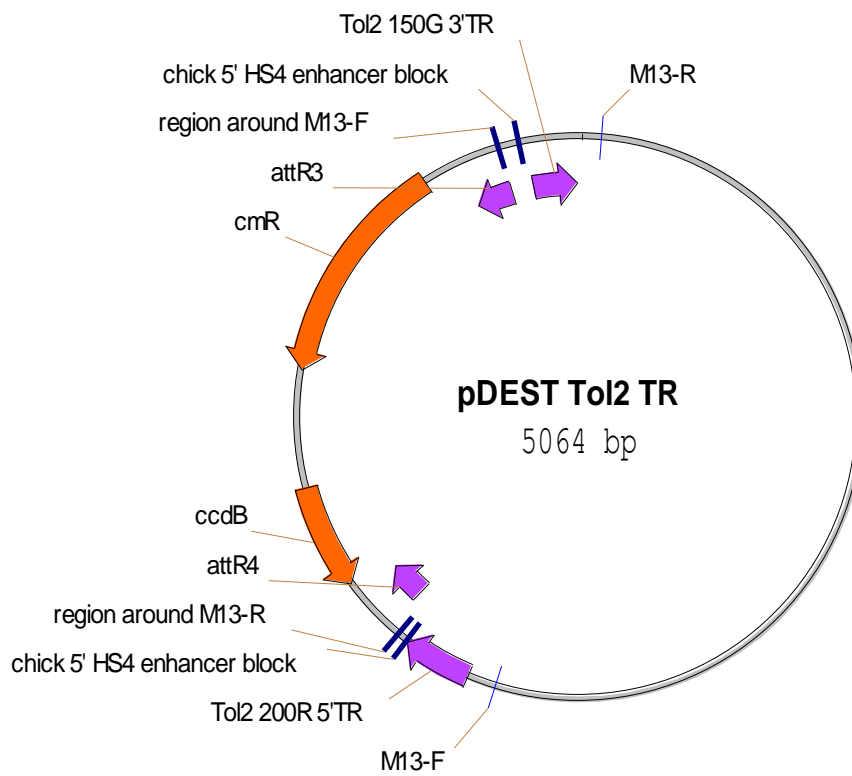
Figure 2.2 Map of destination vector pDEST Tol2 TR
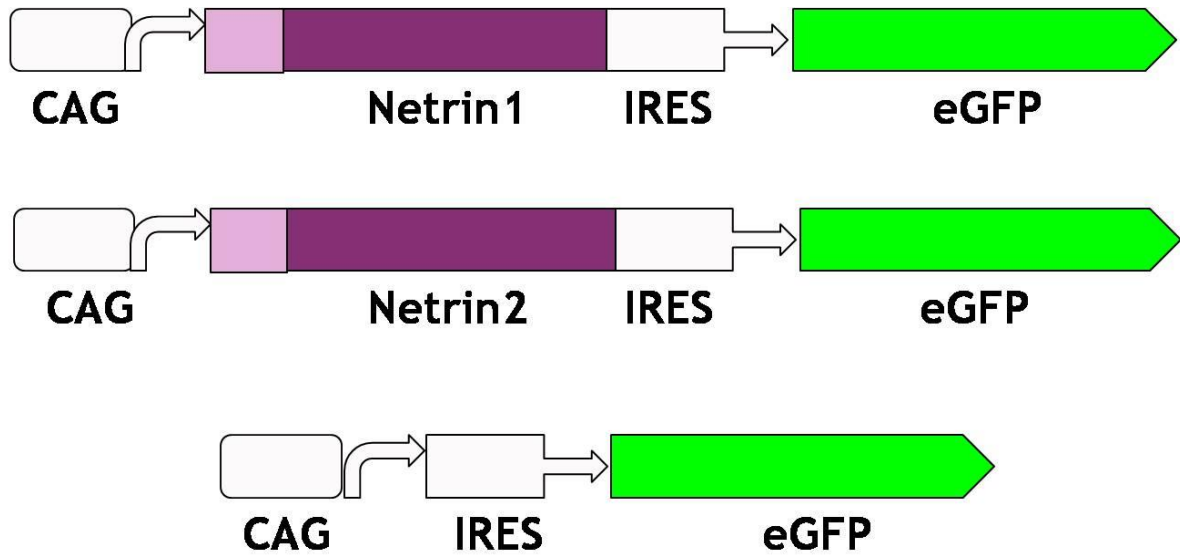
Figure 2.3 Expression constructs of Netrin1, Netrin2 and GFP control used for electroporation

The expression constructs contain a chick β actin promoter (CAG) in front of the gene, an internal ribosomal entry site (IRES) and enhanced green fluorescent protein (eGFP). The GFP will allow the construct to be visualised in the cell.

2.7.8 Restriction digest

To determine the correct gene had been inserted into the vector, a restriction digest reaction was set up to cut the plasmid into linear DNA of known sizes. Restriction enzymes recognise and bind to a particular DNA sequence of 4-6 base pairs cutting the DNA.

The following reagents were mixed in a 1.5ml reaction tube: 3µl purified plasmid, 0.5µl restriction enzyme (Roche), 1µl Buffer A, B or M (Roche) and 5.5µl dH$_2$O (if using more than one enzyme the volume of H$_2$O used was reduced accordingly).

The reaction tube was incubated for 1 hour at 37°C. 2µl of loading dye was added to each reaction and approximately 6.5µl of the reaction was run on a 1% agarose gel.

2.8 In ovo Electroporation

2.8.1 Preparation of embryos

Fertilised chicken eggs were incubated until HH10/HH11. The eggs were sprayed with ethanol to prevent contamination and sellotape was placed over the top of eggs. Using a sterile needle (Fisher), a hole was placed at one end of the egg and 2-2.5ml of albumin was removed. Another hole was placed, slightly off centre at the top of the egg, through the sellotape. A window was cut into the shell using curved scissors to expose the live embryo. 2-3 drops of PBS + AMP were added to prevent the embryos drying out.

2.8.2 Electroporation

1µl of fast green (Sigma) was added to 5µl of expression construct in a 1.5ml reaction tube and a small quantity was transferred to a fine glass needle and placed into a micromanipulator.

The vitelline membrane was removed from above the embryo with fine forceps. The injection needle was placed inside the neural tube and the construct was injected into the embryo. The platinum cathode (set up on micromanipulator) was placed to the left of the neural tube and the tungsten cathode (hand-held) was placed on the opposite side of the neural tube. 3 pulses of 15V with 20ms width and 50ms space was applied and the electrodes were then removed. A further 1-2 drops of PBS were applied to the embryo; the lid of the egg was replaced and sealed with sellotape. The embryos were then reincubated until the required stage.

2.9 Microarrays

2.9.1 Preparation of embryos

Chick embryos were incubated for the desired stage and harvested (as described 2.2.1) into PBS. The embryo was pinned into a petri dish made with sylguard (Dow corning) for stability. The mesenchyme was removed from around the outside of the rostral neural tube. The dorsal region around the MFB was removed exposing the ventral region. The rostral and caudal regions of the neural tube were removed from around the MFB (Fig 6.3). The tissue sample was pipetted (with no more than 1µl PBS) and placed into 100µl lysis buffer (Stratagene) containing 0.7µl β-Mercaptoethanol. The samples were then vortexed to break up the tissue and stored at -20°C. Three samples at each stage were then sent away for microarray analysis (done in collaboration with Dr David Chambers, King's College London).

2.10 Morpholino Oligonucleotides

A morpholino oligonucleotide (MO) was designed by Gene tools targeting the 5' region of the *Xenopus Laevis* Netrin2 gene.

The sequence used was: GACTCATCTCCCGTAGGTAAACCAT with a Fluorescein tag attached to the 3' end of the MO. To knockdown Netrin2, the MO was injected into the *Xenopus* at the 1-cell stage (by Jordan Price, University of Portsmouth).

Chapter 3

Development of the early axon scaffold in the chick embryonic brain

3.1 Introduction

The early axon scaffold, basic array is set up from longitudinal tracts, transversal tracts and commissures and has been studied in a number of anamniotes and amniotes (see chapter 1.3). Next to the mouse, the chick is the other main amniote model for developmental biology. Yet, in contrast to the mouse, the early axon tracts in the chick brain have not been analysed in the same detail. The first descriptions of axon tracts in the embryonic chick brain date back to the beginning of the 20th century (Mesdag, 1909). Later studies used silver staining (Lyser, 1966; Tello, 1923; Windle and Austin, 1936) and immunohistochemistry (Chédotal et al., 1995) to analyse the tracts further. Different from the mouse, the first neurones differentiating in the embryonic chick brain were located at the MFB and extend axons to form the MLF (Chédotal et al., 1995; Lyser, 1966; Windle and Austin, 1936). The MLF is a highly conserved tract that in all vertebrates analysed, apart from mouse was the first axon tract formed during embryogenesis (reviewed in Ahsan et al., 2007 and see chapter 5), possibly linked with its function in controlling motor behaviour of free-swimming zebrafish larval stages (Gahtan et al., 2002). Subsequent tracts like the DTmesV, the TPOC and the TPC were described to form over the course of the following two days of incubation. While previous

studies agree on the MLF being the first tract in the embryonic chick brain, the identity and timing of subsequent tracts like the DTmesV, TPOC and TPC has been less well characterised.

A detailed description of early axon scaffold anatomy in the embryonic chick brain is lacking, which has made it difficult to analyse gene expression patterns and interpret functional data. Using immunohistochemistry and lipophilic dye tracing a detailed description of the early axon scaffold formation and characterisation of the axon tracts was completed. The analysis has revealed the precise location of the neurones and axons in relation to the prosomeric model (Puelles and Rubenstein, 2003 and see 1.2.5) as they form within the embryonic chick brain.

3.2 Early axon scaffold formation in the chick embryo
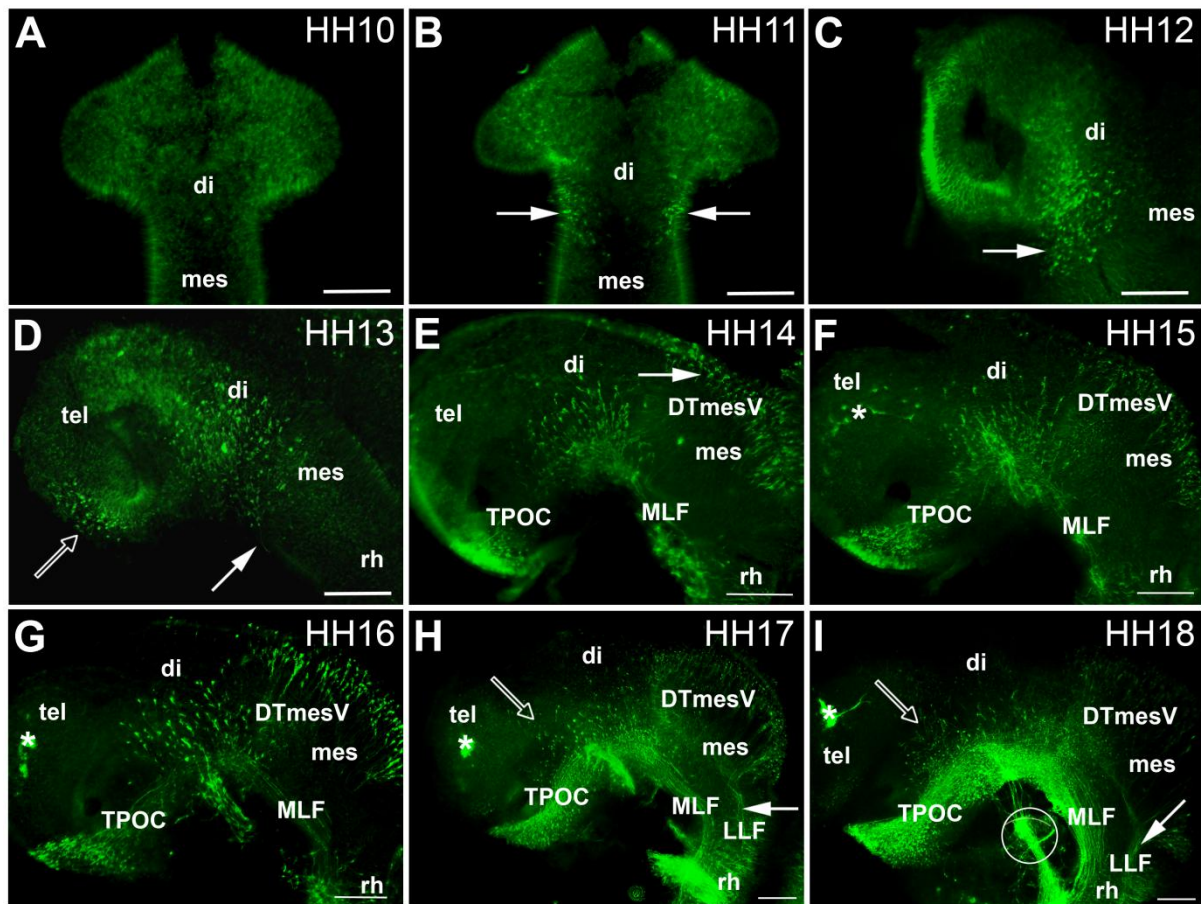
Figure 3.1 Time series of the early axon scaffold in the chick embryonic brain using Tuj1 antibody

A and B are dorsal views of the brain and C-I are lateral views of the brain. A) HH10. No staining of neurones in the mesencephalon. B) HH11. Neurones are located rostral to the MFB, within the diencephalon (arrows). C) HH12. The number of MLF neurones has increased (arrow). D) HH13. Neurones appear in the hypothalamus that will give rise to the TPOC (unfilled arrow). MLF neurones have started to project axons caudally (arrow). E) HH14. The MLF axon tract is visible close to the ventral floor plate and the TPOC neurones have started to project their axons. The DTmesV neurones are located along midline of the dorsal mesencephalon. F) HH15. Many of the axon tracts are well established and the three MLF neurone populations are well distinguished. G) HH16. The number of neurones and axons are increasing. H) HH17. There are neurones located in the alar diencephalon projecting axons ventrally towards the TPOC (unfilled arrow). The LLF is pioneered from the DTmesV axons (arrow). I) HH18. More alar neurones are present in the diencephalon (unfilled arrow). The LLF is pioneered from the DTmesV (arrow). F-I asterisks show the olfactory placodes.

tel, telencephalon; di, diencephalon; mes, mesencephalon; rh, rhombencephalon

To determine when and where the first neurones were differentiating and projecting their initial axons, whole brains of chick embryos between HH10 and HH18 have been analysed by immunofluorescence, using βIII-tubulin as a pan-neural marker (Tuj1 antibody). Labelling of neurones was not detected in the brain at HH10 (Fig 3.1A). The first neurones were labelled in the p1 region of the diencephalon at HH11 (Fig 3.1B and Fig 3.7A) and there was an increase in the number of neurones in the diencephalon at HH12 (Fig 3.1C). The position of these neurones ventrally and close to the MFB identifies them as the MLF neurones. During HH13, there is an increase in the number of neurones in the diencephalon and some of these neurones have started to project axons (Fig 3.1D and Fig 3.7B). These axons are projecting caudal to begin forming the MLF tract (Fig 3.1D, filled arrow). Also at this stage, the first TPOC neurones appear in the rostral basal hypothalamus at HH13 (Fig 3.1D, unfilled arrow) and begin projecting axons caudally towards the MFB (Fig 3.1E). By late HH13 there were neurones labelled along the dorsal midline of the mesencephalon that are likely to be DTmesV neurones (not shown), these neurones start projecting axons ventrally into the mesencephalon during HH14 (Fig 3.1E and Fig 3.7C). The number of MLF, TPOC and DTmesV neurones continues to increase during the following stages. By HH15 (Fig 3.1F), the TPOC axons have projected caudally towards the MFB, however they have not quite reached. The DTmesV neurones located along the dorsal midline of the mesencephalon have projected slightly further into the mesencephalon. The olfactory placode neurones were first labelled at HH15 in the telencephalon (Fig 3.1F, asterisk).

By HH16, the MLF axon tract was well established as a tightly fasciculated tract projecting caudally close to the floor plate and has projected well into the rhombencephalon (Fig 3.1G and Fig 3.7D). The TPOC axons have reached the MLF within the caudal diencephalon and the VLT becomes well established. The DTmesV axons in the mesencephalon have started to turn caudally once they reach the sulcus limitans, remaining in the alar plate. Interestingly, at

this stage there were alar p3 neurones visible that were clearly separate from the MLF neurones. At HH17, these alar p3 neurones were projecting a short distance ventrally towards the TPOC axon tract (Fig 3.1H, unfilled arrow). The DTmesV axons have turned caudally projecting towards the MHB to pioneer the LLF (Fig 3.1H, filled arrow). The ventral longitudinal tract and the DTmesV were the most prominent tracts at HH18 (Fig 3.1I and Fig 3.7E).

3.2.1 The formation of the MLF axon tract

Even though the MLF has been studied in depth in previous studies and recognised as the first tract to form in the chick embryonic brain (Chédotal et al., 1995; Lyser, 1966; Windle and Austin, 1936), there is some discrepancy about when the first neurones appear. The organisation of the neurones contributing to the MLF was also much more complex than previously thought.
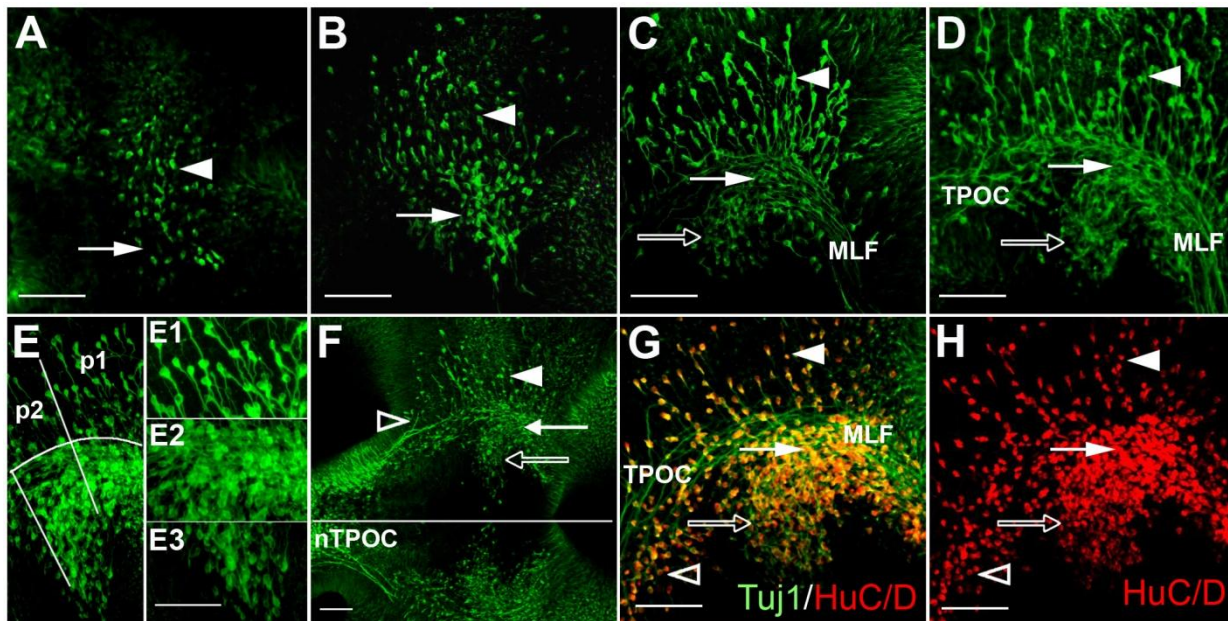
Figure 3.2 Detailed formation of the MLF axon tract in the chick embryonic brain
Lateral view of the whole mount embryo around the MFB

A-D) Time series of the MLF axon tract formation. A) HH11. Neurones are located in the alar (arrowhead) and basal (arrow) plates of the caudal diencephalon. B) HH13. The number of MLF neurones in the alar (arrowhead) and basal (arrow) plates of the diencephalon has increased and some neurones have begun projecting axons. C) HH14. The MLF neurones are becoming organised into three populations, dorsal (arrowhead), central (arrow) and ventral (unfilled arrow). The MLF axon tract is becoming established and projecting axons towards the MHB. D) HH15. The MLF neurones are clearly organised into the dorsal (arrowhead), central (arrow) and ventral (unfilled arrow) populations. E) High magnification images of the three MLF neurone populations in a HH15 chick embryo. E1, E2 and E3 are a small section of each neurone population, highlighting the neurones only. E1) Disperse dorsal population of neurones. E2) Dense central population of neurones. E3) Dense ventral population of neurones.

F) Overview of the longitudinal tract in a HH15 chick embryo, formed from the MLF and TPOC on both sides of the brain, the floor plate is indicated by line. In the hypothalamus, the TPOC neurones differentiate and project axons caudally towards the MFB (unfilled arrowhead). In the caudal diencephalon, the MLF was formed from three separate populations of neurones, the dorsal population (arrowhead), the central population (arrow) and the ventral population (unfilled arrow). The nMLF neurones project their axons caudally towards the rhombencephalon.

G and H) Description of the MTT in HH16 chick embryo. Double-labelling of the early axon scaffold with Tuj1 (green) and HuC/D (red). MTT neurones located rostral to the MLF neurones project their axons caudally with the TPOC axon tract (arrowhead unfilled). In the caudal diencephalon, the MLF was formed from three separate populations of neurones, the dorsal population (arrowhead), the central population (arrow) and the ventral population (unfilled arrow).

p1, prosomere 1; p2, prosomere 2

Scale bars, 100µm, except E scale bar, 50µm
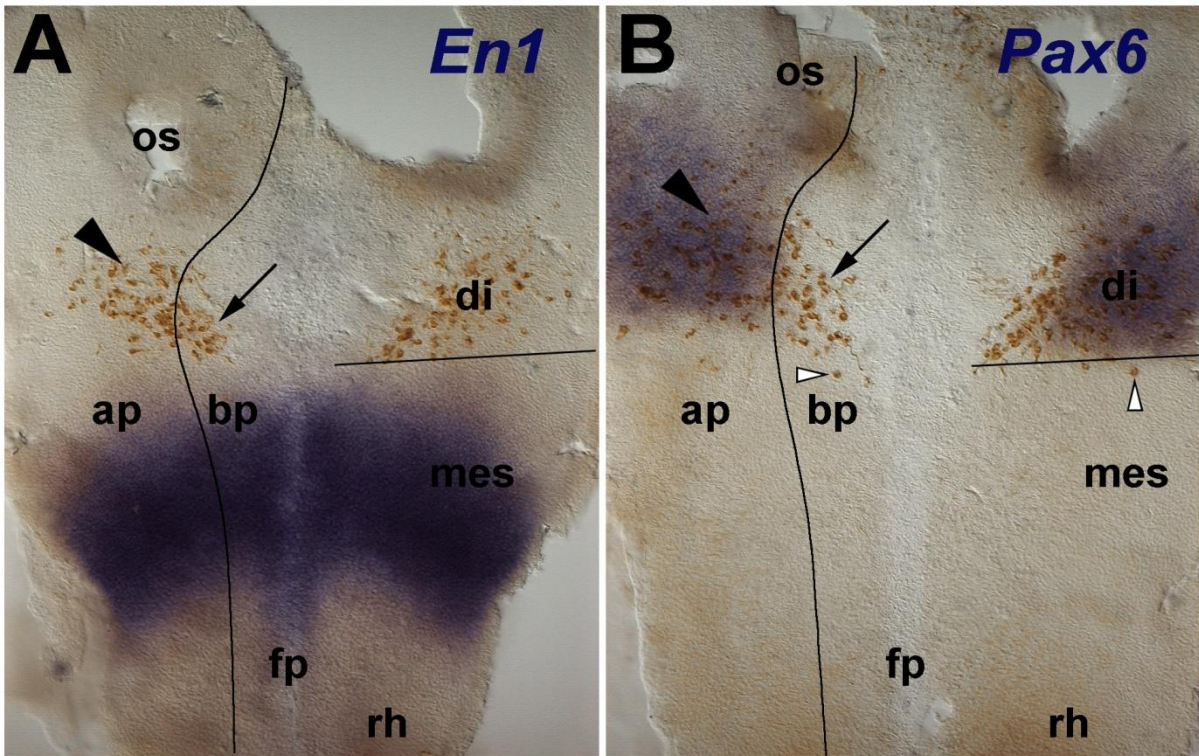
Figure 3.3 MLF neurones are located within the caudal diencephalon
Ventral view of the whole mount embryo

A) HH12. En1 expression within the mesencephalon, marking the caudal edge of the MFB. MLF neurones are located within the diencephalon and are present in both the alar (arrowhead) and basal (arrow) plates. B) HH12. Pax6 expression within the alar plate of the diencephalon marking the rostral edge of the MFB. Pax6 marks the MLF neurones located in the alar plate (arrowhead) and MLF neurones were located in the basal plate (arrow). There were some scattered neurones that differentiate in the mesencephalon (white arrowheads).

ap, alar plate; bp, basal plate; di, diencephalon; fp, floor plate; rh, rhombencephalon; os, optic stalk

The first MLF neurones appear at HH11 in the chick embryonic brain (Fig 3.1B and Fig 3.2A). These neurones were located within the alar and basal plate of the p1 domain in the caudal diencephalon (Fig 3.3A, B). While these first neurones appeared scattered, by HH13 the MLF neurones were starting to become organised into three separate populations and neurones were now located in p2 (Fig 3.2C). These neuronal populations are defined by their position and later the growth of the axons. The dorsal and central population of neurones were visible at HH13 (Fig 3.2C) while the more densely arranged ventral population has yet to form a tight bundle which is seen at HH14 (Fig 3.2C, unfilled arrow). The MLF neuronal populations were well defined by HH15 (Fig 3.2D). The population of neurones dorsal to the MLF axon tract, were dispersed predominately in the alar plate of p1 and some neurones in p2 and first project their axons ventrally, before turning to project caudally into the MLF axon tract (Fig 3.2E, E1). The centrally located population of neurones were present in the basal plate of p1 and p2 and project their axons directly caudal into the MLF axon tract (Fig 3.2E, E2). The population of neurones ventral to the MLF were located predominantly in the basal plate of p2 and first project their axons dorsally, before turning to project caudally in the MLF axon tract (Fig 3.2E, E3). The MLF axons form a tightly fasciculated tract projecting caudally in the basal plate, close to the floor plate into the rhombencephalon.

The MLF is a highly conserved tract and along with the TPOC pioneers the major longitudinal tract that projects along the ventral midline in the basal plate (Fig 3.2F).

3.2.2 The formation of the TPOC axon tract

The TPOC is a major contributor to the VLT, however it has been poorly characterised in the embryonic chick brain. The first TPOC neurones were present in the basal rostral hypothalamus at HH13 (Fig 3.4A). The TPOC axons began projecting caudally at HH14 and by HH15 had projected through the prosencephalon (Fig 3.4B). Interestingly there were neurones visible at HH15, which were projecting their axons rostrally using the TPOC axon

tract towards the nucleus of the TPOC (nTPOC) (Fig 3.4B). By HH17, the nTPOC consists of a very dense population of neurones, but retrograde labelling of the TPOC from the MFB revealed individual TPOC neurones (Fig 3.4C). When the caudal TPOC axons were labelled with DiI at HH18 (Fig 3.4D) there were two contralateral neurones that were labelled which appear to project axons across the midline and follow the TPOC axon tract. There was no indication that an established commissure had formed, however it may appear at a later stage. Labelling with the Tuj1 pan-neural antibody clearly shows the formation of the nTPOC and the initial projection of axons however, it was not clear where the TPOC axons were projecting once they reached the MFB and it was difficult to determine whether they project continuously into the MLF axon tract. As no specific antibody has been found for the TPOC, DiI and DiO were used to specifically label the TPOC from the hypothalamus and the MLF from the caudal mesencephalon. By HH18, when the TPOC axons have reached further caudal, the TPOC axons clearly project dorsally to the MLF axon tract at the MFB, with very little intermingling of axons (Fig 3.4E, F). The TPOC axons are reaching caudally into the mesencephalon and these axons are likely to reach the MHB. When the TPOC growth cones are labelled caudally in the mesencephalon, the DiI diffuses through the axons, showing these axons have come from neurones located in the rostral hypothalamus (Fig 3.4F).

3.2.3 Formation of the MTT

While the main contributors to the VLT were the MLF and TPOC (Fig 3.2F), another population of neurones arose rostral to p3 at HH15 (Fig 3.4B). These neurones were located rostrally and well separated from the ventral population of MLF neurones in the ventral diencephalon (Fig 3.2G, H). The location of these neurones identifies them as the mammillotegmental tract (MTT). The MTT is likely to be a follower tract, which follows the path of the VLT.
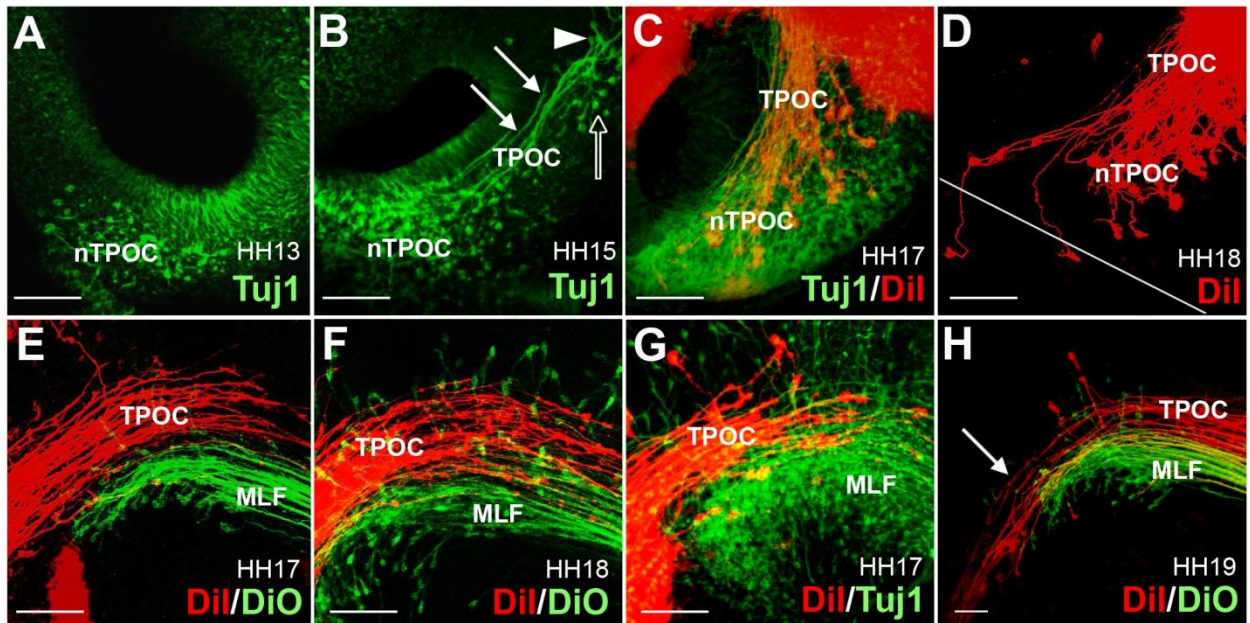
Figure 3.4 TPOC neurones are located within the basal hypothalamus and project axons caudally to form the VLT

Lateral views of the whole mount embryos

A) HH13. The first TPOC neurones differentiate in the rostral hypothalamus of the basal plate. B) HH15. The TPOC neurones have started to project caudally towards the MFB (arrows). There are also neurones located rostral to the nMLF, which are projecting axons back towards the nTPOC (arrowhead). MTT neurones were located rostral to p3 (unfilled arrow). C) HH17. DiI labelling of the TPOC (red) with Tuj1 antibody labelling (green). The nTPOC is now a very dense population of neurones. D) HH18. DiI labelling of caudal TPOC axons. Line indicates anterior midline. Two contralateral neurones appear to be projecting across the midline. E-F) Anterograde labelling of the TPOC from the hypothalamus with DiI (red) and retrograde labelling of the MLF with DiO (green). E) HH17. The TPOC axons are projecting caudally from the rostral hypothalamus and it is clear that when they reach the MLF in the caudal diencephalon they project dorsally, separate from the MLF axon tract. F) HH18. Little intermingling between the MLF and TPOC axon tracts. G) HH17. DiI labelling of the TPOC (red) with Tuj1 antibody labelling (green) of the early axon scaffold tracts. TPOC axons projecting dorsally to the MLF axon tract. H) HH19. DiI labelled TPOC (red) and DiO labelled MLF (green). Both injection sites were done caudally close to the MHB, DiO was injected slightly more ventrally to label the MLF. DiI labelling of the TPOC continues in the rostral hypothalamus (arrow) whereas the DiO labelling of the MLF stops once it reaches the MLF neurones.

Scale bars, 100µm

3.2.4 Formation of the TPC

While the longitudinal tracts are prominent features of the chick embryonic brain, the transversal TPC was not clearly visible in the overview images (Fig 3.1I) due to the density of neurones and axons at this stage, however higher magnification images reveals the TPC axons start projecting around HH18 from the ventral population (Fig 7.8C). While the TPC clearly forms within the diencephalon marking the rostral edge of the MFB forming the PC across the dorsal midline, it was unclear where the TPC neurones were located. As no specific antibody was available to label the TPC, lipophilic dyes DiI and DiO were used to specifically label the TPC and MLF respectively. When DiI was applied in the alar plate along the MFB, the TPC axons were traced back to neurones located ventrally within p1, intermingled with the dorsal and central populations of MLF neurones (Fig 3.5A and Fig 3.5B). The TPC neurones were located just caudal to the ventral population of MLF neurones (Fig 3.5A, arrow). As the TPC neurones were located within the MLF axon tract it was difficult to determine when the neurones first differentiate (Fig 3.2H). When the TPC neurones were labelled with DiI in the basal plate, the TPC axons were clearly visible projecting dorsally away from the ventral midline (Fig 3.5C, arrowheads to show the growth cones). While the ventral TPC neurones were visible with lipophilic dyes, immunofluorescence with Tuj1 revealed there were neurones that were likely to contribute to the TPC located at the dorsal midline at HH16 (Fig 3.5E). By HH21 the PC was formed and many axons were crossing the midline at the MFB (Fig 3.5F).

3.2.5 Axons crossing the ventral midline

In anamniotes, such as zebrafish and *Xenopus,* axons cross the ventral midline at the MFB forming the VC (e.g. Anderson and Key, 1996; Chitnis and Kuwada, 1990). In the chick embryonic brain by HH17, the first axons appeared to be projecting towards the ventral midline at the MFB (Fig 3.5D) and were fully crossed by HH21 (Fig 3.5H, filled arrow).

Some of these axons projecting caudal to the nMLF were likely to be the tecto-bulbar axons (Fig 3.5H, unfilled arrow).

3.2.6 Formation of the DTmesV tract

Unlike in most anamniote brains, the DTmesV is a prominent structure in the amniote brain during the formation of the early axon scaffold (Easter et al., 1993). In the chick embryonic brain, the first DTmesV neurones appear towards the end of HH13. These neurones differentiate in the alar plate along the dorsal midline of the mesencephalon and begin projecting axons ventrally at HH14 (Fig 3.6A). The axons continue projecting ventrally until HH16, where they start turning caudally at the sulcus limitans (Fig 3.6C). The DTmesV axons that have turned, pioneer the LLF and project caudally into the rhombencephalon. At later stages, the DTmesV axons were joined by tecto-bulbar axons in the alar plate that project ventrally from the alar plate of the mesencephalon towards the MLF axon tract (Fig 3.6E, arrow) and project across the ventral midline.

Since it has been reported in the mouse embryonic brain that the TPOC axons joined the DTmesV in the mesencephalon (Mastick and Easter, 1996), the spatial relation between the DTmesV and TPOC was investigated. In some HH16 and HH17 embryos there were stray axons projecting out of the longitudinal bundle towards the DTmesV (Fig 3.6D, arrow), but by HH18 the two tracts were well separated.
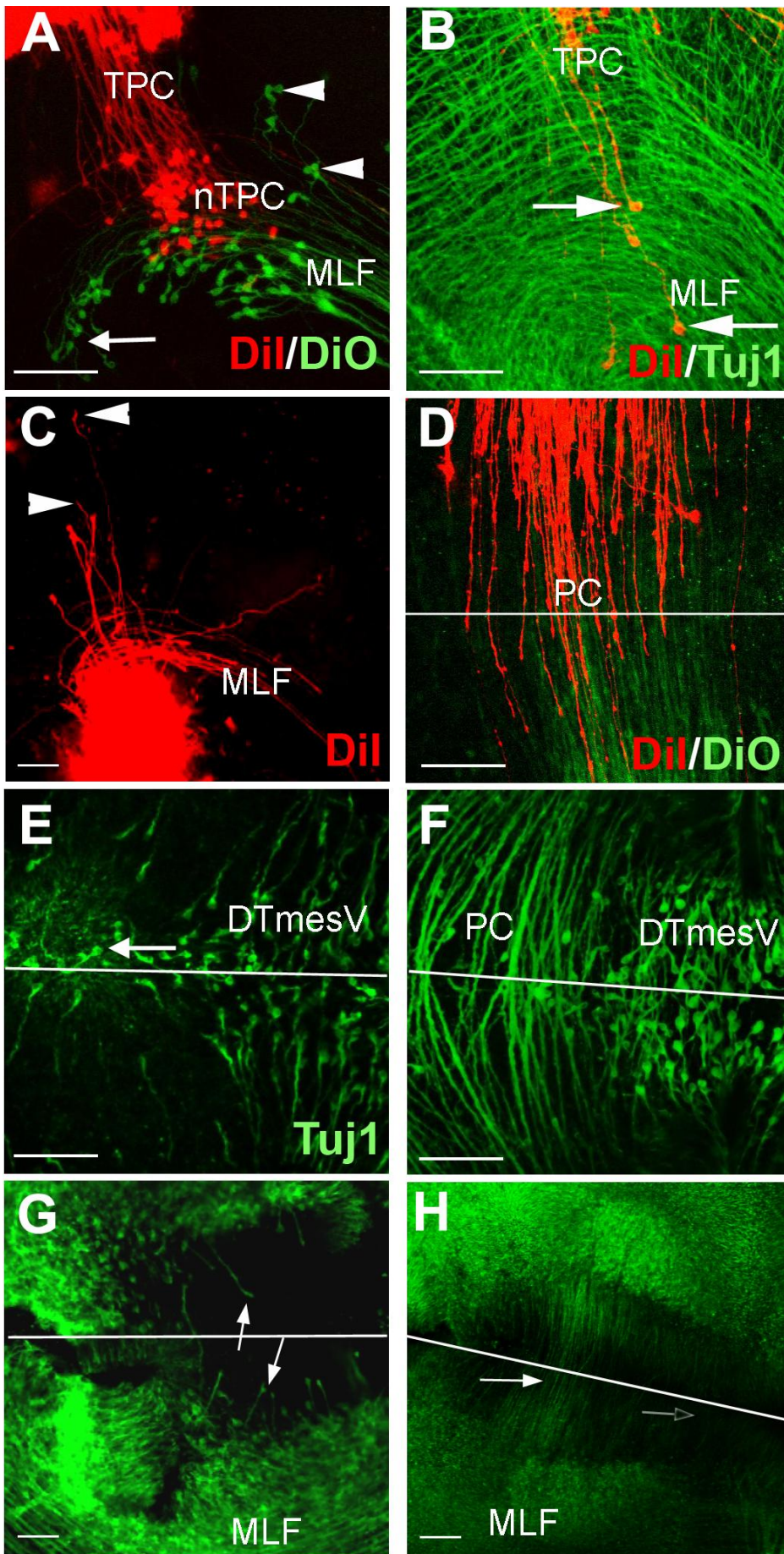
Figure 3.5 The tract of the Posterior Commissure (TPC) neurones are located both dorsally and ventrally along the MFB and the Ventral Commissure (VC) forms along the ventral midline close to the MFB
A-C) lateral views of whole mount brain. D-F) dorsal views around the MFB of whole mount brain. G-H) ventral views around the MFB of whole mount brain.

A) HH19. The TPC axons are labelled with DiI (red) and the MLF axons are labelled with DiO (green). The TPC neurones are located ventrally within the MLF axon tract and project their axons dorsally towards the dorsal midline to form the PC. The central population of MLF neurones were labelled as well as the ventral population of MLF neurones (arrow). Some mesencephalic neurones were labelled projecting axons ventrally into the MLF axon tract (arrowheads). B) HH18. The TPC is labelled with DiI (red) and the axon tracts have been labelled with Tuj1 antibody (green). The TPC neurones that are located within the MLF axon tract (arrows) project axons dorsally. C) HH18. The TPC neurones have been labelled with DiI (red) at the basal MFB. Growth cones can clearly be seen projecting axons dorsally towards the dorsal midline (arrowheads). Some MLF axons have also been labelled as the TPC neurones and MLF neurones are in very close association with each other. D) HH22. The TPC neurones were labelled at the basal MFB with DiI and DiO to show the PC axons reach the midline (indicated with line) and cross the ventral midline to the other side of the brain. E) HH16, one or two dorsally located TPC axons have crossed the dorsal midline. F) HH21, the PC is well formed and many axons have crossed the dorsal midline. G) HH17, VC axons have just started to project across the ventral midline (arrows). H) HH21 VC axons have crossed the ventral midline (filled arrow) and tecto-bulbar axons from the alar mesencephalon have crossed the ventral midline (unfilled arrow).

Scale bars, 100μm
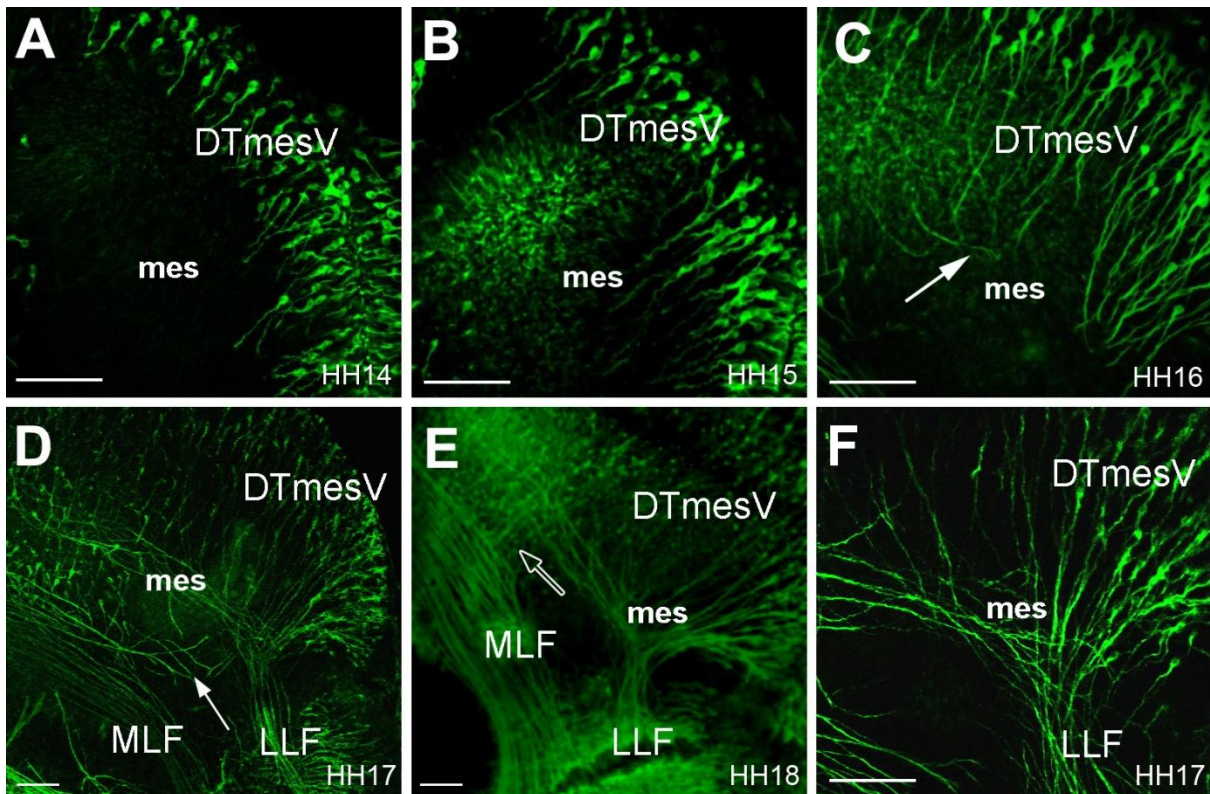
Figure 3.6 Formation of the DTmesV axon tract in the dorsal mesencephalon
Lateral views of the whole mount embryonic mesencephalon

A) HH14. DTmesV neurones have differentiated along the dorsal midline of the mesencephalon and have begun projecting their axons ventrally. B) HH15. DTmesV neurones are continuing to project axons ventrally. C) HH16. DTmesV axons have started turning caudally at the sulcus limitans (arrow). D) HH17. The DTmesV has pioneered the LLF that is projecting axons into the hindbrain towards the trigeminal nerve. Some MLF or TPOC axons are projecting off route towards the DTmesV (arrow). E) HH18. There are no axons projecting from the MLF towards the DTmesV. There are also tecto-bulbar axons present in the mesencephalon (unfilled arrow). F) HH17. The LLF is pioneered from the DTmesV at the MHB.

mes, mesencephalon

Scale bars, 100μm

3.3 Discussion

The early axon scaffold in the chick embryonic brain is set up from neurones located in specific regions of the brain that project axons into an unknown environment to act as a scaffold to allow follower axons to reach their correct target. A detailed time series has been described to show the formation of the early axon scaffold in the embryonic chick brain over the first 4 days of incubation (Fig 3.7).

3.3.1 The formation of the ventral longitudinal tract (VLT)

The most prominent tract in the embryonic chick brain is the VLT that will connect the prosencephalon to the spinal cord. The first neurones were detected in the alar and basal plates of p1 in the diencephalon at HH11 (Fig 3.7A), which was earlier than previously described (Chédotal et al., 1995; Lyser, 1966; Windle and Austin, 1936). These neurones gave rise to the MLF that became organised into three separate populations that all contribute axons to the MLF tract. These populations were located dorsally in the alar plate of p1 with some neurones in p2, centrally in the basal plate of p1 with some neurones in p2 and ventrally in the basal plate of p2 with some neurones in p1 (Fig 3.7C, D, E). A similar distinction has already been noted with neurones in p1 associated with the area fasciculi longitudinalis medialis (aflm), the basal p2 with the area tuberculi posterioris (atp) and in the p1 alar plate with the area commissuralis (acom) (Puelles et al., 1987). The central population of MLF neurones has also been described as the interstitial nucleus of Cajal (INC) (Chédotal et al., 1995; Molle et al., 2004).

The TPOC neurones first appeared at HH13 located ventrally in the rostral basal hypothalamus, consistent with the previous description of neurones located in the area

retrochiasmatica (Puelles et al., 1987). The TPOC axons project caudally reaching the MLF in p1 at HH16; however what was not clear was whether the TPOC axons intermingle with the MLF axons to form the VLT once they reach the MFB. There has been some debate in the literature whether the MLF and TPOC form the VLT as a continuous tract, which seems to be the case in zebrafish and *Xenopus* embryonic brains (Key and Anderson, 1999; Wilson et al., 1990). The TPOC has also been described in the mouse embryonic brain as an alar tract continuous with the DTmesV (Easter et al., 1993). Axon tracing with lipophilic dyes revealed in the chick embryonic brain, the TPOC axons project adjacent but dorsal to the MLF axon tract that projects as a tight bundle along the ventral midline. The TPOC axons mark the alar/basal boundary as shown by Nkx2.2 expression (Fig 6.2B) remaining well separated from the DTmesV and LLF. Expression of Nkx2.2 shows the TPOC axons remain within the basal plate (not shown). It was also unclear in the literature (Lyser, 1966; Windle and Austin, 1936) how far into the mesencephalon the TPOC axons project. The TPOC axons reach the MHB and mostly likely extend further into the rhombencephalon. The MLF axons were more tightly fasciculated than the TPOC axons in zebrafish, which was similar in chick (Ross et al., 1992).

The MTT also projected into the VLT and their neurones were located ventrally but slightly more rostral to the MLF. The MTT neurones first appeared at HH15, consistent with the neurones located in the area mammillaris lateralis (Puelles et al., 1987), which was slightly earlier than previously described at the 37-somite stage (HH19; Windle and Austin, 1936) as a small fascicle.

3.3.2 Formation of the DTmesV

The DTmesV looked similar to that described in mouse (Easter et al., 1993 - another amniote); however in the chick embryonic brain it was more organised. Lyser (1966) described neurones beginning to differentiate along the dorsal midline of the mesencephalon

in HH13⁻ embryos, while these were not mentioned in an earlier study (Windle and Austin, 1936). In this study, neurones were first detected during late HH13 embryos and projecting axons ventrally by HH14, similar to other studies (Chédotal et al., 1995; Puelles et al., 1987). The DTmesV is a prominent feature of the amniote mesencephalon, however this is initially lacking in some anamniotes. At early embryonic stages the DTmesV has been described in medaka (Ishikawa et al., 2004) and cat shark (Kuratani and Horigome, 2000), but not in zebrafish (Chitnis and Kuwada, 1990), turbot (Doldan et al., 2000) or *Xenopus* (Hartenstein, 1993). The role of the DTmesV was discussed in detail in the introduction (1.3.3) and in terms of evolution in chapter 5 (5.8.5).

Tecto-bulbar axons were detected at HH18. These axons, while originating in the dorsal mesencephalon and grow ventrally, take a different route to the DTmesV axons and continue projecting to the ventral midline where they will cross.

3.3.3 Formation of commissures

Commissures are a prominent feature of the anamniote brain, however they form later in amniote development. In zebrafish and *Xenopus*, four early commissures have been described, the AC and POC in the rostral prosencephalon, the PC along the MFB and the VC at the floor of the MFB (Anderson and Key, 1999; Chitnis and Kuwada, 1990; Wilson et al., 1990). Similar to mouse (Easter et al., 1993), commissures were not identified in the rostral prosencephalon of early chick embryos. Consistent with previous studies, some axons were crossing at the chick anterior midline from 3 or 4 fibres (Windle and Austin, 1936) this would suggest the beginning of the POC that is present in zebrafish (Chitnis and Kuwada, 1990; Wilson et al., 1990) and *Xenopus* (Hartenstein, 1993); however it was not as well formed by HH18.

In contrast to the rostral commissures, the TPC and VC form early in amniote development. The first TPC axons were crossing the dorsal midline from neurones located dorsally at

HH16. In mouse lipophilic dyes revealed TPC neurones were also located ventrally (Mastick and Easter, 1996). Axon tracing with lipophilic dyes in the chick embryonic brain revealed the TPC neurones were located at the ventral MFB as well. The TPC neurones were intermingled with the MLF neurones, but the TPC axons projected dorsally across the midline. Neurones were first found to differentiate at HH16, in the area commissuralis (Puelles et al., 1987) suggesting this is when the first ventral TPC neurones differentiate, due to the intermingling of the TPC and MLF neurones it was difficult to determine without using a specific marker for the TPC or birth-dating.

In zebrafish and other anamniotes as well as mouse, the VC formed across the ventral midline at the MFB (Anderson and Key, 1999; Mastick and Easter, 1996; Ross et al., 1992). In chick, axons were projecting towards the ventral midline around the MFB at HH17 and by HH21 a large number of these axons had crossed. In contrast to anamniotes, the chick VC does not form a compact commissure and axons were spread over the floor of the caudal diencephalon and rostral mesencephalon.

This detailed description of the early axon scaffold in the chick embryonic brain will provide valuable for comparison with other vertebrates and will allow the conservation of the early axon scaffold to be analysed. Chapter 5 discusses the conservation of the early axon scaffold in more detail between different vertebrates. The molecular mechanisms involved in the formation of the early axon scaffold are poorly understood. As the MLF neurones have now been shown to be strictly diencephalic, possible molecular markers analysed will need be expressed in the diencephalon not just as part of the midbrain arcs (discussed further in chapter 6).

A — di, mes — HH11

B — p2, p1, nMLF, mes, nTPOC — HH13

C — p3, p2, p1, sp, DTmesV, mes, TPOC, MLF — HH14

D — p3, p2, p1, sp, PC, DTmesV, mes, TPOC, MTT, MLF — HH16

E — p3, p2, p1, sp, PC, TPC, DTmesV, mes, p3 alar neurones, TBT, TPOC, MTT, VC, LLF, MLF, rh — HH18
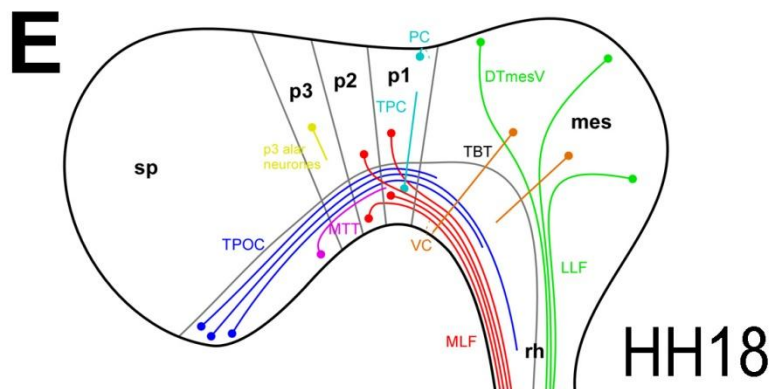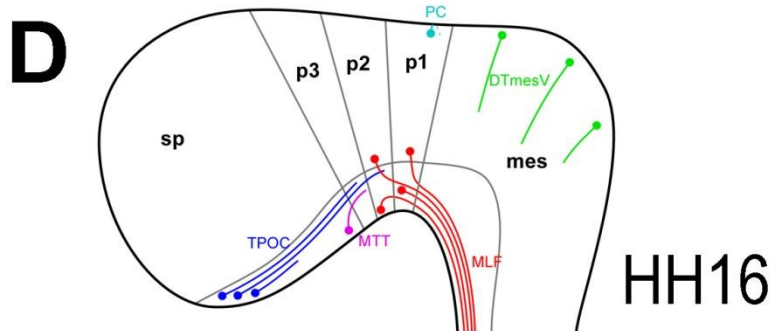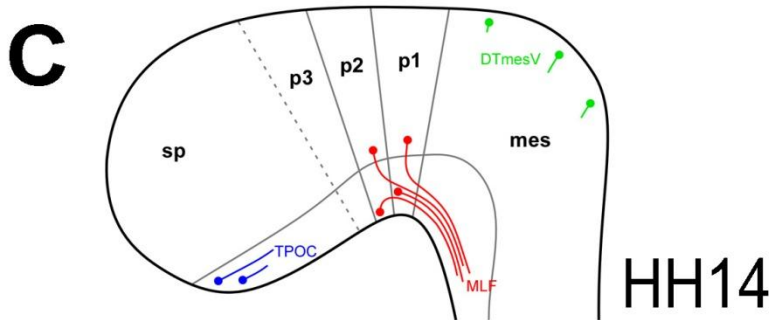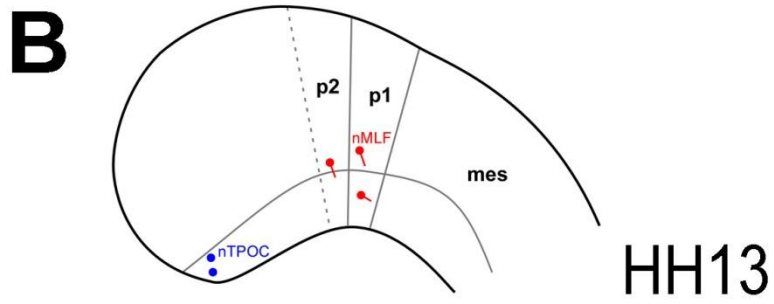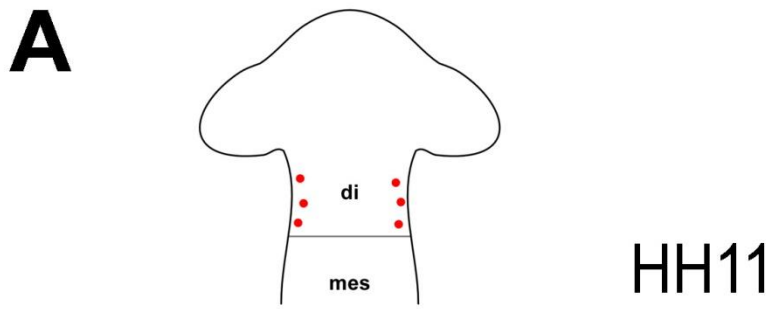
Figure 3.7 Schematics showing the overview of early axon scaffold formation in the embryonic chick brain

A) HH11. The first MLF neurones appear within the diencephalon. B) HH13. While the MLF neurones begin to project axons, the TPOC neurones arise in the rostral basal hypothalamus. C) HH14. The TPOC axons have now started to project axons caudally through the secondary prosencephalon and the MLF axons are projecting tightly, close to the ventral midline. The DTmesV neurones have appeared along the dorsal midline of the mesencephalon and started to project axons ventrally. D) HH16. The TPOC axons have reached the MLF in p1. Rostral to the MLF neurones, the MTT neurones have arisen in the basal plates. These axons will initially follow the TPOC axons to join the VLT. E) HH18. The early axon scaffold is well formed. The TPC neurones also appear in basal plate of p1 and projects axons dorsally along the MFB.

di, diencephalon; mes, mesencephalon; pros, prosencephalon; p1, prosomere 1; p2, prosomere 2; p3, prosomere 3; sp; secondary telencephalon; rh, rhombencephalon; tel, telencephalon;

Chapter 4

Comparison of antibodies and fixatives in embryonic vertebrate brains

4.1 Introduction

At the beginning of the 20th Century, a number of studies were done using silver-impregnated serial sections to analyse early axon development in the embryonic brain by scientists such as Mesdag, Tello, Windle and Herrick (Herrick, 1937; Mesdag, 1909; Tello, 1923; Windle and Austin, 1936). The silver staining method involved impregnating fixed tissue with silver nitrate that was stained black when subjected to a reducing agent. While these studies correctly noted the position of some tracts, other tracts like the DTmesV were not documented. Furthermore, factors like the variability of the silver staining method and the lack of a defined staging system (until Hamburger and Hamilton, 1951) led to discrepancies between the different studies. Since then, the development of new, more specific methods has allowed more complete and detailed descriptions of the early axon scaffold tracts. The methods used now include immunohistochemistry using neuronal specific antibodies and the ability to trace individual tracts with lipophilic dyes.

