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Early expression of axon guidance molecules in the embryonic chick mesencephalon and pretectum

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ABSTRACT Early axon tracts in the developing vertebrate brain are established along precise paths. Yet, little is known about axon guidance processes at early stages of rostral brain development. Using whole mount *in situ* hybridisation in combination with immunohistochemistry, we have analysed the expression patterns of Slits, Netrins, Semaphorins and the respective receptors during the formation of the early axon scaffold, particularly focusing on the pretectal-mesencephalic boundary. Many of these guidance molecules are expressed in close correlation with the growing tracts, and the nuclei of the corresponding neurons often express the respective receptors. The expression patterns of Slits and Netrins implicate them with the positioning of the longitudinal tracts along the dorsoventral axis, while Semaphorins could provide guidance at specific choice points. Our study provides a catalogue of gene expression for future studies on axon guidance mechanisms in the early brain.

KEY WORDS: *early axon scaffold, netrin, slit, semaphorin*

Introduction

The first differentiating neurons in the embryonic vertebrate brain form an early scaffold of axon tracts (Chedotal *et al.*, 1995; Chitnis and Kuwada, 1990; Doldan *et al.*, 2000; Easter *et al.*, 1993; Hartenstein, 1993; Ishikawa *et al.*, 2004; Mastick and Easter, 1996; reviewed in Nieuwenhuys, 1998; Wilson *et al.*, 1990). The early axon scaffold is made up of several longitudinal, transversal and commissural tracts including the medial longitudinal fascicle (MLF) and the posterior commissure (PC), both originating from the midbrain-forebrain border (MFB) region (reviewed in Ahsan *et al.*, 2007). It has been hypothesised that the early axon scaffold sets up major pathways in the brain by forming substrates for future follower axons (Chitnis and Kuwada, 1991, reviewed in Hjorth and Key, 2002). Disruptions in the formation of these early tracts would therefore lead to the miswiring of tracts developing later on. In line with this idea, removing early neurons in the ventral MFB region results in later PC axons extending along aberrant pathways (Chitnis and Kuwada, 1991; Patel *et al.*, 1994). Likewise, the early MLF axons have been implicated with the formation of the reticulospinal projection in the hindbrain (Hernandez-Montiel *et al.*, 2003). Within the chick brain, several other tracts develop early that have not been considered in the descriptions of the early axon scaffold development in anamniotes (Fig. 1). In particular, neurons of the mesencephalic nucleus of the trigeminal nerve and the tectobulbar tract contribute axons to the lateral longitudinal fascicle (LLF) that is evident in the chick brain from Hamburger & Hamilton (HH) stage 17. Motorneurons in the oculomotor nucleus are also present early in the mesencephalon of chick embryos.

The axons of the early axon scaffold follow precise paths, suggesting that their projection is controlled by guidance cues. Most long-range axon guidance signalling is attributed to members of just three, highly conserved families of secreted guidance molecules, Netrins, Slits, and Class3 Semaphorins (reviewed in Chilton, 2006; Cooper, 2002; Dickson, 2002). Vertebrate Netrins were initially isolated as floor plate-derived factors that promote the extension of commissural axons in the spinal cord (Serafini *et al.*, 1994). Subsequent studies showed that they can act as either a chemorepellent or chemoattractant, depending on the receptors present on the developing axons (reviewed in Moore *et al.*,

Abbreviations used in this paper: E, embryonic day (of incubation); HH, Hamburger & Hamilton stage; LLF, lateral longitudinal fascicle; MFB, midbrain-forebrain border; MHB, midbrain-hindbrain border; MLF, medial longitudinal fascicle; nMLF, nucleus of the MLF; PC, posterior commissure.

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2007; Tessier-Lavigne and Goodman, 1996). Binding of Netrin to DCC (Keino-Masu *et al.*, 1996) or its paralogue, Neogenin (Vielmetter *et al.*, 1994), mediates a chemoattractive response, while binding to Unc5 (Leonardo *et al.*, 1997) alone or in combination with Neogenin leads to a repulsion of developing axons. Interestingly, DCC has not been described in the chick, despite the conservation in other vertebrates. Slits are chemorepulsive axon guidance molecules, best known for their role in preventing the re-crossing of commissural axons at the midline of *Drosophila* (Tear *et al.*, 1993). In vertebrates, three Slit homologues and two homologues of the Robo receptor have been identified (Brose *et al.*, 1999; Vargesson *et al.*, 2001). Along with the divergent receptor Rig-1, they are involved in controlling commissure formation in the spinal cord (reviewed in Dickson and Gilestro, 2006). The secreted Class3 Semaphorins, initially termed "Collapsins", were first identified due to their ability to collapse growth cones (Luo *et al.*, 1993; Luo *et al.*, 1995). They form a large family of guidance molecules with seven members identified in amniotes alone, but appear restricted to vertebrates (reviewed in Raper, 2000). The Class3 Semaphorins bind to the Neuropilin receptors (He and Tessier-Lavigne, 1997; Kolodkin *et al.*, 1997), which lack an intracellular domain and therefore have to form a complex with the Plexin co-receptors to mediate signal transduction (Takahashi *et al.*, 1999; Tamagnone *et al.*, 1999). Two Neuropilins and several Plexins have been found in vertebrates (reviewed in Raper, 2000).