To analyse the early axon scaffold in different vertebrates a comparative pan-neural antibody was required, which labels all the axons and neurones that form the early axon scaffold within the rostral neural tube. Previous studies have tried to compare the early axon scaffold

(Barreiro-Iglesias et al., 2008; Nieuwenhuys, 1998) but a side-by-side comparison of the major model organisms has yet to be done and they have used various antibodies making this difficult to analyse, as different antibodies label different antigens. As well as finding a comparative antibody, different fixatives were analysed to see if this improved the quality of the antibody staining. Antibodies that were selected label neuronal cell components (see table 2.1) and were used for immunohistochemistry on whole-mount embryos. Optimal staining was where labelling of the axon tracts and neurones was clear and the fluorescence was bright but with little background staining.

4.2 Comparison of fixatives

Fixatives are used to preserve biological tissue and prevent proteins from decaying or being digested. Fixing is also useful as it hardens the tissue making the embryos easier to handle. Formaldehyde-based fixatives (MEMFA and 4% PFA/PBS) provide good tissue preservation and prevents the tissue shrinking. However, they work by cross-linking protein, which can mask the epitope for the antibody. Therefore, many antibodies work less efficiently or not at all in formaldehyde-fixed tissue. 4% PFA/PBS allows easier preparation of the embryos compared to other fixatives. Two non-formaldehyde fixatives (Dent's and Mirsky's) were tried in addition to examine if their use led to improved labelling. Mirsky's is an aldehyde based fixative and Dent's is a DMSO/methanol mix, not including any aldehydes.

Cat shark, *Xenopus*, Chick and mouse embryonic brains were analysed with a range of pan-neural antibodies and fixatives at different stages as described in table 4.1 (chick), table 4.2 (*Xenopus*), table 4.3 (mouse) and table 4.4 (cat shark). A detailed description of the antigen each antibody labels is present in table 2.1.

Fixatives / Antibodies	4%PFA/PBS	MEMFA	Dent's	Mirsky's
RMO-270	Labels all the tracts very clearly	Labels all the tracts very clearly	Labels all the tracts clearly	Labels all the tracts clearly
Zn-12	The axon tracts are labelled, however weakly. There is lots of background labelling	The axon tracts are labelled, however weakly. There is lots of background labelling	The axon tracts are labelled, however weakly. There is lots of background labelling	The axon tracts are labelled, however weakly. There is lots of background labelling
Tuj1	Labels all the tracts very clearly	Labels all the tracts very clearly	Labels all the tracts clearly, but with slightly more background	Labels all the tracts clearly, but with slightly more background
SV2	The axon tracts are labelled but very weakly. There is lots of background labelling	The axon tracts are labelled but very weakly. There is lots of background labelling	Labelling of the dorsal axon tracts is better than labelling ventrally. There is lots of background labelling	Labelling of the dorsal axon tracts is better than labelling ventrally
HNK-1	The axon tracts are labelled but very weakly. There is lots of background labelling	The axon tracts are labelled but very weakly.	Labelling of the dorsal axon tracts is better than labelling ventrally. There is lots of background labelling	Labelling of the dorsal axon tracts is clearer. There is lots of background labelling
6-11B-1	Labelling is weak	Labelling is weak	No tracts labelled	No tracts labelled
HuC/D	No labelling	Labels the neuronal cell bodies very clearly	No labelling	Some neuronal cell bodies are labelled, however very weakly

Table 4.1 Comparison of pan-neural antibodies with different fixatives in chick embryos

The optimum fixative for chick depends on the primary antibody used. For example, in the chick embryonic brain, HNK-1 labelled the axon tracts most effectively when the embryos were fixed in Mirsky's, however the ventral tracts were labelled weakly in comparison to the DTmesV. HuC/D labelled the neurones most effectively when the embryos are fixed in MEMFA. Despite the possible problem of epitope masking, formaldehyde based 4% PFA/PBA and MEMFA have proven to be the optimum fixatives for many of the antibodies used in the chick embryos. MEMFA does cause the chick embryos to whiten slightly, but this does not seem to affect labelling, the embryos were also slightly harder to prepare compared to 4% PFA/PBS as the tissue is softer. For chick embryos using Mirsky's makes preparing the embryos slightly harder than using the other fixatives as the embryos flatten especially at earlier stages.

Fixatives / Antibodies	4%PFA/PBS	MEMFA	Dent's	Mirsky's
RMO-270		No labelling		
Zn-12		Clear labelling of axon tracts	Clear labelling of axon tracts	
Tuj1		No labelling		
SV2		Clear labelling of axon tracts		
HNK-1	Clear labelling of axon tracts	Clear labelling of axon tracts		Labelling of axon tracts
6-11B-1		Clear labelling of axon tracts		
HuC/D		Clear labelling of the neuronal cell bodies		

Table 4.2 Comparison of pan-neural antibodies with different fixatives in *Xenopus* embryos

Blanks indicate antibody was not tested with fixative.

MEMFA was the optimum fixative for all antibodies labelling the early axon tracts in *Xenopus* embryos (table 4.2). 4% PFA/PBS was only tried with HNK-1 and did not improve the quality of the antibody labelling compared to MEMFA. Mirsky's could not be used as it

caused the embryos to disintegrate, particularly at younger stages. Dent's also did not improve the labelling of the axon tracts. Tuj1 and RMO-270 labelling did not work.

Due to limited embryo resources, cat shark and mouse embryos were only fixed with 4% PFA/PBS and MEMFA as these fixatives worked most effectively for many of the antibodies in chick and *Xenopus*.

In the mouse embryonic brain, most of the antibodies that were tried worked most effectively in MEMFA fixed embryos (table 4.3). SV2, HNK-1 and Zn-12 did not work at all. For cat shark (Table 4.4) there was no labelling for RMO-270, Zn-12 and HNK-1 although only MEMFA was used as the fixative. Tuj1 and HuC/D were tried with MEMFA and labelled the axons clearly. SV2 was only tried with 4% PFA/PBS and showed a similar result to chick in which the axons were labelled weakly.

Fixatives / Antibodies	4%PFA/PBS	MEMFA
RMO-270		Tracts were labelled weakly and there was lots of background
Zn-12		No labelling of axon tracts
Tuj1		Labels all the tracts very clearly
SV2	No labelling of axon tracts	No labelling of axon tracts
HNK-1	No labelling of axon tracts	
6-11B-1		Labelling of axon tracts is weak with lots of background
HuC/D	Clear labelling of the neuronal cell bodies, with some background	Clear labelling of the neuronal cell bodies

Table 4.3 Comparison of pan-neural antibodies with different fixatives in mouse embryos

Blanks indicate antibody was not tested with fixative.

Fixatives / Antibodies	4%PFA/PBS	MEMFA
RMO-270		No labelling of axon tracts
Zn-12		No labelling of axon tracts
Tuj1	Labels all the tracts very clearly	Labels all the tracts very clearly
SV2	Labelling of axon tracts but is very weak with lots of background	
HNK-1		No labelling of axon tracts
6-11B-1		
HuC/D		Clear labelling of the neuronal cell bodies

Table 4.4 Comparison of pan-neural antibodies with different fixatives in cat shark embryos

Blanks indicate antibody was not tested with fixative.

4.3 Comparison of pan-neural markers in embryonic vertebrate brains

Previous studies have used different antibodies for different vertebrates, making a direct comparison of results difficult. Previous antibodies used that have been used to label the early axon scaffold were: acetylated tubulin (6-11B-1) in *Xenopus* (Anderson et al., 2000; Anderson and Key, 1996), zebrafish (Chitnis and Kuwada, 1990; Wilson et al., 1990), cat shark (Kuratani and Horigome, 2000), medaka (Ishikawa et al., 2004) and turbot (Doldan et al., 2000). HNK-1 in Zebrafish (Hjorth and Key, 2002; Metcalfe et al., 1990; Ross et al., 1992; Wilson et al., 1990) and medaka (Ishikawa et al., 2004). Neurofilament (RMO-270) in chick (Hunter et al., 2001; Molle et al., 2004; Schubert and Lumsden, 2005). βIII tubulin in mouse (Easter et al., 1993; Mastick and Easter, 1996) and chick (Chédotal et al., 1995). The cell adhesion molecules: NOC-1 has been used in *Xenopus* (Anderson and Key, 1999; Anderson and Key, 1996) and BEN has been used in chick (Chédotal et al., 1995). Zn-12 has been used in zebrafish (Metcalfe et al., 1990). A range of pan-neural antibodies were tried in cat shark, *Xenopus*, chick, and mouse with the aim of finding an antibody that could be used to label the early axon scaffold across species.

4.3.1 Antibody concentrations

In the chick embryonic brain Zn-12, SV2, HNK-1 and 6-11B-1 all label axon tracts, however weakly with lots of background. The concentrations of these primary and secondary antibodies were increased to try to improve the labelling of axons.

	Zn-12	SV2	HNK-1	6-11B-1
Primary antibody	1:100	1:100	1:500	1:100
Secondary antibody	1:100	1:100	1:100	1:100
	1:500	1:500	1:500	1:500
Primary antibody	1:20	1:20	1:100	1:20
Secondary antibody	1:100	1:100	1:100	1:100
	1:500	1:500	1:500	1:500
Optimum concentrations	1:100/1:100	1:100/1:500	1:100/1:100	1:20/1:100

Table 4.5 Range of concentrations used to find optimal working conditions

Increasing the concentration of HNK-1 and the secondary antibody improved the labelling of the axon tracts. Increasing the concentration of SV2 caused more background to be present. Increasing the concentration of 6-11B-1 and the secondary antibody has improved the labelling of axon tracts particularly of the DTmesV. Increasing the concentration of the secondary antibody with Zn-12 improved the labelling of the axon tracts. Even though increasing the antibody concentration has improved the labelling of the axon tracts, it is still not the same quality of labelling as with RMO-270 or Tuj1.

4.3.2 Antibodies used in the vertebrate embryonic brain

Most of the antibodies label axon tracts but have limitations. HuC/D was the only antibody that labelled clearly in all the vertebrate species used, however it only labels the neuronal cell bodies. RMO-270 labels clearly in chick (Fig 4.1A), however does not label any axon tracts in *Xenopus* (Fig 4.2A) or cat shark (Fig 4.4A). In mouse, the axon tracts were labelled weakly with lots of background (data not shown). Tuj1 labels clearly in the chick embryonic brain (Fig 4.4C), mouse (Fig 4.3A) and cat shark (Fig 4.4C), however the antibody failed to label any tracts in the *Xenopus* embryonic brain (Fig 4.2C).

Zn-12 and SV2 label the axon tracts really clearly in *Xenopus* (Fig 4.2B, D) however; in chick (Fig 4.1B, D), the labelling of axon tracts is weak with lots of background. In chick, labelling of the axon tracts with SV2 was improved slightly by using rabbit serum (Sigma) instead of goat serum. Zn-12 and SV2 does not label any axon tracts in mouse. In cat shark, Zn-12 (Fig 4.4B) does not label any tracts while SV2 labels the tracts, but weakly (Fig 4.4D).

HNK-1 is the optimal antibody for detecting the axon tracts in *Xenopus* (Fig 4.2E), but in chick (Fig 4.1E), this antibody only labelled neurones in the dorsal region really clearly, while the ventral tracts were barely visible. HNK-1 did not label any tracts in mouse (Fig 4.3C) or cat shark (Fig 4.4E). 6-11B-1 labels axon tracts weakly in chick and mouse and labels the axon tracts really clearly in *Xenopus* embryos.

Other antibodies that were tested in chick and *Xenopus* were CYN-1, 4H6, 40E-C, GAD-6, α-TH, GABA, BEN, Pax6, Pax7 and 23.4.5 (data not shown). These antibodies resulted in either weak labelling or no labelling at all. As these antibodies were not useful, they were not tested in cat shark or mouse.
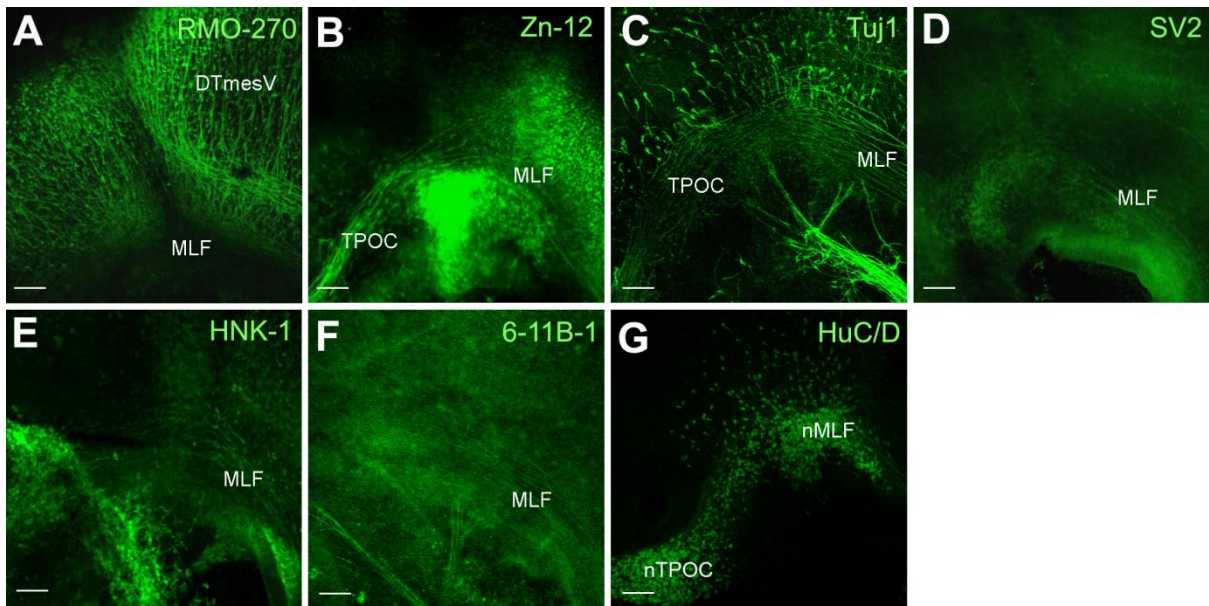
Figure 4.1 Comparison of antibodies in the HH17 chick embryonic brain
Lateral views of whole mount embryos. Scale bars, 100μm

All images are focussed on the ventral region of the embryonic brain around the MFB. A) RMO-270 fixed with 4% PFA/PBS. Labelling of the axon tracts is clear. B) Zn-12 fixed with MEMFA. Labelling of the axon tracts is weak with lots of background. C) Tuj1 fixed with MEMFA. Optimal staining of axon tracts. D) SV2 fixed with 4% PFA/PBS. Labelling of axons tracts is weak. E) HNK-1 fixed with Mirsky's. Ventral labelling of axon tracts is weak. F) 6-11B-1 fixed with MEMFA. Ventral labelling of axon tracts is weak. G) HuC/D fixed with MEMFA. Labelling of neuronal cell bodies is clear.
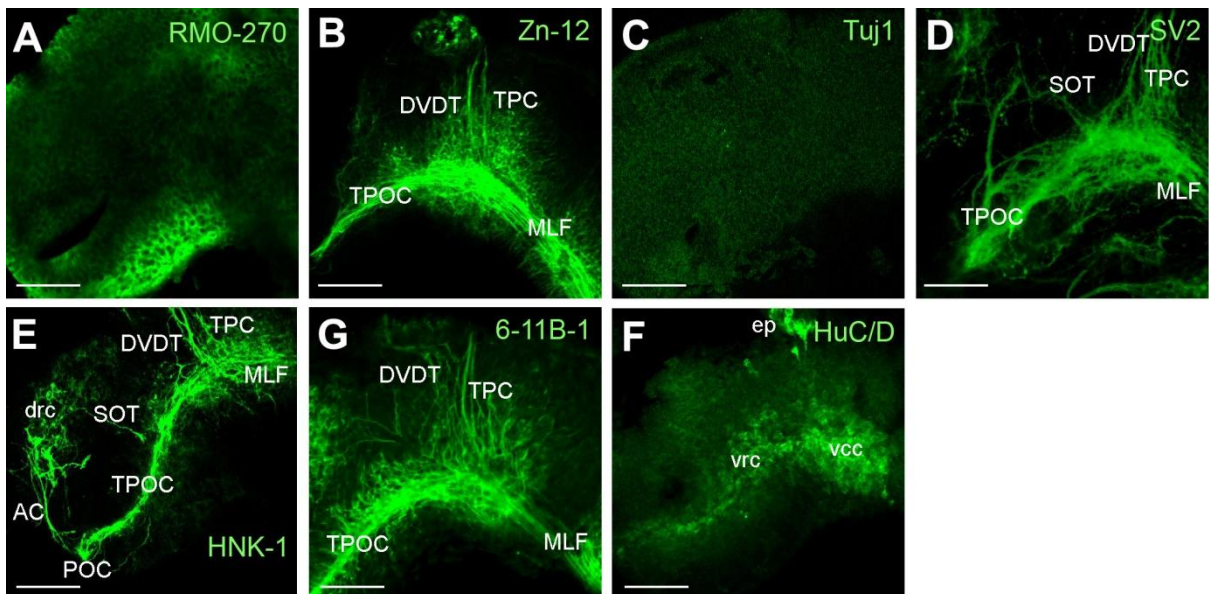
Figure 4.2 Comparison of antibodies in the stage 32 *Xenopus* embryonic brain
Lateral views of whole mount embryos. Scale bars, 100μm

All embryos were fixed with MEMFA. A) RM0-270, no labelling of axon tracts. B) Zn-12, axon tracts labelled clearly. C) Tuj1, no labelling of axon tracts. D) SV2, clear labelling of axon tracts. E) HNK-1, clear labelling of axon tracts. F) 6-11B-1, clear labelling of axon tracts. G) HuC/D, clear labelling of the neuronal cell bodies.
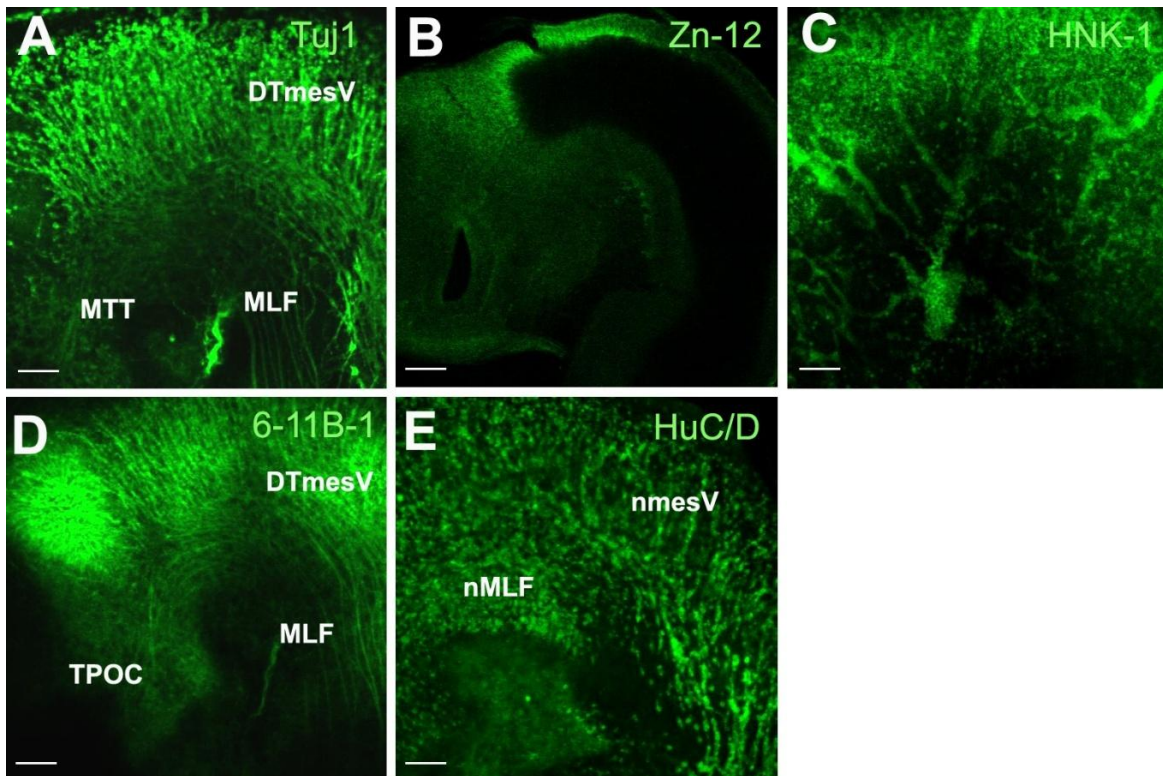
Figure 4.3 Comparison of antibodies in the mouse embryonic brain
Lateral views of whole mount embryos. Scale bars, 100μm

A) E10 Tuj1 fixed with MEMFA. Axon tracts are labelled clearly. B) E9 Zn-12 fixed with 4% PFA/PBS. No axon tracts labelled. C) E10 HNK-1 fixed with 4% PFA/PBS. No axons are labelled. D) E10 6-11B-1 fixed with MEMFA. The axon tracts have been labelled nicely, however there is still quite of background compared with Tuj1. E) E10 HuC/D fixed with MEMFA. The neuronal cell bodies have been clearly labelled.
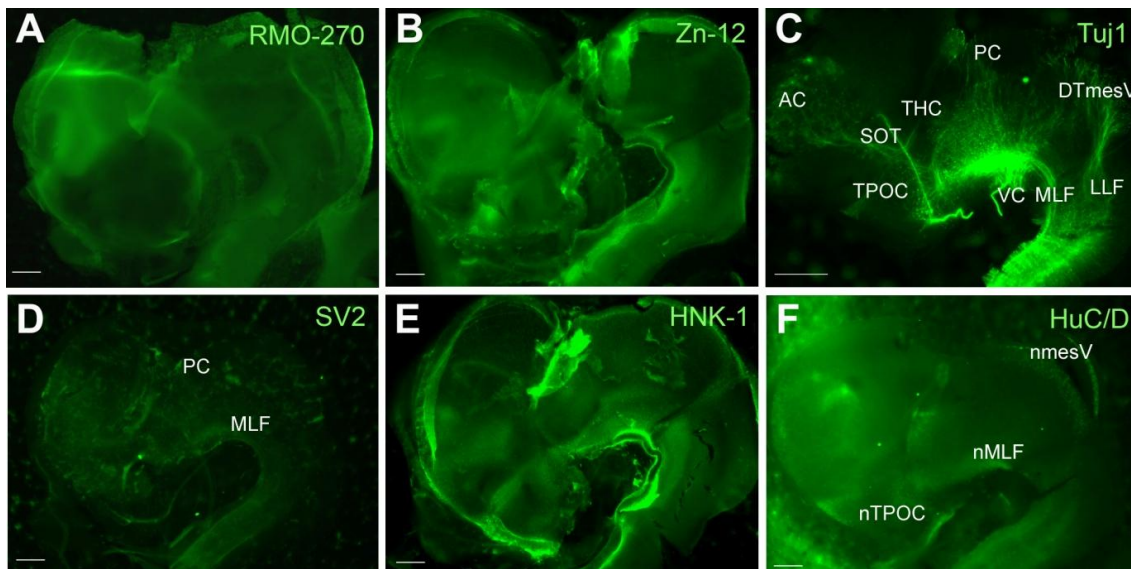
Figure 4.4 Overview showing the comparison of antibodies in the cat shark embryonic brain
Lateral views of whole mount embryos. Scale bars 200μm

A) RMO-270 fixed with MEMFA. No axon tracts labelled. B) Zn-12 fixed with MEMFA. No axon tracts labelled. C) Tuj1 fixed with MEMFA. All the early axon scaffold tracts are labelled clearly. D) SV2 fixed with 4%PFA/PBS. Labels the axon tracts weakly. E) HNK-1 fixed with MEMFA. F) HuC/D fixed with MEMFA labels the neuronal cell bodies really clearly.

4.4 Discussion

A range of antibodies and fixatives have been used to determine optimum labelling of axon tracts to find an antibody that could be used for the comparison of the early axon scaffold in various vertebrate embryonic brains. Optimum labelling of the axons and neurones that were present in the early embryonic brain with the antibody was necessary, otherwise axons were likely to be missed due to weak staining.

Cat shark	*Xenopus*	Chick	Mouse
HuC/D	HuC/D	HuC/D	HuC/D
Tuj1	HNK-1	Tuj1	Tuj1
	6-11B-1	RMO-270	
	SV2		

Table 4.6 Summary of antibodies that labelled neuronal components well in the vertebrate brain

As the summary in table 4.6 shows that even though a relatively large number of antibodies were tested, few worked well. HuC/D will be a useful antibody as it labelled the neuronal cell bodies in all the vertebrates: cat shark, *Xenopus,* chick, and mouse. As it only labelled the cell bodies and not the axon tracts, it could not be used as the comparative antibody. The antibodies that labelled the axons tracts clearly in *Xenopus* (HNK-1, 6-11B-1 and SV2) either labelled weakly or not at all in the other vertebrates. Tuj1 labels the differentiating and mature neurones, but not the neuronal precursor cells (Lee et al., 1990) clearly in cat shark, chick and mouse but does not label any axon tracts in *Xenopus.* Tuj1 did not label any axon tracts in the *Xenopus* embryonic brain as βII tubulin is expressed instead of βIII tubulin. RMO-270 only labels axon tracts clearly in chick. RMO-270 does not label any axon tracts in *Xenopus* as neurofilament-M protein expression occurs after the early axon scaffold is established (Gervasi and Szaro, 1997).

As pan-neural antibodies recognise the same neurones and axons, Tuj1 can be used as the comparative antibody for cat shark, chick, and mouse and HNK-1 will be used for *Xenopus*

Chapter 5

Comparison of the early axon scaffold in embryonic vertebrate brains

5.1 Introduction

The early axon scaffold is a common feature of all vertebrates, making it a highly conserved structure throughout evolution and has been characterised in many studies, particularly in zebrafish and mouse (eg, Chitnis and Kuwada, 1990; Mastick and Easter, 1996). Other studies have attempted to compare the anatomy of the early axon scaffold (Barreiro-Iglesias et al., 2008; Easter et al., 1993; Nieuwenhuys, 1998) however, a direct comparison of these early neurones and tracts in the major model organisms was lacking. Many of the early axon tracts have been poorly characterised and there was still confusion over the nomenclature and homology of these tracts.

5.1.1 Vertebrate evolution

Vertebrates first evolved 542 million years ago, diverging from chordates, in marine waters and are defined by a vertebral column and cranium in which the head contains paired sensory organs such as the eyes and ears (Kardong, 2009). Vertebrates are split into two groups: the agnathans (non-jawed) and the gnathostomes (jawed).

The jaws of gnathostomes derived from the anterior pharyngeal arches during development allowing vertebrates to process larger food. The first major branches of the gnathostomes

were the chondrichthyes that have a cartilaginous skeleton (e.g. cat shark) and fish that have a bony skeleton. The bony fish evolved either into ray-finned (teleosts e g., zebrafish, medaka and turbot) and lobe-finned. From the lobe-finned fish, tetrapods evolved in which vertebrates made the transition from water to land and the first amphibians arose. Amniotes such as birds and mammals have evolved so that the embryos are surrounded by an extraembryonic membrane to protect the embryo (allowing eggs to be laid on dry land) and prevent it drying out. Mammals have evolved further to protect their young, providing nutritional and respiratory needs through a placenta.

5.1.2 Vertebrates used for comparison

The formation of the early axon scaffold has been studied here in the jawed vertebrates: cat shark, *Xenopus*, zebra finch, chick and mouse embryos. Apart from cat shark and zebra finch these vertebrates are major model organisms, they include a variety of amniotes and anamniotes. Chick, zebra finch and mouse are examples of amniotes. Chick was used as it was an example of birds as well as being a major developmental model organism. The early axon scaffold has also been poorly characterised. Zebra finch was used as it provides a direct comparison between different birds and will be interesting to analyse as it is commonly used as a model for studying nuclei involved in vocalisations (Sanes et al., 2006). Mouse provided a good example of a mammal and development appears similar to human. Cat shark and *Xenopus* are examples of anamniotes. *Xenopus* was an example of amphibians and previous studies have described the early axon scaffold in the embryonic *Xenopus* brain (Hartenstein, 1993) however a detailed time series was missing. Cat shark was not a model organism but was a representation of cartilaginous fish at the beginning of the evolutionary tree of vertebrates that will be used in this study (Fig 5.1), which makes it an interesting vertebrate to study. The analysis of the early axon scaffold in the cat shark embryonic brain will help determine the evolutionary conservation of the early axon scaffold, which forms in all

vertebrates. Even though mouse and *Xenopus* had previously been studied in detail it will be useful to compare these results with that of the previous studies.

5.1.3 Homology of early axon tracts

Even though the early axon scaffold has been studied in detail, there had been confusion over the homology and terminology of the axon tracts (Table 5.1). The formation of the DDT and VDT in the prosencephalon of the medaka embryonic brain is an example where there is still confusion over homology (Ishikawa et al., 2004). The DDT is most likely the equivalent of the TPOC due to its location and timing during development. The POC axons also form closely with this tract. The VDT is likely to be the equivalent of the MTT. Apart from the MLF, TPOC and TPC that are present in all the vertebrates studied, many of the other axon tracts only form in some of the vertebrates while the early axon scaffold is set up. Some of these tracts will appear later in development or simply do not form at all. Some of these tracts may have been over looked, due to specificity of the axon labelling method or timing of tract development.

Lamprey (Barreiro-Iglesias et al., 2008)	Cat shark (Kuratani and Horigome, 2000)	Zebrafish (Chitnis and Kuwada, 1990; Ross et al., 1992; Wilson et al., 1990)	Medaka (Ishikawa et al., 2004)	Turbot (Doldan et al., 2000)	*Xenopus* (Anderson and Key, 1999; Hartenstein, 1993)	Chick (Chédotal et al., 1995; Molle et al., 2004)	Alligator (Pritz, 2010)	Mouse (Mastick and Easter, 1996)
MLF	MLF	VLF/MLF	FLM	MLF	FLM/VLT	MLF	MLF	MLF
TPOC	TPOC	TPOC	DDT	TPOC	TGT/TPOC	TPOC	TPOC	TPOC
	trMesV		DMT			mesV/MTN	dtrmesV	tmesV
	DVDT	DVDT			DVDT			
SOT	SOT	SOT/TT	TT	SOT	SOT		SOT	SOT
TPC	CP	TPC	PC	TPC	TPC	TPC	TrPC	TPC
POC	POC	POC	POC	POC	POC		POC	
	AC	AC		AC	AC		AC	AC
VTC		VC	CA	VTC	VC		VTC	VTC
			VDT					MTT
	THC	THC						THC

Table 5.1 Tracts present in various vertebrates as described by previous studies

The aim was to find an antibody that labels all the early axon tracts in the different vertebrates analysed since different antibodies/antigens may have selective specificity for certain neurones/tracts, or may label neurones at different stages of differentiation (Chapter 4). Tuj1 was found to label the early axon tracts in all species except *Xenopus* so was used to label cat shark, zebra finch, chick and mouse. HNK-1 was used instead to label the axon tracts in *Xenopus*. HuC/D is a pan-neuronal marker, which labels the neuronal cell bodies in all the vertebrate species studied here, but cannot be used as a comparative antibody as it only labels the cell bodies and does not label the axons. It will however be useful to compare the formation of the neuronal populations.
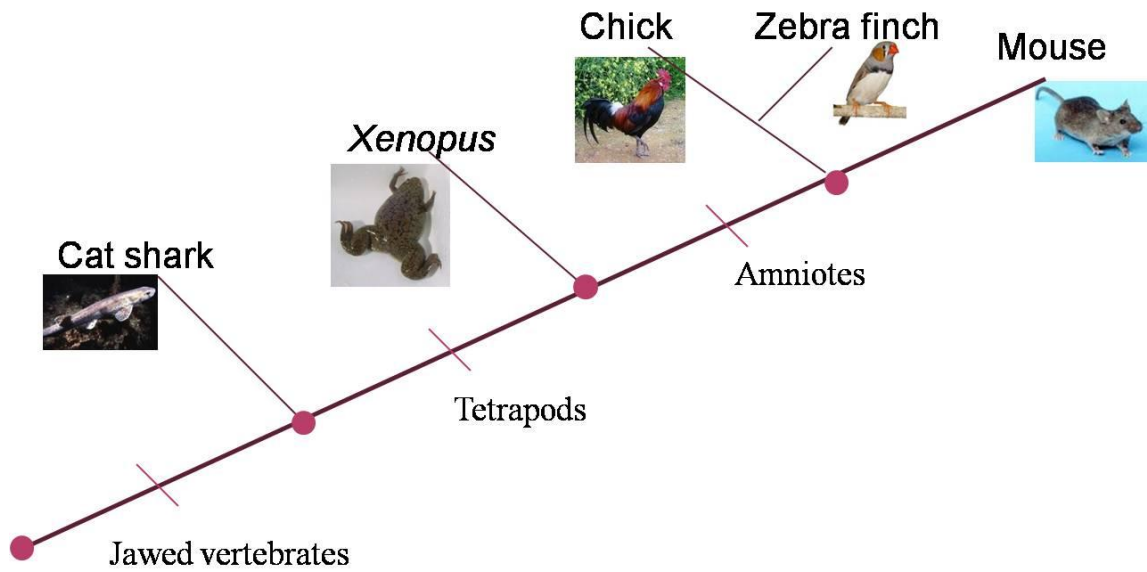
Figure 5.1 Evolutionary tree of the vertebrates used in this study

The common ancestor all these vertebrates share was when the divergence was made for the jawed vertebrates. The first branch is cartilaginous fish and cat shark was used as an example. The next major divergence was for the tetrapods, when vertebrates made the transition from water to land and *Xenopus* is the first example of this group as it is an amphibian. The next divergence to be made was the amniotes. Chick and zebra finch were used as an example of birds and mouse was used as an example of mammals.

5.2 Development of the *Xenopus* early axon scaffold

The MLF neurones appeared first in the embryonic *Xenopus* brain at stage 22 around the MFB (Fig 5.2A and Fig 5.3A). This population has been termed the ventrocaudal cluster (vcc) (due to similar development in the zebrafish, which is also an anamniote; (Ross et al., 1992). The MLF neurones started projecting axons at stage 23 (Fig 5.2B). Neurones also appeared in the epiphysis and the dorsorostral cluster (drc) population in the dorsal telencephalon (Fig 5.2B). By stage 25, the MLF neurones have projected axons further caudally to form a tight bundle projecting along the floor plate (Fig 5.2C). The TPOC axons have started projecting axons both rostrally and caudally from the ventrorostral cluster (vrc). The vrc forms from a chain of neurones in the rostral diencephalon extending from the rostral end of the neural tube (Taylor, 1991), to a larger cluster located close to the MFB. Axons have begun projecting from the drc, pioneering the AC. By stage 27, the TPOC axons have pioneered the POC in which axons project across the anterior midline, ventral to the optic stalk and will connect the contralateral TPOC axon tracts (Fig 5.2D). The DVDT projected a single axon from the epiphysis, ventrally towards the VLT. The number of drc neurones has increased and some AC axons have projected towards the anterior midline, dorsal to the optic stalk. The number of neurones and axons are increasing through stages 28 (Fig 5.2E and Fig 5.3C) and stage 30 (Fig 5.2F) with no new tracts arising at stage 28. The TPC was beginning to form during stage 30 (Fig 5.2F). By stage 32, the early axon scaffold was well established with the addition of the SOT pioneered from the drc and VC across the ventral midline (Fig 5.2G). The TPC axons have crossed the dorsal midline to form the PC. The DLL formed from neurones located in the rhombencephalon and axons project caudally. It was detected from stage 25 (Fig 5.2C), however was not clear in all the images.
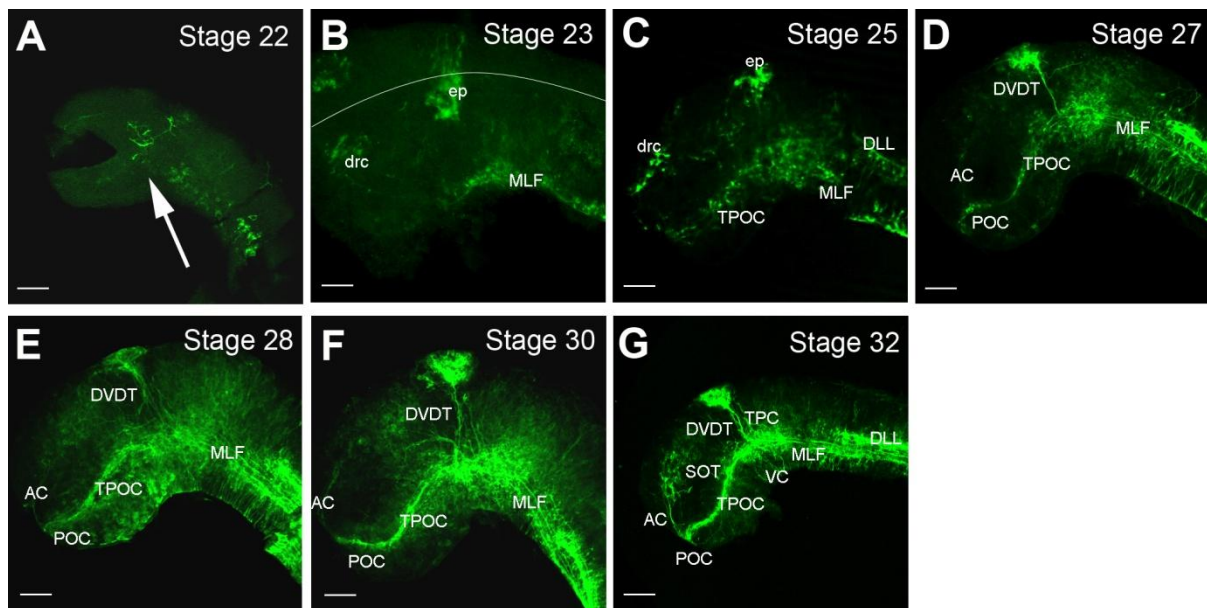
Figure 5.2 Time series of the early axon scaffold in the embryonic *Xenopus* brain using HNK-1
Lateral views of the whole mount neural tubes. Scale bars, 100μm

A) Stage 22. The first MLF neurones arose forming the vcc population. B) Stage 23. The MLF neurones start projecting axons caudally. There were neurones present in the epiphysis. Some neurones were present in the dorsal telencephalon forming the drc population. Line indicates the dorsal midline. C) Stage 25. The vrc population has also formed and started projecting TPOC axons. The DLL is present in the rhombencephalon. D) Stage 27. TPOC axons are projecting from the vrc. The rostral TPOC axons cross the midline to form the POC. There are also a few axons projecting from the drc, beginning to pioneer the AC. A single DVDT axon has projected from the epiphysis and has reached the VLT. E) Stage 28. There was no additional axon tracts formed. F) Stage 30. The TPC was beginning to form. G) Stage 32. The early axon scaffold is well formed. The SOT, TPC and VC are now present.

ep, epiphysis
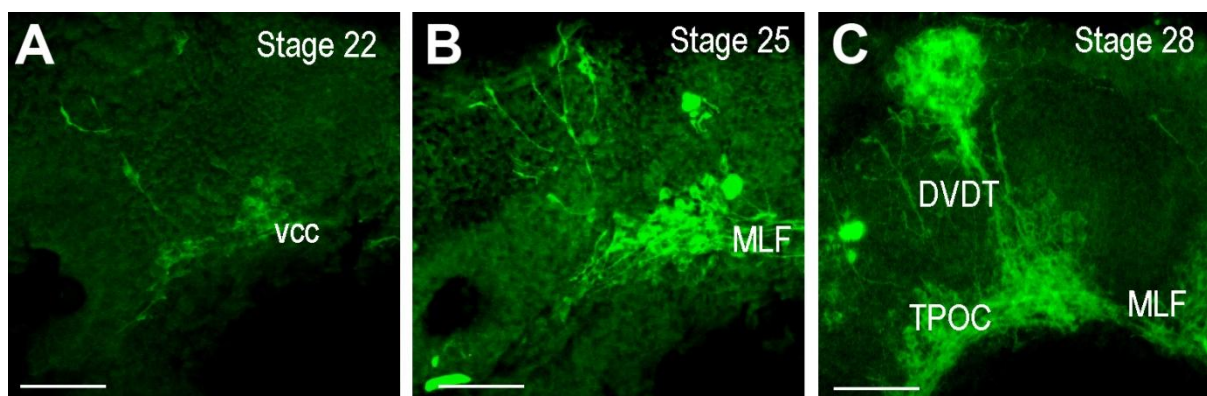
Figure 5.3 Detailed formation of the MLF in the *Xenopus* embryonic brain
Lateral views of the whole mount neural tubes. Scale bars, 100μm

A-C) Axon tracts labelled with pan-neural antibody SV2. A) Stage 22. The first neurones arise at the MFB, forming the vrc, which will give rise to the MLF. B) Stage 25. The MLF neurones are projecting axons caudally. Some TPOC neurones are located rostrally that form the vrc. C) Stage 28. The MLF was well established. The MLF axons form a tightly fasciculated tract projecting along the ventral floor plate. The DVDT single axon tract has reached the vcc after projecting ventrally from the epiphysis. The TPOC axons have projected axons caudally to form the VLT.
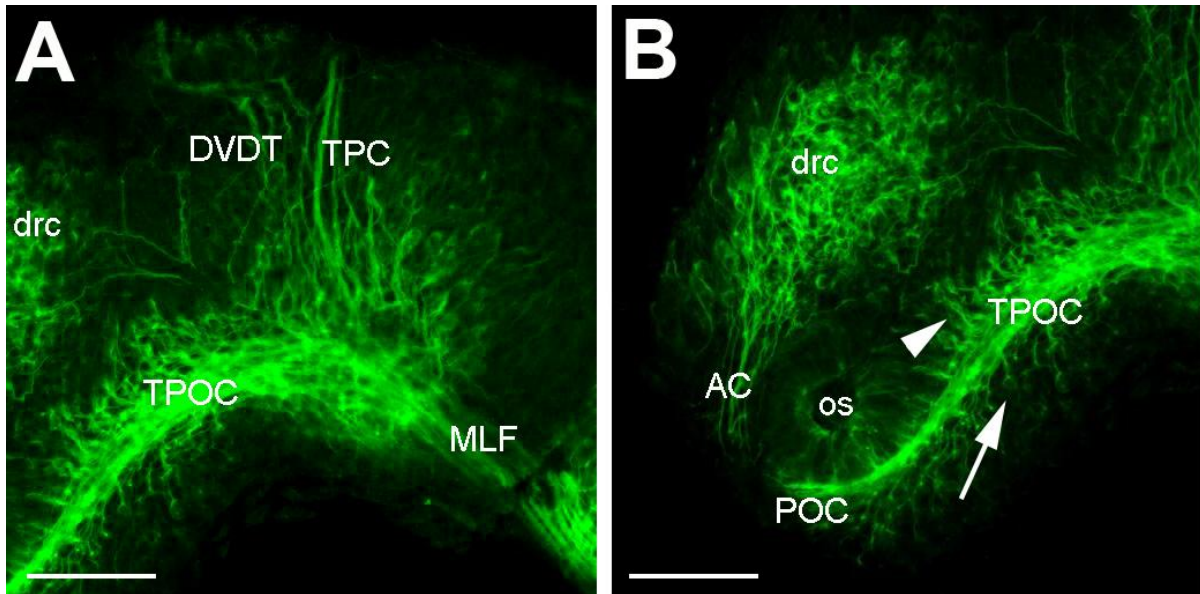
Figure 5.4 Detailed description of the established early axon scaffold in the *Xenopus* embryonic brain
Lateral views of the whole mount neural tubes. Scale bars, 100μm

A and B) Stage 32 *Xenopus* embryo labelled with 6-11B-1 pan-neural antibody (acetylated tubulin). A) MFB region. The vcc is projecting axons caudally in a tight bundle to form the MLF. The vrc is projecting axons to form the TPOC that also projects as a tight bundle. B) Prosencephalon. The vrc projects axons from a dorsal population (arrowhead) and a ventral population (arrow) rostrally and caudally to form the TPOC. The rostrally projecting TPOC axons cross the anterior midline ventral to the optic stalk (os). The drc is a large population of neurones in the dorsal telencephalon. The AC axons are projecting rostrally and dorsal to the os to cross the midline.

At Stage 32, the early axon scaffold was well established (Fig 5.4). The VLT was the most prominent tract in running along the floor plate. The TPOC neurones were located both ventral (Fig 5.4B, arrow) and dorsal (Fig 5.4B, arrowhead) to the axon tract and axons appeared to project directly into the MLF axon tract. The AC and POC tracts were well separated as they both crossed the anterior midline, the AC axons project dorsally to the optic stalk and the POC project ventrally to the optic stalk. In Fig 5.4A the VC was not visible, which was most likely due to the preparation of the embryo and the SOT has not formed yet.

5.3 Formation of the early axon scaffold in the cat shark embryonic brain

The early axon scaffold has been studied in the cat shark species, *Scyliorhinus torazame* by Kuratani and Horigome (2000) but only shows the formation at an older stage (Stage V) once the early axon scaffold appeared to be fully established. Here the early axon scaffold was characterised in the cat shark species, *Scyliorhinus canicula*.

The first neurones to differentiate in the cat shark embryonic brain gave rise to the MLF at stage 18 (Fig 5.5A, arrow). The MLF neurones began projecting axons caudally at stage 19 (Fig 5.5B). By stage 21, the MLF axon tract forms a tight bundle that projects along the floor plate towards the rhombencephalon. A population of scattered neurones have arose at stage 21 located rostral to the MLF neurones (Fig 5.5C, arrow). Some of these neurones were projecting caudally to the MLF and some appear to be projecting dorsally. The MLF was still the only prominent tract in the cat shark brain at stage 22 (Fig 5.5D), along with the scattered neurones. By stage 23, the MLF axons have projected well into the rhombencephalon and the scattered neurone population was continuing to increase (Fig 5.5E). There were also neurones located in the dorsal telencephalon that will give rise to the drc. Between stages 23 and 25,

the early axon scaffold develops rapidly. By stage 25 (Fig 5.5F) the number of axon tracts present has increased and the early axon scaffold was well formed with follower axons likely to be present.

5.3.1 Detailed description of MLF formation in the embryonic cat shark brain

As previously discussed the first neurones to arise in the embryonic cat shark brain at stage 18 were most likely located rostral to the MFB and will give rise to the MLF (Fig 5.6A, arrow). The MLF neurones began to project axons caudally at stage 19 (Fig 5.6B). At stage 20 the MLF continued to project axons caudally (Fig 5.6C) and by stage 21 the MLF had formed a tight bundle that projects along the floor plate into the rhombencephalon (Fig 5.6D). Also evident at stage 20 (Fig 5.6C, unfilled arrow) and stage 21 (Fig 5.6D, unfilled arrow) were a scattered population of neurones located just rostral to the MLF. Some of these neurones appeared to be projecting caudally into the MLF axon tract, however some were projecting dorsally. At stage 23 (Fig 5.6E), the MLF was well established and appears to be formed from a possible two populations of neurones (Fig 5.7B, C). One population was located centrally (Figure 5.7B, C filled arrow) and the other population was located more dorsally (Figure 5.7B, C unfilled arrow). The central population was tightly clustered and appeared to be contributing the most axons to the MLF. The dorsal population was more scattered. There were axons projecting dorsal to the MLF axon tract and appeared to be a separate tract (Fig 5.7B, arrowhead). This axon tract was most likely forming from the scattered neurones located rostral to the MLF.
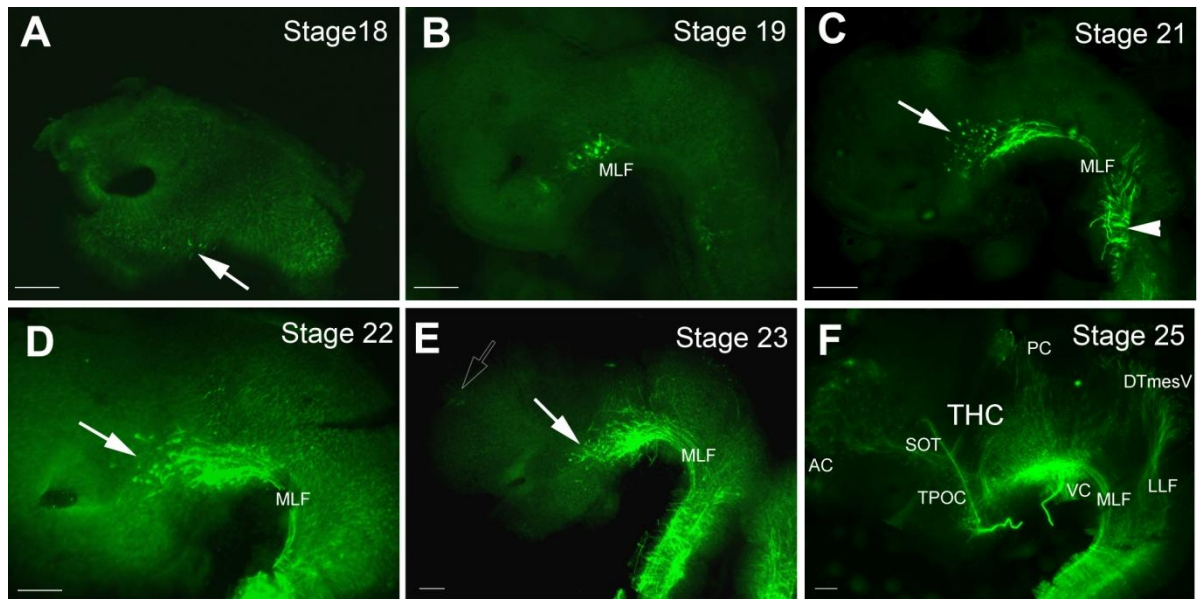
Figure 5.5 The formation of the early axon scaffold in the cat shark embryonic brain
Lateral views of the whole mount embryos. Scale bars, 200µm

A) Stage 18. The first neurones appear that will give rise to the MLF (arrow). B) Stage 19. The first axons are being projected from the MLF neurones. C) Stage 21. The MLF axons have formed a tight bundle projecting along the floor plate towards the rhombencephalon. Scattered neurones appeared rostrally to the MLF neurones (arrow). There are also neurones in the rhombencephalon that do not appear to form part of the early axon scaffold (arrowhead). D) Stage 22. The MLF is still the prominent tract, with scaffold neurones projecting axons (arrow). E) Stage 23. The MLF neurones have projected well into the rhombencephalon. The scattered neurone population has increased in size (filled arrow). Neurones are also located in the dorsal telencephalon (unfilled arrow). F) Stage 25. The number of tracts that appear in the brain has increase dramatically and the early axon scaffold is well established.
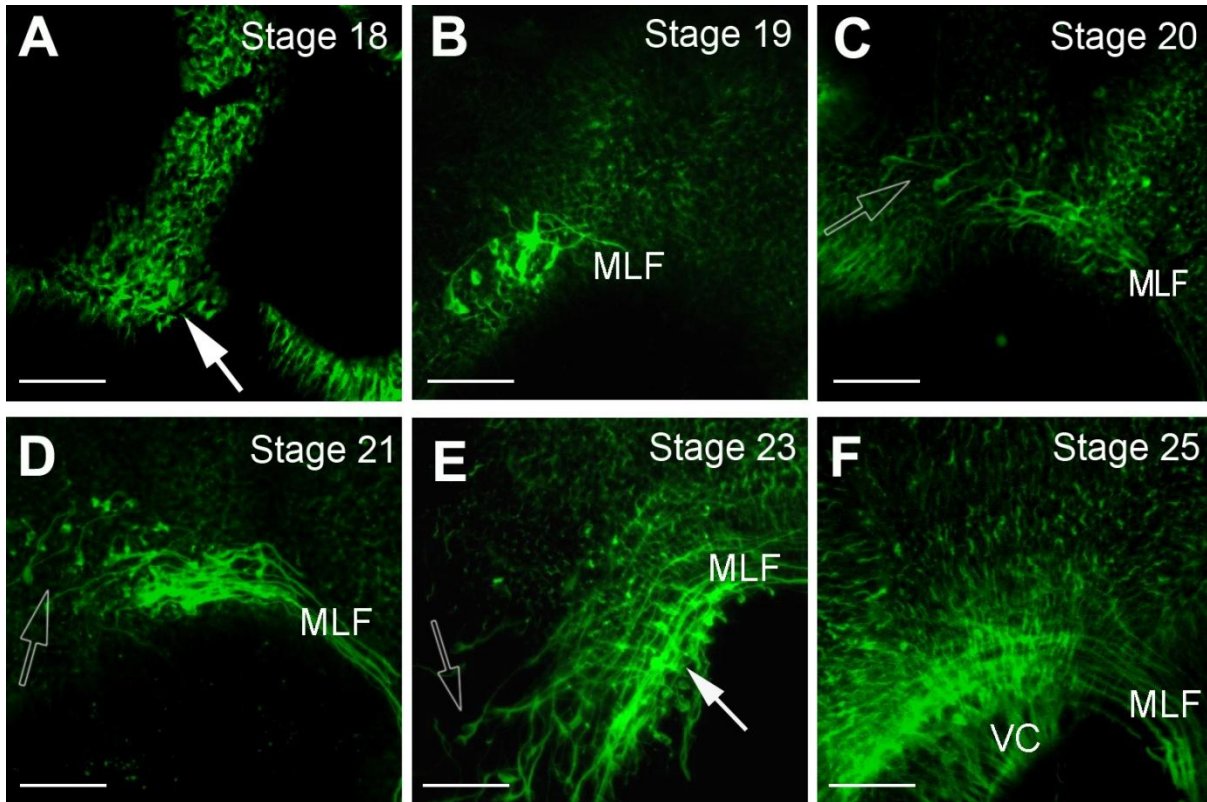
Figure 5.6 Detailed MLF formation in the embryonic cat shark brain
Lateral views of the whole mount embryos. Scale bars, 100µm

A) Stage 18. The first MLF neurones appear (arrow). B) Stage 19. The MLF neurones are projecting their first axons caudally. C) Stage 20. The MLF axons have projected further and there are also scattered neurones located rostral to the MLF (unfilled arrow). D) Stage 21. The MLF axons have formed a tight bundle. The scattered neurones are projecting into the MLF (unfilled arrow) as well as some neurones projecting dorsally. E) Stage 23. The MLF has increased in number of axons and neurones. There are also more scattered neurones present (unfilled arrow). F) Stage 25. The MLF is well established. The VC is also present.
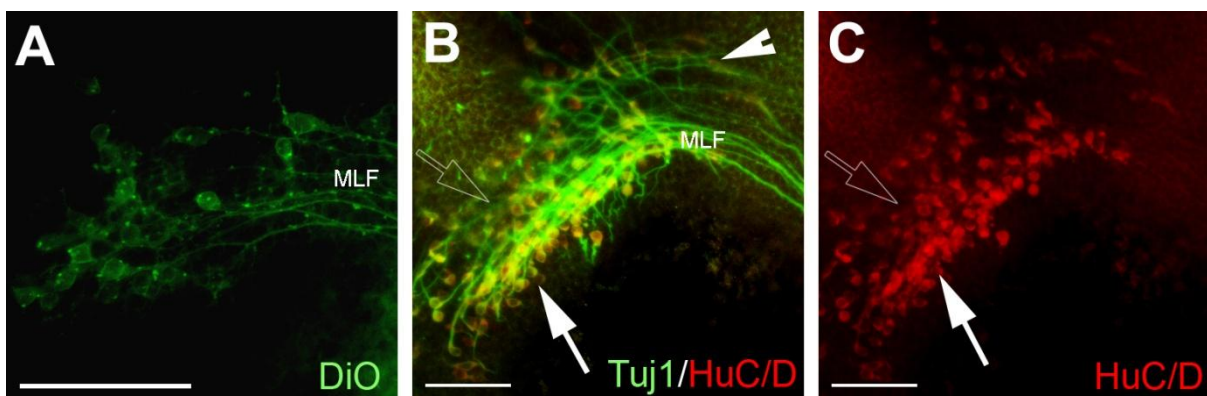
Figure 5.7 The MLF population of neurones in the cat shark embryonic brain
Lateral views of the whole mount embryos. Scale bars, 100µm

A) Stage 22 MLF labelled with DiO. B and C) Stage 23 double labelling with Tuj1 and HuC/D. There appears to be two populations of MLF neurones. One located dorsally (unfilled arrow) and a tight cluster of centrally located neurones along the ventral midline (filled arrow). B) There are also axons projecting ventrally to the MLF (arrowhead) that appears to be a separate axon tract.

5.3.2 Formation of the DTmesV, DVDT and TPOC in the embryonic cat shark brain

Although it was not clear from the overview image at stage 23 (Fig 5.5E), but higher magnification images revealed there were also neurones located in the hypothalamus, a small cluster of neurones in the region of the epiphysis and neurones were located along the dorsal midline in the mesencephalon (Fig 5.8). The neurones that appeared along the dorsal midline of the alar plate in the mesencephalon gave rise to the DTmesV (Fig 5.8A). There were 3-4 neurones located dorsally at the epiphysis (Fig 5.8B). These neurones have started project axons ventrally pioneering the DVDT. Only one of these neurones had projected an axon ventrally almost reaching the MLF (Fig 5.8B, unfilled arrow). The TPOC forms from neurones located in the rostral hypothalamus (Fig 5.8C). These neurones have started to project axons but have not formed the TPOC axon tract yet.
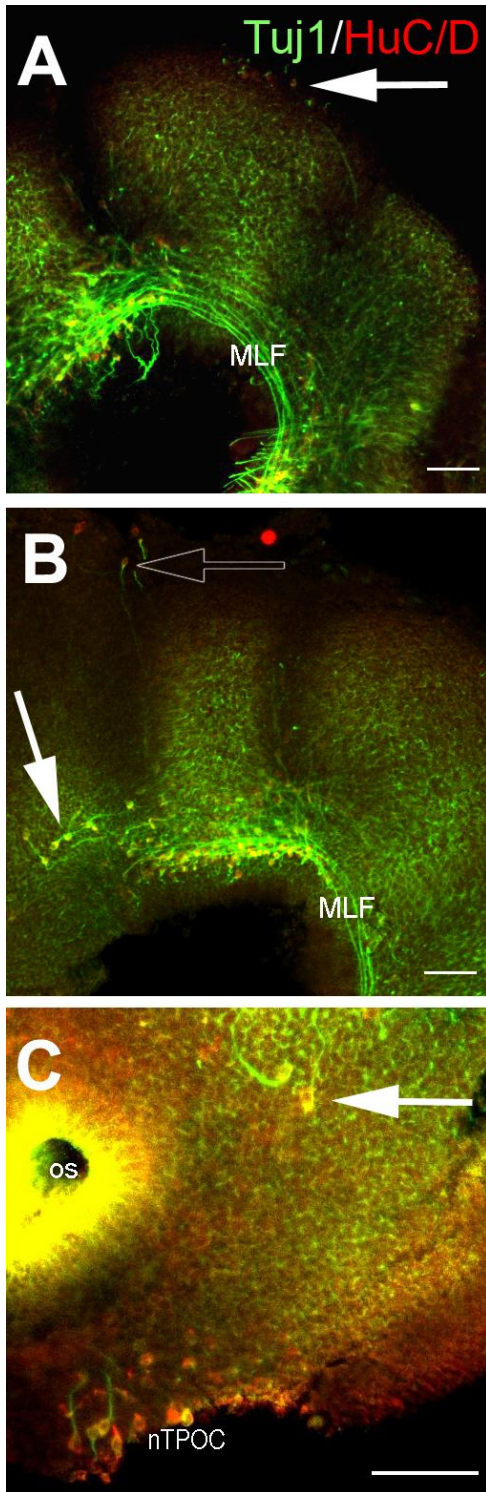
Figure 5.8 Development of axon tracts in the embryonic cat shark brain

Stage 23 cat shark embryonic brain (lateral view) double labelled with Tuj1 (green) and HuC/D (red). A) Neurones arising along the dorsal midline of the mesencephalon (arrow). Some neurones have projected axons to begin forming the DTmesV. The MLF axon tract is well formed. B) 3-4 neurones appeared dorsally at the epiphysis and one neurone has projected its axon ventrally (unfilled arrow), almost reaching the MLF. C) Basal hypothalamus, where the TPOC neurones arise and start to project axons. Neurones were also located caudally and are most likely scattered neurones (filled arrow). Scale bars 100μm
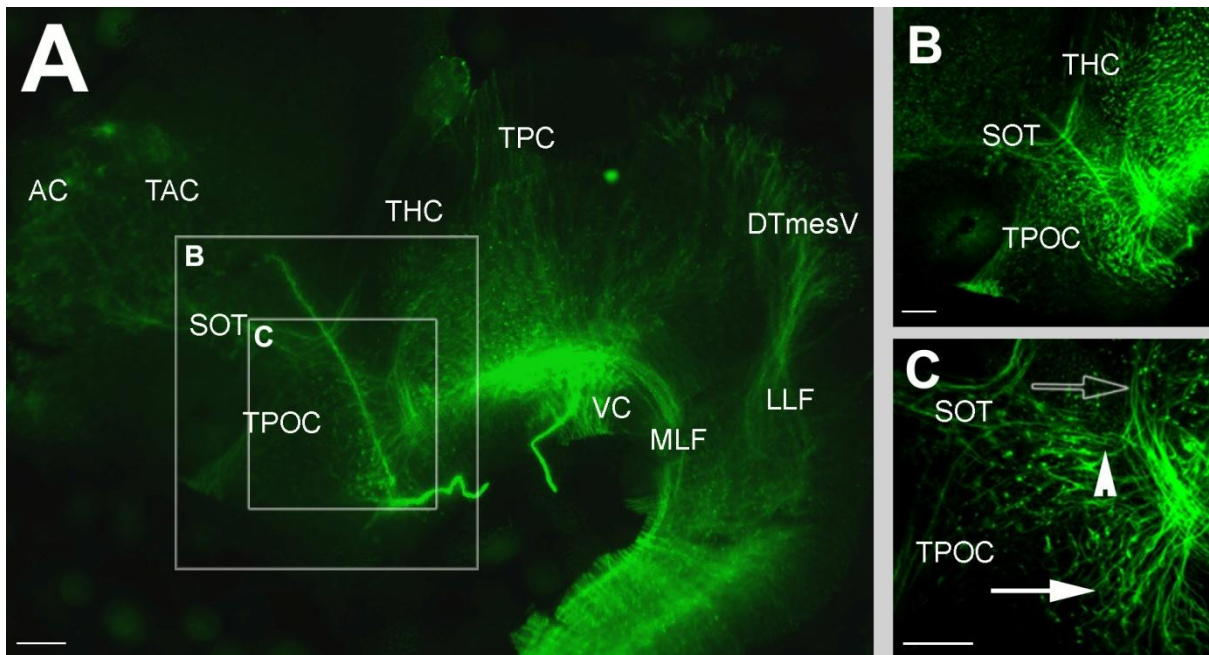
Figure 5.9 Description of the early axon scaffold at stage 25 in the embryonic cat shark brain (lateral view)

A) Overview of the early axon scaffold. All axon tracts are well established. Boxes indicate magnified images in B and C. Scale bar, 200μm. B) Prosencephalon. The TPOC, SOT and THC are well established. Scale bar, 100μm. C) An axon tract appears to be projecting dorsally before turning to project slightly caudally (unfilled arrow). Scattered population of neurones are projecting axons ventrally (filled arrow). The TPOC axons have turned at a right angle and are projecting ventrally (arrowhead). Scale bar, 100μm.

5.3.3 Detailed description of the established early axon scaffold in the embryonic cat shark brain

At stage 25 (Fig 5.9), the early axon scaffold was very well established and most likely, there were follower axons already present using the scaffold. As the early axon scaffold was so well established, it made it difficult to determine the origin of some of these tracts. The MLF originates from neurones located close to the MFB and projects axons caudally along the floor plate into the rhombencephalon (Fig 5.9A). The DTmesV originates from neurones located along the dorsal midline of the mesencephalon and projects axons ventrally to pioneer the LLF that projects into the rhombencephalon (Fig 5.9A). The TPOC originates from neurones located in the rostral hypothalamus and first projects axons dorsally, while remaining ventral to the optic stalk, before turning almost at a right angle to project caudally once it reaches the SOT (Fig 5.9C). The TPOC axons were projecting towards the tract located rostral to the MLF (Fig 5.9C, arrowhead). Therefore, it appeared that the VLT forms predominately from the MLF in the cat shark embryonic brain, with scattered neurones possibly contributing. The dorsal telencephalon contains a large population of neurones that appears to be homologous to the *Xenopus* and zebrafish drc (Fig 5.9A). The drc neurones project axons pioneering the AC that cross the anterior midline, dorsal to the optic stalk. The SOT axons also projected from a population of neurones located within the drc and projects axons ventrally to join the TPOC. An unknown axon tract was projecting in a curved route rostral to the MLF (Fig 5.9C, unfilled arrow). It was unclear where the neurones were located but it would appear most likely they were located ventrally and project axons dorsally before turning to a more caudal route. A population of neurones, which were most likely the scattered neurones, seen at earlier stages (Fig 5.9C, arrow) appeared in a fan shape and projected axons caudally into the MLF. The TPC projects along the MFB (Fig 5.9A); however, it was unclear where the TPC neurones were located. The location of the TPC along

the MFB would suggest the MLF neurones are both diencephalic and mesencephalic by stage 25. The DVDT axon was likely to still be present at stage 25, however labelling of the tract was not clear in the overview image (Fig 5.9A). The THC was projecting axons in the dorsal telencephalon connecting the habenular and the drc (Fig 5.9A). This tract most likely contains SM axons as well as these axons follow the same route. The VC forms across the ventral midline at the MFB, with some axons present in the diencephalon and some in the mesencephalon (Fig 5.9A).

5.4 Comparison of the early axon scaffold in chick and zebra finch

The chick has been studied in detail in chapter 3; therefore, it would be interesting to compare the formation of the early axon scaffold in another species of bird, such as zebra finch.

As the chick and zebra finch are both avian species, the formation of the early axon scaffold was expected to be very similar, which indeed it is (Fig 5.10). The developmental stages of zebra finch have not been described yet. Hence, zebra finch and chick were compared at equivalent stages of early axon scaffold development. The images used were from the same zebra finch embryo (Fig 5.10A, C, E) and was comparable to the chick embryo at HH16.

The main axon tracts: MLF, TPOC, DTmesV and LLF that were present at HH16 in the chick embryonic brain were also present in the zebra finch embryonic brain (Fig 5.10A, C, and E). The MLF neurones were present in the caudal diencephalon, rostral to the MFB (Fig 5.10A, B) and project axons caudally into the rhombencephalon. The MLF appears to form from three populations of neurones in the zebra finch as it does in the chick; the dorsal population (Fig 5.10A, B arrowhead), the central population (Fig 5.10A, B filled arrow) and the ventral population (Fig 5.10A, B unfilled arrow). The MTT was present in the zebra finch diencephalon rostral to the MLF neurones (Fig 5.10A, B, unfilled arrowhead). The TPOC in

zebra finch formed in the same location as it does in chick, the rostral basal hypothalamus and projected axons caudally towards the MLF at the MFB to form the VLT (Fig 5.10C). There were also neurones present that were projecting axons rostrally along the TPOC (Fig 5.10C, arrow) like they do in chick (Fig 3.4B, arrowhead). The DTmesV formed from neurones located at the dorsal midline of the mesencephalon and projected ventrally before turning caudally to pioneer the LLF in the zebra finch (Fig 5.10E) like it does in the chick (Fig 5.10F).

The comparison of the early axon scaffold in the chick and zebra finch embryonic brains would suggest that the early axon scaffold formation was very highly conserved within the species of birds, which was unsurprising.
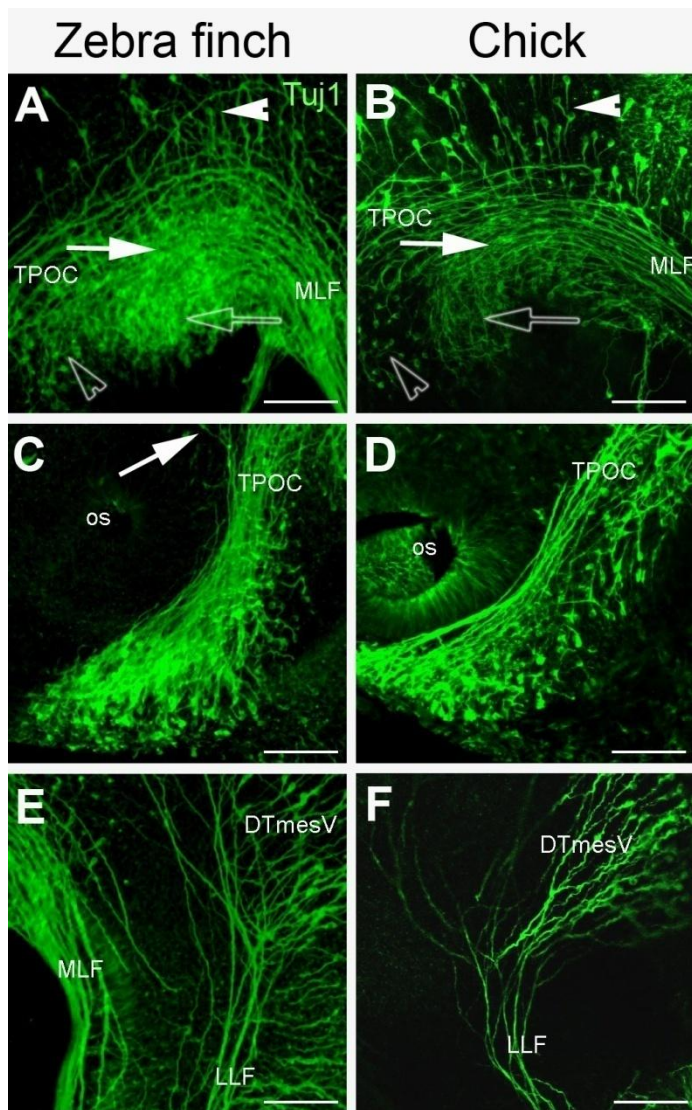
Figure 5.10 Comparison of the main tracts to form the early axon scaffold in chick and zebra finch
Lateral views of the whole mount embryos. Scale bars, 100μm

A-B) The formation of the MLF in the caudal diencephalon. A) Zebra finch, MFB region. The MLF forms from three populations of neurones. One population was located centrally (filled arrow), one dorsal (arrowhead) and one ventral (unfilled arrow) to the MLF axon tract. MTT neurones are located rostrally to the MLF neurones (unfilled arrowhead). B) Chick, HH16, MFB region. The MLF forms from three populations of neurones: the dorsal population (arrowhead), the central population (filled arrow) and the ventral population of neurones (unfilled arrow). The MTT neurones are located rostrally to the MLF (unfilled arrowhead). C-D) The formation of the TPOC in the rostral basal hypothalamus. C) Zebra finch. TPOC neurones project axons caudally towards the MFB. Some neurones located further caudally are projecting axons rostrally (arrow). D) Chick, HH16. TPOC neurones project axons caudally towards the MFB. E-F) The formation of the DTmesV in the dorsal mesencephalon. E) Zebra finch. The DTmesV neurones project axons from neurones located along the dorsal midline of the mesencephalon. These axons project ventrally before turning caudally and pioneering the LLF. F) Chick, HH16. The DTmesV neurones project axons from neurones located along the dorsal midline. These axons project ventrally before turning caudally and pioneering the LLF.

os; optic stalk

5.5 Description of the early axon scaffold in embryonic vertebrate brains

The early axon scaffold in all vertebrates was formed by the differentiation of neurones in specific regions of the embryonic brain, which then project axons to form the scaffold tracts that will be used by later, follower axons. All the vertebrates studied here followed this pattern by setting up a scaffold of longitudinal, transversal and commissural axon tracts.

The early axon scaffold in anamniotes was formed from eight main tracts: MLF, TPOC, DVDT, TPC, SOT, POC, AC and VC (Fig 5.11I, J). The early axon scaffold in amniotes however forms from five main tracts: MLF, TPOC, TPC, DTmesV and LLF, with the PC as the main commissure (Fig 5.11K, L).
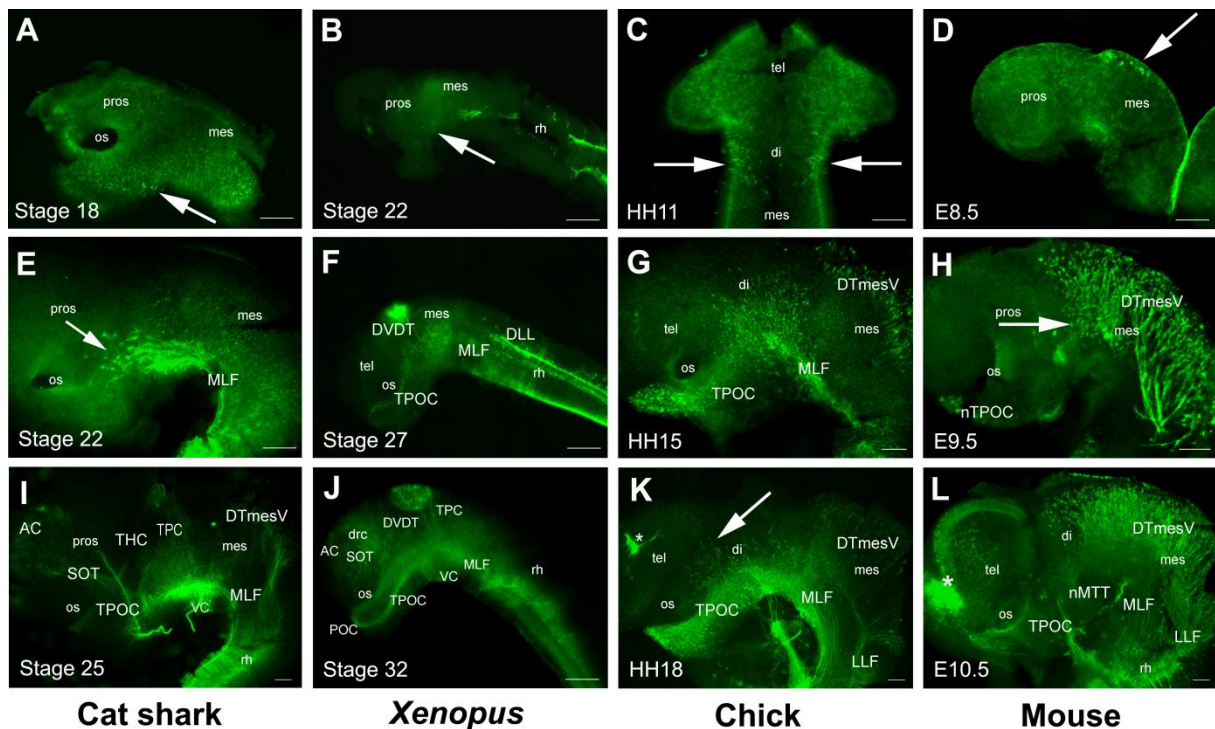
Figure 5.11 Comparison of the early axon scaffold in vertebrate embryonic brains using βIII tubulin antibody Tuj1 (cat shark, chick and mouse) and HNK-1 (*Xenopus*)

A) Cat shark, Stage 18. The first MLF neurones appear at the MFB (arrow). B) *Xenopus*, Stage 22. The first MLF neurones appear at the MFB (arrow). C) Chick, HH11. The first MLF neurones appear rostral to the MFB (arrows). D) Mouse, E8.5. The first DTmesV neurones appear along the dorsal midline of the mesencephalon (arrow). E) Cat shark, Stage 22. The MLF has projected axons into the rhombencephalon and scattered neurones are located rostral to the MLF axon tract (arrow). F) Xenopus, Stage 27. The MLF neurones are projecting axons caudally and the TPOC neurones are present and projecting axons caudally. The epiphysis also contains neurones that pioneer the DVDT. The DLL is also present that forms from neurones located in the rhombencephalon. G) Chick, HH15. The MLF neurones have projected axons caudally towards the MHB. As well as the MLF axon tract, the TPOC and DTmesV neurones have also started projecting axons. H) Mouse, E9.5. The DTmesV is well-formed and beginning to pioneer the LLF at the MHB. There are ventral neurones located in the mesencephalon that are likely to form the MLF (arrow). TPOC neurones are present in the hypothalamus. I) Cat shark, Stage 25. The early axon scaffold is well formed. J) *Xenopus,* Stage 32. The early axon scaffold is well formed. K) Chick, HH18. The early axon scaffold is well established and becoming much more complex. There are scattered neurones located in the diencephalon (arrow). L) Mouse, E10.5. The early axon scaffold is well established. Asterisk in K and L marks the olfactory bulb.

di, diencephalon; mes, mesencephalon; os, optic stalk; pros, prosencephalon; tel, telencephalon; rh, rhombencephalon

Scale bars, 200µm

5.5.1 Comparison of VLT formation

The ventral longitudinal tract formed from the MLF and TPOC was the most conserved tract throughout evolution and was present in all these vertebrates studied here as well as in previous studies. The MLF neurones were the first neurones to appear just rostral to the MFB in cat shark, at stage 18 (Fig 5.11A), *Xenopus*, at stage 22 (Fig 5.11B) and chick at HH11 (Fig 5.11C). Although the mouse differs, in which the DTmesV neurones appear first at E8.5 along the dorsal midline (Fig 5.11D), the MLF neurones arose slightly later at the MFB around E9.5 (Fig 5.11H). The MLF projects axons caudally in a tightly fasciculated bundle along the floor plate towards the rhombencephalon. In cat shark, at stage 22 the MLF axons were projecting caudally to form a tight bundle along the floor plate towards the rhombencephalon, this was still the only axon tract present in the brain apart from scattered neurones (Fig 5.11E, arrow), whereas the other vertebrates have developed more tracts (Fig 5.11F, G, H). In comparison to the chick and mouse, the cat shark and *Xenopus* MLF axon tract forms from a much smaller and densely packed population of neurones. The TPOC forms from neurones located in the rostral basal hypothalamus in cat shark, at stage 23 (Fig 5.8C), *Xenopus* at stage 25 (Fig 5.2C), chick at HH13 (Fig 3.4A) and mouse at E9.5 (Fig 5.11H). The TPOC axons project ventral to the optic stalk and caudally towards the MFB where it reaches the MLF to form the VLT connecting the prosencephalon with the mesencephalon, except in cat shark. The cat shark TPOC axons project along the same path ventral to the optic stalk caudally, however appears not to project directly with the MLF (Fig 5.9C). When the early axon scaffold becomes established, the VLT was the most prominent tract in the cat shark, *Xenopus* and chick embryonic brains.

5.5.2 Formation of the DTmesV

The mouse differs in which the DTmesV was the most prominent tract (Fig 5.11L). The DTmesV was a large structure that formed in the mesencephalon of the cat shark (Fig 5.11I), chick (Fig 5.11K) and mouse (Fig 5.11L) however, it was clearly missing in the *Xenopus* brain (Fig 5.11J). The DTmesV neurones appeared along the dorsal midline and projected axons ventrally before turning caudally at the sulcus limitans to pioneer the LLF. Mouse differs from the other vertebrates where some DTmesV neurones were also located in p1 (Mastick and Easter, 1996). The DTmesV axons will eventually enter the trigeminal nerve (Hunter et al., 2001). The DTmesV in the cat shark appear smaller than the DTmesV present in the chick or mouse. The organisation of the cat shark and chick DTmesV was much clearer than in the mouse and the axons remained well separated from the MLF. By E10.5, the MLF and DTmesV are not clearly separated tracts in the mouse (Fig 5.11L).

5.5.3 Comparison of commissural formation

In anamniotes, commissures were pioneered throughout the formation of the early axon scaffold, whereas in amniotes most commissures formed later. The TPC was another highly conserved transversal tract that forms in the caudal diencephalon marking the MFB. The TPC had already formed in the cat shark by stage 25 (Fig 5.11I) and *Xenopus* by stage 32 (Fig 5.11J) however; it was unclear in the chick embryo (Fig 5.11K) and was not yet present in the mouse at E10.5 (Fig 5.11L). In mouse, the TPC forms at E10.5 (Mastick and Easter, 1996), but was not clear in this figure. The TPC has been described to project from two populations of neurones in anamniotes, one located dorsally and one located ventrally at the MFB (eg, Ross et al., 1992). In amniotes, the TPC neurones were located ventrally and project axons dorsally where the axons will cross the midline (mouse; Mastick and Easter, 1996 and chick; Fig 3.5A, B). In chick there were also neurones located dorsally that appeared to contribute axons to the TPC (Fig 3.5E). It was unclear in the cat shark and *Xenopus* where the TPC

neurones were located. The AC in the dorsal telencephalon and VC at the ventral midline of the rostral mesencephalon were present in both the cat shark (Fig 5.11I) and *Xenopus* (Fig 5.11J). These commissures were not yet visible in the chick or mouse (Fig 5.11K, L). In chick the VC begins projecting axons to the ventral midline at HH17 (Fig 3.5G) but it was unclear in these whole-mount preparations. In mouse the VC forms from circumferential descending axons which cross the ventral midline at E10.5 (Mastick and Easter, 1996). The POC forms early in the *Xenopus* (Fig 5.11J), but it was not clear in the other vertebrates. In chick, some axons cross the midline at HH18 (Fig 3.4D) so the same was likely to happen in cat shark and mouse.

5.5.4 Differences in early axon scaffold formation

As an anamniote, it would be expected that the early axon scaffold structure of the cat shark would be most similar to *Xenopus*. However, the structure was surprising as it shared both features with the anamniotes and the amniotes. Rostrally cat shark was similar to *Xenopus*, consisting of the AC, TAC, SOT, POC and DVDT (Fig 5.11I) that were clearly missing in most amniotes during early axon scaffold formation. Cat shark does form a DTmesV axon tract that was predominantly found in amniotes at early stages. Cat shark and *Xenopus* both contain a large population of neurones (drc) located in the dorsal telencephalon, which will give rise to many tracts; this population was clearly missing in the chick and mouse.

The DVDT that was present in both the anamniotes analysed here (cat shark, Fig 5.11I and *Xenopus*, Fig 5.11F, J) was clearly lacking in both the amniotes (chick, Fig 5.11K and mouse, Fig 5.11L). The DVDT projects axons from neurones located at the epiphysis that project ventrally to join the TPOC, where they turn and project rostrally (Wilson and Easter, 1991). The SOT that forms from the drc in cat shark and *Xenopus* (Fig 5.11I, J) was missing in chick at HH18 (Fig 5.11K) and mouse at E10.5 (Fig 5.11L). The cat shark formed the THC in the dorsal telencephalon (Fig 5.11I) that was not present in the other vertebrates studied here.

The mouse early axon scaffold was not as well organised and the tracts were not as clearly distinguished as the other vertebrates.

5.6 Detailed formation of the MLF in vertebrate embryonic brains

In all vertebrates studied, the MLF appeared to arise from a population of neurones located rostral to the MFB. In cat shark (Fig 5.12A), *Xenopus* (Fig 5.12B) and chick (Fig 5.12C) the MLF arises first, whereas in mouse it arises slightly later after the DTmesV (Fig 5.12D). The MLF neurones in all these vertebrates project axons caudally in a tightly fasciculated tract along the floor plate and into the rhombencephalon. In cat shark, the MLF was formed from two populations of neurones (Fig 5.7B, arrows) and in chick, the MLF forms from three populations of neurones (Fig 5.12G, arrows). In *Xenopus* and mouse, the number of neuronal populations was unclear. In mouse (Fig 5.12H), it was already difficult to determine which neurones will form the MLF as there were already so many DTmesV neurones scattered throughout the mesencephalon. The neurones located most ventrally have not projected any axons yet (Fig 5.12D, H, arrow); by E10.5 (Fig 5.12L), the MLF axon tract has now formed but was not clearly separated from the DTmesV. The MTT which was unclear in the overview image (Fig 5.11K) was present in chick rostral to p3 (Fig 5.12K, arrow) and mouse (Easter et al., 1993). The MTT neurone population was located just rostral to the MLF neurones. Cat shark and *Xenopus* appeared to be missing the MTT.
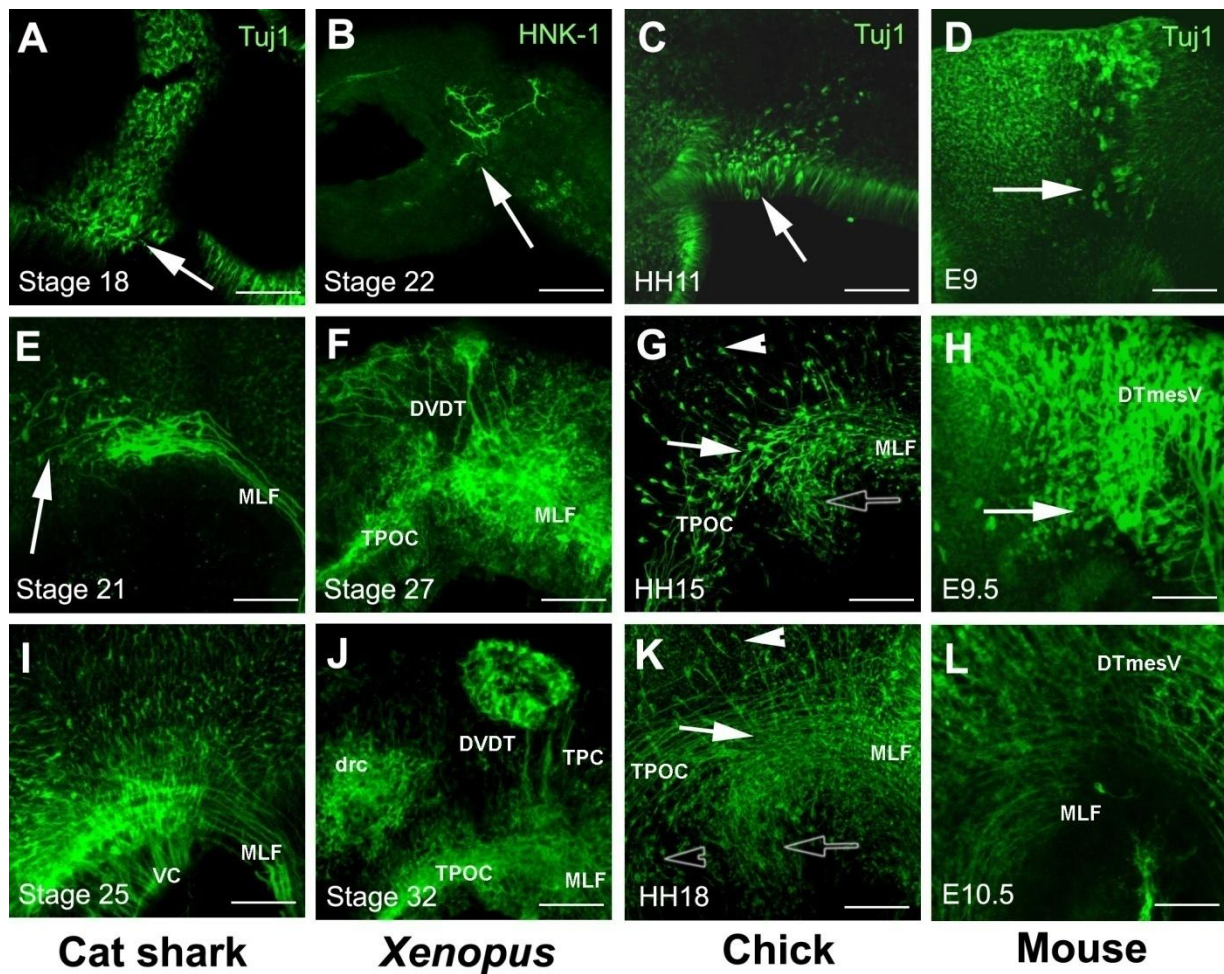
Figure 5.12 Comparison of MLF axon tract formation in the vertebrate embryonic brain
Lateral views of the whole mount embryos. Scale bars, 100μm

A) Cat shark, stage 18. The first MLF neurones appear (arrow). B) *Xenopus,* stage 22. The first MLF neurones appear (arrow). C) Chick, HH11. The first MLF neurones appear (arrow). D) Mouse, E9. The first MLF neurones appear ventral to the DTmesV neurones (arrow). E) Cat shark, stage 21. The MLF neurones have projected axons caudally. Scattered neurones were present, rostral to the MLF (arrow). F) *Xenopus,* stage 27. The MLF axons have projected caudally to form a tightly fasciculated tract. G) Chick, HH15. The MLF was formed from three populations of neurones: central (arrow), ventral (unfilled arrow) and dorsal (arrowhead) H) Mouse E9.5. The number of neurones located ventrally has increased (arrow), however the MLF axon tract does not appear to be established yet. I) Cat shark, stage 25. The MLF was well established. J) *Xenopus,* Stage 32. The MLF was well established. K) Chick, HH18. The MLF was well established. The central (arrow), dorsal (arrowhead) and ventral (unfilled arrow) are still clearly defined. MTT neurones were located rostrally to the MLF neurones (unfilled arrowhead). L) Mouse, E10.5. The MLF was well established, making it difficult to determine where the neurones are located.
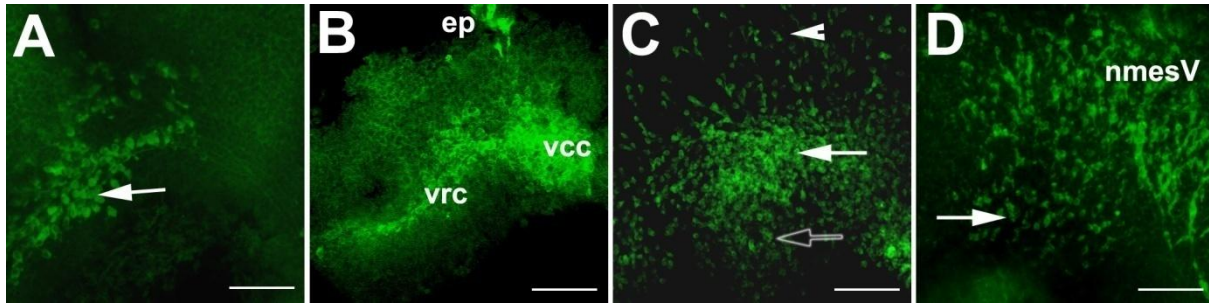
Figure 5.13 Comparison of neuronal clusters using HuC/D antibody
Lateral views of the whole mount embryos. Scale bars, 100µm

A) Stage 23, cat shark. MLF neurones located ventrally (arrow). B) Stage 32, *Xenopus*. The vcc and vrc neurones were located ventrally in the basal plate and neurones were located dorsally in the epiphysis (ep). C) HH17, chick. MLF neurones were located in p1 and p2. The MLF was organised into three populations of neurones: the dorsal population (arrowhead), central population (filled arrow) and ventral population (unfilled arrow). D) E9.5, mouse. MLF neurones were located ventrally (arrow) and DTmesV neurones located throughout the mesencephalon and p1.

5.7 Development of neuronal clusters

In the cat shark (Fig 5.13A) and *Xenopus* (Fig 5.13B) embryonic brains the MLF neurones appeared much more densely packed than in the chick (Fig 5.13C) or mouse (Fig 5.13D). There were fewer neurones in the cat shark and *Xenopus* embryonic brains than in chick or mouse. The neurones in the *Xenopus* brain appeared much larger than in the other vertebrates. In *Xenopus,* there were vrc neurones located throughout the basal diencephalon (Fig 5.13B), with a large population just rostral to the vcc. The vrc will project axons caudally to form the TPOC. These neurones were located more caudal within the hypothalamus than the TPOC neurones in the cat shark, chick or mouse.

5.8 Discussion

For the first time a direct comparison of the early axon scaffold has been described here. An overview highlighting similarities and differences has been shown, as well as a detailed description of the MLF. In addition, the development of the cat shark and *Xenopus* early axon scaffold has been shown in a detailed time series for the first time.

5.8.1 Conservation of axon tracts

As the early axon scaffold has been shown to appear in non-jawed vertebrates (e.g.; lamprey) and jawed vertebrates (e.g.; zebrafish, chick and mouse), it suggests these tracts appeared before the divergence of non-jawed and jawed vertebrates. The axon tracts that were conserved between the non-jawed and jawed vertebrates are the MLF, TPOC, SOT, TPC, POC and SM (Barreiro-Iglesias et al., 2008). The most conserved tract that formed was the VLT that consisted of the TPOC and MLF. The TPOC arises from neurones located in the rostral basal hypothalamus and the MLF arises from neurones located at the MFB. Both axon

tracts project axons caudally within the basal plate. The TPOC axons project ventrally to the optic stalk and connect the prosencephalon and mesencephalon. A VLT like structure also forms in amphioxus suggesting this tract has been highly conserved and maintained throughout evolution (Lacalli et al., 1994).

The location of the MLF neurones has been described in detail in the chick brain. It was more difficult to determine for the other vertebrates (cat shark, *Xenopus* and mouse) an exact location without analysing Pax6 *in situ* hybridisations and correlating that with the position of the MLF neurones. In chick, the MLF neurones were located in p1 and p2 so were strictly diencephalic (Fig 3.3). In mouse (Mastick et al., 1997), and cat shark (Derobert et al., 2002) Pax6 has been shown to mark the MFB boundary. In zebrafish (Hjorth and Key, 2001), the location of the MLF neurones has been shown to be located within the Pax6 expression domain in the diencephalon. It was most likely that the MLF neurones in all vertebrates were positioned within the diencephalon as they were in chick. In mouse, the MLF neurones have been shown to form in p1 and the mesencephalon (Macdonald et al., 1994; Mastick and Easter, 1996), although this has not been shown in relation to Pax6 expression.

The TPC was another highly conserved vertebrate transversal tract. In zebrafish the first axons started projecting at 20hpf (Hjorth and Key, 2002) and was formed from two populations. One population was located dorsally and projects axons ventrally and the other population was located ventrally and projects axons dorsally. In chick, the TPC was also formed from a dorsal and ventral population however they all project axons dorsally to cross the midline, which also occurs in mouse (Mastick and Easter, 1996).
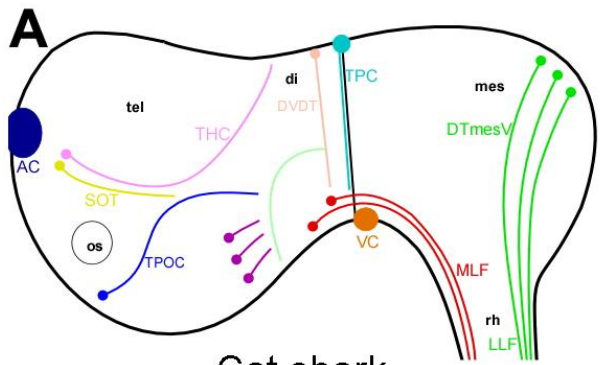
A Cat shark Stage 25
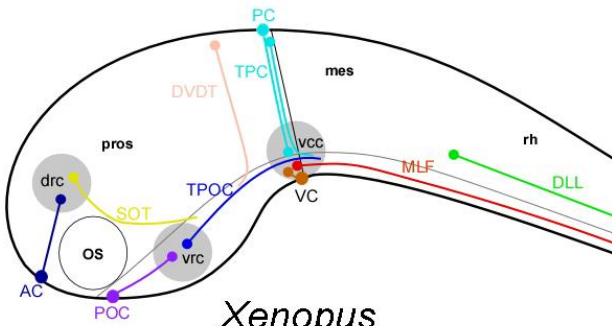
Xenopus Stage 32
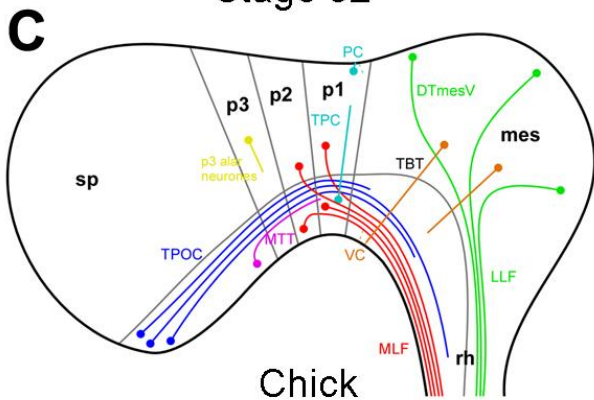
C Chick HH18
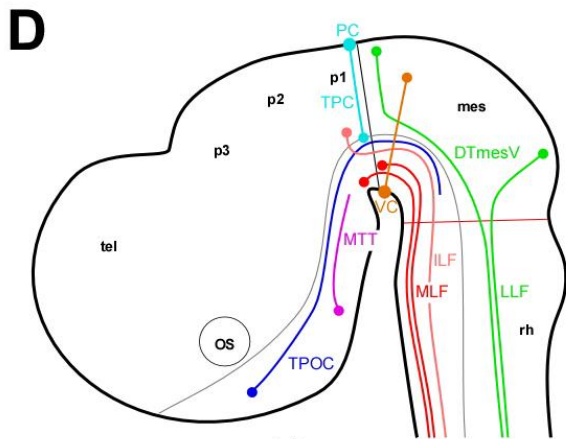
D Mouse E10.5

Figure 5.14 Schematics showing the established early axon scaffold in the vertebrate brains

A) Cat shark, stage 25 (80 somites). B) *Xenopus* stage 32 (26 somites). C) Chick HH18 (31 somites). D) Mouse E10.5 (23-30somites).
Transversal red lines mark prosomeric boundaries. Longitudinal red line marks the alar/basal boundary. The boundaries for cat shark and *Xenopus* are unclear and further *in situ* analysis is required to confirm these boundaries.

di, diencephalon; mes, mesencephalon; pros, prosencephalon; p1, prosomere 1; p2, prosomere 2; p3, prosomere 3; sp, secondary prosencephalon; os, optic stalk; rh, rhombencephalon; tel, telencephalon;

5.8.2 Establishment of the early axon scaffold

Even in a relatively short developmental time of the early axon scaffold formation the brain has increased in complexity and this will continue throughout the development of the embryo. The early axon scaffold is a concept in which initial axons set up a scaffold for later, follower axons however, it was unclear when the early axon scaffold has been set up and then the brain starts making these complex connections. The early axon scaffold in zebrafish has been described as being fully established at 24hpf (Chitnis and Kuwada, 1990; Wilson et al., 1990). In chick the early axon scaffold was established when the four main tracts TPOC, MLF, DTmesV and LLF as well as the TPC were formed at around HH20 (Fig 7.5A). Other tracts present in the chick embryonic brain were the MTT, VC and alar p3 neurones. At HH20, the early axon scaffold has become very complex and due to the number of axons and neurones, it becomes difficult to distinguish new tracts forming. The mouse early axon scaffold was established at E10.5 and in *Xenopus* at stage 32. At stage 25, in comparison to the other vertebrates the early axon scaffold was well established in cat shark but due to the complexity of the axon tracts, this could be slightly later and the early axon scaffold was actually established before this. What was clear was that all vertebrates formed a scaffold that will be used by later follower axons. They all contain a ventral longitudinal tract that was highly important for follower axons even at early stages.

5.8.3 Possible functions of the MLF axon tract

A possible reason for the difference in tract timing could be due to the timing in which the embryos were exposed to their surrounding environment (David McLean: personal communication). So for example, zebrafish and *Xenopus* embryos were exposed a lot sooner than the cat shark or chick embryos that were protected by an egg until hatching. As the MLF has been suggested to be involved in swimming movement and escape mechanisms in zebrafish (Gahtan et al., 2002) it could be the MLF was required first to set up this pathway

to allow the embryo to move. The formation of the MLF first could be lost during evolution as it would not be as important for embryos to move early, like the mouse in which it was protected in the womb like all mammals.

5.8.4 Role of early axon scaffold as pioneering tracts

Even during the formation of the early axon scaffold, tracts were already being used by follower axons for example, the TPOC axon tract was used by the SOT in anamniotes (Anderson and Key, 1999) and the MTT also uses the TPOC in amniotes (Fig 3.4B). The SOT projects ventrally and when it encounters the TPOC axons the SOT axons turn caudally and project along the TPOC (Anderson and Key, 1999). In anamniotes, most of the transversal tracts (SOT, DVDT and TPC) use the VLT. The POC and VC were pioneered from TPOC axons (Anderson and Key, 1999). Therefore, it could be that in fact the VLT forms the initial axon scaffold and many of the other axon tracts are simply follower axons. This would explain why the VLT tract has been so well conserved through evolution.

5.8.5 DTmesV and evolution of the jaw

The DTmesV has been shown to enter the trigeminal nerve and is required for jaw formation in the chick embryonic brain (Chédotal et al., 1995; Hunter et al., 2001), therefore we would expect this tract to appear in all jawed vertebrates. In some vertebrates, it forms as part of the early axon scaffold particularly in mouse where it was the most prominent tract. In most anamniotes the DTmesV has been shown to form later in development, for *Xenopus* at around stage 47, near the time when tadpoles begin to filter feed (Kollros and Thiesse, 1985; Pratt and Aizenman, 2009) and zebrafish at 3-5 days post fertilisation (Kimmel et al., 1985). Among anamniotes, only the cat shark and medaka (Ishikawa et al., 2004) have evidence of this tract during the formation of the early axon scaffold. In mouse, the DTmesV forms first at E8.5 and the axons do not enter the trigeminal nerve until E15.5 (Mastick and Easter, 1996). As it takes a relatively long time for the DTmesV axons to project and enter the

trigeminal nerve, this could be a possible reason for it to form first. During embryonic development in amniotes the formation of the MLF was not as vital for the escape mechanisms that were required in zebrafish (e.g. Gahtan and O'Malley, 2001) and most likely *Xenopus* therefore the MLF would not be needed as early in mouse.

In chick the DTmesV neurones convey information from the jaw muscles to help determine positions of the lower and upper jaws to coordinate biting and mastication (Hunter et al., 2001) therefore we would not expect the lamprey to have a DTmesV structure, as it is a non-jawed vertebrate. Barreiro-Iglesias et al, (2008) suggests the DLL (or DLT) that forms very early in development of some vertebrates along with the MLF in the rhombencephalon (Ross et al., 1992) was equivalent to the DTmesV in mouse and LLF in chick. The DTmesV pioneers the LLF and were present in both the mouse and chick. This would suggest the DLL is not homologous to the DTmesV as its origin was located in the rhombencephalon and it has not been pioneered from another tract. The DLL axons may however contribute axons to the trigeminal nerve.

In mouse, the DTmesV neurones appear in both p1 and the mesencephalon (Mastick and Easter, 1996) whereas it is strictly mesencephalic in chick and cat shark.

5.8.6 Differences in early axon scaffold formation

A major difference in the formation of the early axon scaffold was that some tracts appear during the formation of the early axon scaffold in some vertebrates however, they appear to form later after the early axon scaffold has formed in other vertebrates (Table 5.2).

The MLF was the first tract to form in all these vertebrates except mouse where the DTmesV was the first tract to form and was highly prominent in the brain throughout development. Although in mouse the VLT was not as prominent as it appeared in other vertebrates.

	Sea-lamprey (Barreiro-Iglesias et al., 2008)	Cat shark My results	Zebrafish (Ross et al., 1992)	*Xenopus* My results (Hartenstein, 1993)	Chick My results	Mouse My results (Easter et al., 1993; Nural and Mastick, 2004)
Axon Tract	Developmental stage of appearance					
MLF	E7-E8	Stage 18	16hpf	Stage 22	HH11	E9.5
TPOC	E12	Stage 23	17hpf	Stage 24-25	HH13	E9.5
DTmesV		Stage 23	2-5dpf	Stage 47	HH14	E8.5
TPC	P1	Present at stage 25	20hpf	Stage 30	HH18	E10.5
SOT	E12	Present at stage 25	20hpf	Stage 32		E11.5
POC	P2-P3	Present at stage 25	22hpf	Stage 27		
AC		Present at stage 25	22hpf	Stage 28		
DVDT		Stage 23	22hpf	Stage 26-27		
VC	E8-E9	Present at stage 25	18hpf	Stage 32	HH17	E10.5
MTT					HH15	E10.5
DLL	E7-E8		20hpf	Stage 25		

Table 5.2 Difference in appearance of the early axon scaffold neurones and tracts

The DVDT was present in some vertebrates but not in others. It arises from neurones in the epiphysis and initially projects a single axon ventrally and when the axon encounters the TPOC, the axon turned rostrally. In amniotes such as chick, alligator and mouse, the DVDT was not present, loss of this axon tract could be due to a change in function of the pineal gland from a photosensitive role to a hormonal role (Kardong, 2009).

The SOT and AC arise from neurones located in the drc in the telencephalon of cat shark, zebrafish and *Xenopus*. A similar structure of neurones has not been shown in amniotes. The SOT has been shown to arise later in chick (Ichijo and Kawabata, 2001) and mouse at E11.5 (Nural and Mastick, 2004). Emx3 was expressed by the drc neurones and required for differentiation of the drc neurones in zebrafish and has been identified in *Xenopus tropicalis* (Viktorin et al., 2009). Therefore suggesting the Emx3 gene was not lost during the divergence of the tetrapods but lost only in some tetrapods (Derobert et al., 2002). This maybe a possible reason why there were no drc neurones present in the amniote brain, as Emx3 was not present for the differentiation of these neurones.

The POC forms early in zebrafish and *Xenopus* and was used as a scaffold by axon projections from the retina guiding them to the rectum (Easter and Taylor, 1989; Wilson et al., 1990). The POC was likely to form across the anterior midline in chick (Fig 3.4G).

The MTT axon tract appeared in the chick and mouse embryonic brains, but not in cat shark or *Xenopus* embryonic brains. In mouse, the MTT neurones were located rostral to p3 in the basal plate and form the VLT along with the MLF (Mastick and Easter, 1996), which was different to what was shown in chick as the MTT followed the TPOC axon tract. In mouse, the TPOC was regarded as an alar tract that was continuous with the DTmesV (Mastick and Easter, 1996) whereas in chick it has been shown to be a basal tract along the dorsal edge of Nkx2.2 expression (Shimamura et al., 1995 and Fig 6.2B) forming the VLT along with the MLF.

In the amniote embryonic brain, fewer axon tracts formed during early axon scaffold development than in the anamniote brain. This could be due to a difference in developmental time and the complexity of higher amniote vertebrates. It may have been advantageous during evolution to have fewer tracts involved in the guiding of follower axons and provide less room for error. This could also be due to the transition from land to water. As chick and mouse are land based, they initially form fewer early axon scaffold tracts than *Xenopus* or alligator that are land and water based.

The size of the forebrain was notably larger in the cat shark compared to *Xenopus,* another anamniote. This has been suggested to be due to an increased importance of olfactory information (Kardong, 2009) as well as increasingly complex behaviours and muscle control.

Understanding the anatomy and formation of the early axon scaffold in these vertebrates will help analyse molecular interactions that occur within the brain. Molecular interactions between signalling molecules, transcription factors and axon guidance molecules are important for the correct positioning of the early axon scaffold neurones and axons.

Chapter 6

Cell fate specification of the Medial Longitudinal Fascicle

6.1 Introduction

Investigations into dorsoventral patterning in the spinal cord have shown signalling molecules and transcription factors are required for the specification of neurones at the correct time, in the correct location (Briscoe et al., 2000; Ericson et al., 1997 and introduction 1.2.3). The midbrain arcs appear to constitute a similar dorsoventral patterning mechanism in the ventral mesencephalon (Agarwala et al., 2001), suggesting the organisation and differentiation of the early axon scaffold could be controlled by similar molecular mechanisms to ensure correct development.

6.1.1 The medial longitudinal fascicle (MLF)

The MLF neurones first arise at HH11 in the embryonic chick brain, rostral to the MFB in the alar and basal plates of p1 and were strictly diencephalic (Fig 3.2A, Fig 3.3). The MLF axons project caudally towards the rhombencephalon in a tightly fasciculated tract along the floor plate. While the anatomy has now been described in depth, little was known about the specification of the MLF neurones or the control of their outgrowth. As the MLF was the first axon tract to arise

in the brain and was highly conserved (Fig 5.12), with a role in the visual and movement functions of the zebrafish larvae (Gahtan and O'Malley, 2003), this makes it an interesting model for neuronal specification in higher vertebrates.

6.1.2 Homeodomain transcription factors

The homeobox genes Emx2 and Sax1 have already been shown to be involved in the formation of the early axon scaffold, in particular the MLF (Schubert and Lumsden, 2005). When Sax1 was overexpressed using the CAβ-Sax1 construct, the MLF axon tract appeared enlarged and the axons were no longer organised in a tight bundle. The TPC transversal tract was reduced when Sax1 was overexpressed (Schubert and Lumsden, 2005). Overexpression of Sax1 also had an effect on the expression of Emx2 and Six3 in which their expression was lost from the ventral mesencephalon. The CAβ-VP16Sax1 construct replaces the transrepressor domain of Sax1 and diminishes the function of Sax1 by acting as a transactivator of Sax1 target genes therefore acting as a dominant-negative regulator. This resulted in the MLF becoming diminished and leads to upregulation of Emx2 expression in the dorsal mesencephalon, while the expression of Six3 was normal. As Sax1 has an effect on Emx2 and Six3 expression, this provide evidence that homeobox genes in the ventral mesencephalon can regulate each other (Schubert and Lumsden, 2005) like specific pairs of class I and class II homeobox genes in the spinal cord. While cross-repulsive expression in the spinal cord regulates gene expression in the progenitor cells, Sax1 and Emx2 were expressed in the mantle layer, presumably by differentiating neurones (Ahsan et al., 2007).
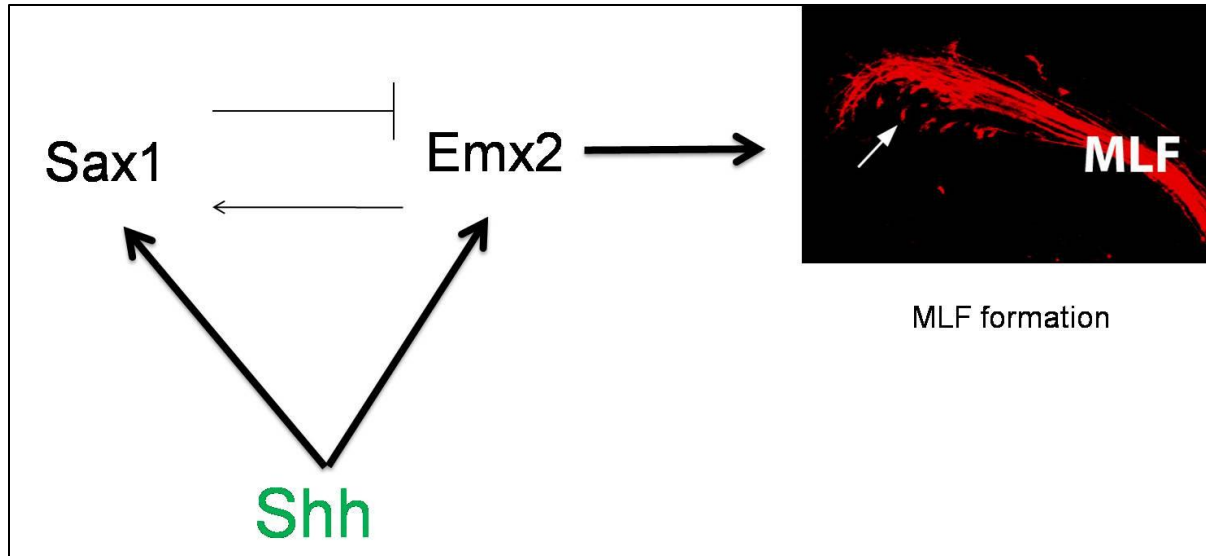
Figure 6.1 Regulation of MLF formation by Shh

Shh expression by the floor plate regulates expression of homeobox genes Sax1 and Emx2. Regulations between these genes are involved in the formation of the MLF, which forms from neurones located at the MFB (arrow, DiI image, MLF axons labelled red). This is a working hypothesis that will require further investigation and finding genes involved in specification will begin unravelling the problem (Schubert and Lumsden, 2005).

Transcription factors control differential gene expression and hence are key players in the specification of cell fate. As a first attempt to find genes involved in MLF specification, transcription factors known to be expressed within the diencephalon where the MLF neurones were located will be analysed. In a separate approach, candidate genes involved in the specification of neuronal cells into a MLF fate will be identified using microarrays.

6.2 Analysis of homeodomain transcription factors expressed in the mesencephalon

The homeobox genes Emx2, Nkx2.2, Sax1 and Six3 have been shown to be expressed in the chick ventral diencephalon and mesencephalon (Ahsan et al., 2007; Schubert and Lumsden, 2005). To test whether the expression of these genes correlates specifically with MLF neurones, *in situ* hybridisation for these genes was combined with immunohistochemistry using Tuj1 to visualise the axons and neurones (Fig 6.2). Emx2 and Six3 were expressed in a longitudinal stripe that appeared to overlap with the position of the MLF axon tract (Fig 6.2A, D). Nkx2.2 was expressed as a longitudinal stripe throughout the prosencephalon and mesencephalon (Fig 6.2B), along the dorsal edge of the TPOC marking the alar-basal boundary (Shimamura et al., 1995). The expression of Sax1 was not as a longitudinal stripe like the other transcription factors but clustered around the MFB (Fig 6.2C, arrowhead). Sax1 expression overlaps with Emx2 and Six3 and appears to be closely associated with the location of the MLF and TPC neurones. What was clear was that the expression of Emx2, Nkx2.2, Sax1 and Six3 was not spotty and therefore did not correlate specifically with the MLF neurones. These genes were likely to be regional markers that affect the MLF as a secondary effect.

The homeobox genes studied here were first expressed much later in development than the appearance of the first MLF neurones at HH11. This would also suggest these homeobox genes were not involved in the cell-fate determination of the MLF. The focus of gene expression studies have been in the mesencephalon, however as MLF neurones have been shown to be strictly diencephalic (Fig 3.3), patterning of transcription factor expression needs to be investigated in this region. Sax1, Emx2, Six3 and Pax6 were expressed in p1 (Schubert and Lumsden, 2005).
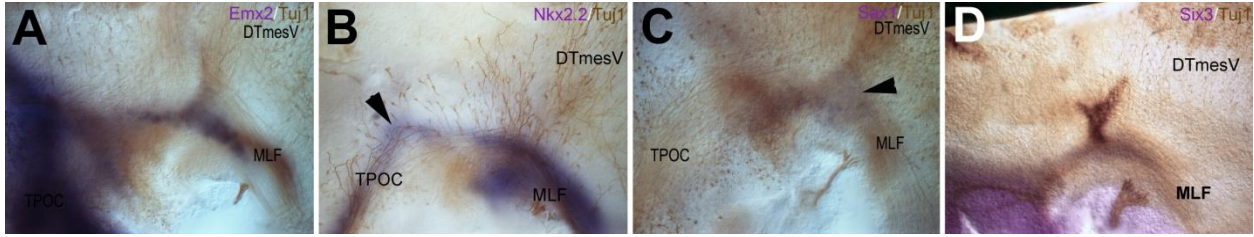
Figure 6.2 Expression of transcription factors ventrally around the MFB

A) HH18, Emx2 is expressed throughout the telencephalon and as a single stripe in the diencephalon and mesencephalon. B) HH18, Nkx2.2 is expressed as a longitudinal stripe throughout the telencephalon, diencephalon and mesencephalon. There is a curve in the expression (arrow) at the ZLI (p2/p3 boundary). C) HH18, Sax1 expression is clustered within the basal plate around the MFB (arrowhead). C) HH18 Six3 is expressed in a single longitudinal strip in the ventral mesencephalon and diencephalon.
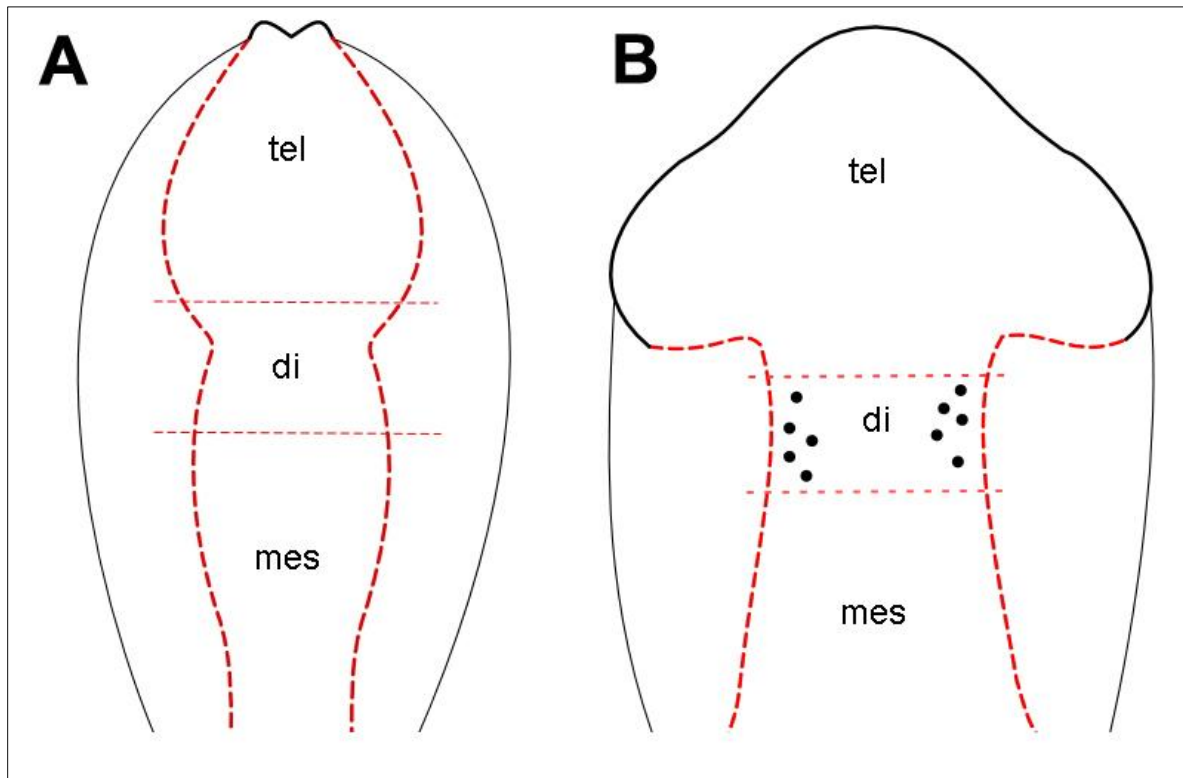
Figure 6.3 Preparation of (A) HH9 and (B) HH11 chick embryonic brains

Dashed red line indicates where the embryo was dissected. Tissue was removed from around the MFB, at HH9 and HH11. The mesenchyme was removed from around the outside of the rostral neural tube. The dorsal region around the MFB was removed exposing the ventral region. The rostral and anterior brain was removed around the MFB (more details 2.9.1).