While the course of the early tracts in the embryonic vertebrate brain has been documented in several species including the chick (Chedotal *et al.*, 1995), the molecular mechanisms governing axon guidance along individual pathways of the early axon scaffold are less well understood. Previous investigations in *Xenopus* have implicated Netrin and Semaphorin signalling in the formation of the ventral commissure (Anderson *et al.*, 2000a; Anderson *et al.*, 2000b), while Slits are involved in the fasciculation of the MLF in chick (Molle *et al.*, 2004) and formation of the tract of the postoptic commissure in zebrafish (Devine and Key, 2008). Yet, generally little is known about the expression of guidance molecules in the rostral brain in relation to the developing tracts. A study in zebrafish highlighted the correlation of gene expression patterns with axon tracts (Hjorth and Key, 2001), but investigated patterning genes rather than axon guidance molecules. The aim of this study was to analyse the expression of the major groups of axon guidance molecules within ventral pretectum and mesencephalon, and to correlate their expression pattern with the position of neurons and axon tracts. Our results show that Netrins, Slits and Semaphorins and their receptors are already expressed during the initial outgrowth of the first axons in the rostral brain and indicate possible guidance mechanisms for the longitudinal tracts.

Results

To investigate the expression of axon guidance molecules within the ventral mesencephalon and pretectum, the temporal and spatial expression of several families of axon guidance molecules, including Slits, Netrins and Class3 Semaphorins, were visualised using *in situ* hybridisation on embryos incubated for 2 days (E2, HH13-15), 3 days (E3, HH17-18) and 4 days (E4, HH20-21), i.e. between the start of axon tract formation in the brain and the appearance of the fully formed early axon scaffold. *In situ* hybridisation was combined with immunohistochemistry, using an anti-neurofilament antibody, to allow simultaneous visualisation of guidance cue expression and axon tracts.

Netrins and their receptors

The best-characterised vertebrate Netrin receptor is DCC (Keino-Masu *et al.*, 1996), which however has not yet been reported in chick. When our attempts to isolate chick *DCC* by PCR with degenerate primers failed, we queried the chick genome sequence for *DCC* and found evidence for a deletion of the whole *DCC* locus during bird evolution (manuscript in preparation). Hence, with *DCC* apparently missing in chick, Netrin binding relies largely on Neogenin and the second receptor class, Unc5, although recent studies suggest that other proteins like Dscam can also act as Netrin receptors (Andrews *et al.*, 2008). We have analysed the expression patterns of the axon guidance mol-