6.3 Microarray analysis of the midbrain-forebrain boundary (MFB)

Microarrays allow the analysis of large numbers of genes to be analysed at the same time to detect differences in gene expression when comparing tissue samples. This method could enable genes specifically expressed by the MLF neurones to be identified by taking tissue only from the ventral region around the MFB where the MLF neurones are located at different stages (Fig 6.3).

At HH9, there were no neurones present in the chick embryonic brain and it was assumed the MLF neurones would not be specified before this stage. The first neurones appear at HH11 so by comparing these stages for gene expression, candidate genes involved in the cell-fate determination of the MLF neurones should be identified.

In collaboration with Dr David Chambers (King's College London), a microarray analysis of HH9 and HH11 ventral diencephalon/mesencephalon was performed. The total RNA extracted from the brain tissue (Fig 6.4 and Fig 6.5) was used to generate cDNA fluorescently labelled probes. The cDNA probes were hybridised to complementary sequences on the GeneChip Chicken Genome array (Affymetrix), which contains 32,773 transcripts corresponding to 28,000 chick genes. Following hybridisation of the two differently labelled cDNA populations the fluorescence levels for each gene were compared to highlight genes whose expression is down-or-upregulated between HH9 and HH11.
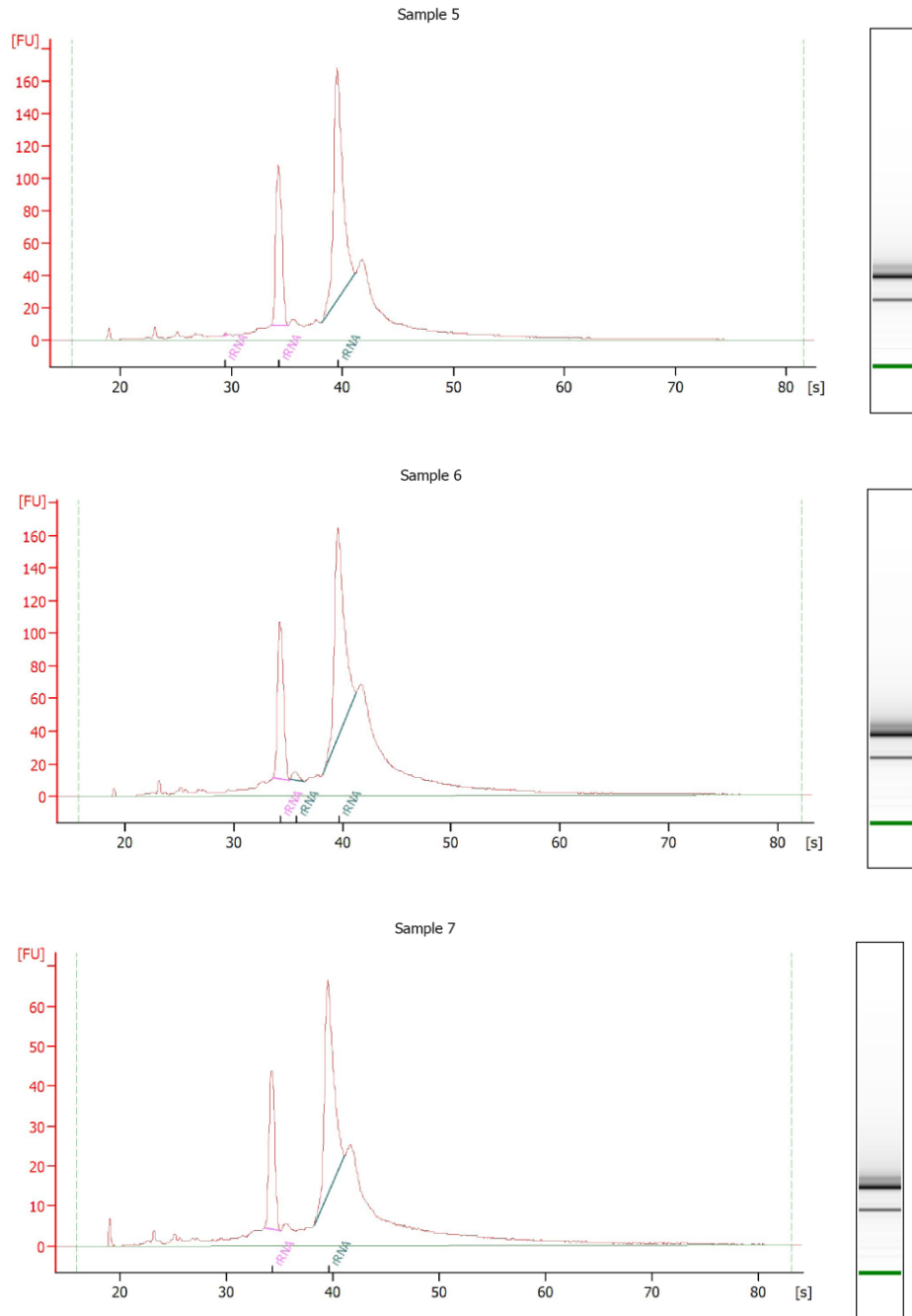
Figure 6.4 Graphs to show quality of RNA from HH9 samples to be used for microarray

Clean bands on the gels show RNA was present in the sample.
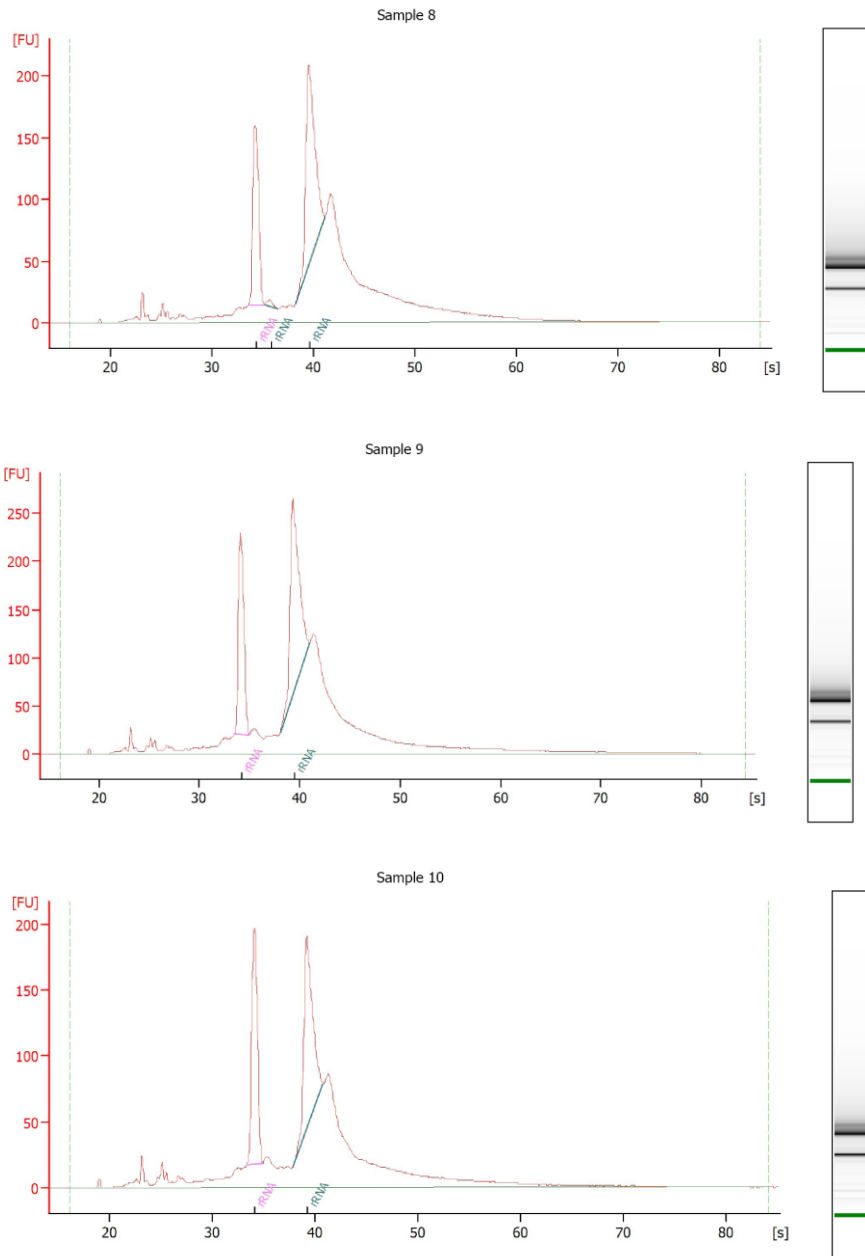
Figure 6.5 Graphs to show quality of RNA from HH11 samples to be used for the microarray

Clean bands on the gels show RNA was present in the sample.
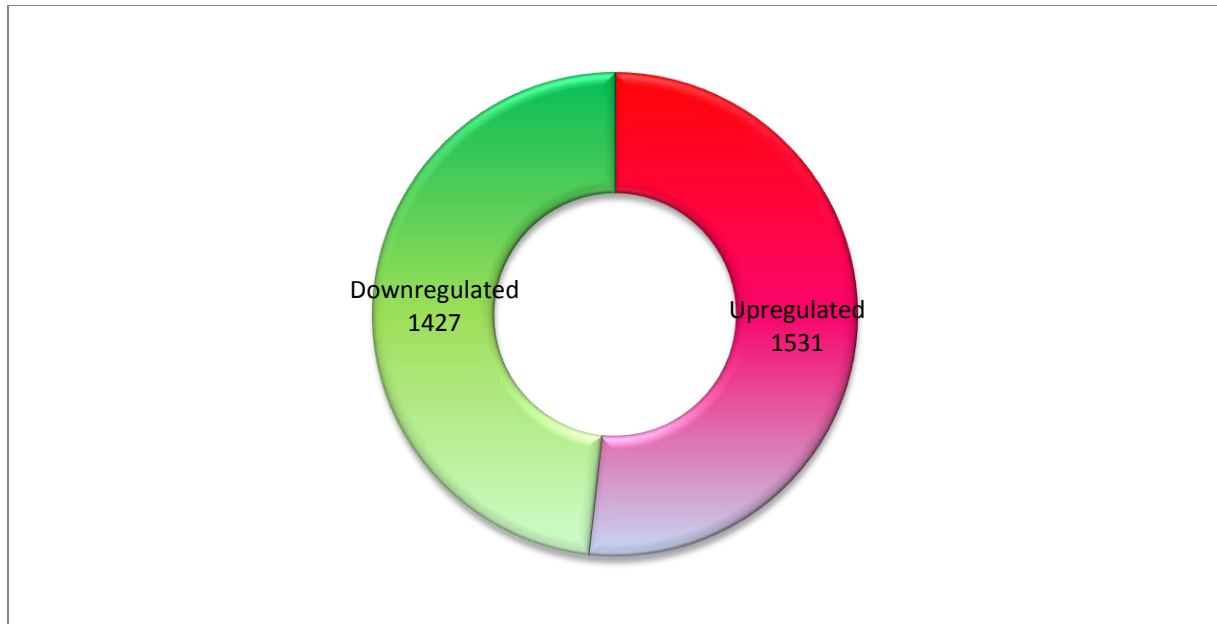
Figure 6.6 Doughnut representing the total number of genes upregulated and downregulated in this microarray

1427 genes were downregulated (green) and 1531 genes were upregulated. The key candidate genes were located above 2-fold (dark red 335 genes) and below 0.5-fold (dark green 352 genes).
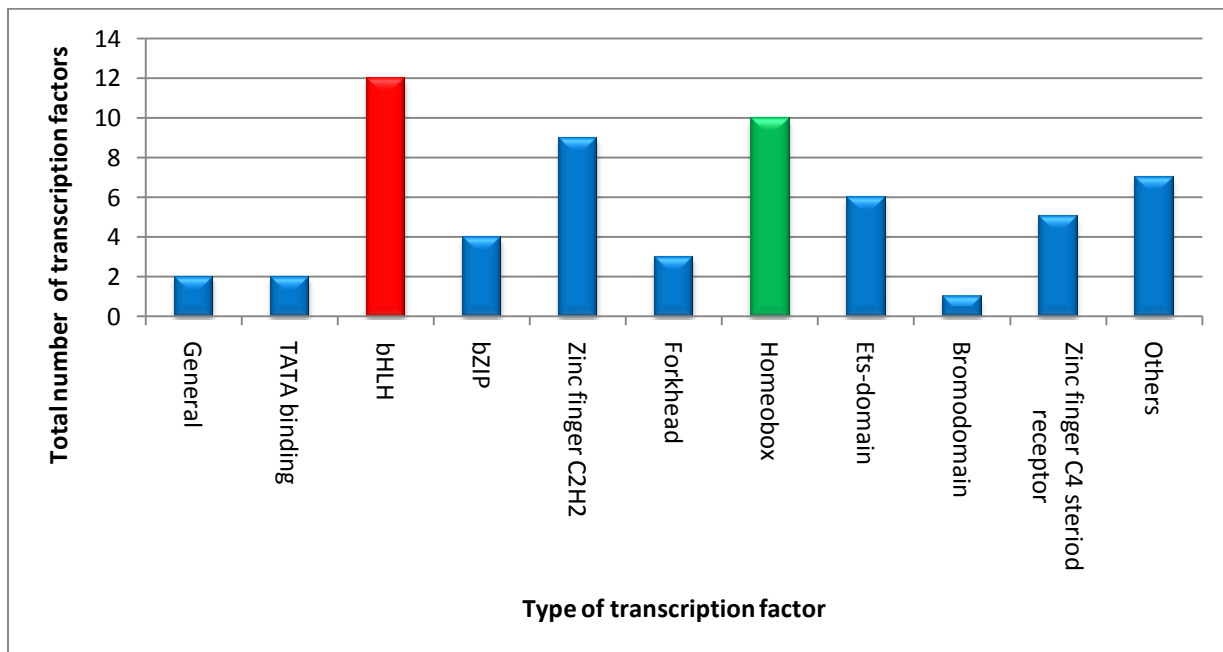
Figure 6.7 Number of different types of transcription factors that were upregulated

A total of 61 transcription factors were upregulated (3.98%).

In total, 2958 genes were detected to have a change in gene expression between HH9 and HH11 in the region of the embryonic chick brain tissue analysed. In total, 1531 genes were upregulated and 1427 genes were downregulated. The best candidate genes for involvement in MLF specification were those upregulated more than 2-fold (335 possible candidates, Fig 6.6).

6.3.1 Types of genes upregulated

As well as identifying specifically expressed genes, the microarray will also pick up genes that were more generally switched on at HH11 in the region around the MFB. Many of the genes identified in the microarray, were patterning molecules and transcription factors that have already been shown to be involved in differentiation of neurones. Surprisingly the highest fold change in the microarray was that of haemoglobin, epsilon 1 (HBE1) and many of the other higher fold change were genes belonging to the haemoglobin family or were markers for vasculogenesis (Table 6.3).

To confirm the differential expression of the genes identified in the microarray screen, the expression patterns of a range of candidate genes by *in situ* hybridisation were analysed. This analysis included a range of transcription factors, signalling molecules and other possible candidates with the aim of identifying a marker for the MLF neurones, which may potentially be involved in cell-fate determination. The signalling molecules analysed were EphA7, Wnt2b, Wnt5a, FGF3 and FGF18.

As transcription factors have already been shown to be involved in the formation of the MLF (Schubert and Lumsden, 2005) and the specification of neurones in the ventral neural tube (Agarwala et al., 2001; Ericson et al., 1997), these genes were of most interest. Although a very small percentage (3.98%) of the genes upregulated in the microarray were transcription factors.

Most of the transcription factors belonged to the bHLH and homeobox families (Fig 6.7 red and green bars respectively). The transcription factors analysed by *in situ* hybridisation were neural bHLH genes NeuroD, Cash1 and Hes5, homeobox genes Dlx5, Gbx2 and Satb1 and other transcription factors Zic1, TFAP2α and Tcf4.

Other genes analysed by *in situ* hybridisation were binding molecules, receptors or genes that were known to be expressed in the ventral mesencephalon as shown by previous studies. Protogenin for example was an interesting gene to investigate as it encodes a type I transmembrane member of the DCC/Neogenin family receptor that is likely to interact with Netrins (discussed further in Chapter 7). As a large number of blood markers were upregulated, Fli1 and Cldn5 were chosen to begin investigating the role of these blood markers and analyse the formation of the vascular system around the neural tube in the chick embryo.

In the following image panels, (Figures 6.9-6.13) expression of each gene was shown throughout the entire chick embryo at HH9, HH11 and HH14 and at higher magnification of the brain vesicles. Anatomical landmarks for the *in situ* images were indicated by schematic representation (Fig 6.8).

Accession number	Gene	Gene title	Fold change	Function
BU265081	Wnt2b	Wingless-type MMTV integration site family, member 2B	127.3	Secreted signalling factor
BX933381	Satb1	Special AT-rich sequence binding protein 1	29.33	Homeobox transcription factor
NM_205504	Cx40/Cx42	Connexin 40	24.11	Gap junction transmembrane channel
X53701	CRABPI	Cellular Retinoic Acid Binding Protein 1	16.18	Lipid binding protein/transporter
NM_204412	ASCL1/Cash1	achaete-scute complex homolog 1	7.47	bHLH transcription factor
BX930360	TAC1	Tachykinin precursor 1	5.44	Neuropeptide
BU338683	Zic1	Zinc finger protein of the cerebellum 1	5.372	Transcription factor
BU209507	EphA7	Ephrin type-A receptor 7	4.826	Receptor
NM_204887	Wnt5a	Wingless-type MMTV integration site family, member 5A	4.573	Secreted signalling factor
NM_204190	Mab21L2	Mab21-like 2	4.57	Developmental protein
NM_205094	TFAP2α	Transcription factor AP-2 alpha	4.449	Transcription factor
BU199562	NeuroD1	Neurogenic differentiation 1	3.617	bHLH transcription factor
L11264	Nrg1	Neuregulin1	3.567	Signalling
BU227549	Hes5	Similar to hairy and enhancer of split 5	3.452	Anti-neural bHLH transcription factor
NM_204159	Dlx5	Distal-less homeobox 5	3.39	Homeobox transcription factor
AJ719959	Fli1	Friend leukaemia virus integration 1	3.214	Ets-domain transcription factor
BU263942	FGF3	Fibroblast growth factor 3	3.12	Secreted signalling factor
BU326284	Jag2	Jagged 2	3.103	Calcium ion binding
NM_204714	FGF18	Fibroblast growth factor 18	3.044	Secreted signalling factor
ENSGALT00000006954	PRTG	Protogenin	2.903	Receptor
BU112702	Tcf4	Transcription factor 4	2.888	Transcription factor (TATA binding)
NM_204201	Cldn5	Claudin 5	2.637	Cell-cell adhesion
CR523152	SRGAP1	SLIT-ROBO Rho GTPase activating protein 1	2.61	GTPase binding
BX930128	PlxDC2	Plexin domain containing 2	2.492	Transmembrane protein
NM_205068	Gbx2	Gastrulation brain homeobox 2	2.35	Homeobox transcription factor
NM_204171	CHRDL1	Chordin-like 1	1.799	BMP antagonist
AF364045	Slit2	Slit homolog 2 protein	1.614	Axon guidance molecule
	SRGAP3	SLIT-ROBO Rho GTPase activating protein 1		GTPase binding

Table 6.1 Upregulated genes analysed by *in situ* hybridisation

SRGAP3 was not upregulated in the microarray, but was suggested to be expressed by differentiating neurones (Bacon et al., 2009) SRGAP3 was analysed by *in situ* hybridisation as a control to ensure this gene was not upregulated (as it was not identified in the microarray).
Many of the plasmids for these genes were kind gifts from other laboratories (table 2.3).
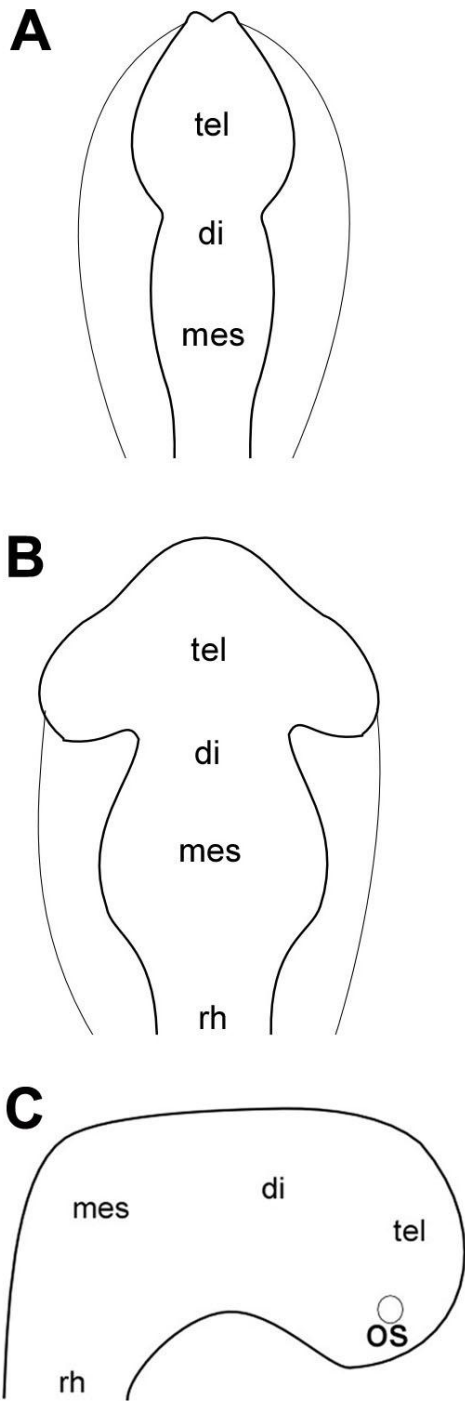
Figure 6.8 Location of brain vesicles as shown by *in situ* hybridisation images

A) HH9 B) HH11 C) HH14. In the higher magnification images, the neural tube has been dissected to view expression inside the brain. Therefore, expression may appear different due to removal of ectoderm and mesenchyme.

di, diencephalon; mes, mesencephalon; os, optic stalk; rh, rhombencephalon; tel, telencephalon;
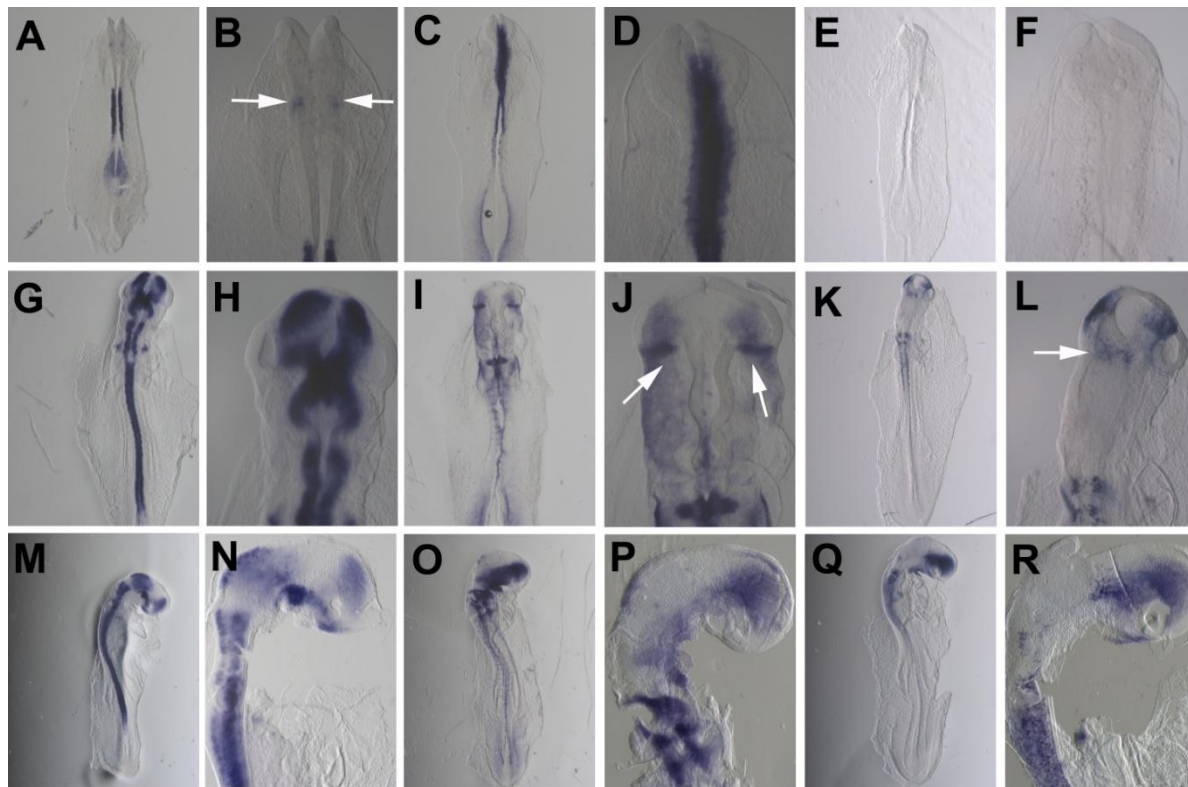
Hes5   TFAP2a   CRABPI
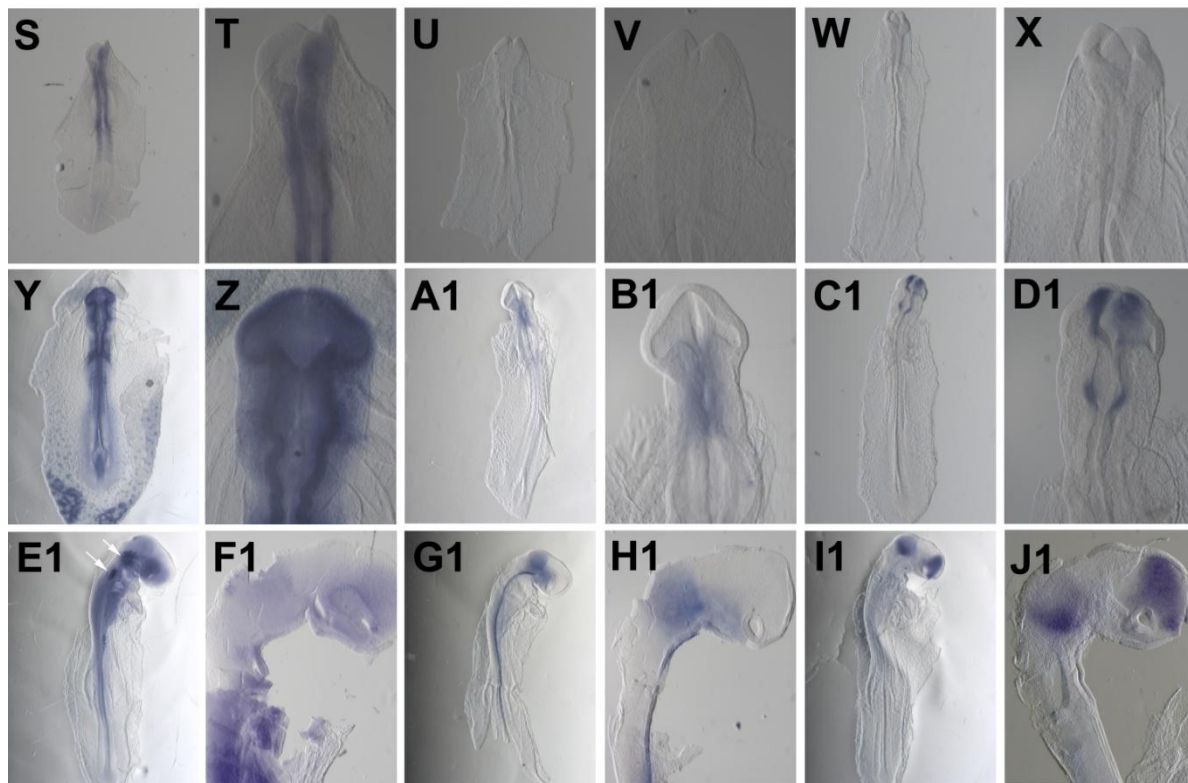
NeuroD1   Cash1   Jag2

Figure 6.9 Expression pattern of genes upregulated in microarray

A-B) HH9 Hes5 expression in the mesencephalon (arrow) and in the spinal cord. G-H) HH11$^+$ Hes5 level of expression has increased throughout the neural tube, excluding the isthmus and optic vesicles. M-N) HH14 Hes5 expression is throughout the alar and basal plates of the mesencephalon and telencephalon but is only expressed in the basal plate of the diencephalon. There is no expression in the isthmus and expression is strong in the rhombencephalon.

C-D) HH9 TFAP2α there is very strong expression throughout the neural tube and no expression in the mesenchyme. I–J) HH11$^+$ TFAP2α expression is becoming more specific. There is expression throughout the mesenchyme and strong patches under the optic vesicles (arrows). Expression is in the presumptive neural crest cells (Shen et al, 1997). In the neural tube, expression is now restricted to the optic vesicles and missing from the diencephalon and mesencephalon. O-P) HH14 TFAP2α expression is located ventrally along the neural tube and in the telencephalon. There is some expression in the roof plate of the caudal mesencephalon.

E-F) HH9$^-$ CRABPI there is no expression in the embryo. K- L) HH11$^+$ CRABPI there is expression rostral to the MFB. The expression is spotty and most likely correlates with the MLF neurones (arrow). There is expression around the optic vesicles and spotty expression further caudally throughout the rhombencephalon. Q-R) HH14 CRABPI expression remains in the diencephalon and rhombencephalon with a spotty appearance.

S-T) HH9 NeuroD there is expression throughout the neural tube, but is missing in the caudal most region of the embryo. Y-Z) NeuroD there is expression is throughout the neural tube and in the extraembryonic membrane. E1-F1) HH14 NeuroD expression is still high throughout the neural tube. On the outside of the neural tube, there are two regions of spotty expression (arrows).

U-V) Cash1 there is no expression. A1-B1) Cash1 expression is located in the diencephalon and mesencephalon. G1-H1) HH14 Cash1 there is expression throughout the ventral neural tube, expression is present in the mesencephalon and ventral diencephalon.

W-X) Jag2 there is no expression. C1-D1) Jag2 expression in the rostral brain is highest in the telencephalon and caudal mesencephalon. I1-J1) HH14 Jag2 expression is restricted to the caudal mesencephalon as an expression gradient and the very rostral telencephalon.
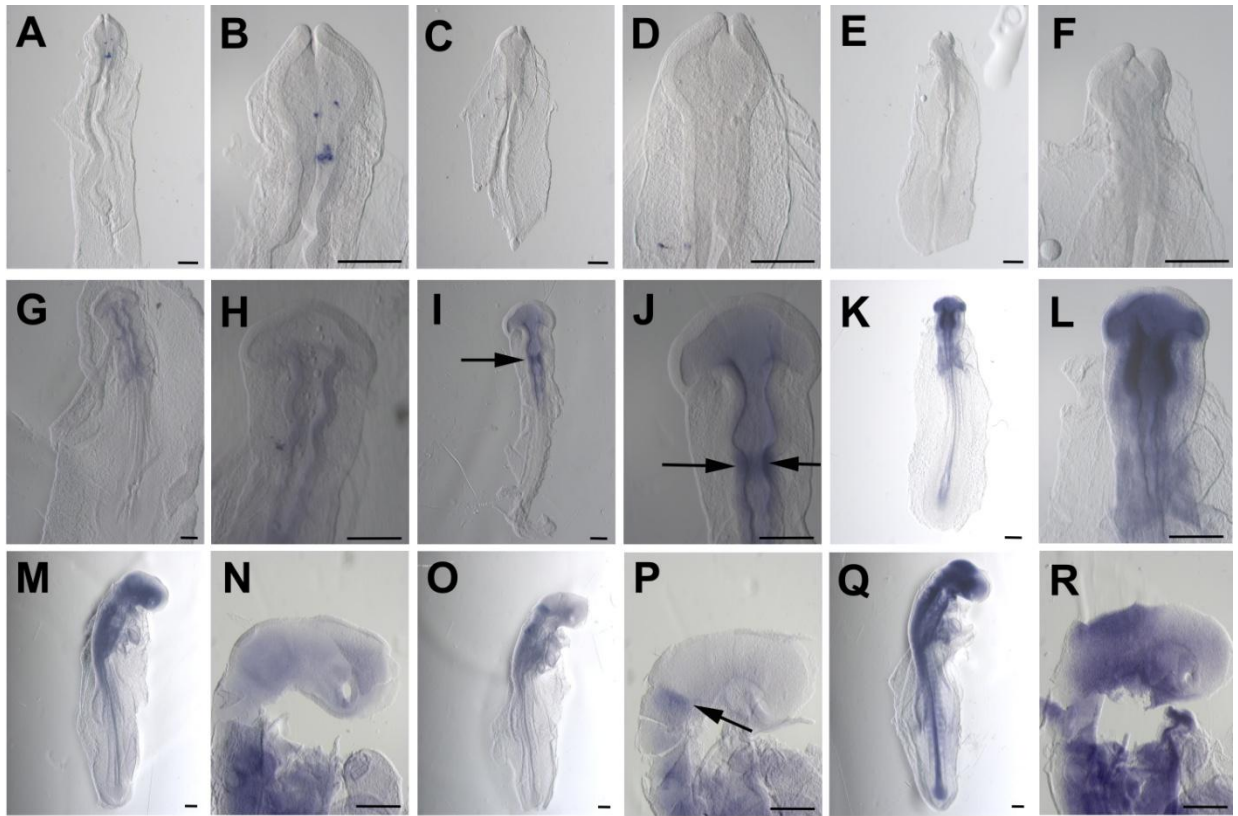
Dlx5

Gbx2

Satb1
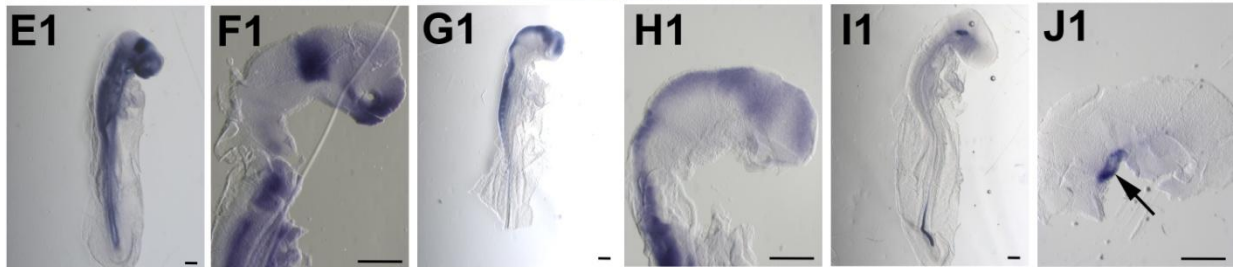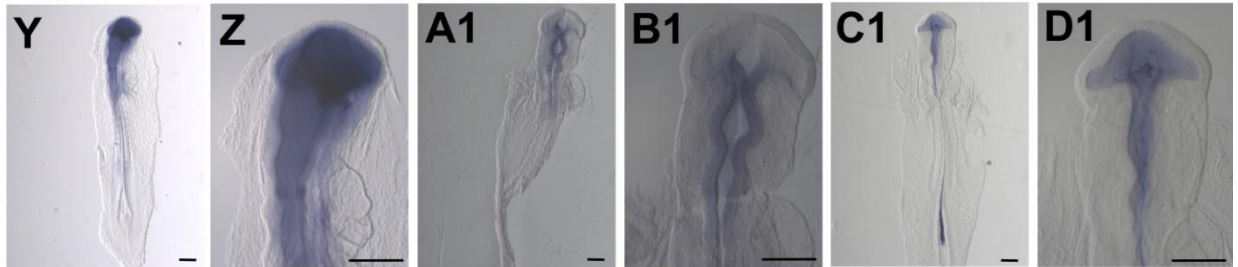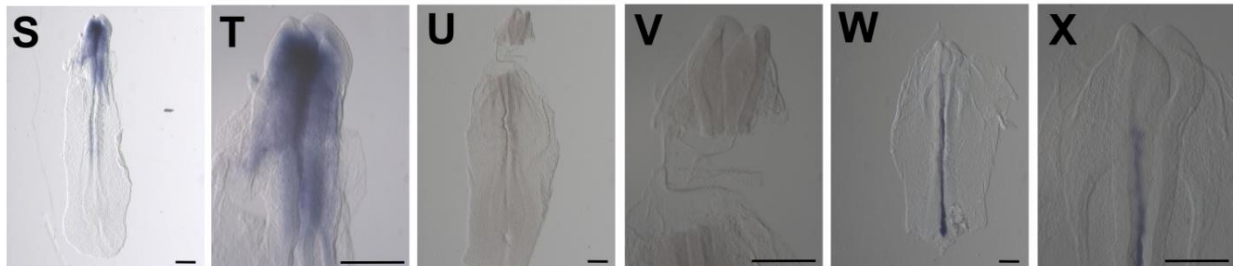
Tcf4

Zic1

Chordin

Figure 6.10 Expression pattern of genes upregulated in microarray

A-B) HH9 Dlx5 there is no specific expression (some unspecific spots present on the outside of the neural tube). -H) HH11$^+$ Dlx5 there is weak expression throughout the brain. M-N) HH14 Dlx5 expression continues throughout the neural tube and is weak throughout the brain.

C-D) HH9 Gbx2 there is no expression. GI-J) HH11$^+$ Gbx2 expression is throughout the rostral neural tube, with expression highest in the MHB (arrows). O-P) HH14 Gbx2 expression is still throughout the neural tube and in the brain expression is restricted to the rhombencephalon marking the MHB (arrow).

E-F) HH9 Satb1 there is no expression. K-L) HH11$^+$ Satb1 there is expression throughout the neural tube. Expression is highest around the diencephalon and mesencephalon. Q-R) HH14 Satb1 expression is high throughout the neural tube. Expression is highest in the dorsal and ventral mesencephalon. There is also expression in the ventral diencephalon and weak expression in the telencephalon.

S-T) HH9 Tcf4 expression is high throughout the brain. Y-Z) HH11 Tcf4 expression becomes highest around the MFB. E1-F1) HH14 Tcf4 expression is throughout the whole neural tube. In the brain, expression has become strongest in the alar plate of the diencephalon and rostral telencephalon.

U-V) HH9$^-$ Zic1 there is no expression (brain is broken caudal to the MFB). A1-B1) HH11 Zic1 is expressed throughout the brain, excluding the telencephalon. G1-H1) HH14 Zic1 expression is restricted along the dorsal midline and in the prosencephalon.

W-X) HH9 Chordin is expressed in the notochord under the neural tube, excluding the prosencephalon. C1-D1) HH11 Chordin expression is clear in the notochord; however, expression in the brain appears to be trapping. I1-J1) HH14 Chordin expression is located ventrally (arrow), highest in the notochord and in the ventral mesencephalon.

Scale bars, 500µm
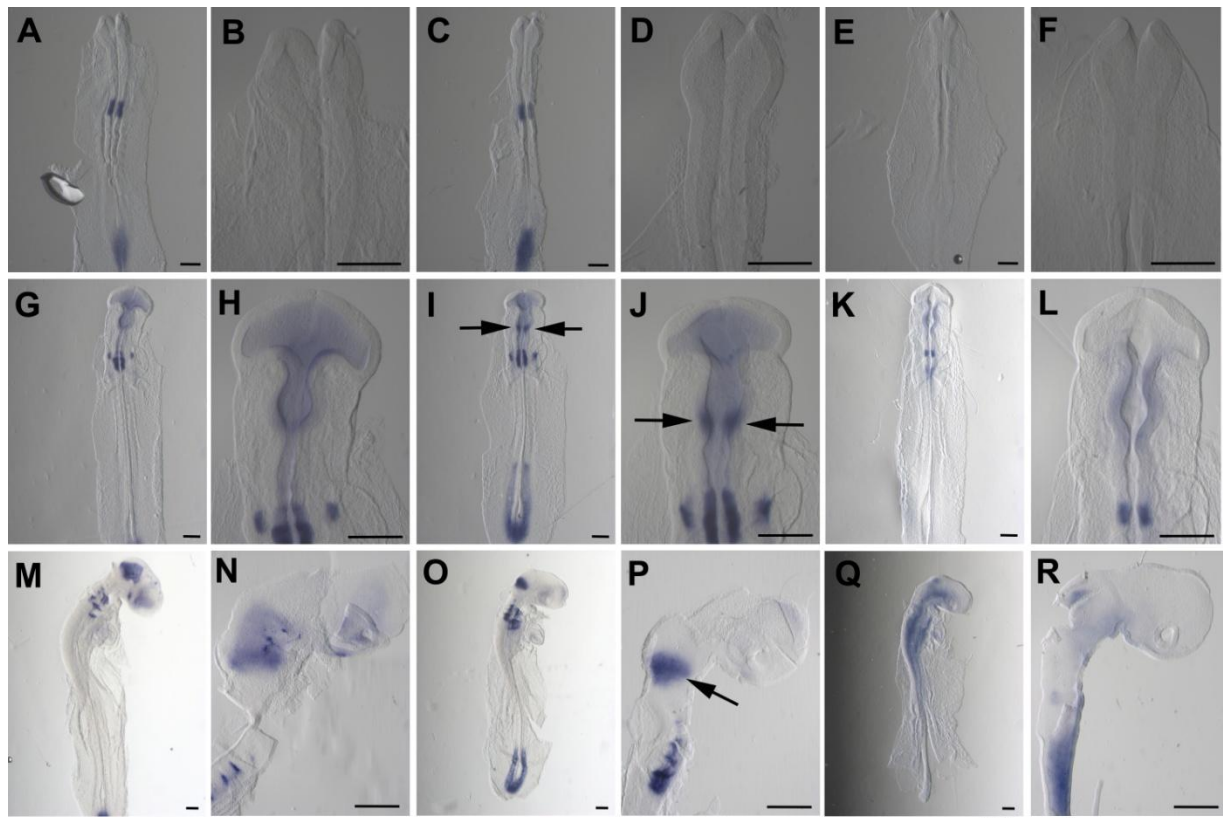
FGF3 **FGF18** **Nrg1**
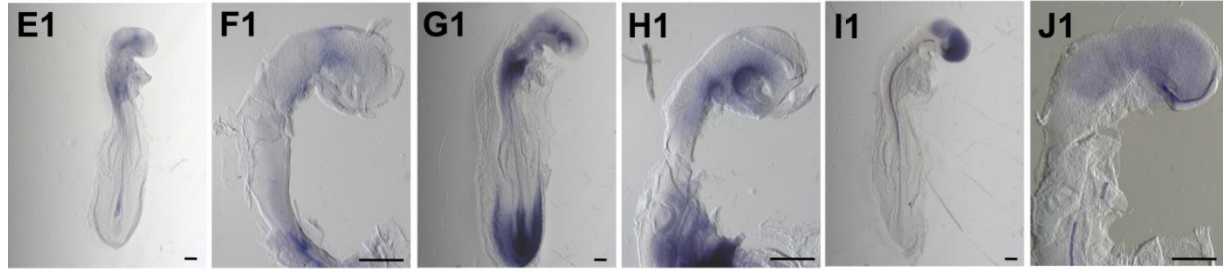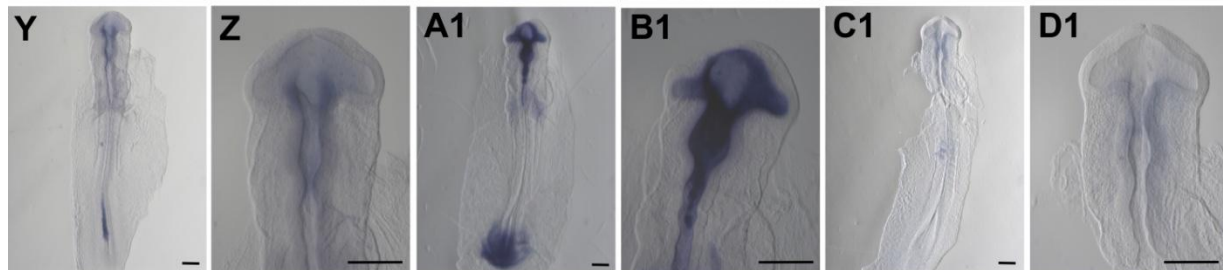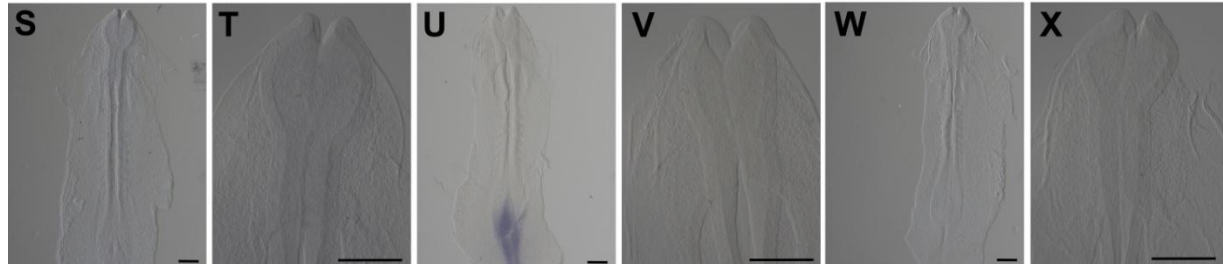
Wnt2b **Wnt5a** **Tac1**

Figure 6.11 Expression pattern of genes upregulated in microarray

A-B) HH9 FGF3 there is no expression in the rostral brain and some expression in the rhombencephalon. G-H) HH11 FGF3 there is expression in the brain, highest around the diencephalon and mesencephalon and in the rhombencephalon. M-N) HH14 FGF3 expression in the brain is restricted to the mesencephalon and some in the telencephalon and rhombencephalon.

C-D) HH9 FGF18 there is no expression in the rostral brain. There is some expression in the rhombencephalon similar to FGF3 expression. I-J) HH11 FGF18 expression appears similar to FGF3. There is some expression in the brain in particular at the MHB (arrows). O-P) HH14 FGF18 expression is restricted to the MHB in the brain (arrows).

E-F) Nrg1 there is no expression in the embryo. K-L) HH11$^+$ Nrg1 there is expression throughout the diencephalon and mesencephalon and specific expression in the rhombencephalon. Q-R) HH14 Nrg1 expression is extended throughout the neural tube. In the brain, expression is restricted ventrally and there is little expression in the telencephalon.

S-T) HH9 Wnt2b there is no expression. Y-Z) HH11 Wnt2b there is expression throughout the brain. E1-F1) HH14 Wnt2b there is expression throughout the neural tube. In the brain, it is restricted ventrally.

U-V) HH9$^-$ Wnt5a there is expression no expression in the brain and some in the caudal neural tube. A1-B1) HH11$^+$ Wnt5a there is expression caudally. The expression in the brain is most likely to be trapping. G1-H1) HH14 Wnt5a there is strong expression around the optic vesicles, at the level of the heart and the caudal most region of the embryo. There is strong expression in the ventral mesencephalon.

W-X) HH9 Tac1 there is no expression. C1-D1) HH11 Tac1 there is some expression throughout the neural tube, in the brain excluding the telencephalon. I1-J1) HH14 Tac1 there is expression throughout the prosencephalon and mesencephalon.

Scale bars, 500μm
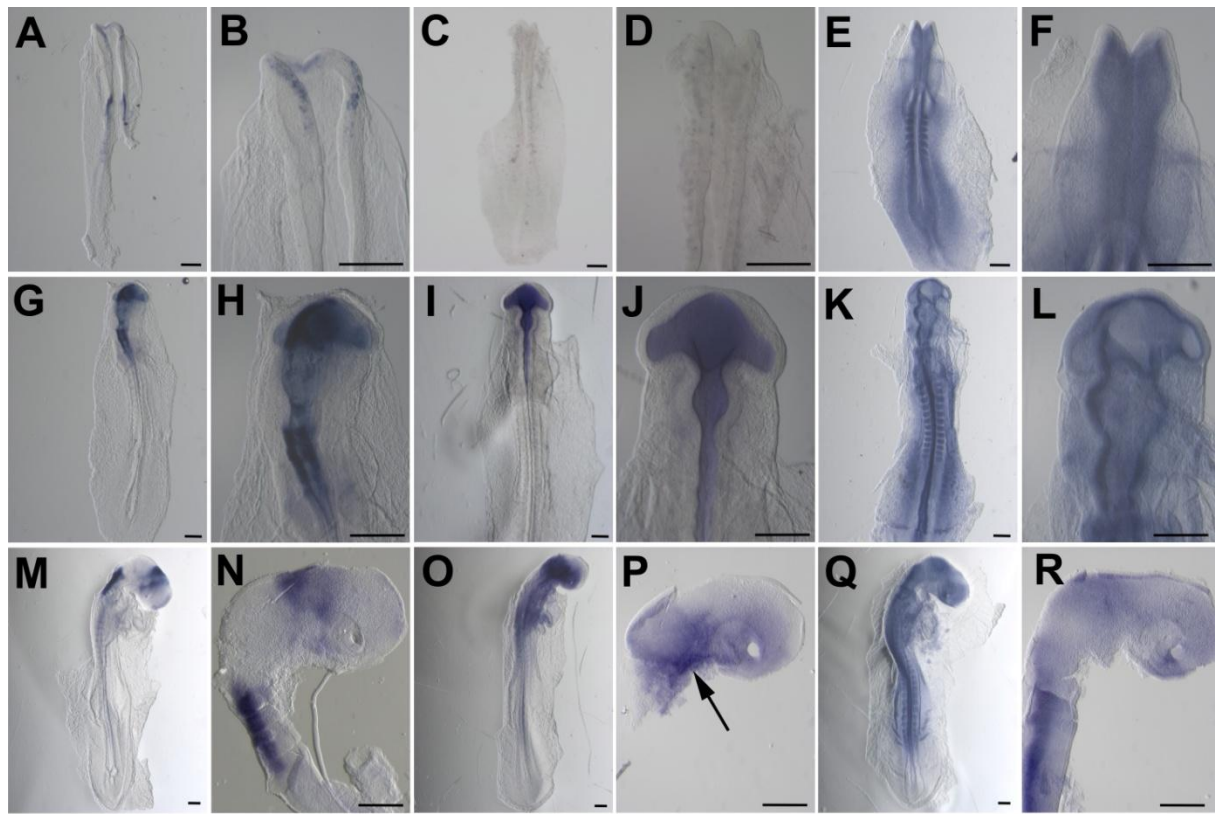
EphA7   PlxDC2   Protogenin
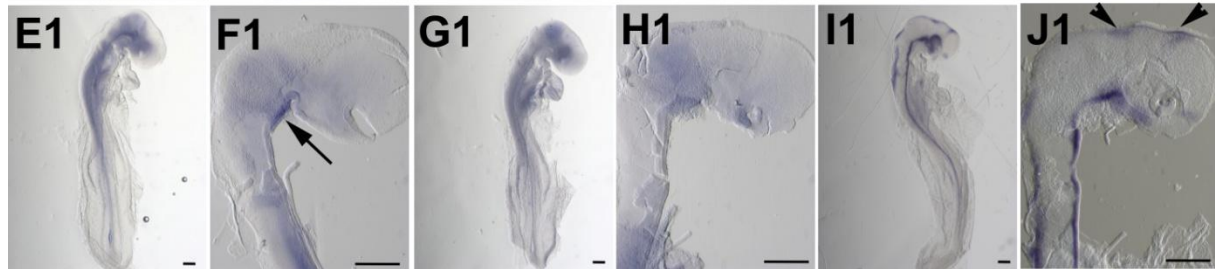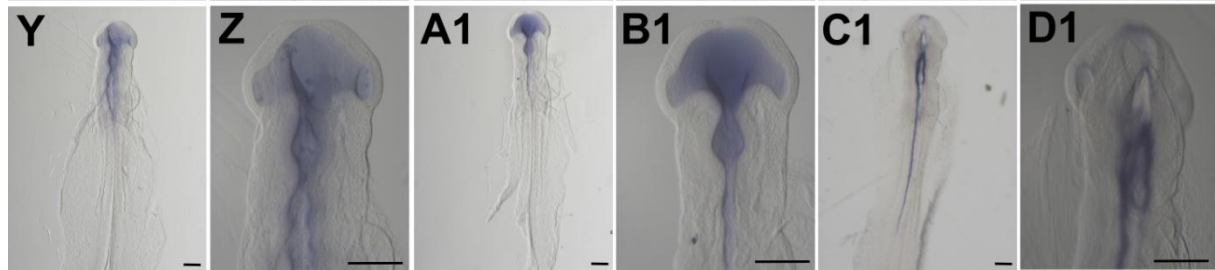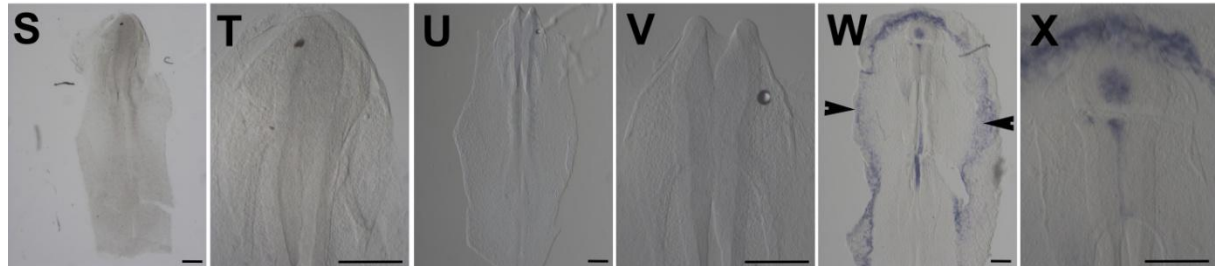
SRGAP1   SRGAP3   Slit2

Figure 6.12 Expression pattern of genes upregulated in microarray

A-B) HH9⁻ EphA7 there is some spotty expression along the rostral neural folds. G-H) HH11 EphA7 there is expression throughout the rostral neural tube, highest in the prosencephalon and rhombencephalon. M-N) HH14 EphA7 expression is restricted to the dorsal diencephalon and rhombencephalon.

C-D) HH9 PlxDC2 there is no specific expression. I-J) HH11^{++} PlxDC2 there is trapping in the neural tube. O-P) HH14 PlxDC2 expression is throughout the neural tube, strongest in the ventral mesencephalon.

E-F) HH9 Protogenin expression is throughout the neural tube, somites and extracellular membrane. K-L) HH11^{+} Protogenin there is expression throughout the whole neural tube, somites and extracellular membrane. Q-R) HH14 Protogenin is expressed throughout the entire neural tube and the brain.

S-T) HH9⁻ SRGAP1 there is no expression. Y-Z) HH11^{+} SRGAP1 there appears to be trapping throughout the brain. E1-F1) HH14 SRGAP1 there is expression throughout the neural tube, excluding the telencephalon. Expression is strongest in the ventral mesencephalon (arrow).

U-V) HH9⁻ SRGAP3 there is no expression. A1-B1) HH11^{+} SRGAP3 there appears to be trapping throughout the brain. G1-H1) HH14 SRGAP3 there is weak expression throughout the neural tube. Expression is highest in the dorsal mesencephalon and around the optic vesicle.

 W-X) HH9 Slit2 (the brain is slightly split at the MFB) there is expression throughout the neural tube and in the extra-embryonic membrane (arrowheads). There is expression throughout the neural tube. C1-D1) HH11^{+} Slit2 expression is throughout the neural tube, excluding most of the neural tube. I1-J1) HH14 Slit2 expression is along the ventral neural tube excluding the telencephalon. There is expression around the eye and there are two patches of expression dorsally in the brain (arrowhead).

Scale bars, 500µm
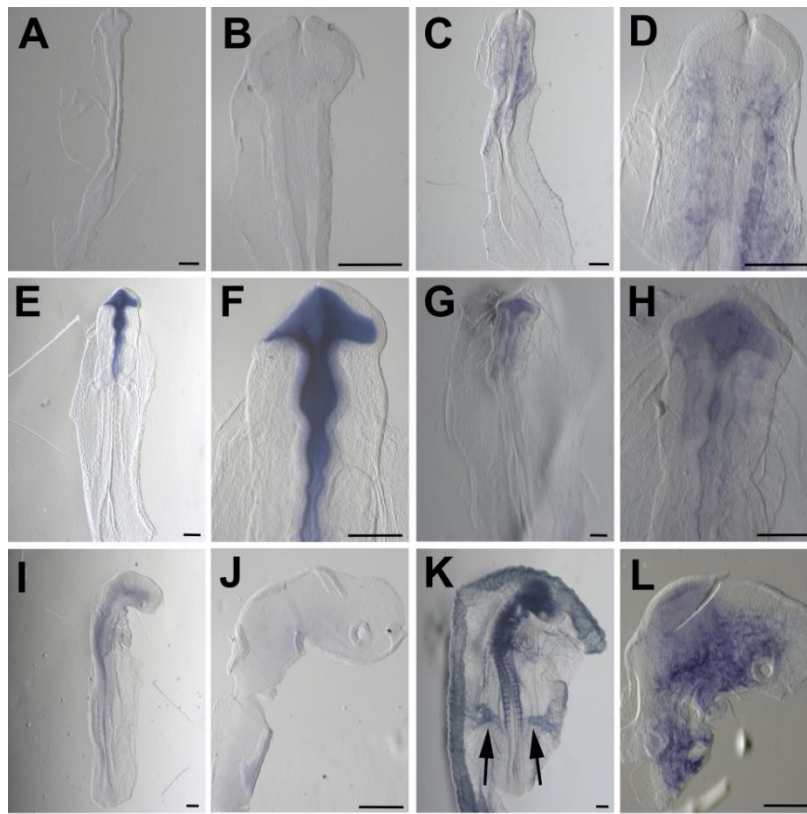
Cx40

Cldn5
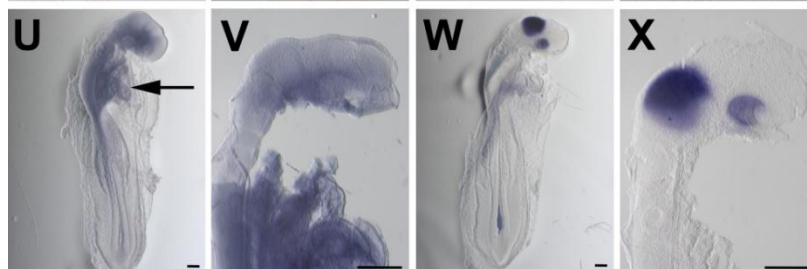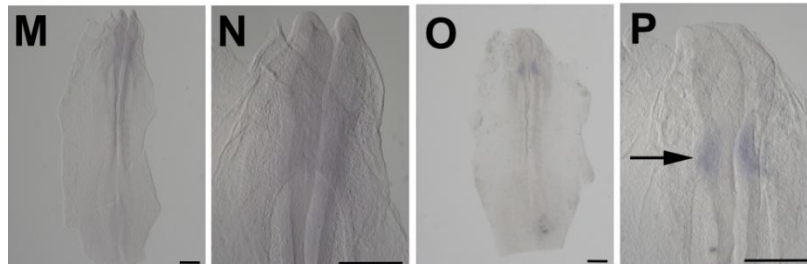
Fli1

Mab21L2

Figure 6.13 Expression pattern of genes upregulated in microarray

A-B) HH9 Cx40 there is no expression. E-F) HH11 Cx40 the left part of the telencephalon is missing due to preparation of the embryo. There is most likely trapping in the rostral neural tube. I-J) HH14 Cx40 expression is present along the ventral neural tube.

C-D) HH9 Cldn5 expression is in the mesenchyme around the neural tube. G-H) HH11$^+$ Cldn5 there is also expression in the mesenchyme, staining in the neural tube is most likely trapping. K-L) HH14 Cldn5 expression is throughout the embryo, in between the somites and in the blood vessels in the extra embryonic membrane (arrows).

M-N) HH9$^-$ Fli1 there is no expression. Q-R) HH11 Fli1 staining appears to be trapping. U-V) HH14 Fli1 there is expression throughout the embryo and in the heart (arrow).

O-P) HH9 Mab21L2 weak expression around the MFB (arrow). S-T) HH11 Mab21L2 there is strong expression in the optic vesicles (arrowhead) and the mesencephalon (arrow). W-X) HH14 Mab21L2 expression is still present in the optic vesicles and is defined to the dorsal mesencephalon. There is also some expression in the caudal neural tube.

Gene	HH9 expression	HH11 expression	HH14 expression
Wnt2b	No expression	Throughout the brain	Dorsal midline of mes and di (weak) ventral di and mes
Satb1	No expression	Throughout the neural tube. Strongest in mes	Dorsal and ventral mes and di with boundary of weak expression in between
Cx40	No expression	Trapping	Weak along ventral midline of neural tube
CRABPI	No expression	Spotty expression at the MFB and in the hindbrain	Spotty expression at the MFB and in the hindbrain
Cash1	No expression	Mes	Mes and ventral di
TAC1	No expression	Mes and di	Trapping in pros. Throughout the mes marking the MHB
Zic1	No expression	Throughout the brain excluding the tel	Along dorsal midline of mes and rh, anterior tel and expansion in di alar plate
EphA7	Expression along the neural folds	Tel and rh	Alar plate of di
Wnt5a	Expression caudally	Trapping	Ventral mes and optic vesicles
Mab21L2	Weak expression in mes	Expression in the optic vesicles and mes	Expression in the optic vesicles and mes
TFAP2α	Expression throughout the neural tube	Mesenchyme	Ventral mes and rh. Dorsal telencephalon
NeuroD	Expression throughout the neural tube	Throughout neural tube	Throughout neural tube. Two patches in the mesenchyme corresponding to cranial ganglia
Nrg1	No expression	Throughout neural tube	Basal plate of mes and patch in alar plate
Hes5	Expression in mes	Throughout the neural tube excluding the isthmus and optic vesicles	Throughout neural tube, excluding isthmus and alar plate of di
Dlx5	No expression	Low expression throughout the brain	Throughout the brain
Fli1	No expression	Trapping	In the heart and neural tube
FGF3	No expression in the brain, further caudal	Throughout the brain and some in rh	Throughout mes
Jag2	No expression	Tel and caudal mes	Tel and gradient from MHB into caudal mes
FGF18	No expression in the brain, further caudal	MHB	Caudal mes marking MHB
PRTG	Expression throughout the neural tube and somites	Throughout neural tube and somites	Throughout neural tube
Tcf4	Expression throughout brain	Throughout brain	Alar plate of di
Cldn5	Expression in mesenchyme not in neural tube	Some trapping in the neural tube	Blood vessels
SRGAP1	No expression	Trapping	Mes
PlxDC2	No expression	Trapping	Ventral mes and somites

Gbx2	No expression	Rh	Rostral rh marking the MHB
CHRDL1	Expression throughout the neural tube	Trapping	Ventral mes
Slit2	Expression throughout the neural tube	Throughout neural tube	Ventral midline of entire neural tube and patches along dorsal midline
SRGAP3	No expression	Mes and optic vesicles	Mes and optic vesicles

Table 6.2 Summary of gene expression

di, diencephalon; mes, mesencephalon; rh, rhombencephalon; tel, telencephalon; MFB, midbrain-forebrain boundary; MHB, midbrain-hindbrain boundary

6.3.2 Correlation of microarray results with in situ hybridisation results

The *in situ* hybridisation analysis showed at HH9 most of the genes were not expressed throughout the chick embryo or in the embryonic chick brain (table 6.2). Mab21L2 and Hes5 were expressed weakly in the mesencephalon and expression became stronger and wider spread within the brain by HH11. TFAP2α, NeuroD, PRTG, Tcf4, CHRDL1 and Slit2 were expressed throughout the neural tube from HH9. Expression of all of these genes appeared to be upregulated at HH11, correlating with the microarray data.

At HH11, there is broad expression throughout the neural tube for most for the upregulated genes. For example, Wnt2b and Zic1 were expressed throughout the brain at HH11 and expression became more specific by HH14 (Fig 6.10A1, B1 and Fig 6.11Y, Z). Genes that had specific expression within the brain at HH11 included Mab21L2, Slit2 and Jag2 (Fig 6.9C1, D1, Fig 6.12C1, D1 and Fig 6.13S, T). Only CRABPI showed a spotty expression in the caudal diencephalon that would appear to correlate with the position of the MLF (Fig 6.9K, L, arrow).

At HH14, many of the genes were expressed within the ventral diencephalon and mesencephalon. CRABPI, Cx40, EphA7, Hes5, Nrg1, PlxDC2, Slit2 Wnt2b and Wnt5a were all expressed in the basal plate of p1 overlapping the location of the MLF neurones. Apart from CRABPI, none of these genes were shown to have expression that would correlate with the MLF neurones.

The vascular markers analysed were Fli1 and Cldn5 (Fig 6.13C, D and Fig 6.13M, N). For both genes, trapping in the neural tube at HH11 masked some of the expression, however Cldn5 expression appeared in the mesenchyme around the neural tube. At HH14, both genes are expressed in the heart (Fig 6.13K, U). Fli1 expression appears to be located in the neural tube

however, Cldn5 expression appears outside the neural tube and expressed throughout the blood vessels (Fig 6.13K, arrows).

6.3.3 Previously uncharacterised genes in the chick embryonic brain

Many of the genes analysed by *in situ* hybridisation have previously had their expression patterns analysed such as Ephs or Hes5, therefore it would be interesting to investigate genes that have not previously been described in the chick embryonic brain. Cldn5 (appendix 1), Mab21L2 (appendix 2), PlxDC2 (appendix 3), Satb1 (appendix 4), SRGAP1 (appendix 5) and SRGAP3 (appendix 6) were successfully cloned from chick cDNA to make RNA *in situ* probes. These genes were all upregulated in the brain between HH9 and HH11, apart from SRGAP3 that had trapping in the brain (table 6.2). As SRGAP3 was not upregulated in the microarray a change in expression between HH9 and HH11 was not expected (Fig 6.12U, V, A1, B1). Although none of these genes were expressed specifically by the MLF neurones, apart from Cldn5 that was a blood marker and Mab21L2, they were expressed ventrally in the diencephalon and mesencephalon. Mab21L2 was an alar plate, mesencephalic marker and expressed in the eye which was similar to expression in the embryonic zebrafish brain (Wong and Chow, 2002).

6.3.4 Expression of CRABP1 in MLF neurones

Expression of CRABPI was upregulated by 16.04 fold and expression at HH11 identified it as possibly being expressed specifically by the MLF neurones due to its spotty expression at the MFB. To analysis this further immunohistochemistry with Tuj1 was used to labelled the neurones and correlate this with the *in situ* pattern of CRABP1. At HH14 CRABPI was expressed specifically by all the MLF neurones (Fig 6.14A, arrows), as well as the DTmesV neurones. At a later stage, MLF axons and neurones were labelled specifically with DiI, by retrograde labelling (Fig 6.14B). The labelled neurones were positive for CRABPI (Fig 6.14A,

arrows). Quite a few CRABPI positive cells were not labelled by DiI, because only a subset of the MLF neurones will have taken up the dye (Fig 6.14A, arrowheads). In addition, CRABPI was also positive in a cluster of neurones labelled rostral to the MLF, likely to be the MTT neurones (Fig 6.14B) and along the dorsal midline of the mesencephalon, likely to be the DTmesV neurones (see appendix 12A). In contrast, the TPOC neurones were negative for CRABPI expression (see appendix 12A). This would suggest CRABPI had a role in the differentiation of neurones but was not specific to the specification of the MLF neurones.
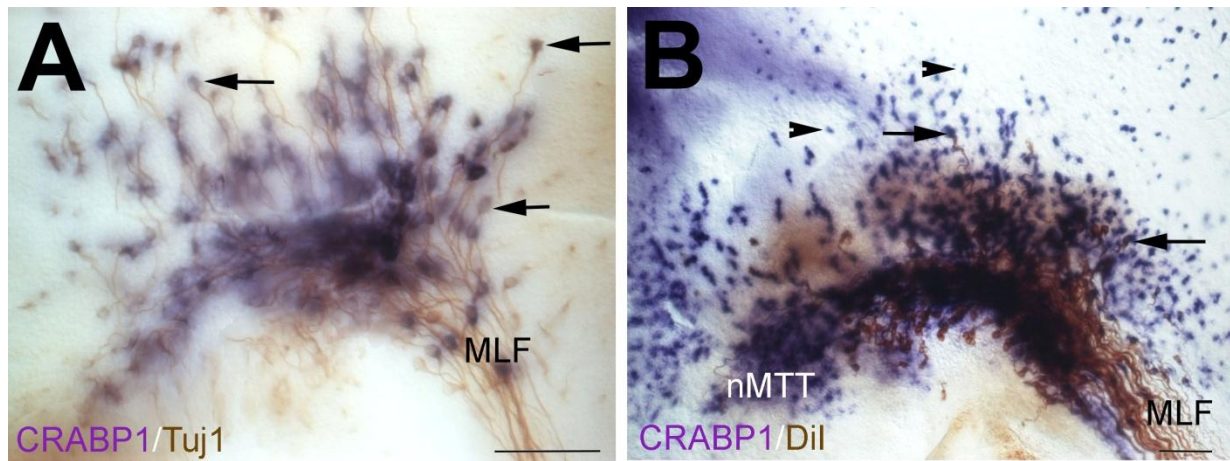
Figure 6.14 Expression of CRABPI by MLF neurones in the chick embryonic brain
Lateral view of whole mount embryo

A) HH14 CRABPI is expressed by the MLF neurones (arrows). B) CRABPI is expressed by the MLF neurones (arrows) as well as other neurones. MLF is specifically labelled using DiI photoconversion. As not all the MLF neurones take up the dye there are many purple spots not labelled with neurones (arrowheads). The MTT neurones also expressed CRABP1.

Scale bars, 100μm

Accession number	Gene	Gene title	Fold change	Function
CD760931	HBE1	Haemoglobin epsilon 1	154.9	Binds and transport oxygen
BX932088	XLKD1	Extracellular link domain containing 1	38	Transporter
CR338842	HBA2	Haemoglobin alpha 2	27.45	Binds and transport oxygen
BU478304	HBA1	Haemoglobin alpha 1	24.75	Binds and transport oxygen
CR390531	Elavl2	ELAV-like neuronal protein 1/Hu-antigen B	19.18	RNA binding
NM_204895	TFAP2B	Transcription factor AP-2 beta	17.9	Transcription factor
NM_205473	NPY	Neuropeptide Y	13	Hormone secretion
NM_204792	Sox10	SRY (sex determining region Y)-box 10	8.909	Transcription factor (TATA binding)
CR386242	Nrg3	Neuregulin 3	8.661	Growth factor
NM_205431	PTPRZ1	Protein tyrosine phosphatase, receptor-type, Z polypeptide 1	7.479	Receptor
ENSGALT00000005670	SLC1A3	Solute carrier family 1 member 3	6.627	Amino acid transporter
NM_205170	Tlx	Nuclear receptor TLX	5.323	Zn-finger C4 steroid receptor
NM_204568	FIGF/VEGF-D	c-fos induced growth factor (vascular endothelial growth factor D)	4.97	Growth factor
NM_205102	GFRA1	GDNF family receptor alpha 1	4.568	Receptor
CR523546	NPAS3	Neuronal PAS domain protein 3	4.406	bHLH Transcription factor
NM_204121	NHLH1/NSCL1	Nescient helix loop helix 1	3.843	bHLH transcription factor
BX929287	RhoJ	Ras homolog gene family, member J	3.752	GTP-binding
NM_205430	EphA3	Ephrin type A receptor 3	3.687	Receptor
BU206789	GABRG2	Gamma-amino butyric acid (GABA) A receptor, gamma 2	3.361	GABA neurotransmitter receptor
BU230100	PTN	Pleiotrophin	3.278	Heparin-binding growth factor
ENSGALT00000016289	BAZ1A	Bromodomain adjacent to zinc finger domain, 1A	3.183	Bromodomain transcription factor
BX932994	Ten-M4	Teneurin-4	3.156	Signal transducer
NM_204503	Beta3	bHLH transcription factor beta3	3.025	bHLH transcription factor
ENSGALT00000017030	Ebf3	Early B-cell factor 3	2.825	bHLH transcription factor
BX935095	PDGFD	Platelet derived growth factor D	2.725	Growth factor
NM_204803	Meis2	Meis1, myeloid ecotropic viral integration site 1 homolog 2 (mouse)	2.698	Homeobox transcription factor
BU200810	ZFHX4	Zinc finger homeodomain 4	2.335	Homeobox transcription factor
NM_205294	RXRG	Retinoid X receptor, gamma	1.926	Zn-finger C4 steroid receptor
AY040529	Id4	Inhibitor of DNA binding 4	1.862	Anti-neural bHLH transcription factor
M76678	BEN/SC1	Activated leukocyte cell adhesion molecule	1.799	Cell adhesion molecule
NM_204504	bHLHB4	Basic helix-loop-helix domain containing class B4	1.69	bHLH transcription factor

BX931132	Robo1	Roundabout 1	1.684	Receptor
BU346190	Dbx2	Developing brain homeobox 2	1.644	Homeobox transcription factor
AF461038	Dmbx1	Diencephalon/mesencephalon homeobox 1	1.633	Homeobox transcription factor
BU263048	TUBB6	Tubulin, beta 6	1.615	GTP-binding
AF075708	Pea3	ETS-domain transcription factor pea3	1.597	Ets-domain transcription factor
AB090235	Nrp2	Neuropilin 2	1.508	Receptor
BU390690	RARB	Retinoic acid receptor, beta	1.473	Zn-finger C4 steroid receptor
NM_205184	EphrinA5	Ephrin A5	1.409	Axon guidance molecule (Repellent)
U23783	EphB6	Ephrin type B receptor 6	1.378	Receptor
NM_204590	Id1	Inhibitor of DNA binding 1	1.376	Anti-neural bHLH transcription factor
NM_204896	Barx2	BarH-like homeobox 2	1.332	Homeobox transcription factor
NM_205059	BRG1	BRG1 protein	1.26	Transcriptional coactivator/SWI/SNF chromatin remodelling protein
AJ720897	TBCD/TITAN	Tubulin folding cofactor D	1.226	Tubulin folding protein

Table 6.3 Other interesting genes

The table highlights the genes that could potentially be involved in MLF specification and blood markers that were regulated. It includes the genes with the highest fold change between HH9 and HH11. Due to time constraints and lack of plasmids, not all of these genes could be analysed. Also many of the genes have not been previously analysed in the chick embryonic brain.

6.4 Discussion

The MLF was the first axon tract to form in the chick embryonic brain, so understanding the molecular mechanisms involved in its formation was part of the key to understanding how the rest of the connections within the brain form. For an MLF neurone to determine this fate, a precursor cell must differentiate into a neuronal cell while becoming specified into its particular fate. To identify candidate genes that could be involved in MLF cell-fate determination, microarray analysis was used to identify genes that have a change in expression between HH9 and HH11, when the first MLF neurones were specified.

In total, 1531 genes were upregulated in the microarray screen, including genes known to be involved in neurone development such as Cash1 and NeuroD, transcription factors such as homeobox genes Dlx5, Gbx2 and Satb1 and signalling molecules such as Wnt5a and FGF3. A number of genes were expected to be upregulated, such as βIII tubulin or neurofilament as they were specifically expressed by neurones at HH11 and not HH9. BEN/SCL is a cell adhesion molecule expressed by neuronal cells (Chédotal et al., 1995) that was upregulated in the microarray (1.799-fold), suggesting the correct region of the brain was dissected and the HH11 sample contained MLF neurones. The microarray analysis did not find βIII-tubulin, which labels the MLF neurones at HH11 or neuropilin1, which has been shown to be expressed specifically by the MLF neurones at HH11(Riley et al., 2009). This could be due to such a small number of cells expressing these genes that the expression level was too low to detect these genes in the microarray. None of the homeobox genes previously analysed Sax1, Emx2, Nkx2.2 or Six3 came up in the array, which provides further evidence these genes were not involved in the cell-fate determination of MLF neurones. Pax6 was downregulated (data not shown), suggesting the

expression becomes restricted to the telencephalon and dorsal diencephalon between HH9 and HH11.

In the *in situ* hybridisation analysis, little or no expression in the brain at HH9 was expected, then by HH11 there would be an upregulation in gene expression. This was true for almost all of the genes (Table 6.2). Where there was expression at HH9, for example protogenin the level of upregulation was very little in comparison to other genes (Table 6.1, 2.903-fold). The top three genes that were upregulated and analysed by *in situ* hybridisation were Wnt2b, Satb1 and Cx40 they all show no expression in the brain at HH9, then by HH11 there was strong expression throughout the neural tube in Wnt2b and Satb1. For Cx40 trapping in the neural tube at HH11 masked some of the expression. At HH14, expression becomes located ventrally in the brain.

6.4.1 Expression of genes around the MFB and MFB

The genes of interest were those expressed around the MFB (Fig 6.15). Wnt5a, Wnt2b and CRABPI were the only genes expressed in both the alar and basal plates of p1 where the MLF neurones are located. Many of these genes were markers for the MHB (Fig 6.16). By HH14, when expression becomes more restricted, many of the genes were expressed in the caudal mesencephalon for example FGF3 and Jag2. Only Tcf4 and EphA7 were markers for the alar diencephalon and no genes analysed by *in situ* hybridisation were specific markers for the basal diencephalon or mesencephalon. Many of these genes, while being expressed in p1 had a broader expression pattern for example Wnt2b and Wnt5a.
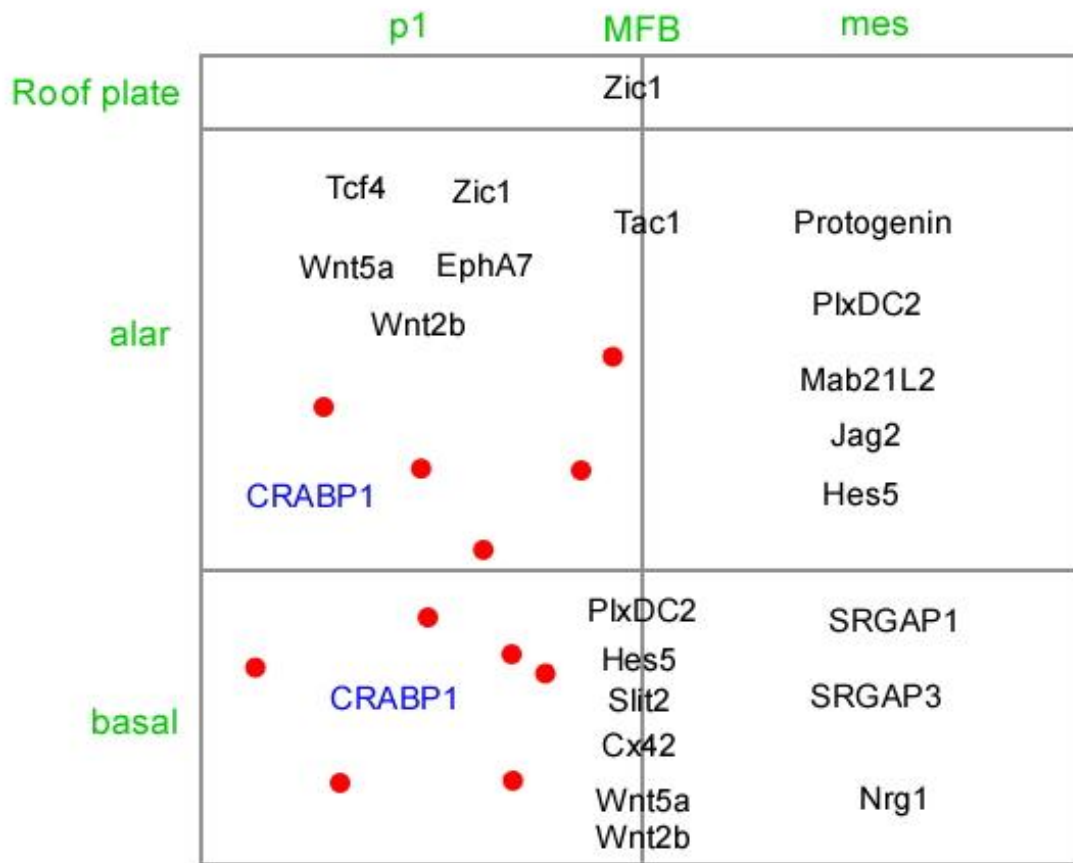
Figure 6.15 Expression of genes around the MFB

Red spots indicate location of MLF neurones within the alar and basal plates of p1. CRABPI was identified as a marker for these neurones. Other genes were identified as being expressed around the MFB.
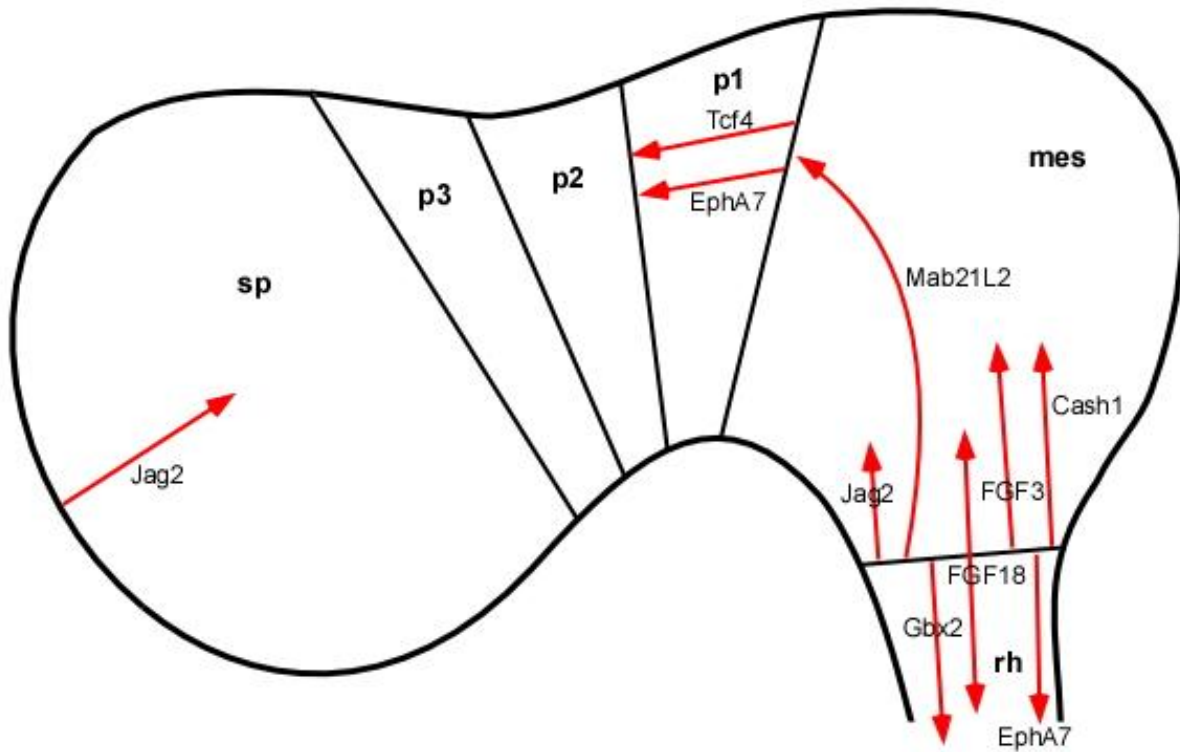
Figure 6.16 Location of genes expressed within specific regions of the brain and marking boundaries

Many of the genes upregulated were markers for the MHB, such as Gbx2 and Cash1. Mab21L2 was a marker for the alar mesencephalon, while Tcf4 and Eph7A were markers for p1.

di, diencephalon; mes, mesencephalon; p1, prosomere 1; p2, prosomere 2; p3, prosomere 3; sp; secondary telencephalon; rh, rhombencephalon; tel, telencephalon;

6.4.2 Identification of Cellular retinoic acid binding protein I (CRABPI)

CRABPI was identified as being specifically expressed by the MLF neurones at HH11. Overall CRABPI was the 14th highest gene to be upregulated by 16.08 fold. CRABPI acts as a transporter of retinoic acid. Retinoic acid has a role in both the AP and DV patterning of the central nervous system (reviewed by Maden, 2002). Retinoic acid is derived from vitamin A and is the component that acts within a cell. Once inside the cell retinoic acid is transported from the cytoplasm to the nucleus by CRABPI or CRABPII. Once in the nucleus, retinoic acid binds to retinoic acid receptors (RAR) and retinoid X receptors (RXR) to switch on expression of retinoic acid response elements (RARE). Retinoic acid has been shown to be involved in patterning of the neural tube as well as differentiation of neurones, upstream and downstream of notch signalling (reviewed by Maden, 2002; Maden, 2007). CRABPI was expressed by the MLF, MTT and DTmesV neurones. The function of CRABPI was likely to transport retinoic acid in these neuronal cells and as CRABP1 was only expressed by some early axon scaffold neurones it would suggest CRABPI has a specific role in some neuronal populations but not in others. In addition, upregulated in the microarray between HH9 and HH11 were retinoic acid targets RARβ and RXRγ (table 6.3) that in mutant mice have severe spatial learning and memory abilities (Chiang et al., 1998). These genes will be important to analyse further to see if they are expressed by the same neurones as CRABP1.

6.4.3 Identification of neurogenesis genes

Genes in the Delta-Notch signalling pathway were identified in the microarray analysis, which was expected as they are involved in the differentiation of neurones (see introduction 1.2.2). Both proneural and anti-neural genes were upregulated, suggesting there were both neurones and

cells not committed to a neuronal fate present in the tissue used for the microarray. Hes and Id genes came up as they are expressed in cells that are not committed to a neuronal fate and much of the tissue dissected would not have a neuronal fate. As an example, Hes5 is a homologue of Drosophila hairy/enhancer of split and functions as an anti-neural bHLH transcription factor. The expression pattern of Hes5 at HH14 was similar to the expression pattern already shown in particular strong expression in the ventral midbrain (Kimura et al., 2004). Hes5 was weakly expressed at HH10, which was seen at HH9.

6.4.4 Identification of transcription factors

The transcription factors upregulated were boundary markers rather than cell-type specific. For example, Tcf4 is a marker for the alar plate of p1 (also shown by Ferran et al., 2007) and Gbx2 marks the MHB. At HH9 Gbx2 was not expressed however, in previous studies Gbx2 was expressed in the rhombencephalon at early stages (Garda et al., 2001). Expression at HH11 and HH14 corresponds with previous data. A possible reason could be that the HH9 embryo was not left in substrate solution for long enough during the *in situ* hybridisation procedure to produce a signal.

6.4.5 Identification of signalling molecules

Signalling molecules have been shown to be involved in AP and DV patterning of the neural tube (see introduction 1.2). FGF18 marks the region around the isthmus and was involved in patterning of the mesencephalon (Sato et al., 2004). Wnt5a was expressed ventrally in the mesencephalon and has been shown to form part of the midbrain arcs, suggesting formation of these arcs could begin early in development (Sanders et al., 2002).

The Ephs are a family of receptor tyrosine kinases which are activated by their ligands, the ephrins, which are membrane bound (Baker and Antin, 2003). They are repulsive axon guidance

molecules as well as being involved in other morphogenetic events during embryonic development such as segmentation of the brain. In this microarray EphA3, EphA7, EphB6 and EphrinA5 were identified as being upregulated. EphrinA5 and Eph7A were co-expressed on the dorsal folds of the neural tube, mutant mice for these genes results in defects during neural closure (Holmberg et al., 2000). Eph7A expression at HH9$^-$ and HH11 was consistent with previous data (Baker and Antin, 2003). EphA3 was expressed throughout the neural folds from HH6-HH9 and then becomes restricted to the prosencephalon, r3 and r4 (Baker and Antin, 2003). At later stages, EphA3 was localised ventrally in the brain and was expressed along the MFB. EphA3 was also expressed in the heart and developing vitelline vein plexus from HH11. EphrinA5 is involved in retinal axon guidance and topographic mapping, and as these axons pass through the diencephalon to the tectum, it is not surprising Ephrin A5 was upregulated (Frisen et al., 1998).

6.4.6 Identification of blood markers

Surprisingly many of the upregulated genes were blood markers. As the neural tube does not contain blood vessel precursor cells, the markers identified were from cells located outside the neural tube. This was most likely due to not all of the mesenchyme being removed from around the neural tube during the dissections of the tissue samples, particularly at HH11. This suggests that the vascular system is already forming around the neural tube by HH11, it also indicates that the blood vessels could be more tightly associated with the neural tube than at HH9 or are entering the neural tube already. The peri-neural vascular plexus initially forms around the CNS and blood vessels then invade the neural tube (reviewed by Bautch and James, 2009). The initial appearance of the vascular system in the chick embryonic brain is not known and further analysis of these blood markers will give a clearer indication of timing. Cldn5 and Fli1 were analysed

here. Cldn5 forms part of the endothelial tight junctions (Morita et al., 1999) and was already expressed in the mesenchyme around the brain at HH9. This would suggest that there were already blood cells present and the vascular system was starting to form. What is unknown is how early the blood vessels penetrate into the neural tube. While several signalling pathways including Notch, Ephrins, Wnts and Semas have been shown to be involved in neurovascular development. VEGF-A is specifically required for communication between the CNS and vascular system allowing blood vessels to penetrate the neural tube wall (James et al., 2009).

6.4.7 Conclusion

As CRABPI was not expressed specifically by MLF neurones, it is unlikely to be involved in the specific cell fate determination of these neurones, ensuring the differentiated cell becomes a MLF neurone and not any other type of neurone. As no other genes analysed by *in situ* hybridisation showed a specific correlation with the MLF neurones, the upregulated genes identified in this microarray will need continued analysis. However, the specification of the early axon scaffold neurones may be slightly different in the ventral diencephalon to that which occurs in the spinal cord. It could be that a combination of patterning genes and differentiation genes are required within the neuronal cells, as well as in a broader area to allow the specification of the MLF neurones. Therefore, CRABPI would be required to produce specification of different neurones in response to different patterning signals. This is a working hypothesis that will require overexpression studies to begin understanding the full mechanism.

Chapter 7

Axon guidance in the embryonic chick brain: Netrin1 and Netrin2

7.1 Introduction

The early axon scaffold is set up to act as a pathway for later, follower axons. These axons are pioneering and project into an unknown environment, where there are no other axons present. When these axons first begin projecting, they require directional cues from their environment to follow the correct path. Axon guidance molecules are involved in chemoattraction or chemorepulsion of the pioneering axons in the embryonic brain along their correct route towards their destination (see 1.5).

7.1.1 Axon guidance role of Netrin1 and Netrin2

Netrins are bifunctional axon guidance molecules and the receptors involved in axon guidance are DCC, Neogenin and Unc5 (Chisholm and Tessier-Lavigne, 1999). DCC and neogenin are involved in mediating the attraction of axons (see introduction 1.5.4). Unc5 was identified in *C.elegans* (Leung-Hagesteijn et al., 1992) as necessary for mediating the repulsion of axons and the homologues Unc5A-D (Unc5H1-Unc5H4) have been identified in vertebrates (Engelkamp, 2002; Leonardo et al., 1997).

Netrin1 and Netrin2 were first purified from the floor plate of the embryonic chick brain (Serafini et al., 1994) and shown to be homologous to UNC-6 which is a laminin related protein found in *C.elegans* (Hedgecock et al., 1990; Ishii et al., 1992). The role of Netrin1 has been shown by Serafini et al., (1994) and Kennedy et al., (1994) to attract spinal cord

commissural axons to the ventral midline in the chick embryo as well as promoting outgrowth of these axons. Netrin2 also plays a role in the attraction of spinal cord commissures. In mice lacking Netrin1 the commissural axons in the spinal cord do not migrate towards the floor plate (Serafini et al., 1996). The role of Netrins in commissure formation is highly conserved in bilateria, since similar functions to vertebrate Netrins has been shown for C.elegans UNC-6 (Hedgecock et al., 1990; Ishii et al., 1992) and *Drosophila* NetrinA and NetrinB (Harris et al., 1996; Mitchell et al., 1996).

7.1.2 Netrin1 and Netrin2 expression

Netrin1 and Netrin2 expression has been shown in the embryonic zebrafish brain (Macdonald et al., 1997). Netrin1 and Netrin2 show similar expression patterns between the AC and POC in the rostral telencephalon, although Netrin1 expression was more diffuse than Netrin2. Netrin1 expression was similar to that shown in chick (see 1.5.4 and 7.2), throughout the ventral neural tube, however the expression was not identical as neither the notochord or somites expressed Netrin1 in zebrafish (Strahle et al., 1997). When Netrin1was ectopically expressed in the zebrafish brain there was no effect on the early axon scaffold, even though Netrin1 shares high conservation with the chick Netrin1 (Lauderdale et al., 1997). Netrin1 was also expressed throughout the ventral neural tube in *Xenopus*. In the embryonic *Xenopus* brain, Netrin1 expression was not as diffuse as the expression in zebrafish and there was no expression between the AC and POC in *Xenopus*. When Netrin1 was knocked down in *Xenopus* the SOT and VC were affected (Wilson and Key, 2006).

Netrin1 and Netrin2 as well as their receptors (neogenin and Unc5) have been shown to be expressed within the mesencephalon of the embryonic chick brain (Riley et al., 2009). Netrin1 was expressed within the floor plate and basal plate. Netrin2 was expressed throughout the brain, caudally and rostrally to the MFB. Caudal to the MFB, Netrin2

expression was restricted to the rostral half of the mesencephalon dorsal to and overlapping the LLF. Rostral to the MFB, Netrin2 expression was restricted to the prosomeres p1 and p2. The expression of Netrin1 and Netrin2 suggests a role in the formation of the early axon scaffold.

Based on their expression pattern within the chick embryonic brain, Netrin1 and Netrin2 were selected as candidate molecules for repelling the tract of the posterior commissure (TPC) axons along their correct path from the ventral MFB to the dorsal midline. To clarify the function of Netrin signalling within the brain, a more detailed expression analysis of Netrin1, Netrin2 and Unc5H4 was required in both chick and other vertebrates. The role of axon guidance molecules can be investigated by ectopically expressing the gene of interest. In ovo electroporation can be applied to the chick embryo to study the function of Netrin1 and Netrin2 by gain-of-function. Gain-of-function (overexpression) has been used similarly to show Sema3A (see 1.5.5) has a role in the repulsion of MLF neurones, preventing them from projecting rostrally into the telencephalon (Riley, 2008).

7.2 Analysis of Netrin axon guidance molecules

The posterior commissure (PC) is a prominent commissure that crosses the dorsal midline in the caudal diencephalon, allowing communication between the two halves of the brain. While some TPC neurones were located at the dorsal MFB, a large cluster of TPC neurones were located ventrally (in p1), rostral to the MFB. The neurones project axons dorsally towards the dorsal midline where the axons cross (Fig 3.5A, B). Even though the ventral TPC neurones were intermingled with the MLF neurones, they still projected along very separate tracts (Fig 3.5A, B). Therefore, there must be different guidance cues present to ensure the TPC axons project dorsally away from the ventral midline and in a tightly fasciculated tract. The TPC

projects along the rostral edge of the MFB in between the expression domains of Netrin2 (Riley et al., 2009). This suggests Netrin2 plays a role in guiding the TPC axons by chemorepulsion from the nTPC towards the dorsal midline where the axons cross to form the PC.

7.2.1 Expression of Netrin1, Netrin2 and Unc5

Netrin1 was expressed ventrally in the floor plate through the mesencephalon and up to the diencephalon (Fig 7.1A). Netrin2 expression in the brain was much more complex. There were two distinct channels where no Netrin2 expression occurred at the MFB (Fig 7.1B, black arrow) and at the ZLI located in the diencephalon between p2/p3. (Fig 7.1B, white arrow). Netrin2 expression was not present in the floor plate and dorsally at the roof plate.

If Netrin1 and Netrin2 were in fact guiding the TPC axons, then the TPC neurones located ventrally should be expressing Unc5, as this is the receptor involved in the repellent activity of Netrins. Unc5H4 expression was located at the ventral MFB (Fig 7.1C, arrow) which appeared to be overlapping the TPC neurones. Unc5H4 was expressed by the TPC neurones (Fig 7.2A). This was determined by injecting the TPC with DiI and then photoconverting the dye so the TPC was the only tract labelled in the embryonic brain, followed by Unc5H4 *in situ* hybridisation.
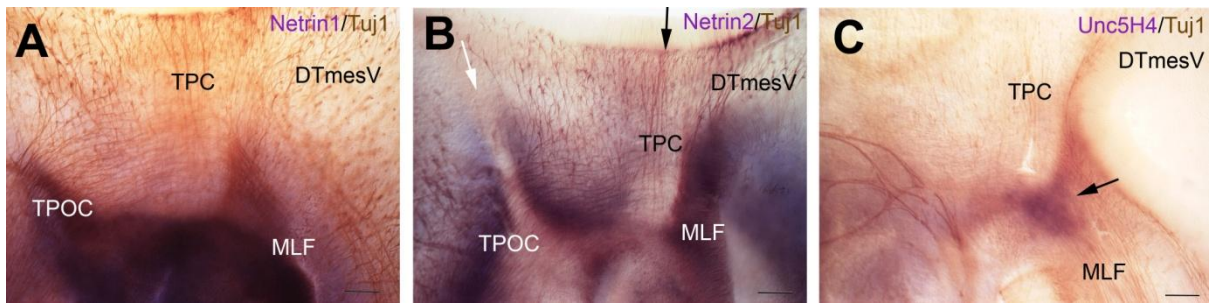
Figure 7.1 The expression patterns of Netrin1, Netrin2 and their receptor Unc5H4 with NBP/BCIP substrate
Lateral view of whole mount embryonic brain. Scale bars, 100μm

A) HH19. Netrin1 expression in the floor plate and basal plate of the diencephalon and mesencephalon. B) HH19. Netrin2 expression throughout the alar plate of the diencephalon and mesencephalon, excluding the most dorsal region. There are two channels within the brain that do not expression Netrin2 (arrows) as well as the floor plate and roof plate. Expression was not present ventrally, overlapping with Netin1 expression. C) HH17. Unc5H4 expression at the ventral MFB (arrow).

7.2.2 Comparison of Unc5 receptors

The *in situ* probe was made from an EST (expressed sequence tag) sequence to detect the Unc5H4 mRNA and the Unc5H4 antibody was used to detect the protein. As the mRNA was made by the cell bodies, only the neurones actively transcribing this gene should be labelled by *in situ* hybridisation. The protein was translated from the mRNA and the Unc5H4 receptor gets transported from the cell body to the growth cone. Therefore the cell bodies, axons and growth cones should be labelled by the antibody. Unc5H4 mRNA was expressed at the ventral MFB, correlating to the position of the TPC neurones (Fig 7.2A, arrows and appendix 12B). When the Unc5H4 antibody was used to label the TPC, it appeared most of the neurone populations (nMLF, nMTT, nmesV) and associated axons present in the brain were labelled apart from the TPOC and the TPC (Fig 7.2B). This discrepancy of mRNA and protein detection would suggest the probe used for *in situ* hybridisation has cross reacted with the mRNA of one, some or all of the other Unc5 receptors (Unc5H1-3).

When the Unc5 sequences were compared, the level of conservation was high, suggesting cross labelling of the Unc5H4 probe with the other receptors (Table 7.1 and appendix 7). The Unc5H4 EST was most similar to the Unc5H4 sequence (91.4%) which was expected however, the EST also showed high similarity to the other Unc5 sequences.
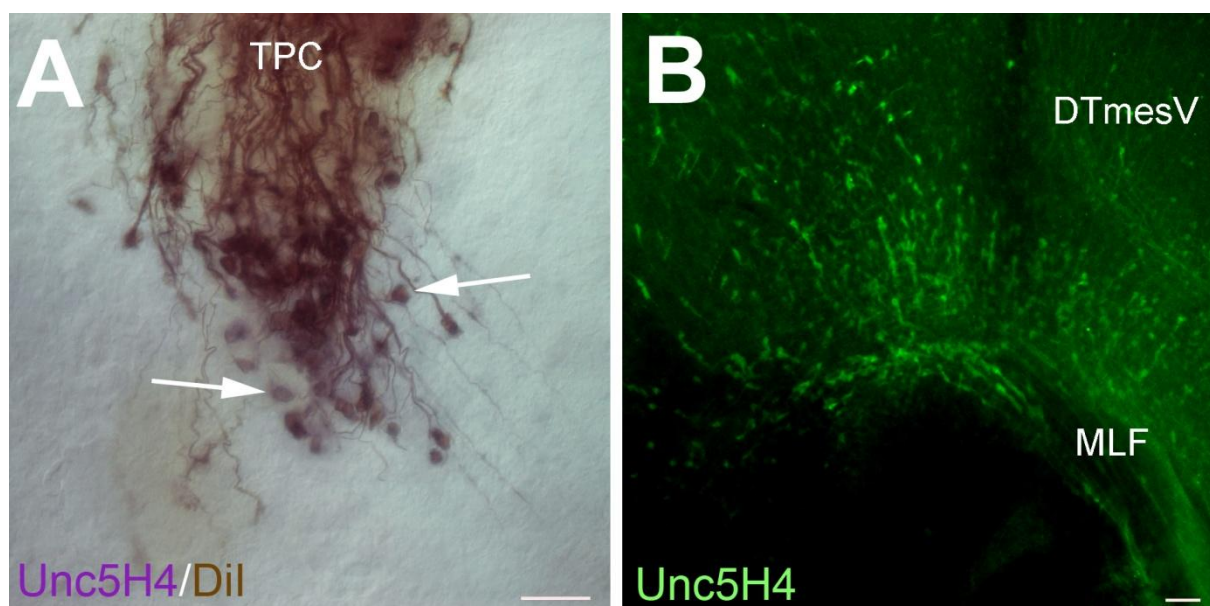
Figure 7.2 Expression of Unc5H4 *in situ* probe and Unc5H4 antibody
Lateral view of whole mount embryonic brain. Scale bars, 100µm

A) Labelling of the TPC with DiI (photo converted to a brown substrate), followed by Unc5H4 *in situ* hybridisation (light purple). The TPC neurones are located ventrally and project axons dorsally towards the dorsal midline. The TPC neurones appear to be expressing Unc5H4 (arrows). B) HH19. Unc5H4 antibody labelling in the ventral diencephalon and mesencephalon. The MLF and DTmesV neurones and axons are labelled.

	Unc5H1	Unc5H2	Unc5H3	Unc5H4
Unc5H1		66.9	65.4	64.6
Unc5H2	66.9		85	63.1
Unc5H3	65.4	85		62
Unc5H4	64.6	63.1	62	
Unc5H4 EST	61.9	59.8	60.3	91.4

Table 7.1 Percentage of conservation between the Unc5 receptors and the Un5H4 EST used for the *in situ* probe

The Unc5H4 EST showed the highest percentage of conservation with the Unc5H4 sequence (91.4% - red). The other receptor sequences (Unc5H1-Unc5H3) also showed a high percentage of conservation (above 50%). This would suggest possible cross reaction with the Unc5H4 and all the Unc5 receptors.

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7.3 Over-expression of Netrin1 and Netrin2 expression constructs

7.3.1 Production of Netrin1 and Netrin2 expression constructs

If Netrin signalling is involved in the formation of the early axon scaffold, then ectopic expression of Netrin1 and Netrin2 at early embryonic stages should lead to defects in the early axon scaffold (particularly the TPC). To determine whether Netrin1 and Netrin2 were guiding the TPC, overexpression constructs of these guidance molecules that should affect the path of the TPC in the chick embryonic brain were produced using gateway multisite cloning.

For the overexpression experiments of axon guidance molecules, Netrin1 (1821bps) and Netrin2 (1764bps) full-length coding regions were isolated from chick cDNA by PCR using flanking primers followed by nested primers with the gateway cloning sequence.

The Netrin1 and Netrin2 fragments were then inserted into entry vectors using donor vector pDONR221 with gateway cloning technology. Once the vectors had been transformed and purified, a restriction digest was done to check the Netrin1 and Netrin2 fragments inserted into the vector were the correct size. For Netrin1 the restriction enzyme ApaI was used and for Netrin2, restriction enzymes BamHI and BglII were used.

Sequencing confirmed that the entry vectors contained the correct, full coding sequences for Netrin1 and Netrin2 (appendix 8 and 9). They were subsequently used as middle entry vectors and combined with a 5' entry vector containing the beta-actin promoter/CMV enhancer/beta-globin intron cassette, a 3' entry vector containing the IRES-eGFP-polyA cassette. The destination vector pDEST TOL2TR was used to produce the expression vector. Once the expression vector was produced, two restriction digests (Netrin1 with ApaI and with BamH1 and Netrin2 with BamH1 and with BglII) were done with each axon guidance molecule to check the recombination had worked successfully.

When the expression constructs were electroporated into the embryo, the cells around the target area will take up the construct. Some of the cells targeted will already express the gene, however many will not and any cell that has taken up the construct will now express the gene. Here, the aim was to express Netrin1 and Netrin2 ectopically in the commissural pretectum (rostral to the MFB in p1) where Netrins were not normally expressed to observe the effect on the formation of the TPC.

7.3.2 Positive controls of Netrin1 and Netin2 in the chick spinal cord

The Netrin1 and Netrin2 expression constructs were first electroporated into the chick spinal cord at E2 and re-incubated until E4 to check the constructs were working. This was used as a positive control test as it was already known Netrin1 and Netrin2 have an attractive effect on the spinal cord commissures (Kennedy et al., 1994; Serafini et al., 1996; Serafini et al., 1994). GFP was used to visualise which cells have taken up the constructs and therefore were over-expressing Netrin1, Netrin2 or the control. After harvesting the embryos, the axon tracts and cells containing the expression construct were visualised by double-labelling immunofluorescence with antibodies Tuj1 (mouse) and GFP (rabbit).
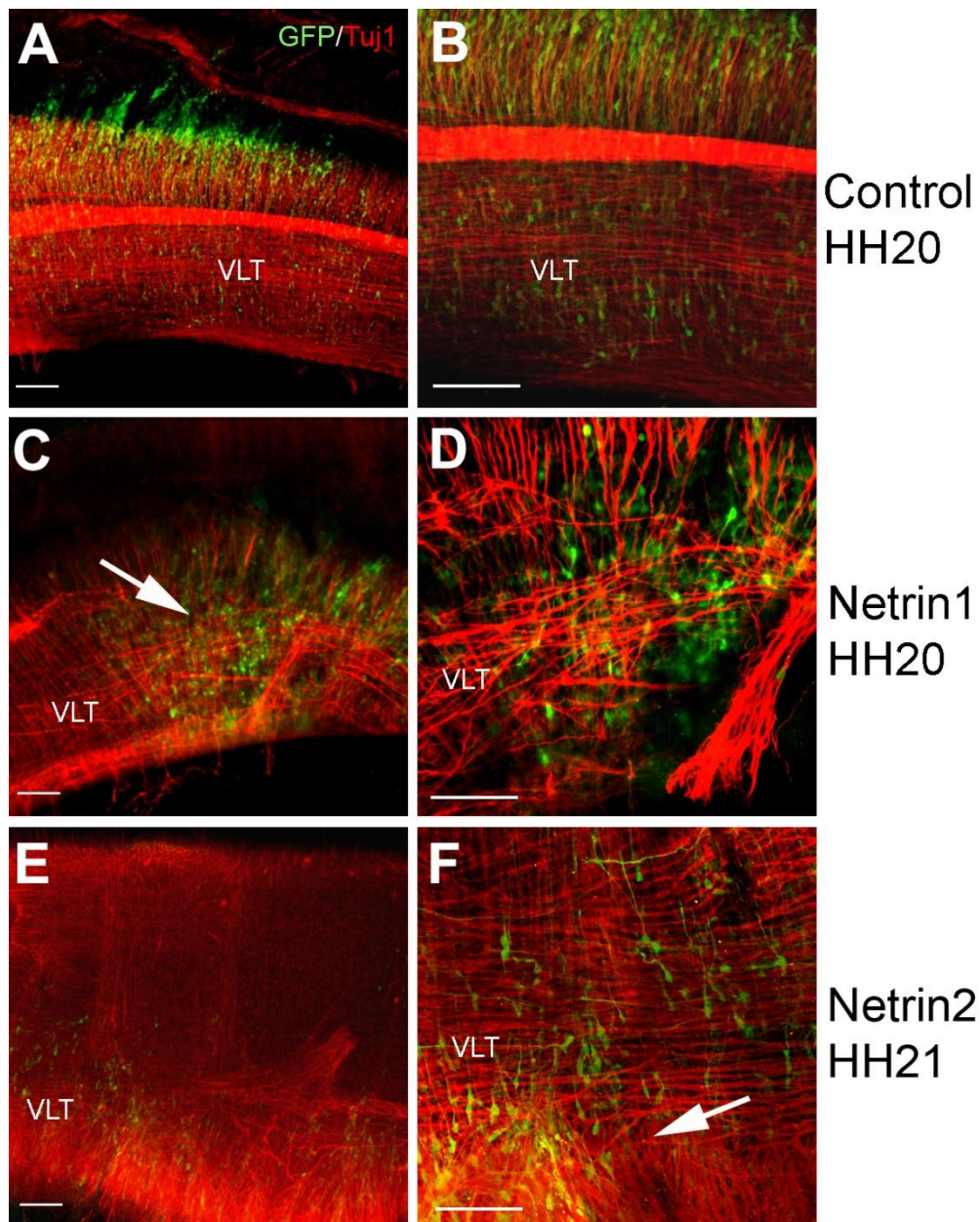
Figure 7.3 Netrin1 and Netrin2 are required for the attraction of commissures and longitudinal tracts in the chick embryonic spinal cord
Lateral view of whole mount spinal cords. Scale bars, 100µm

A-F) Dorsal is top of image and ventral is bottom of image. Commissural axons are projecting from the dorsal midline to the ventral midline where they will cross. The ventral longitudinal tract (VLT) is running along the floor plate. A) HH20 GFP control. Axon tracts are highly organised. B) HH20 GFP control, higher magnification of A. C-D) HH20 Netrin1 overexpression was indicated by green GFP labelling in the ventral spinal cord. Commissural axons and longitudinal axons are affected (arrow). D) Higher magnification of C. E-F) HH21 Netrin2 overexpression was indicated by green GFP labelling in the ventral spinal cord. Commissural axons and longitudinal axons are affected (arrow). F) Higher magnification of E. Some of the ventral longitudinal tracts were affected (arrow).

When Netrin1 and Netrin2 were electroporated into the ventral spinal cord, the axon tracts were clearly affected by the ectopic expression of these guidance molecules and the commissural axons and the ventral longitudinal tract (VLT) were misrouted (Fig 7.3C-F).

The control GFP vector showed no phenotype when it was expressed throughout the spinal cord (Fig 7.3A, B). All the commissural axons were projecting dorsally to the floor plate where they will cross in a highly organised way. The ventral longitudinal tract was running along the floor plate. Netrin1 (Fig 7.3C, D) showed a stronger phenotype than Netrin2 (Fig 7.3E, F). When Netrin1 was overexpressed the projection of the ventral longitudinal tract was disrupted when the axons encounter the region of Netrin1 overexpression. The axons were attracted to this region (Fig 7.3C, arrow). The commissures were also affected. Following Netrin2 overexpression the ventral longitudinal tract was disrupted when the axons encountered the region of overexpression. However, the phenotype was not as severe like with Netrin1, as fewer axons are affected. These results showed that the Netrin1 and Netrin2 expression constructs were working.

7.3.3 Over-expression of Netrin1and Netrin2 in the chick embryonic brain

The Netrin1 and Netrin2 expression constructs were electroporated into the embryonic chick brain targeting the MFB at E2 and the embryos were re-incubated until E4.