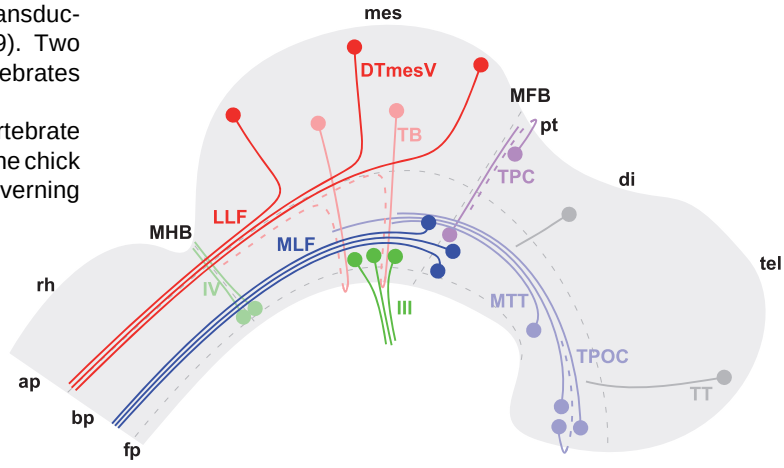

Fig. 1. Schematic representation of early brain organisation in the chick. *The diagram depicts regions, neuron clusters and axon tracts in the early embryonic chick brain at E4. The prominent ventral longitudinal bundle (blue) is made up of the TPOC originating in the hypothalamus, the MTT from the ventral diencephalon, and the MLF from the ventral midbrain-forebrain border. At this stage, all axons in the ventral bundle project caudally. The LLF in the mesencephalon is formed of ipsilateral axons from the DTmesV and contralateral axons from the TB (red). The DTmesV neurons are located along the dorsal midline of the mesencephalon and project their axons ventrally along the tectum before turning sharply caudally. The TB fibres extend from the tectum ventrally across the ventral midline before joining the contralateral LLF. Just rostral to the MFB, transversal axons form the TPC (pink). Neurons contributing to the TPC are located dorsally and ventrally in the caudal pretectum. Somatic motor neurons at the MHB (trochlear) and in the mesencephalon (ocular) yield two of the cranial nerves (green). The tracts that are the focus of this study (MLF, DTmesV, III) are shown in darker colour. Key: III (oculomotor nerve), IV (trochlear nerve), ap (alar plate), bp (basal plate), di (diencephalon), DTmesV (dorsal tract of the mesencephalic nucleus of the trigeminal nerve), fp (floor plate), LLF (lateral longitudinal fascicle), MFB (midbrain-forebrain border), MHB (midbrain-hindbrain border), MLF (medial longitudinal fascicle), mes (mesencephalon), MTT (mamillotegmental tract), pt (pretectum), rh (rhombencephalon), TB (tectobulbar tract), tel (telencephalon), TPC (tract of the posterior commissure), TPOC (tract of the postoptic commissure), TT (telencephalic tract).*