Due to the different efficiencies of the electroporation, there were varying levels of GFP expression in each embryo. These levels have been characterised from expression level 1 to expression level 5 (Fig 7.4). At expression level 1 there was no GFP expression in the embryonic brain and at expression level 5 there was expression throughout the entire brain. When the embryos were electroporated the DNA moved towards the positive (anode) electrode which was placed on the left side of the neural tube (the negative electrode was placed on the right of the neural tube). Therefore only the right side of the brain took up the construct and misexpressed the gene, while the left side will act as a control (change due to positioning of the brain at later stages).
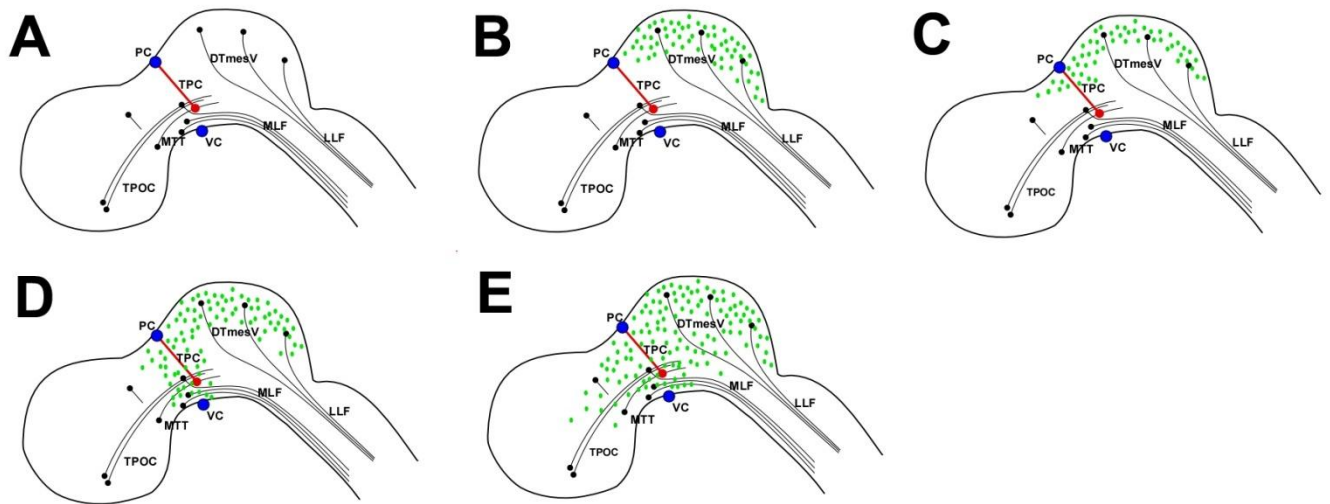
Figure 7.4 Determination of GFP expression in electroporated chick embryonic brains

A) Expression level: 1. No expression throughout the brain. B) Expression level: 2. Expression is in the mesencephalon only. C) Expression level: 3. Expression is in the mesencephalon and along the dorsal MFB. D) Expression level: 4. Expression is along the whole MFB. E) Expression level: 5. Expression is throughout the whole brain. The TPC is highlighted in red. Green spots represent the expression of GFP.
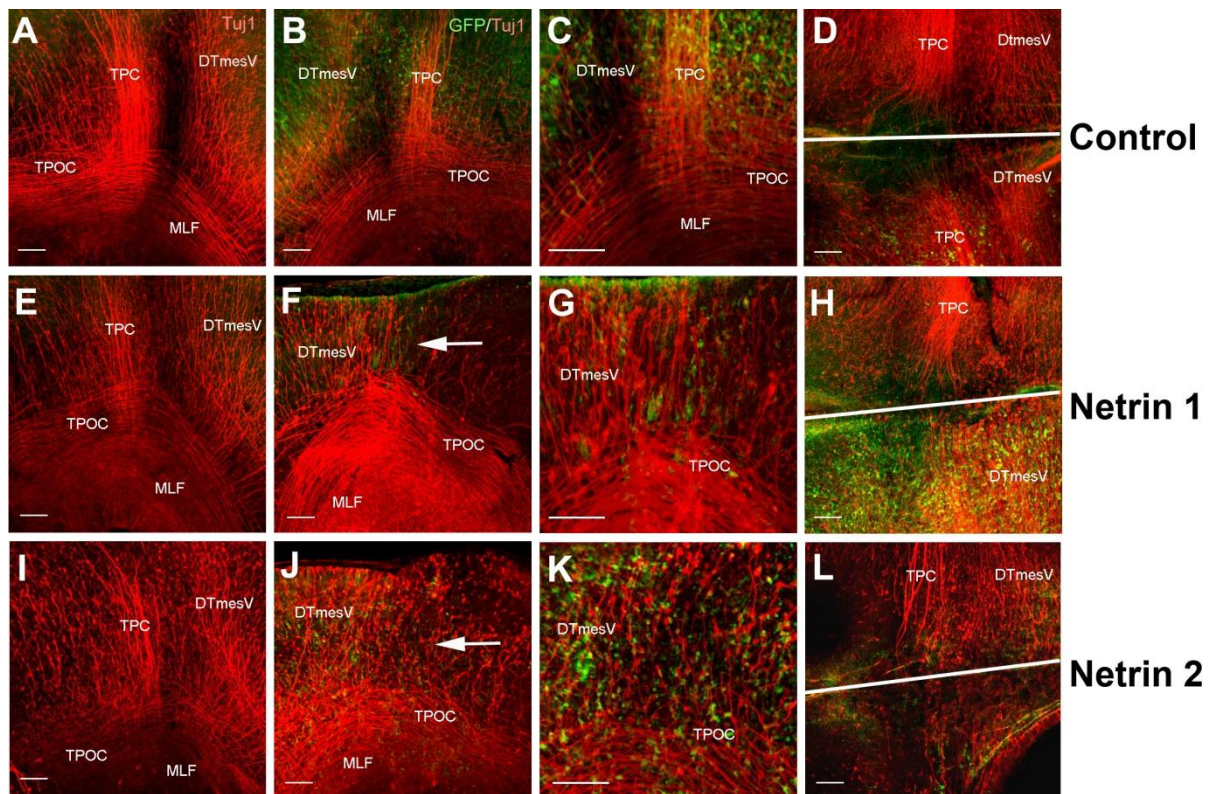
Figure 7.5 Over-expressing Netrin1 and Netrin2 in the chick embryonic brain results in the loss of the TPC at the MFB

Lateral view of whole mount embryos. Scale bars, 100μm

A-D) HH20 GFP control. A) Left side of the brain and therefore no GFP expression. TPC is present. B) Right side of the brain and GFP expression level 4. The TPC is present. C) Right side of the brain, higher magnification of B. The TPC is present. D) Dorsal view of the midline (indicated by line) and GFP expression is only present on one side. The TPC axons are projecting towards the midline on both sides. E-H) HH20 Netrin1. E) Left side of the brain and therefore no GFP expression. The TPC is present. F) Right side of the brain and GFP expression level 4. The TPC is missing along the MFB (arrow). The VLT also appears to project closer to the DTmesV. G) Right side of the brain, higher magnification of F. The TPC is missing along the MFB. H) Dorsal view of the midline (indicated by line) and GFP expression is only present on one side. The TPC axons are only projecting towards the midline on one side. I-L) HH20 Netrin2. I) Left side of the brain and therefore no GFP expression. The TPC is present. J) Right side of the brain and GFP expression throughout the brain. Expression level 5. The TPC is missing from the MFB (arrow). K) Right side of the brain, higher magnification of J. The TPC is missing from the MFB. L) Dorsal view of the midline (indicated by line) and GFP expression is only present on one side. The TPC axons are only projecting towards the midline on the side without GFP expression.

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When Netrin1 (Fig 7.5F, G) and Netrin1 (Fig 7.5J, K) were overexpressed in the chick embryonic brain the TPC axons failed to project from their neurones located ventrally at the MFB. In both the controls (Fig 7.5A-D) and the non-electroporated left side of the brain (Fig 7.5E, I) the TPC was present along the rostral edge of the MFB.

The dorsal preparations (Fig 7.5D, H and L) showed that the TPC axons on both sides project to the midline in the GFP control (Fig 7.5D). In Netrin1 (Fig 7.5H) and Netrin2 (Fig 7.5L) overexpressed embryos, the TPC was clearly projecting towards the midline on the non-electroporated side of the brain but on the other side where there was GFP expression the TPC axons were missing.

The severity of the phenotype depended on the level of GFP expression (Fig 7.7). In the control embryos, the TPC was present at all expression levels (Fig 7.7A; 1-5). When expression of Netrin1 and Netrin2 was low (1-3) the TPC was either present or only slightly affected (Fig 7.6F, H). As the expression level increased to 4 the percentage of embryos with the TPC present decreases (Fig7.7B, C). At expression 5, the majority of the embryos have no TPC (Fig 7.6N, P). When expression levels of the Netrin1 expression construct was 5 (throughout the brain) 60% of the embryos have no TPC and 40% have an affected TPC. When expression levels of the Netrin2 expression construct was 5 (throughout the brain) 66.7% of the embryos have no TPC and 33.3% have an affected TPC.
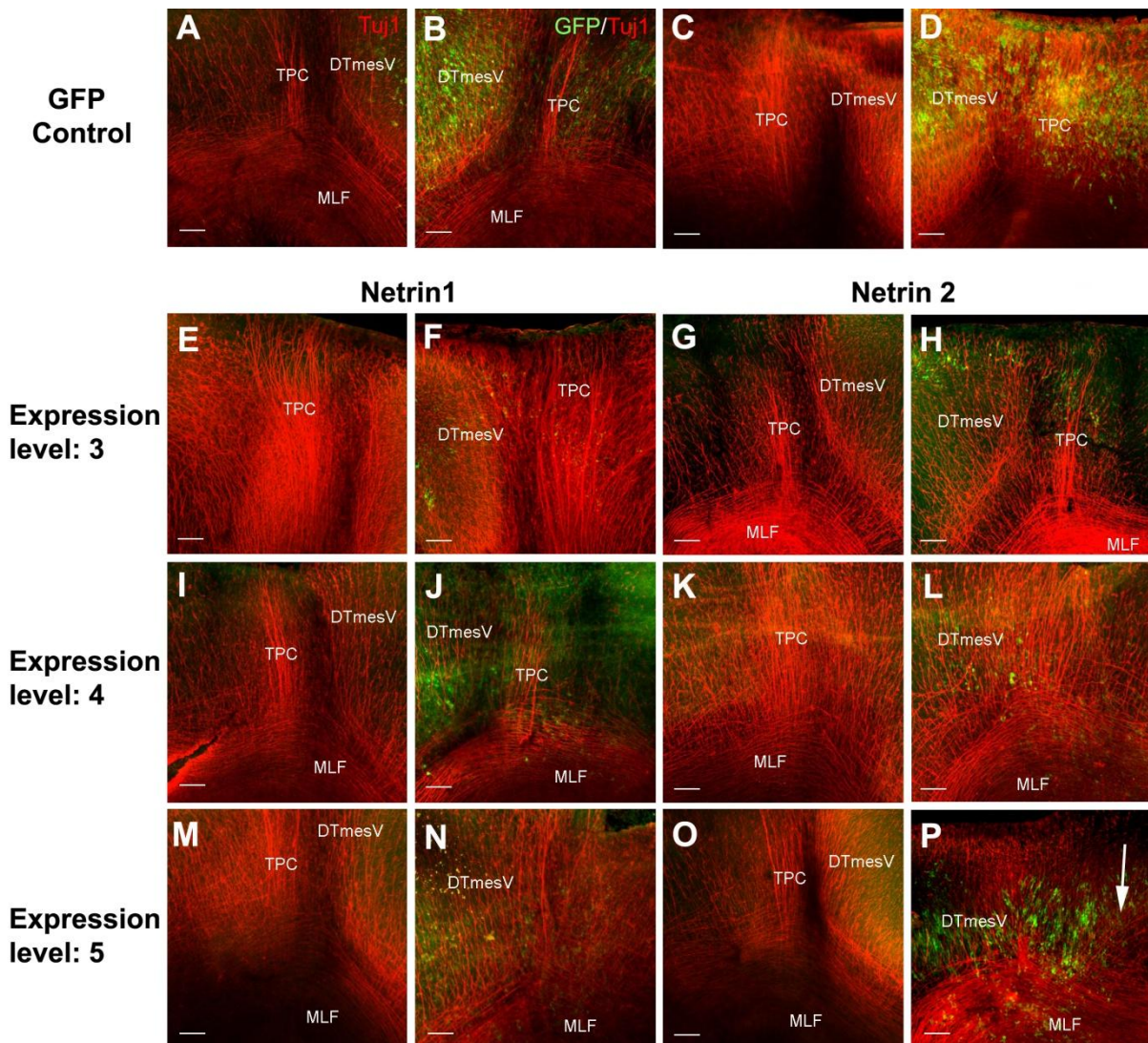
Figure 7.6 Varying expression levels of GFP affects the severity of the phenotype of the TPC
Lateral view of whole mount chick embryonic brains. Scale bars, 100μm

A-D) HH20 GFP controls. A and C) Non-electroporated side of the brain, all axon tracts including the TPC are present. B and D) High levels of GFP on the electroporated side of the brain. All axon tracts including the TPC are present. E and F) HH21 Netrin1 expression level 3. TPC axons are present on the electroporated side, however there appears to be fewer axons and they are less fasciculated. G and H) HH20 Netrin2 expression level 3. TPC axons are present on both sides. I and J) HH21 Netrin1 expression level 4. TPC axons are present on the electroporated side; however the axon tract appears wider and less fasciculated. K-L) HH21 Netrin2 expression level 4. There appears to be fewer TPC axons on the electroporated side. M-N) HH21 Netrin1expression level 5. There appears to be no TPC axons on the electroporated side. O-P) HH19 Netrin2 expression level 5. There appears to be no TPC axons along the MFB. Some axons are projecting dorsally away from the region of Netrin2 misexpression (arrow).
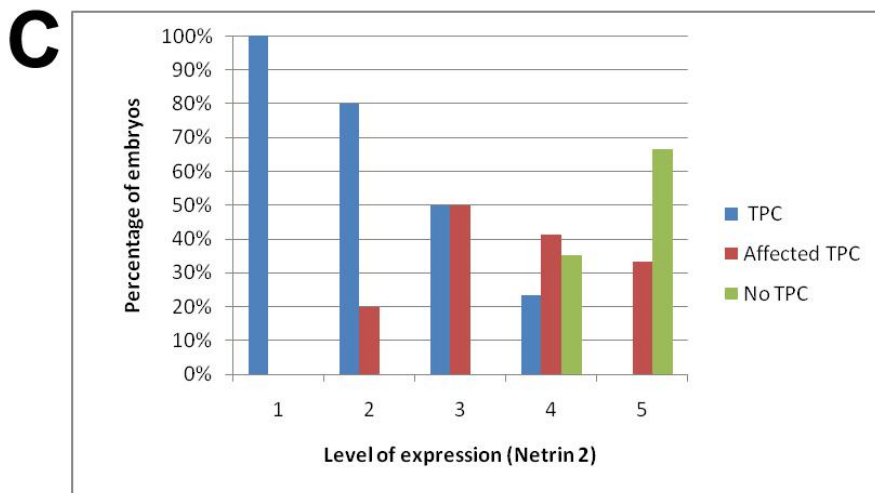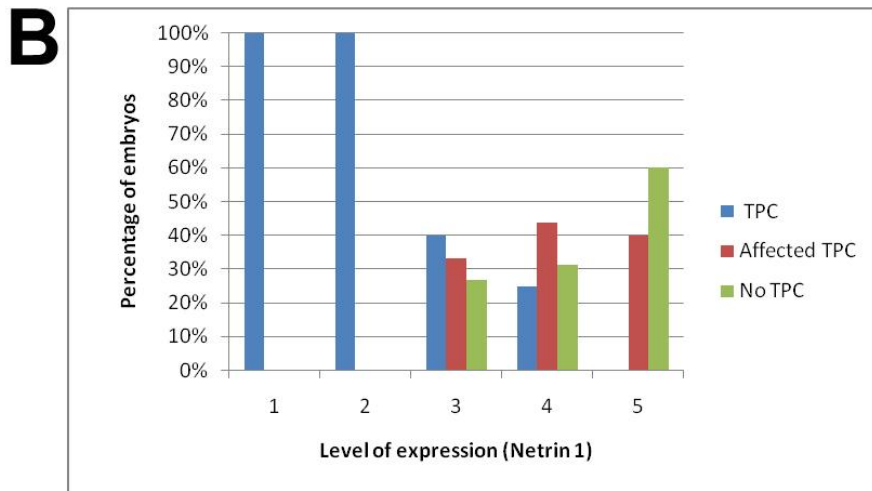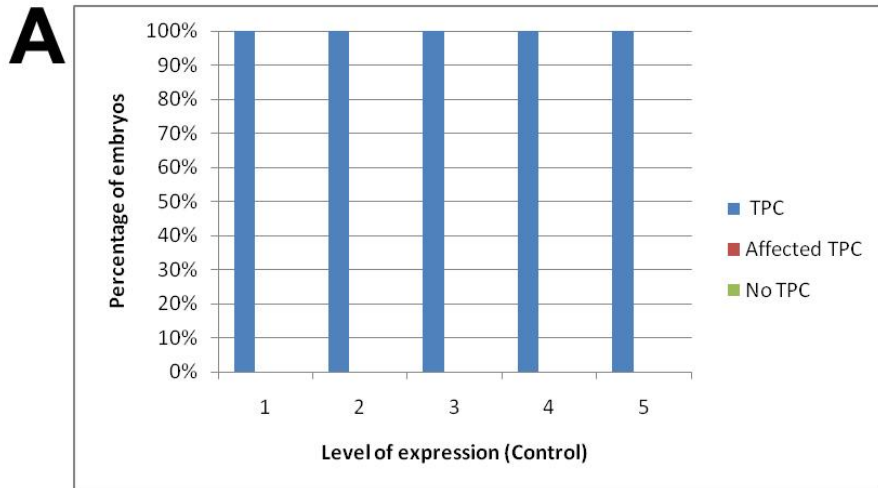
Figure 7.7 Quantification analysis showing percentages of embryos affected by expression construct at different levels of expression

A) GFP Controls. n=20 B) Netrin1 over-expression. n=48 C) Netrin2 over-expression. n=47. Blue bars show the percentage of embryos with TPC still present. Red bars show percentage of embryos with TPC affected. Green bars show percentage of embryos with no TPC present. n = number of chick embryos analysed.

7.3.4 Netrin1 and Netrin2 effect on the ventral longitudinal tract (VLT)

As the VLT appeared to be enlarged in some of the Netrin1 overexpressed embryos at E4 (Fig 7.5F), chick embryos injected with Netrin1 or Netrin2 constructs were harvested a day earlier at E3, to see if Netrin1 or Netrin2 had an effect on any of the other early axon scaffold tracts. It was also been suggested that Netrin1 would have an effect on the MLF but Netrin2 would not (Riley, 2008).

Netrin1 appeared to have an effect on the VLT, as the gap between the VLT and DTmesV was smaller (Fig 7.8B, arrow). The TPOC and MLF axons were likely to be attracted towards the Netrin1 expression. As Netrin2 was already expressed dorsal to the MLF, it would not be expected to have an effect on the VLT. The TPC began to project TPC axons at HH18 from the ventrally located neurones in the non-electroporated side (Fig 7.8C) and on the electroporated side where Netrin2 was overexpressed the TPC axon were missing (Fig 7.8D, arrow).
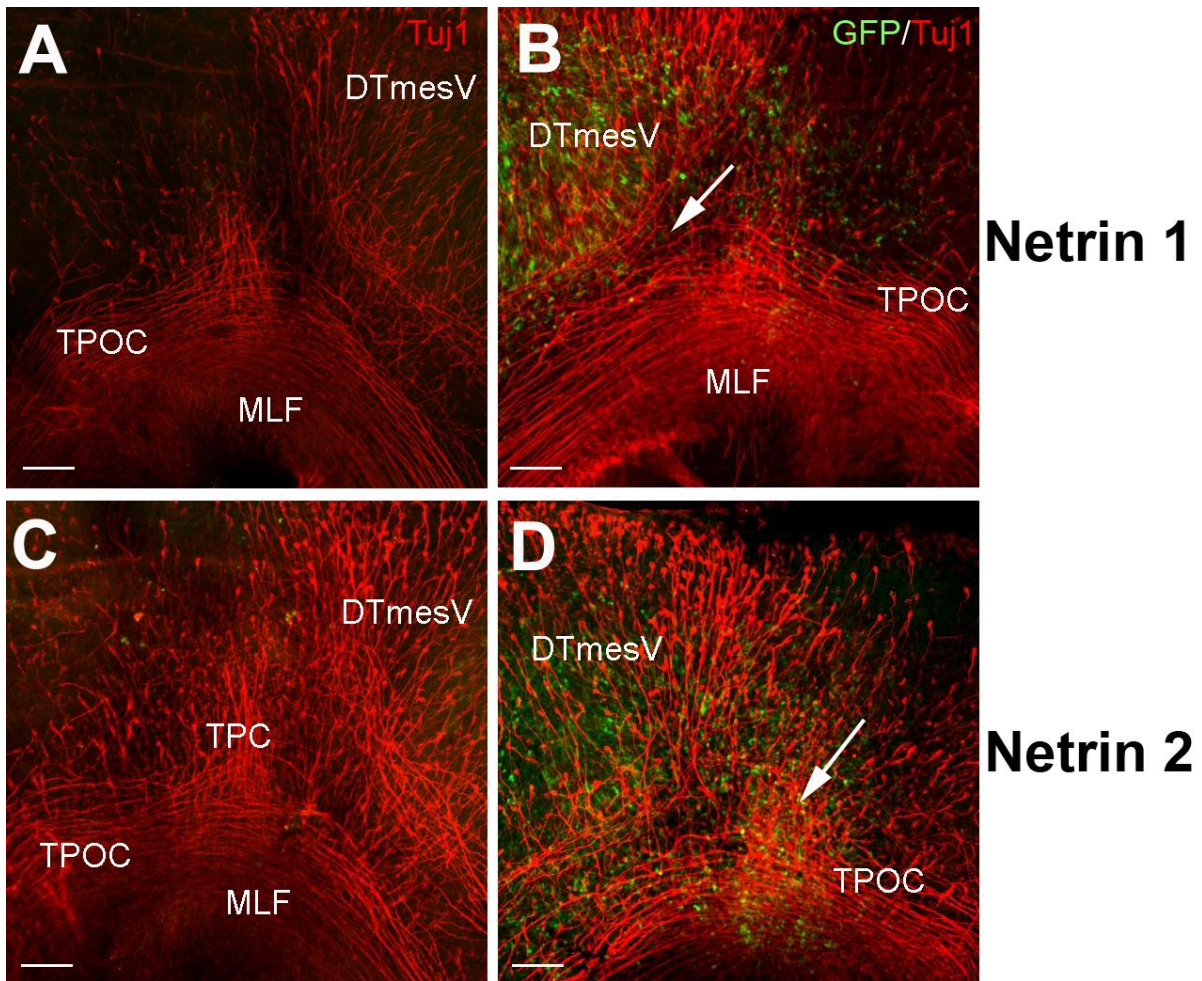
Figure 7.8 Possible effect of overexpressing Netrin1 and Netrin2 on the VLT as well as the TPC at E3

A-B) HH18 Netrin1 the gap between the VLT and DTmesV appears smaller when compared to the non-electroporated side (arrows). C-D) HH18 Netrin2 the TPC is missing (arrow) the MLF and TPOC appear to be projecting normally.
Scale bars, 100μm

7.4 Knockdown of Netrin2 in the Xenopus embryonic brain

Netrin1 expression was present throughout the floor plate of the *Xenopus* neural tube (de la Torre et al., 1997). The Netrin2 sequence in *Xenopus Laevis* has not been previously identified. The sequence for *Xenopus tropicalis* Netrin2 was found by comparison to the chick Netrin2 sequence. The *X.tropicalis* sequence then used to search for the sequence in *X.Laevis*. The sequences were highly conserved (appendix 10 and 11) which suggests the expression pattern and function of Netrin2 would be similar. Netrin2 was expressed in the somites (Fig 7.9A) in the *Xenopus* stage 32 embryo. Netrin2 was also specifically expressed within the hindbrain (Fig 7.9B, white arrows) and in the rostral telencephalon (Fig 7.9B, white arrowheads). The expression where the TPC axon tract is located is less clear and there does not appear to be a channel like in the chick brain (Fig 7.9B, black arrow).

To confirm the role of Netrin1 and Netrin2 in the guidance of the TPC, *Xenopus* was used as a model to knockdown the role of Netrin2 using a morpholino oligonucleotide (MO). The Netrin2 MO was injected in the *Xenopus* egg at different concentrations (11ng, 22ng and 44ng) at the 1-cell stage (done in collaboration with Jordan Price, University of Portsmouth). The MO should block ribosome binding and prevent any Netrin2 protein being translated. The phenotype of the early axon scaffold was analysed by immunohistochemistry to label the axon tracts using the HNK-1 antibody.
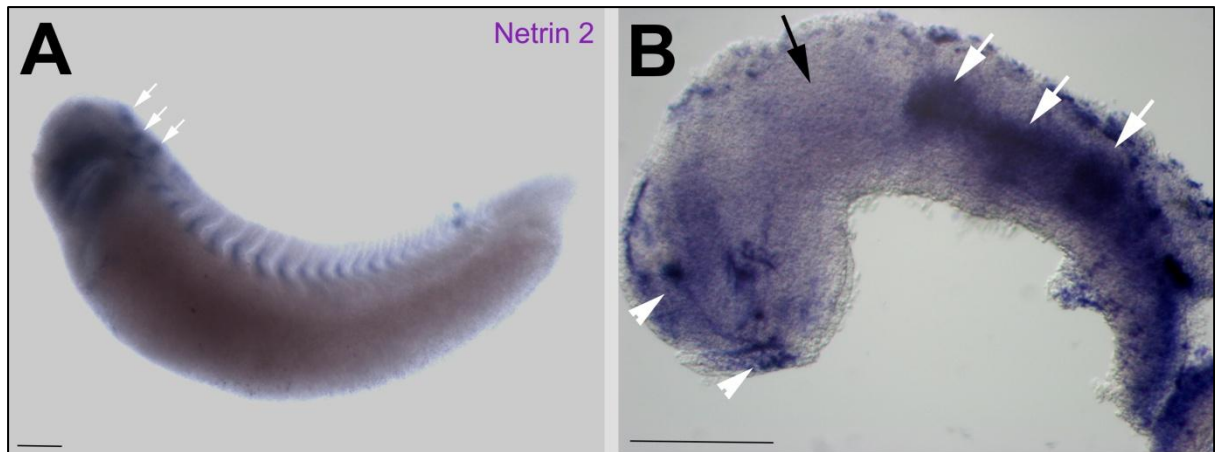
Figure 7.9 Expression of Netrin2 in the *Xenopus* embryo

A) Stage 32 whole-mount *Xenopus* embryo (*in situ* done by Jordan Price). Netrin2 is expressed in the somites and there are three spots of expression in the hindbrain (white arrows). There appears to be staining within the rostral brain. B) Dissected neural tube of the rostral region of the *Xenopus* embryonic brain, lateral view. The expression of Netrin2 in the hindbrain is very strong (white arrows). There also appears to be expression in the very rostral region of the brain that most likely correlate to the POC and AC (white arrowheads). There does not appear to be a clear channel like in the chick brain where the TPC axon tract is present (black arrow).

Scale bars, 500µm
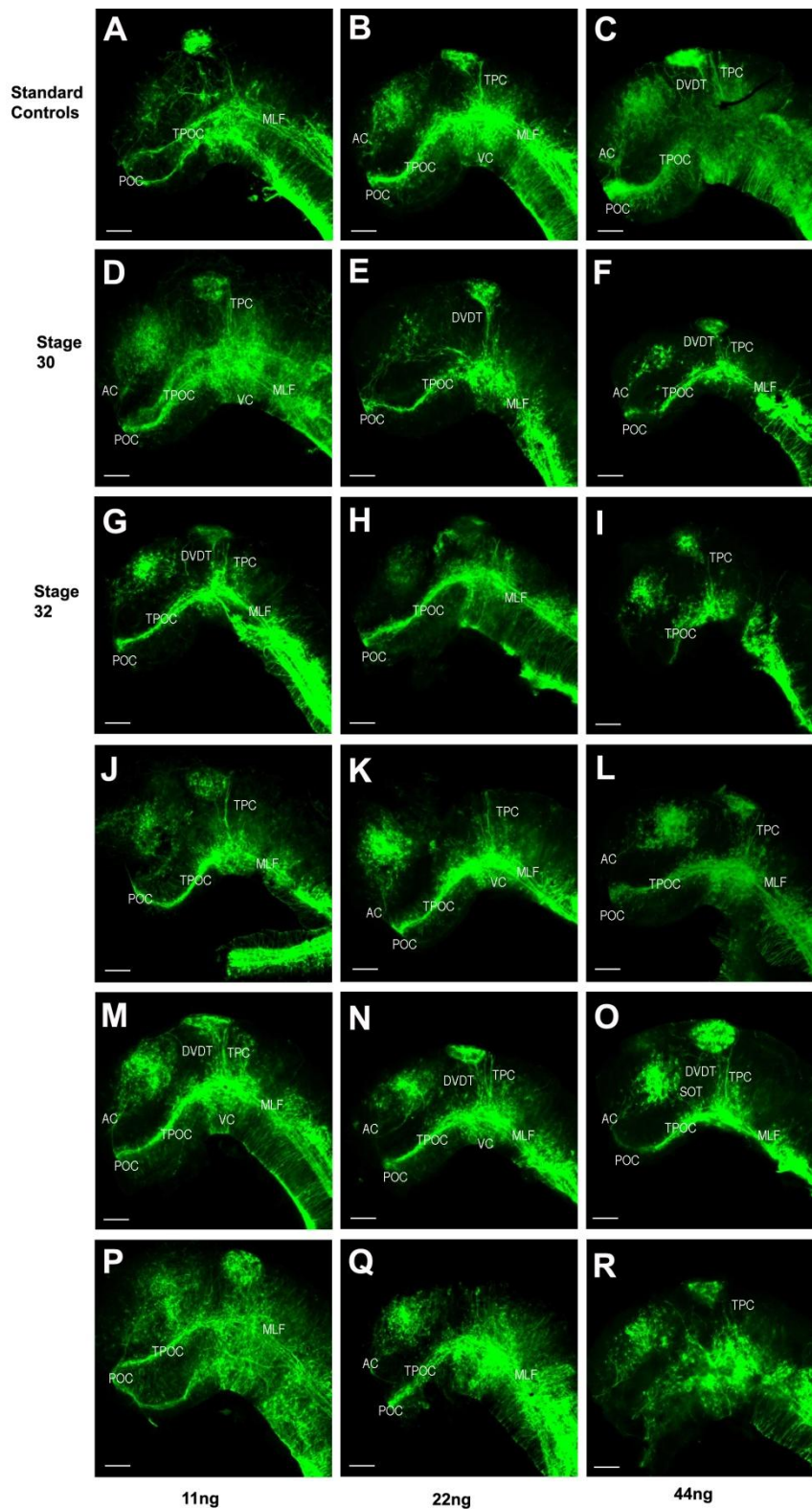
Figure 7.10 Morpholino injections of Netrin2 has no apparent phenotype on the TPC or the other early axon scaffold tracts

Lateral view of whole mount *Xenopus* neural tube. Scale bars, 100μm

A-C) Standard controls stage 32 the early axon scaffold is well established. A) 11ng B) 22ng C) 44ng D-F) Netrin2 MO stage 30 the TPC is beginning to form and the early axon scaffold appears normal. D) 11ng E) 22ng F) 44ng G-R) Netrin2 MO stage 32 the early axon scaffold is well established and appears normal. G, J, M, N) 11ng H, K, N, Q) 22ng I, L, O, R) 44ng.

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When the early axon scaffold formation in Netrin2 MO injected *Xenopus* embryos was compared with standard control injected *Xenopus* embryos, no obvious phenotype was observed (Fig 7.10). Even at different concentrations, the TPC axons and the other early axon scaffold tracts remained unaffected.

7.5 Expression of Netrin1 in the cat shark embryonic brain

To further investigate the conservation of the Netrin genes, both Netrin1 and Netrin2 were attempted to be cloned from the cat shark, *Scyliorhinus canicula*. The Netrin1 sequence was obtained using the elephant shark genome and comparison with other species. Netrin1 was successfully cloned from cat shark cDNA by Constandinos Carserides and then used to make the *in situ* hybridisation probe. The expression pattern showed Netrin1 was also expressed throughout the floor plate, a pattern that was consistent in other organisms. In the embryonic cat shark brain, Netrin1 expression was throughout the ventral neural tube (Fig 7.11A). There was also expression in the pharyngeal pouches (Fig 7.11A, arrows) and a patch located caudally in the neural tube (Fig 7.11A, arrowhead). In the brain, Netrin1 was expressed along the floor plate and expression was more diffuse into the basal plate (Fig 7.11B). There was no expression throughout the alar plate and the secondary telencephalon.
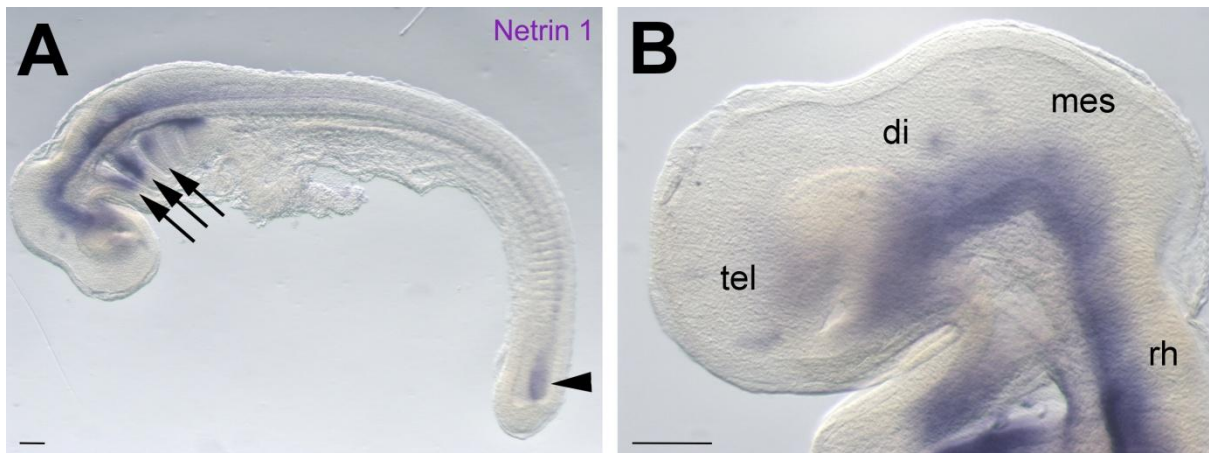
Figure 7.11 Netrin1 expression in the cat shark embryo

A) At stage 23 Netrin1 is expressed throughout the floor plate. Expression is highest in the brain and weakens throughout the spinal cord. There is strong expression in the first, second and third pharyngeal pouches (arrows) and strong expression in the most caudal part of the neural tube (arrowhead). B) Higher magnification of the brain (A) Netrin1 is expressed in the floor plate and diffused in the basal plate from the rostral diencephalon through the rhombencephalon.

Scale bars, 500μm

7.6 Discussion

The formation of the TPC has to be tightly regulated, in particular the pathway the axons take as the TPC neurones were intermingled with the MLF neurones. The choice points the TPC axons must take were to initially project dorsally (not caudally with the MLF). The axons must then project along the rostral boundary of the MFB in p1 as a tightly fasciculated tract and not cross the MFB into the mesencephalon. Once the axons reached the dorsal midline, they must then cross to form the PC. Due to their expression patterns, Netrin1 and Netrin2 were selected as candidate axon guidance molecules, repelling the TPC axons dorsally along the MFB in the chick embryonic brain.

7.6.1 Functional analysis of Netrin1 and Netrin2 in the chick embryonic brain

The Netrin1 and Netrin2 expression constructs were confirmed to be working by electroporation of the constructs into the spinal cord causing misrouting of the ventral longitudinal axons and the commissures. Netrin2 was expressed at a lower level than Netrin1 in the embryonic chick spinal cord (Kennedy et al., 1994) which would explain why the phenotype in the spinal cord with Netrin2 was not as severe as with Netrin1. GFP control constructs were also used to show the TPC axons were unaffected by the electroporations.

Netrin1 and Netrin2 have been shown by gain-of-function studies to have a repulsive role in the guidance of the TPC to ensure these axons project along the correct path in the chick embryonic brain. When there was a high level of the expression constructs (expression level 4-5) in the brain, the TPC axons would fail to project along the MFB (Table 7.2). When expression of the Netrin1 construct was high (expression level 5), 60% of the embryos had no TPC and with Netrin2, 66.7% of the embryos had no TPC.

Constructs	% No TPC	% Affected TPC	% TPC
Control	0	0	100
Netrin1	60	40	0
Netrin2	66.7	35.3	0

Table 7.2 Percentage of embryos affected when expression level is 5

When there was misexpression around the MFB, it would be expected that the TPC axons were repelled away from the area of overexpression. This was not seen and very often, even though the TPC may have been affected and many embryos contained fewer TPC axons, they were not obviously repelled away from the area of overexpression. The TPC axons did not appear to project along a route that they would not normally take. It was possible that the TPC axons could act as MLF axons and project caudally along the floor plate but this was difficult to determine as the MLF and TPC neurones were intermingled. The phenotype appeared stronger when Netrin1 and Netrin2 overexpression occurred ventrally over the TPC neurones. Not all of the chick embryos showed a phenotype, due to overexpression of the constructs not being in the path of the TPC.

The varying phenotype of Netrin1 and Netrin2 on the TPC axons suggests these axon guidance molecules could be required to act only when the neurone initially projects its axon and then the repulsive effect is weakened as the axons course along the MFB to the midline. Unc5H4 expression could be reduced as the axons project closer to the midline. There may also be an attractant at the roof plate that has a stronger affect on the TPC axons (Fig 7.12).
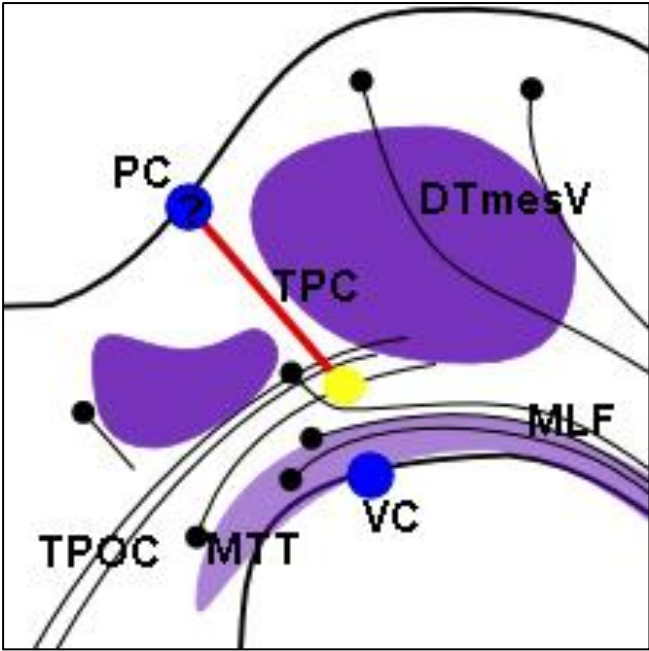
Figure 7.12 Schematic showing the overview of Netrin expression and its effect on the TPC

The TPC axons (red) project dorsally along the MFB to the dorsal midline. The TPC neurones express Unc5H4 (yellow) that would suggest the axons are being repelled by Netrin1 (light purple) and Netrin2 (dark purple). There could possibly be an attractive cue at the dorsal midline attracting the TPC axons.

7.6.2 Unc5 receptor

When comparing the expression of Unc5H4 by *in situ* hybridisation and immunohistochemistry, the pattern appeared slightly differently in the chick embryonic brain. The Unc5H4 *in situ* probe showed expression of mRNA in the TPC and other neurones within the brain, whereas the Unc5H4 antibody did not appear to label the TPC neurones or axons. This could be due to the high level of conservation between the Unc5 receptors and the Unc5H4 EST used for the *in situ* probe and there was a cross reaction between the different Unc5 receptors (therefore the Unc5H4 EST bound to more than one of the Unc5 receptors). To test this immunohistochemistry would need to be done for the other Unc5 receptors (Unc5H1-3) to check if they label the TPC neurones. To prevent cross reaction, an Unc5 probe will need to be designed that does not have high conservation with the other receptors. The Unc5H4 antibody showed the MLF, DTmesV and MTT express the receptor. This would suggest the growth of these axons were also influenced by repulsion of Netrin1 and Netrin2. The overexpression of Netrin1 and Netrin2 did not appear to have a repulsive effect on the MLF, DTmesV and MTT. Initial analysis of Netrin1 appeared to have an attractive role on the MLF. The binding affinities of Netrin1 and Netrin2 to the Unc5 receptor may be different and would need to be analysed further.

Initial experiments with Unc5H4 double stranded RNA (dsRNA) to knock down the function of Unc5H4 appeared to have no effect on the formation of the early axon scaffold (data not shown). This was most likely due to the number of cells targeted were too low to have an effect on the function of Unc5H4. If Unc5H4 was successfully knocked down, the TPC axons would be expected to project along a completely different route.

7.6.3 Functional analysis of Netrin2 in the Xenopus embryonic brain

To confirm the repulsive function of Netrin1 and Netrin2, a Netrin2 MO was designed to knockdown the function of Netrin2 in the *Xenopus* embryo. There was no obvious phenotype

of the early axon scaffold tracts when Netrin2 MOs were injected into *Xenopus* embryos. This could be due to lack of conservation of Netrin2 function or the MO simply did not work. To check the Netrin2 MO was working a fusion protein can be used or immunohistochemistry with a Netrin2 antibody to show no Netrin2 protein was translated.

7.6.4 Conservation of Netrins

Netrin1 has been shown to be expressed in the floor plate of many vertebrates such as zebrafish (Macdonald et al., 1997), *Xenopus* (de la Torre et al., 1997; Wilson and Key, 2006) and mouse (Matise et al., 1999; Serafini et al., 1996). The expression pattern was also true for chick (Riley et al., 2009; Serafini et al., 1994) and cat shark (Fig 7.10). Netrin1 was also expressed in the notochord and floor plate of amphioxus (Shimeld, 2000). As the expression of Netrin1 was conserved we would also expect the function to be conserved. Even though the conservation of the Netrin sequences was high, (Lauderdale et al., 1997) the function of Netrin was possibly not conserved. Netrin1 has no effect on early axon scaffold formation in zebrafish (Macdonald et al., 1997), however Netrin1 has an attractive role in the formation of the early axon scaffold in the *Xenopus* embryonic brain (Wilson and Key, 2006). Any affect on the TPC was not shown due to the selective labelling of the antibody NOC-1 used. It was therefore possible that the Netrin2 MO used in *Xenopus* was working and this would further confirm the function of Netrins was not conserved. Analysis of the early axon scaffold in knockout mice could also be done to confirm the repulsive role of Netrin1 and Netrin2 (Netrin3) on the TPC axons.

7.6.5 Additional Netrin receptors in the embryonic brain

Protogenin has been shown to be a type I transmembrane member of the DCC/Neogenin family (Toyoda et al., 2005). This would suggest protogenin could act as a Netrin receptor, involved in the attraction of axons. Like neogenin, protogenin was also expressed throughout the brain (Fig 6.12Q, R). Initial expression of protogenin in mouse, chick and zebrafish was

similar, however throughout development the expression changes (Vesque et al., 2006), and this would suggest protogenin has slightly different functions that are not conserved like the function of Netrin1 and Netrin2. As the DCC receptor has not been identified in chick (F.R. Schubert, unpublished results), protogenin could have a similar role to DCC in which it binds to Unc5 and mediates a repulsive role. Functional studies are required to determine whether protogenin acts as a Netrin receptor and if it has a role in guiding the early axon scaffold tracts.

Chapter 8

Discussion

8.1 Anatomy of early axon scaffold development

The early axon scaffold is the first neuronal structure to form within the embryonic vertebrate brain. It is important for the guidance of later, follower axons as well as axon tracts that form within the scaffold. While detailed analysis of early axon scaffold formation has been done in various vertebrates such as zebrafish (Chitnis and Kuwada, 1990; Wilson et al., 1990) or mouse (Easter et al., 1993; Mastick and Easter, 1996), detailed analysis was missing in chick and a direct comparison of jawed vertebrates was yet to be done. Here the early axon scaffold, formed from an array of longitudinal, transversal and commissural tracts, was compared in cat shark, *Xenopus*, chick, zebra finch and mouse embryonic brains. The comparative antibody used was Tuj1 that labels βIII tubulin, except in *Xenopus* where associated surface glycoprotein HNK-1 antibody was used. The comparison of these vertebrates highlighted the formation of the early axon scaffold that has remained highly conserved throughout evolution, although there were notable differences in the appearance of some of the axon tracts (Fig 8.1). The comparison of these vertebrates has been discussed in detail in chapter 5.8.

8.1.1 Conservation of the ventral longitudinal tract (VLT)

The VLT, formed from the TPOC and MLF was present in all the vertebrates studied here and the MLF was the most conserved axon tract throughout evolution as well as the first tract to arise in the embryonic vertebrate brain (except in mouse where the DTmesV forms first). This makes the formation of the MLF particularly interesting to study. The MLF neurones in the chick embryonic brain were shown to be located in the Pax6-positive rather than the En1-positive domain and hence were strictly diencephalic. This would suggest that genes expressed as a diencephalic extension of the midbrain arcs (Agarwala and Ragsdale, 2002; Agarwala et al., 2001; Sanders et al., 2002) are important for patterning of these neurones. Among these, the transcription factors Sax1 and Emx2 have previously been shown to be involved in the formation of the MLF (Schubert and Lumsden, 2005). However, due to the late expression of these transcription factors, it was not believed they were involved in the specification of the progenitor cells into a MLF neuronal fate.

8.1.2 Function of the early axon scaffold

The main role of the early axon scaffold tracts during development is to act as a scaffold for the later, follower axons however many of these tracts will play a role in the function of the adult. The function of some of these early axon scaffold tracts has been investigated, however evidence for their function is lacking in higher vertebrates (Table 8.1).

Tract	Vertebrate	Function	Reference
MLF	Zebrafish	Swimming behaviour, visual and escape	(Gahtan and O'Malley, 2003; Gahtan et al., 2002; Sankrithi and O'Malley, 2010)
DTmesV	Chick	Jaw movement	(Hunter et al., 2001)
	Mouse	Jaw movement	(Mastick and Easter, 1996)
POC	Zebrafish	Guidance of optic nerves from retinal ganglion cells into the brain	(Burrill and Easter, 1995)
	Xenopus		(Easter and Taylor, 1989)

Table 8.1 Function of the early axon scaffold tracts

The MLF neurones are involved in visual and movement behaviour in the zebrafish larvae. It is most likely that the MLF has a similar function in other vertebrates, however this is yet to be investigated. There is plenty of evidence in jawed vertebrates to show the DTmesV axons innervate the jaws through the trigeminal nerve at rhombomeres 2. The DTmesV axon tract is specific for the function of jawed vertebrates and is not present in non-jawed vertebrates. The POC has a role in guiding the optic nerves into the brain. The POC also connects the TPOC on both sides of the brain.

A Lamprey

B Cat shark

C *Xenopus*

D Zebrafish

E Turbot

F Medaka

G Alligator

H Chick

I Mouse

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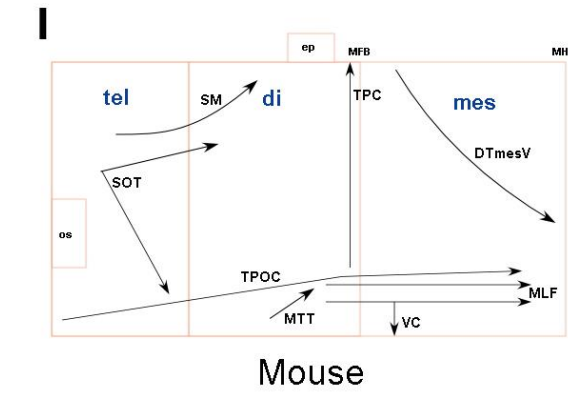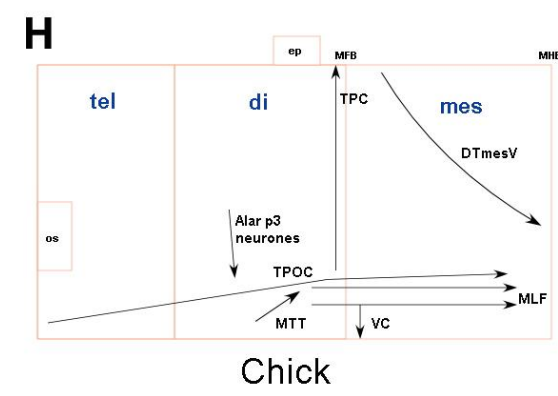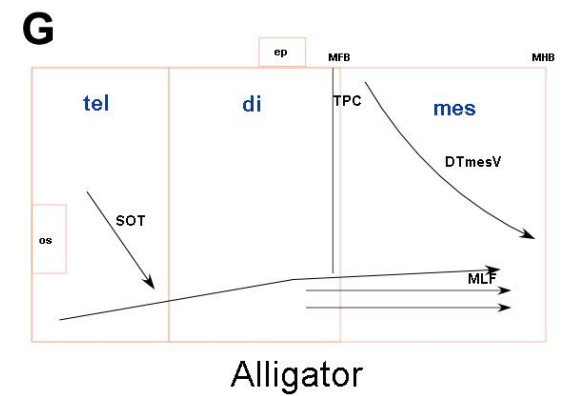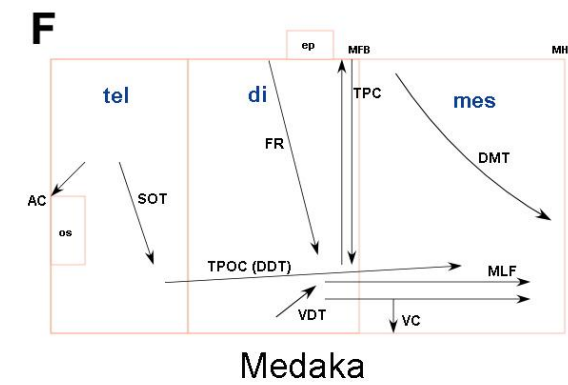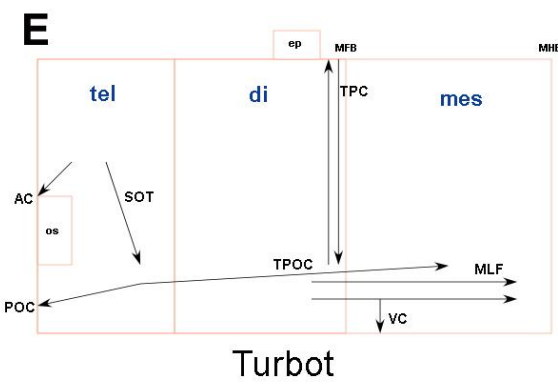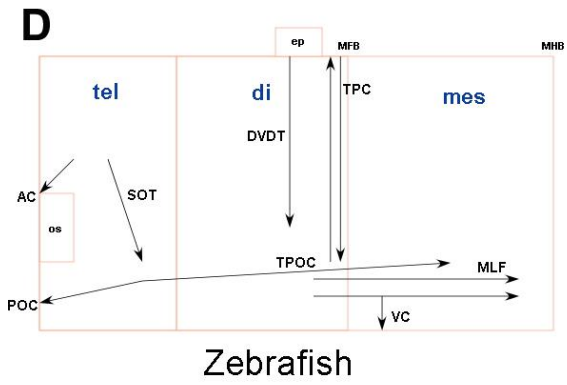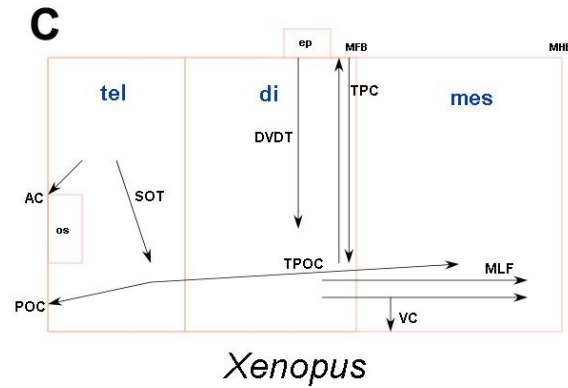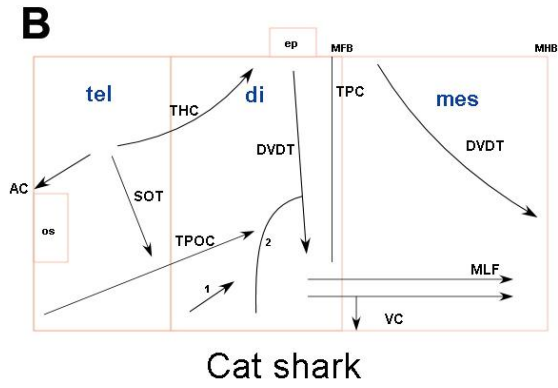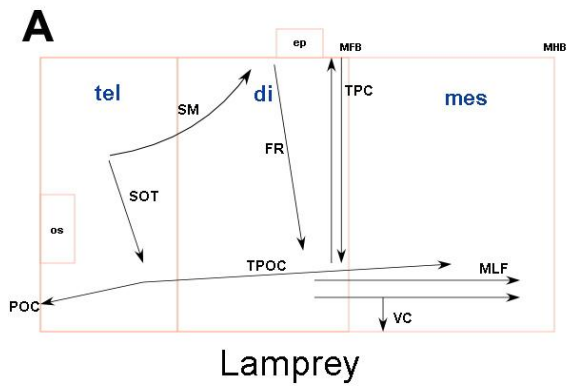
Figure 8.1 Schematic representation of the vertebrate embryonic brain and formation of early axon scaffold tracts

These schematic were done based on the results presented here (Chapter 5) and previous studies. A) Lamprey (Barreiro-Iglesias et al., 2008) B) Cat shark (chapter 5) two of the axon tracts remain uncharacterised (1 and 2). C) *Xenopus* (Chapter 5 Anderson and Key, 1999) D) Zebrafish (Ross et al., 1992) E) Turbot (Doldan et al., 2000) F) Medaka (Ishikawa et al., 2004) G) Alligator (Pritz, 2010) due to figures and lack of explanation in the text it was difficult to determine the exact location of some tracts. H) Chick (Chapter 5). I) Mouse (Chapter 5 Easter et al., 1993; Mastick and Easter, 1996). The SM and SOT axon tracts form later in development after the early axon scaffold is established (Nural and Mastick, 2004).

The most highly conserved tracts were the MLF which forms first in all vertebrates, except mouse where the DTmesV forms first, the TPC which marks the MFB and the TPOC forms the VLT along with the MLF.

The direction of the arrowheads indicates the direction of the axon projection from their neurones (where known).

di, diencephalon; ep, epiphysis; mes, mesencephalon; MFB, midbrain-forebrain boundary; MHB; midbrain-hindbrain boundary; os, optic stalk; tel, telencephalon

8.2 Specification of neurones into MLF fate

Microarray analysis was used to identify genes that were involved in specifying ventral diencephalic cells into a MLF fate between HH9 and HH11 in the chick embryonic brain. A total of 1531 genes were upregulated, 335 of which by 2-fold or above. From these results candidate genes were selected to be analysed further by *in situ* hybridisation. These genes included transcription factors, signalling molecules, receptors and vascular markers. The expression analysis confirms the upregulation at HH11 for most of the genes. However, only CRABPI was found to be expressed specifically by the MLF neurones at HH11. At HH14 CRABPI also labelled other neurones, suggesting it is not involved in specifying neurones to a MLF fate, but plays a more general role in differentiation of neurones (discussed in detail in 6.4.2 and 6.4.7)

8.3 Axon guidance of the early axon scaffold

The guidance of early axon scaffold tracts by axon guidance molecules has been shown in various vertebrates and is a highly conserved mechanism.

8.3.1 Axon guidance of the posterior commissure

A highly conserved transversal tract is the TPC, which was found in all the vertebrates studied (Fig 8.1). The TPC connects the two halves of the brain by crossing the dorsal midline at the MFB. It was particularly interesting that in chick and mouse the MLF and TPC neurones were intermingled ventrally in the caudal diencephalon. Even though these neurones were intermingled, they still projected along very separate paths, the MLF projected caudally along the ventral midline of the mesencephalon and the TPC projected dorsally along the MFB. The guidance of the TPC axons had not been studied previously and the axon

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guidance molecules Netrin1 and Netrin2 were selected as candidate genes for repelling these axons away from the ventral midline and dorsally along the MFB. The receptor Unc5H4 interacts with Netrins and is involved in mediating the repulsion of axons away from the area of Netrin expression (Engelkamp, 2002). Using *in situ* hybridisation, Unc5H4 appeared to be expressed by the TPC neurones suggesting their axons are channelled into their narrow path by Netrins. Ectopic expression of Netrin1 and Netrin2 caused the TPC axons to be missing from the MFB boundary. However, in some of the experiments, the TPC was only slightly affected and contained fewer axons, depending on the level of ectopic expression. In these cases, the TPC axons grew through the Netrin expression without being repelled. This suggests that Netrin1 and Netrin2 only affect the initial outgrowth of the TPC axons and there are other guidance cues involved in the guidance towards and across the midline (Fig 8.2). Netrin1 and Netrin2 may also play a role in the guidance of other axon tracts, in particular Netrin1 may play a role in attracting the MLF neurones to the floor plate (Ahsan et al., 2007). Although the function of Netrins as axon guidance molecules was conserved, the role they play in guiding the early axon scaffold tracts was not likely to be conserved (Lauderdale et al., 1997; Wilson and Key, 2006). As shown in this study with *Xenopus* Netrin2 was not expressed in the alar diencephalon and mesencephalon and knockdown of Netrin2 had no consistent effect on the early axon scaffold.
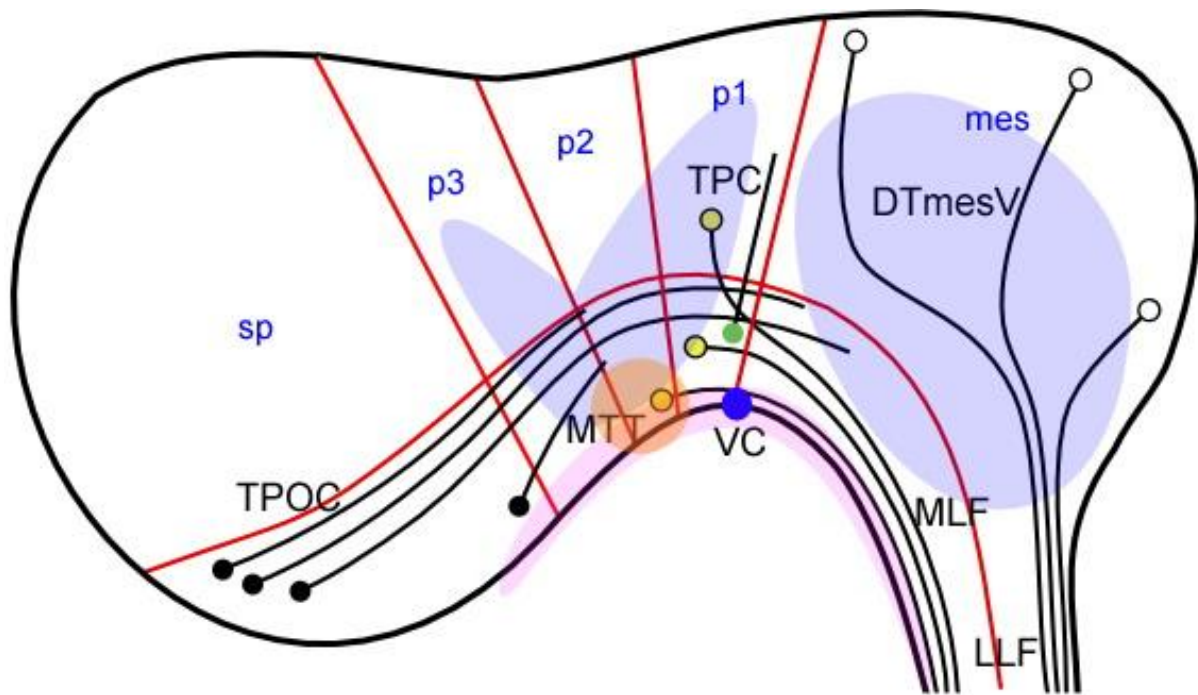
Figure 8.2 Overview of axon guidance in the embryonic chick brain

Netrin1 (pink) and Netrin2 (blue) interact with an Unc5 receptor expressed by the TPC axons (green) to repel the axons dorsally towards the dorsal midline where they cross. Unc5H4 was also expressed by DTmesV neurones (white) and MLF neurones (yellow). Sema3A expression (orange) lies rostral to the MLF neurones and is involved in repulsion of MLF neurones, preventing them entering the telencephalon (Riley, 2008). Sema3A interacts with Neuropilin1 that is expressed specifically by the MLF neurones (yellow). The floor plate expresses a number of axon guidance including Slits, Netrin1 and Shh (pink). Netrin1 and Shh are likely to have an attractive role on the MLF neurones, ensuring they project along the ventral floor plate and Slits prevent them crossing the midline.

mes, mesencephalon; p1, prosomere1; p2, prosomere2; p3, prosomere3; r1, rhombomere1; r2, rhombomere2; sp, secondary prosencephalon

8.3.2 Axon guidance of the MLF

In chick, Slits (Molle et al., 2004) and Sema3A (Riley, 2008) have been shown to be involved in the guidance of the MLF along the ventral midline. Robo1 was expressed by the MLF neurones in mouse (Farmer et al., 2008) and prevents the MLF axons crossing the midline by Slit repulsion. Slit2 was upregulated in the microarray, suggesting expression of this gene was required for MLF guidance when the first MLF neurones differentiate and start projecting at HH12, preventing them from crossing the ventral midline. The SRGAP proteins (SRGAP1 was upregulated in the microarray) bind to Robo1 in mouse to link Slit and Robo to the actin cytoskeleton (Bacon et al., 2009).

8.3.3 Axon guidance of the TPOC

In the embryonic *Xenopus* brain NOC-2, a neural cell adhesion molecule expressed specifically by prosencephalon axon tracts was involved in the guidance of these axons (Anderson and Key, 1999). Slits were involved in the projection of the TPOC in the zebrafish embryonic brain involving the expression of Robo1 and Robo3 in the TPOC neurones (Devine and Key, 2008). Normally slits act in repelling axons away from the midline, but here the role involves keeping tight fasciculation of the TPOC axons. In comparison to the MLF and now the TPC, relatively little is known about the guidance of the TPOC axons in chick embryonic brain.

8.4 Vasculogenesis

The cells within the neural tube require a fresh supply of blood, which is received from blood vessels than form outside the neural tube (Bautch and James, 2009). Many of the genes found

to be upregulated in the microarray analysis were blood markers (see 6.4.6). This would suggest that the vascular system around the neural tube was already forming between HH9 and HH11 of the embryonic chick embryo. The expression of Cldn5 in the mesenchyme around the brain, even at HH9, further supports the early formation of the vascular system. Axon guidance molecules also play a role in guiding blood vessels. Netrins for example have a role in repelling blood vessels through activation of Unc5H2 (Lu et al., 2004).

8.5   Future directions

Even though the TPC was missing when there was high overexpression of Netrin1 and Netrin2 in the chick embryonic brain, the TPC axons were still able to project through the overexpression in some cases. This suggests the guidance of the TPC was more complex and further analysis is required particularly into the possibility of attraction of the TPC axons towards the dorsal midline and other guidance cues involved. Due to a difference in the Unc5H4 pattern with the *in situ* probe and antibody, further analysis will be need to identify the correct Unc5 receptor (Unc5H1-Unc5H4) expressed by the TPC neurones as cross reaction of the *in situ* probe was a possible reason for the different patterns. A knockdown of the Unc5H4 receptor will also give further indication of the role Netrins play in guiding the TPC.

Further analysis of microarray data will be required to find a gene involved in the specification of the MLF neurones. For CRABPI, overexpression experiments in the brain are required to see the effect on differentiation of neurones. If CRABPI is involved in differentiation a possible outcome would be an increase in the number of neurones within the

brain coinciding with the expression of ectopic CRABPI expression. It will be interesting to see which type(s) of neurones will be formed.

Further investigation of the blood/vascular markers identified as upregulated in the microarray analysis will give insight into the early neural-vascular interactions, particularly the timing of the blood vessel invasion into the neural tube.

Many of the genes identified as upregulated, have not previously been analysed in the chick embryonic brain and with little data from other vertebrates, makes these genes particularly interesting to investigate further.

8.6 Conclusion

The early axon scaffold has been shown to be highly conserved through vertebrate evolution. A clearer understanding of the anatomy has allowed molecular mechanism investigations to be interpreted more easily. The timing and positioning of the MLF neurones has raised many questions in its formation, in particular its fate determination. Microarray analysis has begun to understand this, identifying genes that were expressed within the MLF region. CRABPI was expressed specifically by the MLF neurones and other early axon scaffold neurones. The TPC neurones were shown to be intermingled with MLF neurones in the ventral diencephalon. This suggested specific regulation of TPC axon guidance was required and Netrin1 and Netrin2 were shown to be involved in the repulsion of these axons.

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Appendix

1. Cldn5 sequence (see 6.3.3)

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                10        20        30        40        50        60        70        80        90       100
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    ATGGCTTCGGCGGCGGTGGAGATTTTGGGGCTGGGACTGGGCATCCTGGGCTGGGTGGGGGTGATCCTGGCCTGCGGGCTGCCCATGTGGCAGGTGTCAG
Cldn5 cloned  ATGGCTTCGGCGGCGGTGGAGATTTTGGGGCTGGGACTGGGCATCCTGGGCTGGGTGGGGGTGATCCTGGCCTGCGGGCTGCCCATGTGGCAGGTGTCAG

               110       120       130       140       150       160       170       180       190       200
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    CCTTCATCGACGTGAACATCGTGGTGGCGCAGACCATCTGGGAAGGGCTGTGGATGAACTGCGTCGTGCAGAGCACGGGGCAGATGCAGTGCAAGGTGTA
Cldn5 cloned  CCTTCATCGACGTGAACATCGTGGTGGCGCAGACCATCTGGGAAGGGCTGTGGATGAACTGCGTGGTGCAGAGCACGGGGCAGATGCAGTGCAAGGTGTA

               210       220       230       240       250       260       270       280       290       300
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    CGATTCCATCCTGGCGCTGCGGCCGGAGGTGCAGGCGGGCCGGGCGCTCACGGTCATCGTGGCGCTGCTGGGGCTGGTGGCGCTCATGGTCACCGTGGTG
Cldn5 cloned  CGATTCCATCCTGGCGCTGCGGCCGGAGGTGCAGGCGGGCCGGGCGCTCACGGTCATCGTGGCGCTGCTGGGGCTGGTGGCGCTCATGGTCACCGTGGTG

               310       320       330       340       350       360       370       380       390       400
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    GGCGCGCAGTGCACCAACTGCATCCGGCCCGGCAAGATGAAGTCCCGCATCGTGATCGCCGGAGGGACCATCTACATCCTCTGCGGGGTCCTGGTCCTCG
Cldn5 cloned  GGCGCGCAGTGCACCAACTGCATCCGGCCCGGCAAGATGAAGTCCCGCATCGTGATCGCCGGAGGGACCATCTACATCCTCTGCGGGGTCCTGGTCCTCG

               410       420       430       440       450       460       470       480       490       500
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    TCCCGCTCTGCTGGTTCGCCAACATCGTCATCAGCGACTTCTACGACCCCTCCGTGCCGCCGTCCCAGAAGCGGGAGATAGGGGCCGCGCTGTACATCGG
Cldn5 cloned  TCCCGCTCTGCTGGTTCGCCAACATCGTCATCAGCGACTTCTACGACCCCTCCGTGCCGCCGTCCCAGAAGCGGGAGATAGGGGCCGCGCTGTACATCGG

               510       520       530       540       550       560       570       580       590       600
         ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Cldn5 mRNA    CTGGGCGGCCACGGCTCTGCTGCTGCTTTTCGGGGGCTGCCTCATCTGCTGCTGCTCCTGCTTGCAGCGCGACGAGACCTCCTTCCCCGTCAAGTACTCGGCG
Cldn5 cloned  CTGGGCGGCCACGGCTCTGCTGCTGCTTTTCGGGGGCTGCCTCATCTGCTGCTGCTCCTGCTTGCAGCGCGACGAGACCTCCTTCCCCGTCAAGTACTCGGCG

               610       620       630       640       650
         ....|....|....|....|....|....|....|....|....|....|.
Cldn5 mRNA    CCGCGGCGGCCCACCTCCAGCGGCGAGTACGACAAGAAGAACTACGTCTGA
Cldn5 cloned  CCGCGGCGGCCCACCTCCAGCGGCGAGTACGACAAGAAGAACTACGTCCAC
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2. Mab21L2 (see 6.3.3)

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                    10        20        30        40        50        60        70        80        90       100
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Mab21L2 cloned ATGATCGCCGCCCAGGCCAAGCTGGTGTACCAGCTCAACAAGTACTACACGGAGCGCTGCCAGGCGCGCAAGGCGGCCATCGCCNNNNACCATCCGCGAG
Mab21L2 mRNA   ATGATCGCCGCCCAGGCCAAGCTGGTGTACCAGCTCAACAAGTACTACACGGAGCGCTGCCAGGCGCGCAAGGCGGCCATCGCCAAGA-CCATCCGCGAG

                   110       120       130       140       150       160       170       180       190       200
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Mab21L2 cloned GTGTGCAAAGTGGTGTCGGACGTGCTGAAGGAGGTGGAGGTGCAGGAGCCGCGCTTCATCAGCTCGCTGAGCGAGATCGACGCCCGCTACGAGGGGCTGG
Mab21L2 mRNA   GTGTGCAAAGTGGTGTCGGACGTGCTGAAGGAGGTGGAGGTGCAGGAGCCGCGCTTCATCAGCTCGCTGAGCGAGATCGACGCCCGCTACGAGGGGCTGG

                   210       220       230       240       250       260       270       280       290       300
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Mab21L2 cloned AGGTGATCTCGCCCACCGAGTTCGAGGTGGTGCTCTACCTCAACCAGATGGGCGTCTTCAACTTCGTGGACGACGGCTCCCTGCCGGGCTGCGCCGTGCT
Mab21L2 mRNA   AGGTGATCTCGCCCACCGAGTTCGAGGTGGTGCTCTACCTCAACCAGATGGGCGTCTTCAACTTCGTGGACGACGGCTCCCTGCCGGGCTGCGCCGTGCT

                   310       320       330       340       350       360       370       380       390       400
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Mab21L2 cloned CAAGCTGAGCGACGGCCGCAAGCGCAGCATGTCGCTCTGGGTGGAGTTCATCACGGCCTCGGGCTACCTGTCGGCGCGCAAGATCCGCTCCCGCTTCCAG
Mab21L2 mRNA   CAAGCTGAGCGACGGCCGCAAA-GCAGCATGTCGCTCTGGGTGGAGTTCATCACGGCCTCGGGCTACCTGTCGGCGCGCAAGATCCGCTCCCGCTTCCAG

                   410       420       430       440
               ....|....|....|....|....|....|....|....|....|....
Mab21L2 cloned ACGCTGGTGGCCCAGGCCGTGGACAAGTGCAGCTACCGCGACGTGGCAC
Mab21L2 mRNA   ACGCTGGTGGCCCAGGCCGTGGACAAGTGCAGCTACCGCGACGTGGTGA
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3. PlxDC2 (see 6.3.3)

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                  10        20        30        40        50        60        70        80        90       100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       ATGGCGAGGCTGCGGAGAAGCAAACTAGCCGCTGGATTTCTATTACTTTTCCAGTTCCTGAGCGAGCGCTGCCAGCTCGCCGCCGGAGAGACGCCGAGCC
PlxDC2 5' cloned  ATGGCGAGGCTGCGGAGAAGCAAACTAGCCGCTGGATTTCTATTACTTTTCCAGTTCCTGAGCGAGCGCTGCCAGCTCGCCGCCGGAGAGACGCCGAGCC

                 110       120       130       140       150       160       170       180       190       200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       AGAGCCGCGGTGTGCTTTATGAAGTTGTTCAGAGCTTCCCTGGAGTGGAGGAAAATGTGCAAGTGGATGCACGTGTAAATAGCCACAGGTGGAGAAGGCA
PlxDC2 5' cloned  AGAGCCGCGGTGTGCTTTATGAAGTTGTTCAGAGCTTCCCTGGAGTGGAGGAAAATGTGCAAGTGGATGCACGTGTAAATAGCCACAGGTGGAGAAGGCA

                 210       220       230       240       250       260       270       280       290       300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       CTCAGAGTCTCTTAAATCAGTCAACACTAACAGAGCCAGTATGGGGCAGGATTCCTCTGAGCCAGGTGGTTTCACAGATCTGTTGCTTGAAGAGGGACAT
PlxDC2 5' cloned  CTCAGAGTCTCTTAAATCAGTCAACACTAACAGAGCCAGTATGGGGCAGGATTCCTCTGAGCCAGGTGGTTTCACAGATCTGTTGCTTGAAGAGGGACAT

                 310       320       330       340       350       360       370       380       390       400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       GAGAATGCTACCCAGATTGAGGAGGACACAGATCATAATTATTATACTTCAAGGACATATGGCCCATATGATTCTACCAGCCGGGATTTATGGGTCAATA
PlxDC2 5' cloned  GAGAATGCTACCCAGATTGAGGAGGACACAGATCATAATTATTATACTTCAAGGACATATGGCCCATATGATTCTACCAGCCGGGATTTATGGGTCAATA

                 410       420       430       440       450       460       470       480       490       500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TAGACCAAATGGAGAAAGATAAAGTAAAGATTCATGGGATCCTCTCCAATACCCATCGACAAGCAGCAAGAGTGAATCTGTCCTTTGATTTTCCATTTTA
PlxDC2 5' cloned  TAGACCAAATGGAGAAAGATAAAGTAAAGATTCATGGGATCCTCTCCAATACCCATCGACAAGCAGCAAGAGTGAATCTGTCCTTTGATTTTCCATTTTA

                 510       520       530       540       550       560       570       580       590       600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TGGCCATTTTCTACGAGAAATTACAGTGGCAACTGGGGGTTTCATATATACTGGAGAAGTTGTGCATCGAATGCTAACAGCTACACAATATATTGCACCC
PlxDC2 5' cloned  TGGCCATTTTCTACGAGAAATTACAGTGGCAACTGGGGGTTTCATATATACTGGAGAAGTTGTGCATCGAATGCTAACAGCTACACAATATATTGCACCC

                 610       620       630       640       650       660       670       680       690       700
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TTAATGGCAAATTTTGATCCCAGTGTATCAAGAAATTCAACAGTCAGATACTTTGATAATGGCACAGCACTAGTTGTCCAGTGGGACCATGTTCACCTGC
PlxDC2 5' cloned  TTAATGGCAAATTTTGATCCCAGTGTATCAAGAAATTCAACAGTCAGATACTTTGATAATGGCACAGCACTAGTTGTCCAGTGGGACCATGTTCACCTGC

                 710       720       730       740       750       760       770       780       790       800
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       AGGATAATTACAACCTGGGCAGTTTCACTTTTCAGGCCACCCTTCTCAATGATGGGCGTATCATTTTCGGCTACAAAGAAATTCCTGTCGCTGTGACACA
PlxDC2 5' cloned  AGGATAATTACAACCTGGGCAGTTTCACTTTTCAGGCCACCCTTCTCAATGATGGGCGTATCATTTTCGGCTACAAAGAAATTCCTGTCGCTGTGACACA

                 810       820       830       840       850       860       870       880       890       900
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       GATAAGCTCAACCAACCACCCAGTGAAAGTTGGACTATCAGATGCATTTGTGGTTGTGCACAGGATCCAGCAAATTCCCAATGTCCGCAGAAGAACAATT
PlxDC2 5' cloned  GATAAGCTCAACCAACCACCCAGTGAAAGTTGGACTATCAGATGCATTTGTGGTTGTGCACAGGATCCAGCAAATTCCCAATGTCCGCAGAAGAACAATT

                 910       920       930       940       950       960       970       980       990      1000
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TACGAATACCACAGGGTGGAGCTGCAGATGTCAAAGATTACAAATCTGTCAGCTGTTGAAATGATACCTCTTCCAACTTGTCTCCAGTTTAACAGCTGTG
PlxDC2 5' cloned  TACGAATACCACAGGGTGGAACTGCAAATGTCAAAGATTACAAATCTGGCAGCTGTTGAAATGATACCTCTTCCAACTTGGCTCCAGTTTAACAGCTGGG

                1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       GCCCCTGTGTCACTGCCCAGATTGGCTTCAACTGCAGCTGGTGCAGTAAACTCCAAAGATGCTCCAGCGGATTTGACCGTCACCGACAAGATTGGGTAGA
PlxDC2 5' cloned  GCCCCTGGGTCACTGCCCAAATTGGCTTCAACTGGAGCTGGGGCAATAAACTCCAAAGAGGCTCCACCGGATTTGACCGTC-CCGACAAAATTGGGTAAA

                1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       CAGTGGCTGCCCTGAAGAGTCAAAAGATAAGATTTGTGAGAAGAATATAGACACAACTGAAGCACTGCTGTA-CTCTACCACCACTCTTCAACCAACCAC
PlxDC2 5' cloned  CAGGGGCTGCCCTGAAAAATCAAATAAAAAAATTT-TGGGAAAAAATTAAAACCAACTGGAGCCCCCTTGGATCTTTACCACCTTT-TTCA-CCACCCCC

                1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       CACAAAGTTCAGAGTTTTAACAACCACC-AGAGGATTCACCAGCTCCCAGCTGCCAACCAGCCTACCCACAGAAGATGATACCAAGATAGC-GCTGCACC
PlxDC2 5' cloned  CA--AAGTTCAAAGTTT--ACAACCCCCCAGAGG-TTCCCC-GCCCCCCGTGGCCACCGCTCC--CCCAAA-AAGATAA-ACAAAAA-AGCCGC-GCCCC

                1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TAAAAGATAATGGAGCTTCCACAGATGACAGCGCTGCAGAGAAAAAAGGCGGAACAC------TT-CATGCTGGTTTAATCATCGGCATTCTAGTCCTGG
PlxDC2 5' cloned  --AAAGA--ATAGGGGTTCCCCA-A-GAAACGGGT-CGGAAAAAAAGGGCAAATTTCCTGGGGTTACACCCGGGTTT--TCTTCGGCCCCCTCGGGGGGG

                1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       TCCTCATCGTGGCTGCAGCC-ATCC----TC---GTGACTGTCTA-CATGTA---CCATCATCCAAC---ATCAGCAGCCA-GTCTCTTCTTCATAG-AG
PlxDC2 5' cloned  CCCCCTTCGGGTGTAAAGAAGATCCAAAATCAAAGCGTCT-TCTATCAAGGCGGCCCAAAAGGGGACGATATTAA-AGAAAAGTC-CTCCTTTGTAGGAG

                1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
PlxDC2 mRNA       C--GGCGTCCGAGCAGATG---GCCA--GCAAT---GAAATTCAGAAGAGGAT-CTG--GACATCCTG-CA---TATGCTGAAGTGG-AACCA-GTTGG-
PlxDC2 5' cloned  CCCGGGGAAAAAGCTTATCTTAGACACCGCTCTTTTGTTGTTAAAAAGATTTTTCTGTGGATAAGTTAACAATCTATCGTGTC-TGGCAACGACGTTTCT

                1610      1620      1630      1640      1650      1660      1670
           ....|....|....|....|....|....|....|....|....|....|....|....|....|....|..
PlxDC2 mRNA       ------AGAAA--AAGAAGGCTTCATT-----GTAT-CAGAGC------------AATGCTAA-------
PlxDC2 5' cloned  TCCTTCACACCCGAATTAATCTTCCTTCCTCAAGCATACACACCCCCTCTTTCCCGGAATGAAAAAGGTATT
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4. Satb1 (see 6.3.3)

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                 10        20        30        40        50        60        70        80        90        100
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ATGGATCATTTGAACGAGGCAACTCAGGGGAAAGAACATTCAGAAATGTCTAACAATGTAAGCGATCCGAAGGGTCCACCAGCCAAAATTGCGCGCTTGG
Satb1 mRNA       ATGGATCATTTGAACGAGGCAACTCAGGGGAAAGAACATTCAGAAATGTCTAACAATGTAAGCGATCCGAAGGGTCCACCAGCCAAAATTGCGCGCTTGG
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 110       120       130       140       150       160       170       180       190       200
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  AACAGAATGGGAGCCCATTAGGAAGAGGAAGACTTGGAAGTACAGGAACTAAAATGCAAGGAGTGCCTTTAAAACACTCTGGACACCTGATGAAAACTAA
Satb1 mRNA       AACAGAATGGGAGCCCATTAGGAAGAGGAAGACTTGGAAGTACAGGAACTAAAATGCAAGGAGTGCCTTTAAAACACTCTGGACACCTGATGAAAACTAA
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 210       220       230       240       250       260       270       280       290       300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  TATTAGAAAAGGAAGTATGCTTCCAGTTTTCTGTGTAGTGGAACATTATGAAAATGCCATTGAGTACGATTCTAAGGAGGAGCATGCAGAATTTGTGCTG
Satb1 mRNA       TATTAGAAAAGGAAGTATGCTTCCAGTTTTCTGTGTAGTGGAACATTATGAAAATGCCATTGAGTACGATTCTAAGGAGGAGCATGCAGAATTTGTGCTG
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 310       320       330       340       350       360       370       380       390       400
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  GTGAGGAAGGACATGCTTTTCAACCAACTGATCGAAATGGCATTGCTATCCCTTGGATATTCTCACAGCTCTGCTGCCCAAGCTAAAGGGCTTATCCAGG
Satb1 mRNA       GTGAGGAAGGACATGCTTTTCAACCAACTGATCGAAATGGCATTGCTATCCCTTGGATATTCTCACAGCTCTGCTGCCCAAGCTAAAGGGCTTATCCAGG
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 410       420       430       440       450       460       470       480       490       500
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  TTGGAAAGTGGAATCCGGTTCCACTCTCCTATGTGACAGATGCCCCTGATGCTACAGTAGCAGACATGCTGCAAGATGTGTATCATGTGGTCACACTGAA
Satb1 mRNA       TTGGAAAGTGGAATCCGGTTCCACTCTCCTATGTGACAGATGCCCCTGATGCTACAGTAGCAGACATGCTGCAAGATGTGTATCATGTGGTCACACTGAA
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 510       520       530       540       550       560       570       580       590       600
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  AATCCAGTTACACAGTTGCCCTAAACTAGAAGACTTGCCTCCTGAACAATGGTCTCACACGACAGTAAGAAATGCTCTGAAGGACTTACTGAAGGATATG
Satb1 mRNA       AATCCAGTTACACAGTTGCCCTAAACTAGAAGACTTGCCTCCTGAACAATGGTCTCACACGACAGTAAGAAATGCTCTGAAGGACTTACTGAAGGATATG
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 610       620       630       640       650       660       670       680       690       700
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  AACCAGAGTTCATTGGCCAAGGAATGTCCCCTTTCACAGAGTATGATTTCTTCCATTGTGAACAGCACTTACTATGCAAATGTCTCAGCAGCAAAATGTC
Satb1 mRNA       AACCAGAGTTCATTGGCCAAGGAATGTCCCCTTTCACAGAGTATGATTTCTTCCATTGTGAACAGCACTTACTATGCAAATGTCTCAGCAGCAAAATGTC
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 710       720       730       740       750       760       770       780       790       800
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  AGGAATTTGGAAGGTGGTATAAACATTTCAAGAAGGCAAAAGATATGATGGTTGAGATGGATAGCCTTTCTGAACTATCCCAGCAAGGTGCCAACCATGT
Satb1 mRNA       AGGAATTTGGAAGGTGGTATAAACATTTCAAGAAGGCAAAAGATATGATGGTTGAGATGGATAGCCTTTCTGAACTATCCCAGCAAGGTGCCAACCATGT
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 810       820       830       840       850       860       870       880       890       900
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  CAACTTCGGTCAGCAGCCGGTCCCAGGGAACACAGCCGAACAGCCTCCATCCCCTGTTCAGCTTTCTCATGGTAGTCAACCATCAGTTCGGANCCCACTT
Satb1 mRNA       CAACTTCGGTCAGCAGCCGGTCCCAGGGAACACAGCCGAACAGCCTCCATCCCCTGTTCAGCTTTCTCATGGTAGTCAACCATCAGTTCGGACCCCACTT
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 910       920       930       940       950       960       970       980       990       1000
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  CCAAACCTGCACCCTGGACTTGTATCTACTCCCATTAGCCCTCAGCTGGTAAATCAGCAGCTGGTAATGGCCCAGTTGCTGAATCAGCAGTATGCAGTGA
Satb1 mRNA       CCAAACCTGCACCCTGGACTTGTATCTACTCCCATTAGCCCTCAGCTGGTAAATCAGCAGCTGGTAATGGCCCAGTTGCTGAATCAGCAGTATGCAGTGA
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ACAGACTTCTAGCCCAGCAGTCCTTAAACCAACAGTACTTGAACNNCC-TCCTCCT----------------------------------------------
Satb1 mRNA       ACAGACTTCTAGCCCAGCAGTCCTTAAACCAACAGTACTTGAACCACCCTCCTCCTGTCAGTAGATCCATGAACAAGCCGTTGGAGCAGCAGGTCTCAAC
Satb1 cloned 3'  ----------------------------------GAAATCT-----------------------------------------------------------

                 1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ----------------------------------------------------------------------------------------------------
Satb1 mRNA       AAACACAGAGGTGTCTTCCGAAATCTACCAGTGGGTCCGTGATGAACTGAAACGAGCAGGAATCTCACAGGCAGTATTTGCACGTGTGGCTTTTAACCGA
Satb1 cloned 3'  ----------------------------------------------------------------------------------------------------

                 1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ----------------------------------------------------------------------------------------------------
Satb1 mRNA       ACTCAGGGCTTGCTCTCAGAAATCCTCCGAAAGGAAGAGGATCCCAAGACTGCCTCGCAGTCCTTGCTCGTAAACCTTCGGGCTATGCAGAACTTCTTGC
Satb1 cloned 3'  -------------------TCCGAAAGGAAGAGGATCCCAANNNTGCCTCGCAGTCCTTGCTCNTAAACCTTCGGGCTATGCAGAACTTCTTGC

                 1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ----------------------------------------------------------------------------------------------------
Satb1 mRNA       AGCTGCCAGAAGCTGAACGAGATCGAATTTACCAGGATGAAAGGGAAAGGAGCTTGAACGCAGCCTCTGCCATGGGCCCTGCACCTCTCATAAGCACACC
Satb1 cloned 3'  AGCTGCCAGAAGCTGAACGAGATCGAATTTACCAGGATGAAAGGGAAAGGAGCTTGAACGCAGCCTCTGCCATGGGCCCTGCACNTCTCATAAGCACACC

                 1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'  ----------------------------------------------------------------------------------------------------
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Satb1 mRNA        GCCCAGCCGGCCTCCACAAGTCAAAACTGCTACTATTGCTACGGAGAGGAATGGAAAAACAGAAAATAATTCCATGAACATTAATGCTTCCATTTATGAT
Satb1 cloned 3'   GCCCAGCCGGCCTCCACAAGTCAAAACTGCTACTATTGCTACGGAGAGGAATGGAAAAACAGAAAATAATTCCATGAACATTAATGCTTCCATTTATGAT

                        1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        GAGATTCAGCAGGAAATGAAGAGAGCTAAGGTGTCTCAAGCGCTGTTTGCAAAGGTGGCAGCAACCAAAAGCCAGGGATGGCTCTGTGAGCTGTTACGTT
Satb1 cloned 3'   GAGATTCAGCAGGAAATGAAGAGAGCTAAGGTGTCTCAAGCGCTGTTTGCAAAGGTGGCAGCAACCAAAAGCCAGGGATGGCTCTGTGAGCTGTTACGTT

                        1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        GGAAAGAAGATCCCTCACCAGAGAACAGGACCCTCTGGGAGAATCTCTCCATGATCCGAAGGTTCCTCAGTCTTCCTCAGCCTGAACGCGATGCCATTTA
Satb1 cloned 3'   GGAAAGAAGATCCCTCACCAGAGAACAGGACCCTCTGGGAGAATCTCTCCATGATCCGAAGGTTCCTCAGTCTTCCTCAGCCTGAACGCGATGCCATTTA

                        1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        TGAACAAGAAAGCAATGCAGTTCATCACCATGGTGACAGGCCTTCCCACATTATCCATGTGCCAGCAGAACAGATTCAGCAGCAACAGCAGCAGCAGCAA
Satb1 cloned 3'   TGAACAAGAAAGCAATGCAGTTCATCACCATGGTGACAGGCCTTCCCACATTATCCATGTGCCAGCAGAACAGATTCAGCAGCAACAGCAGCAGCAGCAA

                        1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        CAGCAACAACAGCAGCAGCAGCAACAGCAACCGGGTCCCAGACTCCCCCCAAGGCAACCGACGGTAGCATCACCAGCTGAATCTGAGGATGAAAATCGTC
Satb1 cloned 3'   CAGCAACAACAGCAGCAGCAGCAACAGCAACCGGGTCCCAGACTCCCCCCAAGGCAACCGACGGTAGCATCACCAGCTGAATCTGAGGATGAAAATCGTC

                        1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        AGAAACCCCGGCCACGAACAAAGATTTCTGTTGAAGCCCTAGGGATCCTACAGAGTTTCATACAAGATGTGGGCCTGTATCCTGATGAAGAAGCAATCCA
Satb1 cloned 3'   AGAAACCCCGGCCACGAACAAAGATTTCTGTTGAAGCCCTAGGGATCCTACAGAGTTTCATACAAGATGTGGGCCTGTATCCTGATGAAGAAGCAATCCA

                        2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        GACTCTCTCCGCTCAGCTTGACCTGCCCAAGTACACCATCATCAAGTTCTTTCAGAACCAGCGGTATTATCTCAAGCACCACGGAAAGCTGAAGGACAAT
Satb1 cloned 3'   GACTCTCTCCGCTCAGCTTGACCTGCCCAAGTACACCATCATCAAGTTCTTTCAGAACCAGCGGTATTATCTCAAGCACCACGGAAAGCTGAAGGACAAT

                        2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
                  ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------------------------------------------------
Satb1 mRNA        TCTGGGTTGGAGGTAGATGTTGCAGAATACAAGGAAGAAGAGTTGCTCAAGGATTTAGAAGACAGCATCCAAGACAAAAATGCAAACACGCTTTTTTCAG
Satb1 cloned 3'   TCTGGGTTGGAGGTAGATGTTGCAGAATACAAGGAAGAAGAGTTGCTCAAGGATTTAGAAGACAGCATCCAAGACAAAAATGCAAACACGCTTTTTTCAG

                        2210      2220      2230      2240      2250      2260
                  ....|....|....|....|....|....|....|....|....|....|....|....|
Satb1 cloned 5'   ---------------------------------------------------------
Satb1 mRNA        TTAAACTAGAAGAAGAGTTATCGGTAGAAGGGAACACAGAGATTAATGCTGAATTGAAAGACTGA
Satb1 cloned 3'   TTAAACTAGAAGAAGAGTTATCGGTAGAAGGGAACACAGAGATTAATGCTGAATTGAAAGACCAC
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5. SRGAP1 (see 6.3.3)

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                  10        20        30        40        50        60        70        80        90       100
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       ATGTCAACCCCGAGCAGATTCAAAAAGGACAAAGAGATTATAGCGGAGTATGAAAGTCAAGTGAAAGAGATCCGAGCTCAACTCATAGAGCAGCAGAAAT
SRGAP1 cloned 3'' ATGTCAACCCCGAGCAGATTCAAAAAGGACAAAGAGATTATAGCGGAGTATGAAAGTCAAGTGAAAGAGATCCGAGCTCAACTCATAGAGCAGCAGAAAT

                  110       120       130       140       150       160       170       180       190       200
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       GCCTGGAGCAGCAGACTGAAATGAGAGTGCAGCTTCTTCAGGATCTGCAGGACTTCTTCCGCAAGAAGTCGGAAATAGAGATGGAGTATTCCCGAAACCT
SRGAP1 cloned 3'' GCCTGGAGCAGCAGACTGAAATGAGAGTGCAGCTTCTTCAGGATCTGCAGGACTTCTTCCGCAAGAAGTCGGAAATAGAGATGGAGTATTCCCTCTCCAT

                  210       220       230       240       250       260       270       280       290       300
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       GGAAAAATTGGCAGAGAGGTTCATGGCAAAAACAAGAAGTACAAAAGACCATCAGCAGTACAAGAAAGATCAGAACCTCTTATCACCAGTGAATTGCTGG
SRGAP1 cloned 3'' AGCGTGGTTT-TACGGAGCTCTGAGCCTCAGATTCGCCGTAG------CACTAGTTCTTCCAGTGACACCATGAGTACTTTCAA---------GCCCA

                  310       320       330       340       350       360       370       380       390       400
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       TATTTACTCCTGAACCAAGTGAGAAGAGAAAGCAAAGACCATGCAACGTTGAGTGATATCTACCTGAACAACGTCATCATGCGCTTCATGCAGATAAGCG
SRGAP1 cloned 3'' TGGTGGCCCCTAGAATGGGAGTGCAG------CTGAAACCTCCCGCTCTTAGGCCAAAACCTATTGTTCTTC--CAAAAACCAACCCTAGCATAGGGCCT

                  410       420       430       440       450       460       470       480       490       500
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       AAGACTCCACCAGAATGTTCAAGA---AGAGCAAAGAGATTGCATTCCAGCTTCATGAAGACTTAATGAAAGTTCTTAATGAGCTTTACACAGTCATGAA
SRGAP1 cloned 3'' TCCCCTCCTTCCCAGGGTCCAGCAGACAAATCTTGCACAATGCACCCAGCTTTCTTGTACAA--AGTTGGCATTATAAGAAAGCATTGCTTA-TCAATTT

                  510       520       530       540       550       560       570       580       590       600
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       AACATACCACATGTATCATGCAGAGAGCATCAGTGCAGAAAGCAAACTGAAGGAAGCAGAGAAGCAAGA-AGAAAACAGATTGGGAGGTCAGGAGACCC
SRGAP1 cloned 3'' GTTGCAACGAACAGGTCA--CTATCAGTCAAAATAAAATCATTATTTGCCATCCAGCTGATATCCCCTATAGTGAGTCGTATTACATGGTCATAG----C

                  610       620       630       640       650       660       670       680       690       700
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       TGTTTTTCATATACGACTAGAAGACAGGCACCAAAGACGGAGCTCTGTGAAAAAGATCGAAAAAATGAAGGAAAAACGTCAAGCAAAATATTCTGAAAAC
SRGAP1 cloned 3'' TGTTTCCTG---GCAGCTCTGGCCCGTGTCTCAAA-----ATCTCTGATGTTACATTGCACAAGATAAAAATATATCATCATGAACAATA------AAAC

                  710       720       730       740       750       760       770       780       790       800
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       AAGCTGAAATCTATTAAGGCCCGTAATGAATACCTGCTCACCCTTGAAGCAACCAATGCTTCCGTTTTCAAGTATTATATTCACGACCTTTCAGATTTAA
SRGAP1 cloned 3'' TGTCTGCT-TACATAAACAGTAATACAAGGGGTGTTATGAGCCAT-ATTCAACGGGAAACGTCGAGGCCGCG-ATTAAATTC-CAACAT---GGAT---G

                  810       820       830       840       850       860       870       880       890       900
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       TTGATTGTTGTGATCTTGGATACCACGCAAGTCTGAACAGAGCCCTAAGGACGTATCTTTCTGCAGAGTATAACCTTGAAACTTCCAGGCATGAGGGCCT
SRGAP1 cloned 3'' CTGATTTATATGGGTATAAATGG-GCTCGCGATAATGTCGGGCAATCAGG-TGCGACAATCTATCGCTTGTA---TGGGAAGCCCGATGCGCCAGAGTTG

                  910       920       930       940       950       960       970       980       990      1000
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP1 mRNA       TGACATCATAGAAAATGCAGTTGACAGCTTGGAGCCACGAAGTGACAAGC-AGAGATTCATGGAAATGTTCCCCACTGCTTTTTGTCCACCAATGAAATT
SRGAP1 cloned 3'' T----TTCTGAAACATGGCAAAGGTAGCGTTGC-CAATGATGTTACAGATGAGATGGTCAGACTAAACTGGCTGACGGAATTTATGCCTCTTCCGACCAT

                  1010      1020      1030      1040      1050      1060      1070      1080
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
SRGAP1 mRNA       TGAGTTCCAATCTCATATGGGTGATGAGGTTTGTCAAGTCACTGCCCAGCAACCTGTGCAAGCAGAGCTCATGCTTAGATATCAGCAAC
SRGAP1 cloned 3'' CAAGCATTTTATCCGTACTCCTGATGATGCATGGNTACTCACCACTGCGATCCCCGGAAAAACAGCATTC-----CAGGTNTTAGAANA
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6. SRGAP3 (see 6.3.3)

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                 10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  ATGTCCTCGCAGGGCAAGTCCAAGAAGGACAAAGAGATCATCGCGGAGTACGATGCGCAAGTGAAGGAGATCCGCACACAGCTGGTGGAGCAGTTCAAAT
SRGAP3 mRNA       ATGTCCTCGCAGGGCAAGTCCAAGAAGGACAAAGAGATCATCGCGGAGTACGATGCGCAAGTGAAGGAGATCCGCACGCAGCTGGTGGAGCAGTTCAAAT
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  GCCTGGAGCAGCAGTCGGAGTCGCGCCTGCAGCTGCTACAGGACCTGCAGGAGTTCTTCCGCAGGAAGGCCGAGATCGAGCTGGAGTATTCCCGCAGCCT
SRGAP3 mRNA       GCCTGGAGCAGCAGTCGGAGTCGCGCCTGCAGCTGCTGCAGGACCTGCAGGAGTTCTTCCGCAGGAAGGCCGAGATCGAGCTGGAGTATTCCCGCAGCCT
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                210       220       230       240       250       260       270       280       290       300
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  GGAGAAGCTGGCCGAGCGCTTCTCCTCCAAGATCCGCGGCTCCCGTGAGCACCAGTTCAAGAAGGATCAGCACCTCCTCTCCCCGGTGAACTGCTGGTAC
SRGAP3 mRNA       GGAGAAGCTGGCCGAGCGCTTCTCCTCCAAGATCCGCGGCTCCCGTGAGCACCAGTTCAAGAAGGATCAGCACCTCCTCTCCCCGGTGAACTGCTGGTAC
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                310       320       330       340       350       360       370       380       390       400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  CTGGTCCTGACGCAGACCCGCCGGGAGAGCCGGGATCATGCAACTCTGAACGACATCTTCACAAACAACGTCATCGTGCGGCTGTCGCAGATCAGCGAGG
SRGAP3 mRNA       CTGGTCCTGACGCAGACCCGCCGGGAGAGCCGGGATCATGCAACTCTGAACGACATCTTCACAAACAACGTCATCGTGCGGCTGTCGCAGATCAGCGAGG
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                410       420       430       440       450       460       470       480       490       500
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  ATGTCATCAGGCTCTTTAAGAAGAGTAAAGAAATTGGCTTACAGATGCATGAAGAACTCCTGAAAGTTACCAATGAGCTGTACACGGTGATGAAGACCTA
SRGAP3 mRNA       ATGTCATCAGGCTCTTTAAGAAGAGTAAAGAAATTGGCTTACAGATGCATGAAGAACTCCTGAAAGTTACCAATGAGCTGTACACGGTGATGAAGACCTA
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                510       520       530       540       550       560       570       580       590       600
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  CCACATGTATCACGCAGAAAGCATCAGTGCTGAGAGCAAGCTGAAGGAGGCAGAGAAGCAGGAGGAAAAGCAGTTCAACAAGTCAGGGGATGTGAGCGTG
SRGAP3 mRNA       CCACATGTATCACGCAGAAAGCATTAGTGCTGAGAGCAAGCTGAAGGAGGCAGAGAAGCAGGAGGAAAAGCAGTTCAACAAGTCAGGGGATGTGAGCGTG
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                610       620       630       640       650       660       670       680       690       700
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  AACCTTCTGCGGCACGAGGAGAGGCCACAGCGGCGGAGCTCCGTCAAGAAGATTGAGAAGATGAAGGAGAAGAGACAAGCCAAATACTCTGAGAACAAGC
SRGAP3 mRNA       AACCTTCTGCGGCACGAGGAGAGGCCACAGCGGCGGAGCTCCGTCAAGAAGATTGAGAAGATGAAGGAGAAGAGACAAGCCAAATACTCTGAGAACAAGC
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                710       720       730       740       750       760       770       780       790       800
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  TGAAGTGTACTAAAGCCAGGAATGACTACCTGCTGAACCTGGCAGCCACCAACGCAGCTGTCAGTAAATACTATATCCACGATGTCTCTGACCTCATTGA
SRGAP3 mRNA       TGAAGTGTACTAAAGCCAGGAATGACTACCTGCTGAACCTGGCAGCCACCAACGCAGCTGTCAGTAAATACTATATCCACGATGTCTCTGACCTCATTGA
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                810       820       830       840       850       860       870       880       890       900
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  TTGCTGTGACCTGGGGTTCCATGCCAGCCTTGCACGGACCTTCCGGACATACCTGTCTGCTGAGTACAACCTGGANACCTCCCGCCACGAGGGCCTGGAC
SRGAP3 mRNA       TTGCTGTGACCTGGGGTTCCATGCCAGCCTTGCACGGACCTTCCGGACATACCTGTCTGCTGAGTACAACCTGGAGACCTCCCGCCACGAGGGCCTGGAC
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

                910       920       930       940       950       960       970       980       990      1000
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  ATCATTGAGAATGCTGTGGACAACCTGGATGCTCGGAGCGACAAGCACACCATCATGGACATGTGCAATCAGGNCTTCTGCCCTCCACTGAAGTTCGNGT
SRGAP3 mRNA       ATCATTGAGAATGCTGTGGACAACCTGGATGCTCGGAGCGACAAGCACACCATCATGGACATGTGCAATCAGGTCTTCTGCCCTCCACTGAAGTTCGAGT
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

               1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  TCCAGCCCCNNATGGGGGNATGAGGNNTGCCAAGTCAGCGCCCAGCAGCCTGTGCA-----------------------------------------------
SRGAP3 mRNA       TCCAGCCCCACATGGGGGA-TGAGGTGTGCCAAGTCAGCGCCCAGCAGCCTGTGCAGACTGAGCTGCTGATGCGGTACCACCAGCTGCAGTCCCGCCTGG
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

               1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  -----------------------------------------------------------------------------------------------------
SRGAP3 mRNA       CCACCCTCAAGATAGAGAACGAAGAGGTACGAAAAACTCTGGATGCCACAATGCAGACTTTGCAGGACATGCTGACAGTGGAAGATTTTGATGTTTCAGA
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

               1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  -----------------------------------------------------------------------------------------------------
SRGAP3 mRNA       TGCCTTCCAGCACAGTCGCTCCACTGAGTCGGTCAAATCAGCTGCTTCAGAGACCTACATGAGCAAAATAAACATTGCCAAGAGGAGAGCCAACCAACAG
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

               1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'  -----------------------------------------------------------------------------------------------------
SRGAP3 mRNA       GAGACTGAGATGTTCTATTTTACAAAATTTAAAGAGTATTTGAATGGCAGTAACCTTATCACAAAGCTCCAGGCTAAGCACGACTTGCTGAAGCAGACTC
SRGAP3 cloned 35' -----------------------------------------------------------------------------------------------------

               1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         TTGGGGAAGGTGAAAGAGCCGAATGTGGAACAACCAGGCCCCCATGTCTTCCCCCTAAGCCACAGAAAATGAGGAGACCTAGGCCTCTCTCAGTCTATAA
SRGAP3 cloned 35'   ----------------------------------------------------------------------------------------------~~~~~~~~~~


                         1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         TCATAAACTCTTTAACGGCAATATGGAAACGTTCATTAAGGATTCAGGACAGGCCATTCCGCTTGTAGTAGAAAGCTGCATTCGTTACATCAATTTGTAT
SRGAP3 cloned 35'   ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~TNAGGATTCAGGACAGGCCATTCCGCTTGTAGTAGAAAGCTGCATTCGTTACATCAATTTNTAT


                         1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         GGCCTTCAGCAGCAGGGTATTTTCCGAGTTCCTGGCTCACAGGTGGAAGTCAATGACATCAAGAATTCCTTTGAACGAGGTGAAGATCCCCTTGCTGATG
SRGAP3 cloned 35'   GGCCTTCAGCAGCAGGGTATTTTCCGAGTTCCTGGCTCACAGGTGGAAGTCAATGACATCAAGAATTCCTTTGAGCGAGGTGAAGATCCCCTTGCTGATG


                         1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         ATCAAAATGAACGTGACATTAACTCAGTGGCTGGAGTCTTGAAGCTGTATTTCCGAGGACTGGAAAACCCCCTCTTTCCTAAGGAAAGGTTTCAAGATTT
SRGAP3 cloned 35'   ATCAAAATGAACGTGACATTAACTCAGTGGCTGGAGTCTTGAAGCTGTATTTCCGAGGACTGGAAAACCCCCTCTTTCCTAAGGAAAGGTTTCAAGATTT


                         1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    --------------AACTGA--------------------------------------------------------------------------------
SRGAP3 mRNA         GGTATCTACTATAAAAACTGAGAATCCCACCGAGAGGGTGCACCAGATCCAGCAAATCATCGTCACTCTGCCCCGGGCTGTCATCGTTGTCATGAGATAT
SRGAP3 cloned 35'   GGTATCTACTATAAAAACTGAGAATCCCACCGAGAGGGTGCACCAGATCCAGCAAATCATCGTCACTCTGCCCCGGGCTGTCATCGTTGTCATGAGATAT


                         1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         CTGTTTGCTTTCCTCAATCACTTGTCACAGTACAGCGATGAGAACATGATGGATCCCTACAACCTGGCCATCTGCTTTGGGCCCACTCTGATGCACATTC
SRGAP3 cloned 35'   CTGTTTGCTTTCCTCAATCACTTGTCACAGTACAGCGATGAGAACATGATGGATCCCTACAACCTGGCCATCTGCTTTGGGCCCACTCTGATGCACATTC


                         2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         CAGATGGGCAGGATCCGGTGTCCTGTCAAGCCCATGTCAATGAGGTCATCAAAACCATCATCATCAACCATGAGGGCATCTTCCCCAGCCACAGAGAGCT
SRGAP3 cloned 35'   CAGATGGGCAGGATCCGGTGTCCTGTCAAGCCCATGTCAATGAGGTCATCAAAACCATCATCATCAACCATGAGGGCATCTTCCCCAGCCACAGAGAGCT


                         2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         GGAAGGACCTGTCTATGAGAAGTGCATGACTGGAGGGGAGGAGTACTGCGACAGCCCTCACAGTGAGCCAGGCACCATTGATGAAGTTGACCACGACAAC
SRGAP3 cloned 35'   GGAAGGACCTGTCTATGAGAAGTGCATGACTGGAGGGGAGGAGTACTGCGACAGCCCTCACAGTGAGCCAGGCACCATTGATGAAGTTGACCACGACAAC


                         2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         GGCACGGAGCCACACACCAGTGATGATGAAGTAGAGCAGATTGAAGCCATAGCAAAGTTTGACTACGTTGGACGGTCTCCACGAGAGCTGTCCTTTAAGA
SRGAP3 cloned 35'   GGCACGGAGCCACACACCAGTGATGATGAAGTAGAGCAGATTGAAGCCATAGCAAAGTTTGACTACGTTGGACGGTCTCCACGAGAGCTGTCCTTTAAGA


                         2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         AAGGGGCCTCACTCCTCCTGTACCATCGGGCATCAGAAGATTGGTGGGAAGGGAGACACAATGGTGTGGATGGTCTCATACCCCACCAGTACATTGTGGT
SRGAP3 cloned 35'   AAGGGGCCTCGCTCCTCCTGTACCATCGGGCATCAGAAGATTGGTGGGAAGGGAGACACAATGGTGTGGATGGTCTCATACCCCACCAGTACATTGTGGT


                         2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
                    ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
SRGAP3 cloned 5'    ----------------------------------------------------------------------------------------------------
SRGAP3 mRNA         GCAAGACATGGATGATGCCTTCTCCGACAGCCTGAGCCAGAAGGCGGACAGCGAGGCGAGCAGCGGCCCGCTGCTGGATGACAAGGCCTCCTCCAAGAAC
SRGAP3 cloned 35'   GCAAGACATGGATGATGCCTTCTCCGACAGCCTGAGCCAGAAGGCGGACAGCGAGGCGAGCAGCGGCCCGCTGCTGGATGACAAGGCCTCCTCCAAGAAC


                         2510      2520      2530      2540      2550      2560
                    ....|....|....|....|....|....|....|....|....|....|....|....|...
SRGAP3 cloned 5'    -------------------------------------------------------------
SRGAP3 mRNA         GACATCCAGTCTCCGACGGATCACCTCGTGGACTACGGCTTTGGCGGAGTCATGGGCCGGTAG
SRGAP3 cloned 35'   GACATCCAGTCCCCAACGGATCACCTCGTGGACTACGGCTTTGGCGGAGTCATGGGCCGGCAC
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7. Unc5 chick receptor conservation (see 7.2.2)

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                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  ATGAGGGATCGGTGCGCCCAGCAGAGCGTGACGGTGGCCAGCCCTGCATCGGGCGCGTCCCCCGACCTGCTGCCCCACTTCCTGCTGGAGCCCGACGACG
Unc5H2  ATGCCGCCGGCGCGTCGTCTGCTGCTGCCGTTTTTTCTGCTGCTGCTGCTGCCGCTGCATCTGCGTTGGGCGCTGGCGGCGGCGGGGCCTGGAATATAGCG
Unc5H3  ATGGGGAAGGGGCTGGAGGGCACGGCGGCCCGCTGCGGGCTGGGAATGGGATACCTGCTGCACAGCGTGGTGCTCCCGGCACTGGCCGTCCTGGGGGCCA
Unc5H4  ATGTATATTCTGAACTATGTGAAAATTAGCGGCATTGAACTGGGCAGCTATAAACGTTGCCATAGCATGGATCGTCTGCAGGGCGTGTGCTTTATTCGTG
Unc5H4 EST  GGCGTCCGTCCGCATCGCTTATTTGAGGAAAAACTTTGAGCAAGACCCCCNAGGGAAGGAGGTTCCTATTGAAGGGATGATCGTTCTGCACTGCCGGCCC


               110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  TCTACATCGTAAAGAACAAGGCGGTGAGCCTGGCCTGCCGCGCCACCCCCGCCACTCAGATCTACTTCAAGTGCAATGGGGAGTGGGTGCACCAGGGTGA
Unc5H2  AAGTGCTGCCGGATAGCTTTCCGAGCGCGCCGGCCGGAAACCCTGCCGCATTTTCTGCGTGAACCGCAGGATGCGTATATTGTGAAAAACAAACCGGTGGA
Unc5H3  GCCGGCCCGGCTCCGCCGCGCAAGATGATGATTTTTTCATGAACTTCCAGAAACTTTTCCTTCTGATCCTCCAGAGCCATTGCCCCACTTTCTCATTGA
Unc5H4  CGGTGGTGCTGCTGAGCATGCTGCGTCAGCGTGTGAAACTGGTGTATAGCCAGCTGACCAACACCAAAATTAAACAGTGCGAAAAATATGTGCTGAGCTT
Unc5H4 EST  CCCGAGGGCGTCCCTGCAGCTGAGGTGGAATGGCTGAAGAATGAGGAGCCCATAGATTCCAACCTGGATGAGAACATTGACACCAGGGCAGACCACAACC


               210       220       230       240       250       260       270       280       290       300
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  CCACATCACACAGCGCAGCACTGACCGCGGCACCGGGCTGCCCGTGATGGAGGTGCGCATCGAGATCACCCGCCAGCAAGTGGAGAAGCTCTTTGGTCTG
Unc5H2  ACTGGTGTGCCGTGCGAACCCGGCGACCCAGATTTATTTTAAATGCAACGGCGAATGGGTGAACCAGAACGATCATGTGACCACCGAAAGCCTGGATGAA
Unc5H3  ACCCGAAGAAGCTTCATCGTGAAAAACAAGCCTGTGAATCTGTACTGCAAAGCGAGCCCTGCCACGCAGATCTATTTTAAGTGCAACAGTGAATGGGTT
Unc5H4  TCTGAGCCTGAGCGGCAACGATAACAGCGAAGCGCTGCCGGAAAGCATTCCGAGCGCGCCGGGCACCCTGCCGCATTTTATGGAAGAACCGGATGATGCG
Unc5H4 EST  TGATCATCCGGCAGGCGCGTCTGTCCGACTCAGGGAACTACACCTGTATGGCTGCCAATATTGTTGCCAAGAGGAGGAGCATGTCTGCAACTGTCGTGGT


               310       320       330       340       350       360       370       380       390       400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  GAGGAGTACTGGTGCCAGTGCGTGGCGTGGAGCTCCTCCGGCACCACCAAGAGCCAGAAGGCCTTCGTGCGCATCGCCTATCTGCGCAAGAACTTCGAGC
Unc5H2  GTGACCGGCCTGCTGGTGCGTGAAGTGCAGATTGAAGTGAGCCGTCAGCAGGTGGAAGAACTGTTTGGCCTGGAAGATTATTGGTGCCAGTGCGTGGCGT
Unc5H3  CATCAGAAGGATCATGTGGTGGATGAGAGAGTAGATGAAACCTCTGGTCTGATCGTCTGTGAGGTGAGCATCGAGATTTCCCGCCAGCAGGTGGAAGAGC
Unc5H4  TATATTATTATTAAAAGCAACCCGATTGTGCTGCGTTGCAAAGCGATGCCGGCGATGCAGATTTTTTTTAAATGCAACGGCGAATGGGTGCATCAGAACGAAC
Unc5H4 EST  TTATGTGAATGGAGGTTGGTCGTCATGGACTGAGTGGTCCAACTGCAACGCGCGCTGCGGTCGGGGCTGGCAGAAGCGATCGCGGACCTGCACCAACCCT


               410       420       430       440       450       460       470       480       490       500
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  AGGAGCCGACAGCTAGGGAGGTGTCCATCGAGCAGGGTGTTGTGCTGCCGTGCCGCCCTCCCGAGGGCATCCCTCCCGCCGAGGTGGAGTGGCTGCGCAA
Unc5H2  GGAGCAGCGCGGGCACCACCAAAAGCCGTCGTGCGTATGTGCGTATTGCGTATCTGCGTAAAAACTTTGATCAGGAACCGCTGGGCAAAGAAGTGCCGCT
Unc5H3  TCTTTGGACCCGAGGACTACTGGTGCCAGTGTGTCGCCTGGAGCTCAGCTGGCACCACCAAGAGCCGCAAGGCCTACGTCCGCATTGCATATCTCAGAAA
Unc5H4  ATGTGAGCGAAGAAAGCATGGATGAAGCGACCGGCCTGAAAGTGCGTGAAGTGTTTATTAACGTGACCCGTCAGCAGGTGGAAGATTTTCATGGCCCGGA
Unc5H4 EST  GCCCCGCTCAACGGAGGGGCGTTCTGCGAGGGGATGTCCGTGCAGAAGATCACCTGCACTTCTCTTTGCCCTGTGGCATGGAAACTGGGAGGTGTGGAGT


               510       520       530       540       550       560       570       580       590       600
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  TGAGGAGCTGGTGGACCCGGCGCTGGATGCCAATGTCTTGGTGACGCCGGAGCACAGCCTGGTGCTGCGCCAAGCCCGCCTGGCCGACACCGCCAACTAC
Unc5H2  GGAACAGGAAGTGCTGCTGCAGTGCCGTCCGCCGGAAGGCGTGCCGCAGGCGGAAGTGGAATGGCTGCGTAACGAAGATGTGATTGATCCGACCCAGGAT
Unc5H3  GACTTTTGAGCAGGAGCCGCTGGGGAAAGAAGTGTCCCTGGAGCAAGAGGTCCTGCTCCAGTGCCGTCCTCCTGAAGGCATTCCAGTAGCTGAGGTAGAG
Unc5H4  AGATTATTGGTGCCAGTGCGTGGCGTGGAGCCATCTGGGCACCAGCAAAAGCCGTAAAGCGAGCGTGCGTATTGCGTATCTGCGTAAAAACTTTGAACAG
Unc5H4 EST  GAATGGTCTGTGTGCAGCCCGGAGTGCGAGCATTTGAGGGGTTCGGGAATGTATTGCACCAGCGCCACGAAACGGAGGGAAGTACTGCGAGGGCCTGAGCC


               610       620       630       640       650       660       670       680       690       700
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  ACCTGCGTGGCCAAAAACATCGTGGCGCGCCGCCGCCGCAGTGCCTCCGCCGCCATCACCGTCTATGTGAATGGCGGCTGGTCGACGTGGACGCAGTGGTCGG
Unc5H2  ACCAACTTTCTGATTACCATTGATCATAACCTGATTATTAAACAGGCGCGTCTGCTGGATACCGCGAACTATACCTGCATGGCGAAAAACATTGTGGCGA
Unc5H3  TGGCTGAAGAATGAAGAGGTGATCGATCCTGTGGAAGACCGAAATTTTTCATCACCATTGATCACAACCTGATCATCAAGCAAGCCCGGCTTTCCGACA
Unc5H4  GATCCGCAGGGCAAAGAAGTGCCGATTGAAGGCATGATTGTGCTGCATTGCCGTCCGCCGGAAGGCGTGCCGGGCGGAAGTGGAATGGCTGAAAAACG
Unc5H4 EST  AGGAGTCAGAGAACTGCACTGAGGGGCTTTGCATTCCAAGATATAGAGACCGCCAGTGACAAACACGTTGGACTTTCCCCAAACATTGTTTAATGTGAGT


               710       720       730       740       750       760       770       780       790       800
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  GCTGCAGCACCAGCTGCGGACGGGGCTGGCAGAAGCGCAGCCGGACTTGCACCAACCCCACACCCCTCAACGGGGGAGCTTTCTGCGAGGGGCAAAATGT
Unc5H2  AACGTCGTAGCACCACCGCGGCGGTGATTGTGTATGTGAACGGCGGCTGGAGCACCTGGAGCGAATGGACCCCGTGCAACAACCGTTGCGGCCGTGGCTG
Unc5H3  CGGCTAACTACACCTGTGTTGCCAAAAACATTGTGGCCAAAAGGAAAAGCACGACAGCAACTGTGATTGTTGTCTATGTGAATGGAGGCTGGTCTACCTGGAC
Unc5H4  AAGAACCGATTGATAGCAACCTGGATGAAAACATTGATACCCGTGCGGATCATAACCTGATTATTCGTCAGGCGCGTCTGAGCGATAGCGGCAACTATAC
Unc5H4 EST  ATTTCACGCAGTCTGACATCAGCACTCCTGTTTGCAATGGGAACCCATAACTGCTGCAAGCATACTTTAAGACG


               810       820       830       840       850       860       870       880       890       900
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  GCAGAAAAGCGCCTGCACCACCCTCTGCCCAGTGGATGGAGACTGGTCAAAGTGGAGCAAGTGGTCGGTGTGTGGGGCCGAGTGCACACACTGGCGGAGC
Unc5H2  GCAGAAACGTACCCGTACCTGCACCAACCCGGCGCCGCTGAACGGCGGCAGCTTTTGCGATGGCCAGCCGTTTCAGAAAGTGACCTGCACCACCCTGTGC
Unc5H3  CGAGTGGTCAGCGTGCAACAGCCGCTGTGGGAGAGGCTTCCAGAAGCGCACAAGGACCTGCACTAACCCTGCCCCACTCAATGGGGGGCCTTCTGCGAG
Unc5H4  CTGCATGGCGGCGAACATTGTGGCGAAACGTCGTAGCATGAGCGCGACCGTGGTGGTGTATGTGAACGGCGGCTGGAGCAGCTGGACCGAATGGAGCAAC
Unc5H4 EST


               910       920       930       940       950       960       970       980       990      1000
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  CGGGAGTGCTCTGAGCCTGCACCCCGCAACGGAGGTCAGGAGTGCCACGGCCCCGAGCTGGAGACCCACAACTGCACCTCCGAGCTGTGCAGCCCCACCA
Unc5H2  CCGGTGGATGGCGCGTGGACCGAATGGAGCAAATGGAGCGCGTGCAGCACCGAATGCACCCATTGGCGTAGCCGTGAATGCAGCGCGCCGGCGCGCGTA
Unc5H3  GGGCAAAATGTTCAGAAAATAGCTTGCACCACCCTGTGTCCAGTGGATGGCAAATGGACGTCCTGGAGCAAGTGGTCCACTTGTGGCACAGAGTGTACCC
Unc5H4  TGCAACGCGCGTTGCGGCCGTGGCTGGCAGAAACGTAGCCGTACCTGCACCAACCCGGCGCCGCTGAACGGCGGCGCGTTTTGCGAAGGCATGAGCGTGC
Unc5H4 EST


              1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1  CCCCAGGTGCTGAGGACCTGGCGCTGTACGTGGGGCTGATCGCCGTGGCCGTATGCCTGGTGCTGCTGCTGCTGGTGGGCGTGCTGGTGTACTGCCGCAA
Unc5H2  ACGGCGGCAAAGATTGCAGCGGCGGCGCCTGCTGGATAGCAAAAACTGCACCGATGGCCTGTGCCTGCATAACAAACGTGTGCTGAGCGAACCGAAAAGCCA
Unc5H3  ACTGGCGCCGGAGGGAGTGCACAGCTCCGGCCCCGAAGAATGGAGGCAAGGACTGTGAGGGACTGGTGCTGCAGTCTAAGAACTGCACTGATGGGCTCTG
Unc5H4  AGAAAATTACCTGCACCAGCCTGTGCCCGGTGGATGGCAACTGGGAAGTGTGTGGAGCGAATGGAGCGTGTGTGCAGCCCGGAATGCGAACATCTGCGTGTGCG
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Unc5H4 EST


               1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    GAAGGGCGGCCTGGACGCCGATGTGGCAGACTCCTCCATCCTCACCACTGGCTTCCAGCCCGTCAGCATCAAGCCCAGCAAGGCTGACAACCTGCTCACC
Unc5H2    TCTGCTGGAAGCGACCGGCGATGTGGCGCTGTATGCGGGCCTGGTGGTGGCGATTTTTGTGTTTATTGTGATTCTGATGGCGGTGGGCGTGGTGGTGTAT
Unc5H3    CATGCAGGCTGCACCTGACTCGGATGATGTTGCTCTCTACGTGGGGATTGTCATTGCTGTGATTGTGTGCCTGGCTATTTCTGTGGTTGTGGCCCTGTTT
Unc5H4    TGAATGCATTGCGCCGGCGCCGCGTAACGGCGGCAAATATTGCGAAGGCCTGAGCCAGGAAAGCGAAAACTGCACCGAAGGCCTGTGCATTCAGGATAAA
Unc5H4 EST


               1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    ATCCAGCCCGACCTCAGCACCGCCACCATGACCTACCAGGGCTCGCTGTGCCCACGCCAGGACGGCCCTGCCAAGCTCCAGCTCCCCAACGGGCACCTGC
Unc5H2    CGTCGTCGTTGCCGTGATTTTGATACCGATATTACCGATAGCAGCGCGGCGCTGACCGGCGGCTTTCATCCGGTGAACTTTAAAACCGCGCGTCATGATA
Unc5H3    GTCTATCGCAAGAACCACCGTGACTTTGAGTCAGATATTATCGACTCATCGGCGCTAAATGGGGGATTTCAGCCTGTTAACATCAAGGCTGCAAGACAAG
Unc5H4    AAACCGCTGCATGAAATTAAAAGCCAGAACATTGAAACCGCGAGCGATATTGCGCTGTATAGCGGCCTGGGCGCGGCGGTGATTGCGGTGGCGGTGCTGG
Unc5H4 EST


               1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    TGAGCCCGCTGGGTGCCGGGCGGCACACTCTGCACCACAGCTCACCTGCTGCCGAGGGAGCCGACTTCGTGGCCCGGCTCTCCACCCAGAGCTATTTCCG
Unc5H2    ACCCGCAGCTGCTGCATCCGAGCATGCAGCCGGATCTGACCGCGAACGCGGGCGTGTATCGTGGCCCGATGTATGCGCTGCAGGATAGCAGCGATAAAAT
Unc5H3    ACCTCTTGGCAGTGCCACCAGACCTCACTTCTGCTGCAGCCATGTACAGGGGGCCTGTGTATGCCTTGCATGATGTCTCTGATAAAATCCCAATGACCAA
Unc5H4    TGGTGGGCGTGACCCTGTATCGTCGTAGCCAGAGCGAATATGGCGTGGATGTGATTGATAGCAGCGCGCTGACCGGCGGCTTTCAGACCTTTAACTTTAA
Unc5H4 EST


               1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    CTCGCTGCCCCGTGGGCACCGGCCAACATGGCCTACGGCACCTTCAACTTCCTGGGGGGGCGGCTCATGATCCCCAACACAGGTGTCAGCTTGCTGATCCCT
Unc5H2    TCCGATGACCAACAGCCCGCTGCTGGATCCGCTGCCGAACCTGAAAATTAAAGTGTATAACAGCAGCACCACCAGCAGCAGCCCGGGCCTGCATGATGGC
Unc5H3    TTCTCCGATCCTGGACCCACTGCCCAATCTGAAGATTAAAGTTTATAACACCTCTGGAGCAGTCACCCCCCAGGATGAACTCTCTGACTTCTCCTCCAAG
Unc5H4    AACCGTGCGTCAGGGCAACAGCCTGCTGCTGAACAGCAGCATGCAGCCGGATCTGACCGTGAGCCGTACCTATAGCGGCCCGATTTGCCTGCAGGATCCG
Unc5H4 EST


               1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    CCCGACGCAATCCCCCGAGGGAAGATCTACGAGGTGTACCTCACGCTGCACAAGCACGAGGAGGTGAGGCTGCCCCTCGCCGGATGCCAGACGCTGCTGA
Unc5H2    ACCGATCTGCTGGGCGGCATTCCGGCGGTGGGCACCTTTCCGGGCGATAGCAGCAGCCAGTTTGTGAACATGCGTAACAAAGCGCAGCAGGGCAGCCAGC
Unc5H3    CTGTCCCCACAGATTACCCAGTCTCTGTTGGAGAATGAGACTCTGAACGTGAAGAACCAAAGCCTTGCACGGCAAACAGACCCATCCTGCACTGCATTTG
Unc5H4    ATGGATAAAGAACTGATGACCGAAAGCAGCCTGTTTAACCCGCTGAGCGATATTAAAGTGAAAGTGCAGAGCAGCTTTATGGTGAGCCTGGGCGTGACCG
Unc5H4 EST


               1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1    GCCCCATCGTCAGCTGCGGCCCCCCCGGCGTGCTGCTCACCCGCCCCGCCATTAAGGGCATGGGGCCCTGCGTGGAGGCGGGCGCTGAGCATTGA
Unc5H2    ATCTGCTGAGCCTGCCCGCGTGAACATGGCACCAGCGCGAGCGGCCACCTTTAGCTATCTGGGCGGCCGTCTGACCATTCCGGGCACCGGCGTGAGCCTGCT
Unc5H3    GGACCTTCAACTCGTTAGGGGGCCACCTAGTAATTCCTAATTCAGGAGTGAGCTTGCTGATCCCAGCAGGGGCTGTTCCCCAAGGAAGAGTCTATGAAAT
Unc5H4    AACGTGCGGAATATCATGGCAAAGCGCATAGCGGCCACCTTTCCGCATGGCAACGCGCGTGGCTTTGGCACCATTCAGGCGCGTAACAAAGCGGCGTATAT
Unc5H4 EST


               1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2    GGTGCCGCATGGCGCGATTCCGCAGGGCAAATTTTATGAAATTTATCTGGTGATTAACAAAGCGGAAAGCGGCTTTCTGCCGAGCGAAGGCACCCAGACC
Unc5H3    GTATGTGACAGTCCACAGGAAGGAGGGCATGAGACCACCTGTAGAAGACAGCCAGACGCTGCTGACACCAGTGGTGAGCTGTGGCCCACCAGGAGCGCTG
Unc5H4    TCAGAACCTGAGCAGCATTAGCACCCGTAACGAACTGAAAACCACCGCGATTTTTGGCCATCTGGGCGGCCGTCTGGTGGTGCCGAACACCGGCGTGAGC
Unc5H4 EST


               1810      1820      1830      1840      1850      1860      1870      1880      1890      1900
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2    GTGCTGAGCCCGGCGGTGACCTGCGGCCCGACCGGCCTGCTGCTGTGCCGTCCGGTGGTGCTGACCATTCCGCATTGCGCGGATGTGAGCAGCAGCGATT
Unc5H3    CTGACCCGACCCGTTGTGCTGACCATGCACCACTGTGCTGAGCCCAACATGGATGACTGGCAGATCCAGCTCAAGCACCAGGCAGGCCAGGGACCATGGG
Unc5H4    CTGCTGGTGCCGCATGGCGCGATTCCGGAAGAAAGCAGCTGGGAAATTTATCTGGCGATTAACCCGCGTGAAAGCAGCCTGCAGCCGGAAGGCCCGGATG
Unc5H4 EST


               1910      1920      1930      1940      1950      1960      1970      1980      1990      2000
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2    GGATTTTTCAGCTGAAAACCCAGAGCCATCAGGGCAACTGGGAAGAAGTGGTGACCCTGGATGAAGAAACCCTGAACACCCCGTGCTATTGCCAGCTGGA
Unc5H3    AGGATGTAGTGGTGGTCGGGGAGGAAAACTTCACCACTCCATGCTACATCCAGCTGGACCCAGAGGCCTGTCATATCCTGACGGAGACCCTCAGCACGTA
Unc5H4    TGCTGCTGGGCCCGGAAGTGACCTGCGGCCCGAGCGATGTGAGCGTGAGCAGCCCGTTTGCGCTGACCATTCCGCATTGCGCGGAAGTGAACAGCGAACA
Unc5H4 EST


               2010      2020      2030      2040      2050      2060      2070      2080      2090      2100
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2    AGCGAAAAGCTGCCATGTGCTGCTGGATCAGCTGGGCACCTATGTGTTTGTGGGCGAAAGCTATAGCCGTAGCGCGATTAAACGTCTGCAGCTGGCGATT
Unc5H3    CGCCTTGGTGGGACAATCCATCACCAAAGCAGCAGCCAAACGTCTCAAATTGGCCATCTTTGGACCACTGTCCTGTTCCTCACTGGAGTACAGCATCCGC
Unc5H4    TTGGAACATTCATCTGAAAAAACGTACCCAGCAGGGCAAATGGGAAGAAGTGATGAGCGTGGAAGAAGAAACCACCAGCTGCTATTGCCTGCTGGATCCG
Unc5H4 EST


               2110      2120      2130      2140      2150      2160      2170      2180      2190      2200
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2    TTTGCGCCGGCCGATTTGCACCAGCCTGGAATATAGCCTGAAAGTGTATTGCCTGGAAGATACCCCGGATGCGCTGAAAGAAGTGCTGGAACTGGAACGTA
Unc5H3    GTCTACTGCCTCGATGACACACAGGATGCCCTGAAGGAGGTCCTCCAGCTTGAGCGGCAGATGGGTGGGCAGCTGTTGGAGGAACCCAAAACTTTGCATT
Unc5H4    TATGCGTGCCATATTCTGCTGAACAGCTTTGGCACCTATGCGCTGATTGGCGAACCGATTAGCGAATGCGCGGTGCGTCAGCTGAAAGTGGCGGTGTTTG
Unc5H4 EST


               2210      2220      2230      2240      2250      2260      2270      2280      2290      2300
          ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
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Unc5H1
Unc5H2      CCCTGGGCGGCTATCTGCTGGAAGAACCGAAACCGCTGCCGTTTAAAGATAGCTATCATAACCTGCGTCTGAGCATTCATGATATTCCGCATAGCCTGTG
Unc5H3      TTAAGGGAAGTACCCACAACCTGCGCTTATCCATTCATGACATTGCCCACTCTCTCTGGAAGAGCAAACTGCCGGCTAAATACCAGGAGATTCCTTTTTA
Unc5H4      GCTGCCTGAGCTGCAACAGCCTGGATTATAACCTGCGTGTGTATTGCATGGATAACACCCCGTGCGCGTTTCAGGAAGTGGTGCTGGATGAACGTCTGCA
Unc5H4 EST

                 2310      2320      2330      2340      2350      2360      2370      2380      2390      2400
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      GCGTAGCAAACTGCTGGCGAAATATCAGGAAATTCCGTTTTATCATATTTGGAGCGGCAGCCAGCGTGCGCTGCATTGCACCTTTACCCTGGAACGTTAT
Unc5H3      CCACATCTGGAGTGGGTGCCAGAGGAACTTGCACTGCACCTTCACGCTGGAACGATTCAGTCTCAATACCCTGGAGCTCGTCTGCAAACTCTGTGTGCGG
Unc5H4      GGGCGGCCAGCTGCTGGATGAACCGAAACTGCTGCATTTTAAAGGCAACACCTTTAGCCTGCAGATTAGCGTGCTGGATATTCCGCCGTTTCTGTGGCGT
Unc5H4 EST

                 2410      2420      2430      2440      2450      2460      2470      2480      2490      2500
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      AGCCAGGCGAGCACCGAACTGACCTGCAAAATTTGCGTGCGTCAGGTGGAAGGCGAAGGCCAGATTTTTCAGCTGCATGTGACCCTGGGCGAACATGCGA
Unc5H3      CAAGTCGAAGGAGAAGGGCAGATCTTCCAGCTGAACTGCTCAGTATCAGAGGAACCCACTGGCATTGATTATCCCATCATGGATTCAGCAGGCAGCATCA
Unc5H4      ATTAAACCGTTTACCGCGTGCCAGGAAGTGCCGTTTAGCCGTGTGTGGTGCAGCAACCAGAAACCGCTGCATTGCGCGTTTAGCCTGGAACGTTATACCC
Unc5H4 EST

                 2510      2520      2530      2540      2550      2560      2570      2580      2590      2600
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      ACAGCTTTGATACCCTGCATAGCCATAACAGCAGCGCGCCGACCACCCAGCTGGGCCCGTATGCGTTTAAAATTCCGCTGAGCATTCGTCAGAAAATTTG
Unc5H3      CAACGATAGTTGGGCCCAACGCTTTCAGCATCCCCCTCCCAATAAGGCAGAAGCTCTGCAGCAGCCTGGATGCACCCCAGACCCGGGGCCATGACTGGAG
Unc5H4      CGGCGACCACCCAGCTGAGCTGCAAAATTTGCGTGCGTCAGGTGAAAGGCCATGAACAGATTCTGCAGATTCAGACCAGCATTCTGGAAAACGAACGTGA
Unc5H4 EST

                 2610      2620      2630      2640      2650      2660      2670      2680      2690      2700
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      CAACAGCCTGGATGCGCCGAACAGCCGTGGCAACGATTGGCGTCTGCTGGCGCAGAAACTGAGCATGGATCGTTATCTGAACTATTTTGCGACCAAAGCG
Unc5H3      GATGCTGGCCCACAAGCTGAAATTGGACAGGTACCTAAATTATTTTGCTACGAAGTCGAGTCCCACTGGGGTGATCCTGGATCTCTGGGAAGCCCAGAAT
Unc5H4      AACCATTGCGTTTTTTGCGCATGATGATAGCAACTTTCCGGCGCAGATGGGCGCGAAAGCGTTTAAAATTCCGTATAGCATTCGTCAGCGTATTTGCGCG
Unc5H4 EST

                 2710      2720      2730      2740      2750      2760      2770      2780      2790      2800
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      AGCCCGACCGGCGTGATTCTGGATCTGTGGGAAGCGGAACATCAGGATGATGGCGATCTGAACACCCTGGCGAGCGCGCTGGAAGAAATGGGCAAAAGCG
Unc5H3      TTCCCTGATGGCAACCTGAGCATGCTGGCAGCAGTGCTGGAAGAAATGGGACGACATGAAACCGTTGTTTCTTTGGCAGCAGAAGGAAATTACTGA
Unc5H4      ACCTTTGATACCCCGAACGCGAAAGGCAAAGATTGGCAGATGCTGGCGCAGAAAAACAGCATTAGCCGTAACCTGAGCTATTTTGCGACCCAGAGCAGCC
Unc5H4 EST

                 2810      2820      2830      2840      2850      2860      2870      2880      2890      2900
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Unc5H1
Unc5H2      AAATGCTGGTGGTGATGGCGACCGAAGGCGATTGCTAA
Unc5H3
Unc5H4      CGAGCGCGGTGATTCTGGATCTGTGGGAAGCGCGTCATCAGCATGATGGCGATCTGGATAGCCTGGCGTGCGCGCTGGAAGAAATTGGCCGTACCCATAG
Unc5H4 EST

                 2910      2920      2930      2940      2950      2960      2970
            ....|....|....|....|....|....|....|....|....|....|....|....|....|....|...
Unc5H1
Unc5H2
Unc5H3
Unc5H4      CAAAATTAGCGATATTACCGAAACCGAAATTGAAGAACCGGATTTTAACTATAGCCGTCAGAACGGCCTGTAA
Unc5H4 EST
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8. Netrin1 chick (see 7.3.1)

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                      10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  ATGCCGCGGAGGGGCGCGGAGGGGCCGCTCGCCCTGCTGCTGGCGGCCGCGTGGCTGGCACAGCCGCTGCGAGGCGGCTACCCCGGGCTGAACATGTTCG
Ntn1-3 5' cloned ATGCCGCGGAGGGGCGCGGAGGGGCCGCTCGCCCTGCTGCTGGCGGCCGCGTGGCTGGCACAGCCGCTGCGAGGCGGCTACCCCGGGCTGAACATGTTCG
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CCGTGCAGACGGCGCAGCCCGACCCCTGCTACGACGAGCACGGGCTGCCCCGCCGCTGCATCCCGGACTTCGTCAACTCGGCCTTCGGCAAGGAGGTGAA
Ntn1-3 5' cloned CCGTGCAGACGGCGCAGCCCGACCCCTGCTACGACGAGCACGGGCTGCCCCGCCGCTGCATCCCGGACTTCGTCAACTCGGCCTTCGGCAAGGAGGTGAA
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  GGTGTCGAGCACCTGCGGGAAGCCGCCGTCGAGGTACTGCGTGGTGACGGAGAAGGGCGAGGAGCAGGTCCGCTCGTGCCACCTCTGCAACGCCTCCGAC
Ntn1-3 5' cloned GGTGTCGAGCACCTGCGGGAAGCCGCCGTCGAGGTACTGCGTGGTGACGGAGAAGGGCGAGGAGCAGGTCCGCTCGTGCCACCTCTGCAACGCCTCCGAC
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CCCAAGCGCGCCCACCCGCCCTCCTTCCTCACCGACCTCAACAACCCGCACAACCTGACGTGCTGGCAGTCCGACAGCTACGTGCAGTACCCGCACAACG
Ntn1-3 5' cloned CCCAAGCGCGCCCACCCGCCCTCCTTCCTCACCGACCTCAACAACCCGCACAACCTGACGTGCTGGCAGTCCGACAGCTACGTGCAGTACCCGCACAACG
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  TCACCCTCACGCTGTCCCTCGGCAAGAAGTTCGAGGTGACCTACGTGAGCCTGCAGTTCTGCTCGCCGCGCCCCGAGTCCATGGCCATCTACAAGTCCAT
Ntn1-3 5' cloned TCACCCTCACGCTGTCCCTCGGCAAGAAGTTCGAGGTGACCTACGTGAGCCTGCAGTTCTGCTCGCCGCGCCCCGAGTCCATGGCCATCTACAAGTCCAT
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  GGACTACGGCAAGACGTGGGTGCCCTTCCAGTTCTACTCCACGCAGTGCCGCAAGATGTACAACAAGCCGAGCCGCGCCGCCATCACCAAGCAGAACGAG
Ntn1-3 5' cloned GGACTACGGCAAGACGTGGGTGCCCTTCCAGTTCTACTCCACGCAGTGCCGCAAGATGTACAACAAGCCGAGCCGCGCCGCCATCACCAAGCAGAACGAG
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CAGGAGGCCATCTGCACCGACTCGCACACCGACGTGCGGCCCCTCTCCGGCGGCCTCATCGCCTTCAGCACCCTGGACGGCCGCCCCACCGCCCACGACT
Ntn1-3 5' cloned CAGGAGGCCATCTGCACCGACTCGCACACCGACGTGCGGCCCCTCTCCGGCGGCCTCATCGCCTTCAGCACCCTGGACGGCCGCCCCACCGCCCACGACT
Ntn1-3 3' cloned ----------------------------------------------------------------------------------------------------

                     710       720       730       740       750       760       770       780       790       800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  TCGACAACTCGCCCGTGCTGCAGGACTGGGTGACGGCCACCGACATCAAGGTGACCTTCAGCCGCCTGCACACCTTCGGCGACGAGAACGAGGACGACTC
Ntn1-3 5' cloned TCGACAACTCGCCCGTGCTGCAGGACTGGGTGACGGCCACCGACATCAAGGTGACCTTCAGCCGCCTGCACACCTTCGGCGACGAGAACGAGGACGACTC
Ntn1-3 3' cloned ----------------------------------------------------------------~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~

                     810       820       830       840       850       860       870       880       890       900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CGAGCTCGCCCGCGACTCCTACTTCTACGCCGTGTCCGACCTGCAGGTCGGCGGGCGCTGCAAGTGCAACGGGCACGCGTCCCGCTGCGTCCGCGACCGC
Ntn1-3 5' cloned CGAGCTCGCCCGCGACTCCTACTTCTACGCCGTGTCCGACCTGCAGGTCGGCGGGCGCTGCAAGTGCAACGGGCACGCGTCCCGCTGCGTCCGCGACCGC
Ntn1-3 3' cloned ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~---GGCGGGCGCTGCAAGTGCAACGGGCNNNNNNNNCCGCTGCGTCCGCGACCNN

                     910       920       930       940       950       960       970       980       990      1000
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  GACGACAACCTGGTGTGCGACTGCAAGCACAACACGGCCGGGCCCGAGTGCGACCGCTGCAAACCCTTCCACTACGACCGGCCCTGGCAGAGGGCGACCG
Ntn1-3 5' cloned GACGACAACCTGGTGTGCGACTGCAAGCACAACACGGCCGGGCCCGAGTGCGACCGCTGCAAACCCTTCCACTACGACCGGCCCTGGCAGANGGCGACCG
Ntn1-3 3' cloned GACGACAACCTGGTGTGCGACTGCAAGCACAACACGGCCGGGCCCGAGTGCGACCGCTGCAAACCCTTCCACTACGACCGGCCCTGGCAGAGGGCGACCG

                    1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CCCGAGAGGCCAACGAGTGCGTGGCCTGCAACTGCAACCTGCATGCACGGCGCTGCCGCTTCAACATGGAGCTGTACAAGCTGTCGGGCAGAAAGAGCGG
Ntn1-3 5' cloned CCCGANNGGC-AACGANNGCGTGGCCTGCAACTGCAACCTGCATGCACGGCNCTGCC------------------------------------------
Ntn1-3 3' cloned CCCGAGAGGCCAACGAGTGCGTGGCCTGCAACTGCAACCTGCATGCACGGCGCTGCCGCTTCAACATGGAGCTGTACAAGCTGTCGGGCAGAAAGAGCGG

                    1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CGGTGTCTGCCTCAACTGCCGGCACAACACGGCCGGGCGGCACTGCCACTACTGCAAGGAAGGCTTCTACCGCGACCTCAGCAAACCCATCTCCCACCGC
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned CGGTGTCTGCCTCAACTGCCGGCACAACACGGCCGGGCGGCACTGCCACTACTGCAAGGAAGGCTTCTACCGCGACCTCAGCAAACCCATCTCCCACCGC

                    1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  AAGGCCTGCAAAGAGTGCGATTGCCATCCCGTGGGCGCCGCCGGCCAAACCTGCAACCAAACCACGGGGCAGTGTCCATGCAAGGACGGCGTCACCGGCA
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned AAGGCCTGCAAAGAGTGCGATTGCCATCCCGTGGGTGCCGCCGGCCAAACCTGCAACCAAACCACGGGGCAGTGTCCATGCAAGGACGGTGTCACCGGCA

                    1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  TCACCTGCAACCGCTGCGCCAAGGGCTACCAGCAGAGCCGCTCGCCCATTGCCCCCTGCATAAAGATCCCCGCCGCGCCGCCCCCCACAGCTGCCAGCAG
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned TCACCTGCAACCGCTGCGCCAAGGGCTACCAGCAGAGCCGCTCGCCCATCGCGCCCTGCATAAAGATCCCCGCCGCGCCGCCCCCCACAGCTGCCAGCAG

                    1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CACGGAGGAGCCTGCAGACTGTGACTCGTACTGCAAAGCCTCCAAGGGGAAGCTGAAGATCAACATGAAGAAGTACTGCAAGAAGGACTACGCTGTGCAG
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned CACGGAGGAGCCCGCAGACTGTGACTCGTACTGCAAAGCCTCCAAGGGGAAGCTGAAGATCAACATGAAGAAGTACTGCAAGAAGGACTACGCTGTGCAG
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                     1510      1520      1530      1540      1550      1560      1570      1580      1590      1600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  ATCCACATCCTGAAAGCGGAAAAAAATGCCGACTGGTGGAAGTTCACCGTCAACATCATCTCTGTCTACAAACAGGGCAGCAACCGGCTGCGGCGCGGGG
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned ATCCACATCCTGAAAGCGGAAAAAAATGCCGACTGGTGGAAGTTCACCGTCAACATCATCTCCGTCTACAAACAGGGCAGCAACCGGCTGCGGCGCGGGG

                     1610      1620      1630      1640      1650      1660      1670      1680      1690      1700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  ACCAGACCCTGTGGGTGCACGCCAAGGACATCGCCTGCAAGTGCCCCAAGGTGAAGCCCATGAAGAAGTACCTCCTGCTGGGCAGCACCGAGGACTCTCC
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned ACCAGACCCTGTGGGTGCACGCCAAGGACATCGCCTGCAAGTGCCCCAAGGTGAAGCCCATGAAGAAGTACCTCCTGCTGGGCAGCACCGAGGACTCTCC

                     1710      1720      1730      1740      1750      1760      1770      1780      1790      1800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
Netrin-1 mRNA.  CGACCAGAGCGGCATCATCGCGGACAAGAGCAGCCTGGTGATCCAATGGCGGGACACGTGGGCACGGCGGCTGCGGAAGTTCCAGCAGAGGGAGAAGAAG
Ntn1-3 5' cloned ----------------------------------------------------------------------------------------------------
Ntn1-3 3' cloned CGACCAGAGCGGCATCATCGCGGACAAGAGCAGCCTGGTGATCCAATGGCGGGACACGTGGGCACGGCGGCTGCGGAAGTTCCAGCAGAGGGAGAAGAAG

                     1810      1820
                ....|....|....|....|.
Netrin-1 mRNA.  GGGAAGTGTAGGAAGGCGTAG
Ntn1-3 5' cloned ---------------------
Ntn1-3 3' cloned GGGAAGTGTAGGAAGGCGCAC
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9. Netrin2 chick (see 7.3.1)

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                10        20        30        40        50        60        70        80        90       100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   ATGGAGGCCCCTCAGCTCCTGCGCCTGCTGCTCACCACCAGCGTGCTCCGCCTGGCACAGACTGCAAACCCCTTCGTGGCTCAGCAGACACCCCCAGACC
Ntn2-2          ATGGAGGCCCCTCAGCTCCTGCGCCTGCTGCTCACCACCAGCGTGCTCCGCCTGGCACAGACTGCAAACCCCTTCGTGGCTCAGCAGACACCCCCAGACC
Ntn2-1          ATGGAGGCCCCTCAGCTCCTGCGCCTGCTGCTCACCACCAGCGTGCTCCGCCTGGCACAGACTGCAAACCCCTTCGTGGCTCAGCAGACACCCCCAGACC

               110       120       130       140       150       160       170       180       190       200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CCTGCTACGATGAGAGCGGGGCTCCCCGCCGCTGCATCCCCGAGTTCGTCAACGCCGCCTTTGGGAAGGAGGTGCAGGCTTCCAGCACCTGTGGGAAGCC
Ntn2-2          CCTGCTACGATGAGAGCGGGGCTCCCCGCCGCTGCATCCCCGAGTTCGTCAACGCCGCCTTTGGGAAGGAGGTGCAGGCTTCCAGCACCTGTGGGAAGCC
Ntn2-1          CCTGCTACGATGAGAGCGGGGCTCCCCGCCGCTGCATCCCCGAGTTCGTCAACGCCGCCTTTGGGAAGGAGGTGCAGGCTTCCAGCACCTGTGGGAAGCC

               210       220       230       240       250       260       270       280       290       300
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CCCAACACGGCACTGCGATGCCTCGGACCCCCGCCGAGCCCACCCACCCGCCTACCTGACCGACCTCAACACCGCCGCCAACATGACGTGCTGGCGCTCC
Ntn2-2          CCCAACACGGCACTGCGATGCCTCGGACCCCCGCCGAGCCCACCCACCCGCCTACCTGACCGACCTCAACACCGCCGCCAACATGACGTGCTGGCGCTCC
Ntn2-1          CCCAACACGGCACTGCGATGCCTCGGACCCCCGCCGAGCCCACCCACCCGCCTACCTGACCGACCTCAACACCGCCGCCAACATGACGTGCTGGCGCTCC

               310       320       330       340       350       360       370       380       390       400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   GAGACCCTGCACCACCTGCCCCACAACGTCACCCTCACCCTTTCCCTCGGCAAGAAGTTTGAGGTGGTCTACGTCAGCCTCCAGTTCTGCTCGCCCCGGC
Ntn2-2          GAGACCCTGCACCACCTGCCCCACAACGTCACCCTCACCCTTTCCCTCGGCAAGAAGTTCGAGGTGGTCTACGTCAGCCTCCAGTTCTGCTCGCCCCGGC
Ntn2-1          GAGACCCTGCACCACCTGCCCCACAACGTCACCCTCACCCTTTCCCTCGGCAAGAAGTTTGAGGTGGTCTACGTCAGCCTCCAGTTCTGCTCGCCCCGGC

               410       420       430       440       450       460       470       480       490       500
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2 CGGAGTCCACCGCCATCTTCAAGTCCATGGACTACGGCAAGACGTGGGTGCCCTACCAGTACTACTCCTCGCAGTGCCGCAAGATCTACGGCAAGCCCAG
Ntn2-2          CGGAGTCCACCGCCATCTTCAAGTCCATGGACTACGGCAAGACGTGGGTGCCCTACCAGTACTACTCCTCGCAGTGCCGCAAGATCTACGGCAAGCCCAG
Ntn2-1          CGGAGTCCACCGCCATCTTCAAGTCCATGGACTACGGCAAGACGTGGGTGCCCTACCAGTACTACTCCTCGCAGTGCCGCAAGATCTACGGCAAGCCCAG

               510       520       530       540       550       560       570       580       590       600
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CAAGGCCACCGTCACCAAGCAGAACGAGCAGGAGGCGCTGTGCACCGATGGCCTCACCGACCTCTACCCGCTCACTGGCGGCCTCATCGCCTTCAGCACG
Ntn2-2          CAAGGCCACCGTCACCAAGCAGAACGAGCAGGAGGCGCTGTGCACCGATGGCCTCACCGACCTCTACCCGCTCACTGGCGGCCTCATCGCCTTCAGCACG
Ntn2-1          CAAGGCCACCGTCACCAAGCAGAACGAGCAGGAGGCGCTGTGCACCGATGGCCTCACCGACCTCTACCCGCTCACTGGCGGCCTCATCGCCTTCAGCACG

               610       620       630       640       650       660       670       680       690       700
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CTCGACGGGCGGCCCTCGGCCCAGGACTTCGACAGCAGCCCTGTGCTGCAGGACTGGGTGACGGCCACCGACATCCGGGTGGTGTTCAGCCGTCCCCACC
Ntn2-2          CTCGACGGGCGGCCCTCGGCCCAGGACTTCGACAGCAGCCCTGTGCTGCAGGACTGGGTGACGGCCACCGACATCCGGGTGGTGTTCAGCCGTCCCCACC
Ntn2-1          CTCGACGGGCGGCCCTCGGCCCAGGACTTCGACAGCAGCCCTGTGCTGCAGGACTGGGTGACGGCCACCGACATCCGGGTGGTGTTCAGCCGTCCCCACC

               710       720       730       740       750       760       770       780       790       800
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   TCTTCCGCGAGCTGGGGGGCCGCGAGGCTGGCGAGGAGGACGGGGGGGCCGGGGCCACCCCCTACTACTACTCGGTGGGCGAGCTGCAGGTCGGCGGGCG
Ntn2-2          TCTTCCGCGAGCTGGGGGGCCGCGAGGCTGGCGAGGAGGACGGGGGGGCCGGGGCCACCCCCTACTACTACTCGGTGGGCGAGCTGCAGGTCGGCGGGCG
Ntn2-1          TCTTCCGCGAGCTGGGGGGCCGCGAGGCTGGCGAGGAGGACGGGNGGGCCGNGGCCACC----------------------------------------

               810       820       830       840       850       860       870       880       890       900
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CTGCAAGTGCAACGGGCACGCCTCGCGCTGCGTCAAGGACAAGGAGCAGAAGCTGGTGTGTGACTGCAAGCACAACACCGAGGGGCCCGAGTGCGACCGC
Ntn2-2          CTGCAAGTGCAACGGGCACGCCTCGCGCTGCGTCAAGGACAAGGAGCAGAAGCTGGTGTGTGACTGCAAGCACAACACCGAGGGGCCCGAGTGCGACCGC
Ntn2-1          ----------------------------------------------------------------------------------GAGTGCGACCGN

               910       920       930       940       950       960       970       980       990      1000
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   TGCAAGCCCTTCCACTACGACCGGCCGTGGCAGCGGGCCAGCGCCCCGCGAGGCCAACGAGTGCCTGGCCTGCAACTGCAACCTGCACGCTCGGCGCTGCC
Ntn2-2          TGCAAGCCCTTCCACTACGACCGGCCGTGGCAGCGGGCCAGCGCCCCGCGAGGCCAACGAGTGCCTGGCCTGCAACTGCAACCTGCACGCTCGGCGCTGCC
Ntn2-1          TGCAAGCCNNNNNCNTTANGACCGGCCGTGGCAGCGGGCCAGCGCCCCGCGAGGCCAANGAGTGCCTGGCCTGCAACTGCAACCTGCACNNTCGGCGCTGCC

              1010      1020      1030      1040      1050      1060      1070      1080      1090      1100
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   GCTTCAACATGGAGCTGTATAAGCTGTCCGGCAGGAAGAGCGGCGGCGTTTGCCTCAACTGCCGACACAACACGGCTGGGAGGCACTGCCACTACTGCAA
Ntn2-2          GCTTCAACATGGAGCTGTATAAGCTGTCCGGCAGGAAGAGCGGCGGCGTTTGCCTCAACTGCCGACACAACACGGCTGGGAGGCACTGCCACTACTGCAA
Ntn2-1          GCTTCAACATGGAGCTGTATAAGCTGTCCGGCAGGAAGAGCGGCGGCGTTTGCCTCAACTGCCGACACAACACGGCTGGGAGGCACTGCCACTACTGCAA

              1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   GGAGGGCTTCTACCGGGACCTCAGCAAGTCCATCACGGACCGCAAGGCCTGCAAAGCCTGTGACTGCCACCCAGTTGGTGCTGCTGGCAAGACCTGCAAC
Ntn2-2          GGAGGGCTTCTACCGGGACCTCAGCAAGTCCATCACGGACCGCAAGGCCTGCAAAGCCTGTGACTGCCACCCAGTTGGTGCTGCTGGCAAGACCTGCAAC
Ntn2-1          GGAGNGCTTCTACCGGGACCTCAGCAAGTCCATCACAGACCGCAAGGCCTGCAAAGCCTGTGACTGCCACCCGGTTGGTGCTGCCGGCAAGACCTGCAAC

              1210      1220      1230      1240      1250      1260      1270      1280      1290      1300
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   CAAACAACAGGGCAGTGCCCGTGCAAGGACGGCGTGACCGGCCTCACCTGCAACCGCTGCGCCAAGGGCTTCCAGCAGAGCCGCTCGCCTGTGGCCCCCT
Ntn2-2          CAAACAACAGGGCAGTGCCCGTGCAAGGACGGCGTGACCGGCCTCACCTGCAACCGCTGCGCCAAGGGCTTCCAGCAGAGCCGCTCGCCTGTGGCCCCCT
Ntn2-1          CAAACAACAGGGCAGTGCCCGTGCAAGGACGGCGTGACCGGCCTCACCTGCAACCGCTGCGCCAAGGGCTTCCAGCAGAGCCGCTCGCCTGTGGCCCCCT

              1310      1320      1330      1340      1350      1360      1370      1380      1390      1400
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   GCATCAAGATCCCTGCCATCAACCCGACCTCTCTTGTCACCAGCACGGAGGCACCTGCAGACTGTGACTCCTACTGCAAGCCAGCCAAAGGCAACTACAA
Ntn2-2          GCATCAAGATCCCTGCCATCAACCCGACCTCTCTTGTCACCAGCACGGAGGCACCTGCAGACTGTGACTCCTACTGCAAGCCAGCCAAAGGCAACTACAA
Ntn2-1          GCATCAAGATCCCTGCCATCAACCCGACCTCTCTTGTCACCAGCACGGAGGCACCTGCAGACTGTGACTCCTACTGCAAGCCAGCCAAAGGCAACTACAA

              1410      1420      1430      1440      1450      1460      1470      1480      1490      1500
        ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2   GATTAACATGAAGAAGTACTGCAAGAAGGATTACGTGGTCCAAGTGAACATTTTGGAAATGGAGACGGTGGCCAACTGGGCCAAGTTCACCATCAACATC
Ntn2-2          GATTAACATGAAGAAGTACTGCAAGAAGGATTACGTGGTCCAAGTGAACATTTTGGAAATGGAGACGGTGGCCAACTGGGCCAAGTTCACCATCAACATC
Ntn2-1          GATTAACATGAAGAAGTACTGCAAGAAGGATTACGTGGTCCAAGTGAACATTTTGGAAATGGAGACGGTGGCCAACTGGGCCAAGTTCACCATCAACATC
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                        1510       1520       1530       1540       1550       1560       1570       1580       1590       1600
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2  CTCTCTGTCTACAAGTGCCGCGACGAGCGGGTCAAGCGCGGAGACAACTTCTTGTGGATCCACCTCAAGGACCTGTCCTGCAAGTGCCCCAAAATCCAGA
Ntn2-2         CTCTCTGTCTACAAGTGCCGCGACGAGCGGGTCAAGCGCGGAGACAACTTCTTGTGGATCCACCTGAAGGACCTGTCCTGCAAGTGCCCCAAAATCCAGA
Ntn2-1         CTCTCTGTCTACAAGTGCCGCGACGAGCGGGTCAAGCGCGGAGACAACTTCTTGTGGATCCACCTCAAGGACCTGTCCTGCAAGTGCCCCAAAATCCAGA

                        1610       1620       1630       1640       1650       1660       1670       1680       1690       1700
                   ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
chick Netrin2  TCAGCAAGAAGTACCTGGTGATGGGCATCAGCGAGAACTCCACCGACCGGCCGGGACTGATGGCCGACAAGAACAGCCTGGTCATCCAGTGGAGGGACGC
Ntn2-2         TCAGCAAGAAGTACCTGGTGATGGGCATCAGCGAGAACTCCACCGACCGGCCGGGACTGATGGCCGACAAGAACAGCCTGGTCATCCAGTGGAGGGACGC
Ntn2-1         TCAGCAAGAAGTACCTGGTGATGGGCATCAGCGAGAACTCCACCGACCGGCCGGGACTGATGGCCGACAAGAACAGCCTGGTCATCCAGTGGAGGGACGC

                        1710       1720       1730       1740       1750       1760
                   ....|....|....|....|....|....|....|....|....|....|....|....|....
chick Netrin2  CTGGACTCGCCGCCTTCGGAAACTGCAGCGGAGGGAGAAGAAAGGGAAGTGTGTGAAGCCCTGA
Ntn2-2         CTGGACTCGCCGCCTTCGGAAACTGCAGCGGAGGGAGAAGAAAGGGAAGTGTGTGAAGCCC
Ntn2-1         CTGGACTCGCCGCCTTCGGAAACTGCAGCGGAGGGAGAAGAAAGGGAAGTGTGTGAAGCCC
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10. Netrin2 conservation *Xenopus* and chick (see 7.4)

```
                  10        20        30        40        50        60        70        80
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  --------------------------------------------------------------------------------
laevis N2      --------------------------------------------------------------------------------
chick Netrin2  CCGGCACTCCTGGAGTCAGCTGGAACGCAGGTGGCCCGGGATGGCTGGGGGCAGGCAGCTGCTGGGGGGGCCCGGGGCCGC

                  90       100       110       120       130       140       150       160
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  ---------------------------------------ATTTTACCGTTCATTGGTGCAAATGATTAGTACATGTCAGGT
laevis N2      ----------------------------------------------GGGGCCTTCATACAACCCCGGNTAATA
chick Netrin2  CGCGCTGGGCAGCCCGTGCCAGCGGAGATACCCACGTCCCTCCTCCCTCCTGCCCAACCACCACGGGCCAAGCGCCGGTG

                 170       180       190       200       210       220       230       240
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  CTTAAAGTCC----TTTCCCCAAACCATGAAGGAAGATATGGGATCTCTTGAG------GGCTATGTTTTACCTTCGGG
laevis N2      CCCCGGNGCG----GAGGATTAAAGAGCAAGAGGAG-TACCTGTCTTCTAACT------GGCTATGGTTTACCTACGGG
chick Netrin2  CGGCAACGCGTGAAGAGCCCCNNNCATCACGGGACGGAGCCGATCCTGTAAGGAAGGGGAGGCTGCTCCTCGCCCGGGAG

                 250       260       270       280       290       300       310       320
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  A--GATGCCTCATCTGTTCCTTCTTGTTTCATTTTTTCCCATGCTCCCTCACTCCACATCATCTCC---TTTCTCTGGGC
laevis N2      A--GATGAGTCATCTGTTCCTTCTTGTTTCATTTTTTCCCATGCTGCCTCACTCCGCATCTTCTCC---TTTCTCTGGGC
chick Netrin2  GATGGAGGCCCCTCAGCTCCTGCGCCTGCTGCTCACCACCAGCGTGCTCCGCCTGGCACAGACTGCAAACCCCTTCGTGG

                 330       340       350       360       370       380       390       400
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  AGCAGCAGCAACACTCCATTCCAGACCCCTGCTATGATGAGCATGGCCTTCCCCGACGCTGTGTGCCAGAATTTGTCAAT
laevis N2      AACAACAACCGCACTCTATTCCAGACCCCTGCTATGATGAGCATGGCCTTCCCCGACGCTGTGTGCCAGAATTTGTTAAT
chick Netrin2  CTCAGCAG--ACACCC----CCAGACCCCTGCTACGATGAGAGCGGGGCTCCCCGCCGCTGCATCCCCGAGTTCGTCAAC

                 410       420       430       440       450       460       470       480
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  GCAGCATTTAACAAAGAAGTCCAAGCCTCCAGTACCTGTGGGCGTCTGGCAACTCGCCACTGTGATACTACAGATCCTCG
laevis N2      GCAGCATTTAACAAAGAAGTCCAAGCCTCCAGTACGTGTGGGCGTCCGGCAACTCGCCACTGTGATGCTACAGATCCTCG
chick Netrin2  GCCGCCTTTGGGAAGGAGGTGCAGGCTTCCAGCACCTGTGGGAAGCCCCCAACACGGCACTGCGATGCCTCGGACCCCCG

                 490       500       510       520       530       540       550       560
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  GAAGTCGTACCCAGCCTCTTACCTCACTGACCTTAACACTGCTGGCAACATGACCTGCTGGAGATCAGAGACCTTCCCTC
laevis N2      GAAGTCACACCCAGCCTCATATCTCACTGACCTTAACACGGCTGGGAACATGACCTGCTGGCGATCGGAGACCTTCCCTC
chick Netrin2  CCGAGCCCACCCACCCGCCTACCTGACCGACCTCAACACCGCCGCCAACATGACGTGCTGGCGCTCCGAGACCCT---GC

                 570       580       590       600       610       620       630       640
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  GCCACTCTTCACAGAATGTTAGTCTTACCCTCTCGTTGGGAAAGAAATTTGAGGTGACCTATGTCAGCCTTCAGTTCTGC
laevis N2      GTCACTCTTCTCAGAACGTCAGTCTCACCCTCTCGTTGGGAAAGAAATTTGAGGTAACCTATGTGAGTCTTCAGTTCTGC
chick Netrin2  ACCACCTGCCCCACAACGTCACCCCTCACCCTTTCCCTCGGCAAGAAGTTTGAGGTGGTCTACGTCAGCCTCCAGTTCTGC

                 650       660       670       680       690       700       710       720
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  TCTCCCCGTCCAGAATCTGTGGCCATCTTCAAGTCCATGGATTATGGAAAGAGCTGGGTCCCTTATCAGTATTACTCATC
laevis N2      TCTCCCCGTCCAGAATCTGTGGCCATTTTCAAGTCCATGGATTATGGGAAAAGCTGGGTCCCATATCAGTATTACTCATC
chick Netrin2  TCGCCCCGGCCGGAGTCCACCGCCATCTTCAAGTCCATGGACTACGGCAAGACGTGGGTGCCCTACCAGTACTACTCCTC

                 730       740       750       760       770       780       790       800
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  ACAGTGTCGTAAAGTGTATGGCAAACCTAGCCGTGCAAGTGTTACCAAACAAAATGAGCAAGAGGCTTTGTGCACAGATG
laevis N2      ACAGTGTCGTAAAGTGTATGGCAGACCTAGCCGTGCAAGTGTTACCAAACAAAATGAGCAGGAGGCTTTGTGC-------
chick Netrin2  GCAGTGCCGCAAGATCTACGGCAAGCCCAGCAAGGCCACCGTCACCAAGCAGAACGAGCAGGAGGCGCTGTGCACCGATG

                 810       820       830       840       850       860       870       880
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  GACAAACAGAACTTTATCCTCTCACTGGTGGACTAATAGCCTTCAGTACATTAGATGGACGACCTTCTGCCCAGGACCTT
laevis N2      --------------------------------------------------------------------------------
chick Netrin2  GCCTCACCGACCTCTACCCGCTCACTGGCGGCCTCATCGCCTTCAGCACGCTCGACGGGCGGCCCTCGGCCCAGGACTTC

                 890       900       910       920       930       940       950       960
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  GACAACAGCCCTGTTTTGCAGGACTGGCTGACTGCCACAGATATCCGTGTCTTCTTTAGC-------------------
laevis N2      --------------------------------------------------------------------------------
chick Netrin2  GACAGCAGCCCTGTGCTGCAGGACTGGGTGACGGCCACCGACATCCGGGTGGTGTTCAGCCGTCCCCACCTCTTCCGCGA

                 970       980       990      1000      1010      1020      1030      1040
               ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
tropicalis N2  --------------------------------------------------------------------------------
laevis N2      --------------------------------------------------------------------------------
chick Netrin2  GCTGGGGGGCCGCGAGGCTGGCGAGGAGGACGGGGGGGGCCGGGGCCACCCCCTACTACTACTCGGTGGGCGAGCTGCAGG
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11. Netrin2 *Xenopus tropicalis* sequence (see 7.4)

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                        10        20        30        40        50        60        70        80        90       100
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned ATGTTTTACCTTCGGGAGATGCCTCATCTGTTCCTTCTTGTTTCATTTTTTCCCATGCTCCCTCACTCCACATCATCTCCTTTCTCTGGGCAGCAGCAGC
xNetrin2 mRNA   ATGTTTTACCTTCGGGAGATGCCTCATCTGTTCCTTCTTGTTTCATTTTTTCCCATGCTCCCTCACTCCACATCATCTCCTTTCTCTGGGCAGCAGCAGC

                       110       120       130       140       150       160       170       180       190       200
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned AACACTCCATTCCAGACCCCTGCTATGATGAGCATGGCCTTCCCCGACGCTGTGTGCCAGAATTTGTCAATGCAGCATTTAACAAAGAAGTCCAAGCCTC
xNetrin2 mRNA   AACACTCCATTCCAGACCCCTGCTATGATGAGCATGGCCTTCCCCGACGCTGTGTGCCAGAATTTGTCAATGCAGCATTTAACAAAGAAGTCCAAGCCTC

                       210       220       230       240       250       260       270       280       290       300
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned CAGTACCTGTGGGCGTCTGGCAACTCGCCACTGTGATACTACAGATCCTCGGAAGTCGTACCCAGCCTCTTACCTCACTGACCTTAACACTGCTGGCAAC
xNetrin2 mRNA   CAGTACCTGTGGGCGTCTGGCAACTCGCCACTGTGATACTACAGATCCTCGGAAGTCGTACCCAGCCTCTTACCTCACTGACCTTAACACTGCTGGCAAC

                       310       320       330       340       350       360       370       380       390       400
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned ATGACCTGCTGGAGATCAGAGACCTTCCCTCGCCACTCTTCACAGAATGTTAGTCTTACCCTCTCGTTGGGAAAGAAATTTGAGGTGACCTATGTCAGCC
xNetrin2 mRNA   ATGACCTGCTGGAGATCAGAGACCTTCCCTCGCCACTCTTCACAGAATGTTAGTCTTACCCTCTCGTTGGGAAAGAAATTTGAGGTGACCTATGTCAGCC

                       410       420       430       440       450       460       470       480       490       500
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned TTCAGTTCTGCTCTCCCCGTCCAGAATCTGTGGCCATCTTCAAGTCCATGGATTATGGAAAGAGCTGGGTCCCTTATCAGTATTACTCATCACAGTGTCG
xNetrin2 mRNA   TTCAGTTCTGCTCTCCCCGTCCAGAATCTGTGGCCATCTTCAAGTCCATGGATTATGGAAAGAGCTGGGTCCCTTATCAGTATTACTCATCACAGTGTCG

                       510       520       530       540       550       560       570       580       590       600
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned TAAAGTGTATGGCAAACCTAGCCGTGCAAGTGTTACCAAACAAAATGAGCAAGAGGCTTTGTGCACAGATGGACAAACAGAACTTTATCCTCTCACTGGT
xNetrin2 mRNA   TAAAGTGTATGGCAAACCTAGCCGTGCAAGTGTTACCAAACAAAATGAGCAAGAGGCTTTGTGCACAGATGGACAAACAGAACTTTATCCTCTCACTGGT

                       610       620       630       640       650       660       670       680       690       700
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned GGACTAATAGCCTTCAGTACATTAGATGGACGACCTTCTGCCCAGGACCTTGACAACAGCCCTGTTTTGCAGGACTGGCTGACTGCCACAGATATCCGTG
xNetrin2 mRNA   GGACTAATAGCCTTCAGTACATTAGATGGACGACCTTCTGCCCAGGACCTTGACAACAGCCCTGTTTTGCAGGACTGGCTGACTGCCACAGATATCCGTG

                       710       720       730       740       750       760       770       780       790       800
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned TCTTCTTTAGCCGCCCTCACCTTCTAGGAAATAAGGAATCTGAGGCAAGTGATGAAGGCCCAGGTTTCTACTACTCAGTAGGAGAGTTCCAAGTTGGGGG
xNetrin2 mRNA   TCTTCTTTAGCCGCCCTCACCTTCTAGGAAATAAGGAATCTGAGGCAAGTGATGAAGGCCCAGGTTTCTACTACTCAGTAGGAGAGTTCCAAGTTGGGGG

                       810       820       830       840       850       860       870       880       890       900
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned CAGGTGCAAATGCAATGGGCATGCTTCCCGCTGTGGCAAGGATAAGGAGGCTCGCTTGATCTGTGATTGTAAACACAACACAGAGGGCCCAGAAT-GTGA
xNetrin2 mRNA   CAGGTGCAAATGCAATGGNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNTATGTCTCGCAATAAACAGGGCAAAATTGTAA

                       910       920       930       940       950       960       970       980       990      1000
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
xNetrin2 cloned --CCGGTGTAAACCC---TTTCATTATGATCGTCCATG-----GCAGANAGCCACTGCACG-TGAGGCCAATGAATGNN--TGGCCTGCACCTGTAACCT
xNetrin2 mRNA   ATTTAGAGTAAATTCAAATTCTACTCCCATTGCCAAGACATCTGTATAAACAAACCCCTCCCTTCCCCAGTTGATGCTCTTGTTAAACGCTGGGATAAG

                      1010      1020      1030      1040      1050
                ....|....|....|....|....|....|....|....|....|....|.
xNetrin2 cloned GNNCGCCCGGCGCTGTCGCTTTAACATGGAACTCTA---CAAGCTCTCC-
xNetrin2 mRNA   GAGCAGCTTATATTTCCCATTTAAGGTGAATGTCTGGGCCAAGGCAAGTAG
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12. CRABPI and Unc5H4 expression in the chick embryonic brain
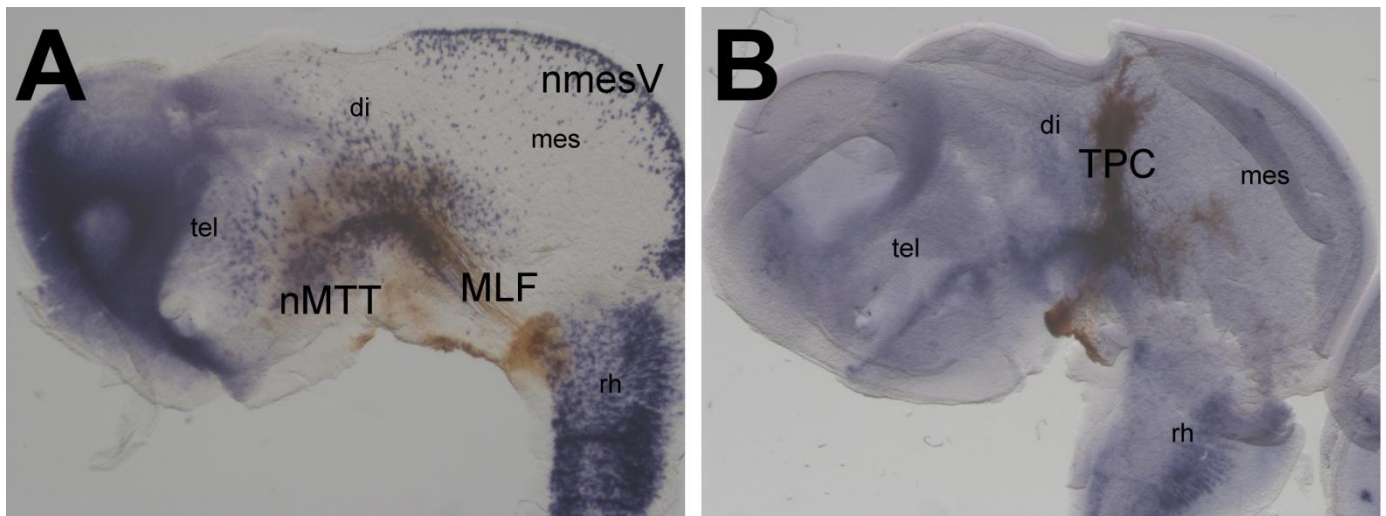
A) MLF labelled specifically with DiI and photoconverted (see 6.3.4). The MLF neurones are specifically labelled with CRABPI as well as the MTT and DTmesV neurones. The TPOC located in the rostral basal hypothalamus do not express CRABPI. There are lots of neurones expressing CRABPI in the rhombencephalon.

B) TPC labelled specifically with DiI and photoconverted (see 7.2.2). The TPC axons appear to express Unc5H4. There is also expression along the basal plate and neurones appear to be labelled in the rhombencephalon.

tel, telencephalon; di, diencephalon; mes, mesencephalon; rh, rhombencephalon