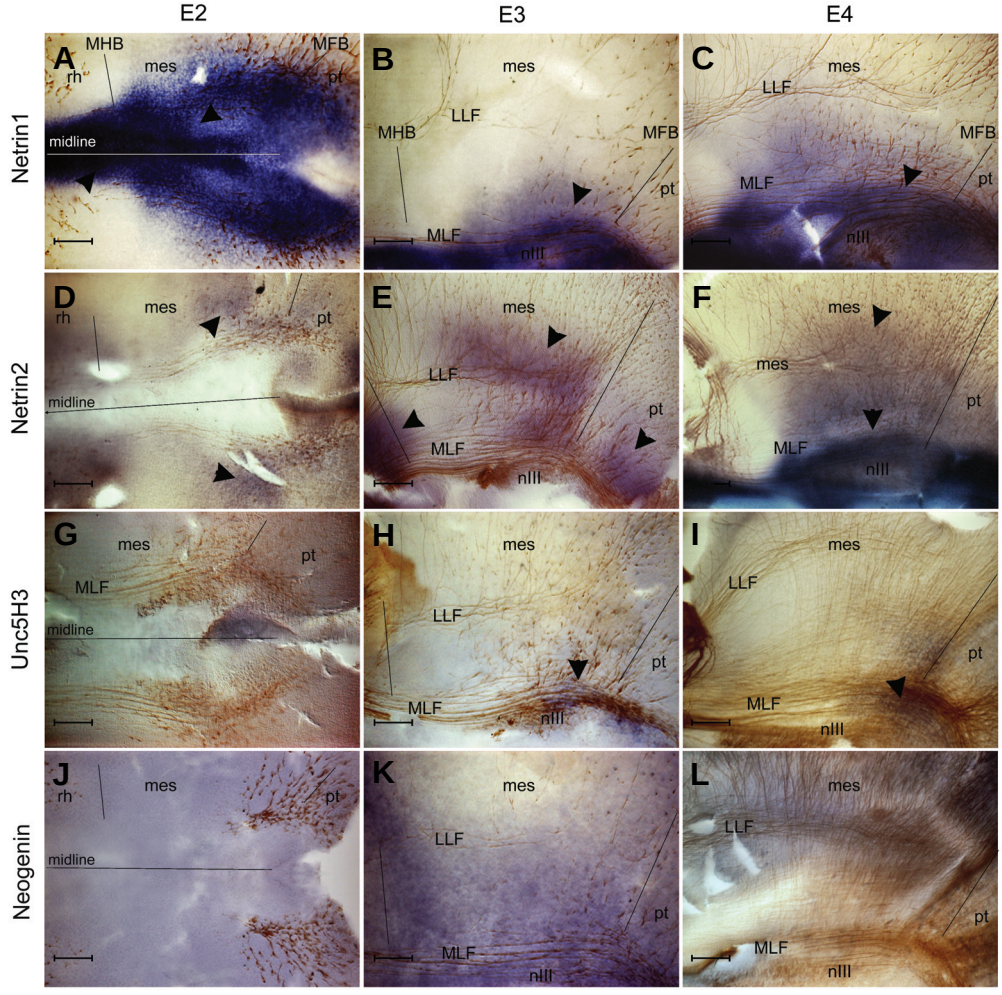

Fig. 2. Expression of Netrins and their receptors. *Embryos at stages E2, E3 and E4 were analysed, using* in situ *hybridisation for the expression of* Netrin1, Netrin2, Unc5H3, *and* Neogenin *(blue staining, black arrowheads) within the ventral mesencephalon. Neurons (brown) were visualised using an anti-neurofilament antibody. In photographs of E2 embryos, rostral is orientated to the right and the ventral midline (line) runs through the middle of the photograph. The images of later stages focus on the ventral midbrain, with rostral to the right and dorsal orientated towards the top. The positions of the midbrain-hindbrain (MHB) and midbrain-forebrain borders (MFB) are indicated by lines. The scale bar is 100 μm.* **(A-C)** *Throughout the developmental stages investigated,* Netrin1 *is expressed from the ventral floor plate up to and including the MLF. Weaker signal appears towards the more dorsal regions of this expression domain, particularly overlapping with MLF axons.* **(D-F)** Netrin2 *expression.* **(D)** *At HH14,* Netrin2 *is expressed in two areas, a small domain in the rostral mesencephalic basal plate, dorsal to the nMLF, and the basal plate of the MHB.* **(E)** *In HH18 embryos,* Netrin2 *expression is seen both rostral and caudal to the midbrain-forebrain boundary, bordering the MFB. The main midbrain expression domain is located in the rostral part of the ventral alar plate, ventrally delimited by the LLF. Further expression is restricted to the basal plate at the caudal most region of the mesencephalon and MHB.* **(F)** *At HH20, expression of* Netrin2 *extends throughout the rostral tegmentum and pretectal region. Stronger expression spans from the floor plate across the MLF tract, whilst weaker expression is observed within the basal plate region of the rostral mesencephalon.* **(G-I)** Unc5H3 *expression.* **(G)** *At HH15, no* Unc5H3 *expression is observed.* **(H,I)** *At HH18 and HH21,* Unc5H3 *expression overlaps with the nMLF.* **(J-L)** Neogenin *expression.* **(J)** *At HH13,* Neogenin *is weakly expressed throughout the mesencephalon.* **(K)** Neogenin *expression continues in the basal plate at HH17, but is reduced in the alar plate of the midbrain.* **(L)** *By HH21,* Neogenin *signals are restricted to the mesencephalic alar plate. Abbreviations: LLF (lateral longitudinal fascicle), MFB (midbrain-forebrain border), MHB (midbrain-hindbrain border), MLF (medial longitudinal fascicle), mes (mesencephalon), nIII (oculomotor nucleus), pt (pretectum), rh (rhombencephalon).*

ecules, *Netrin1* and *Netrin2*, and the receptors, *Unc5H3* and *Neogenin* (Fig. 2) in the ventral mesencephalon. The two Netrin ligands display distinct expression patterns in the midbrain. Throughout the developmental stages investigated, *Netrin1* was expressed as a gradient reaching from the floor plate into the ventral midbrain (Fig. 2 A-C). The *Netrin1* signals in the mesencephalon were confined to floor plate and ventral basal plate, covering the MLF. The expression pattern of *Netrin2* is more complex and dynamic. At HH14, *Netrin2* is expressed in a distinct pattern within the midbrain (Fig. 2D). Caudally, *Netrin2* signals were observed at the ventral midbrain-hindbrain boundary (MHB) and the hindbrain. The rostral expression domain is located dorsal to the nucleus of the MLF (nMLF) in the rostral mesencephalon. At HH18, *Netrin2* is expressed in the rostral half of the mesencephalon dorsal to and overlapping the LLF, and also in a patch abutting the MFB between LLF and oculomotor nucleus (Fig. 2E). In addition, *Netrin2* signals were apparent caudally around the MHB, and rostrally in the pretectum, rostral to the commissural region. By HH20, *Netrin2* is expressed throughout the whole ventral mesencephalon, at high levels in the floor plate, and at lower level between floor plate and LLF (Fig. 2F).

Unc5H3 expression was not detectable at HH15 (Fig. 2G). At HH18, *Unc5H3* is expressed by individual neurons at the MFB, indicated by the speckled pattern of expression (Fig. 2H), while the *Unc5H3* staining broadly covered the area of the nMLF at HH21 (Fig. 2I). *Neogenin* at HH13 appeared to be expressed ubiquitiously at very low levels (Fig. 2J). At HH17, *Neogenin* signals were stronger in the basal plate of the mesencephalon, possibly even absent from the alar plate (Fig. 2K), while at HH21 the staining became restricted to the alar plate, from the LLF up to the roof plate (Fig. 2L).

Slits and Robo receptors

We have analysed the expression of *Slit1*, *Slit2* and *Slit3*, and

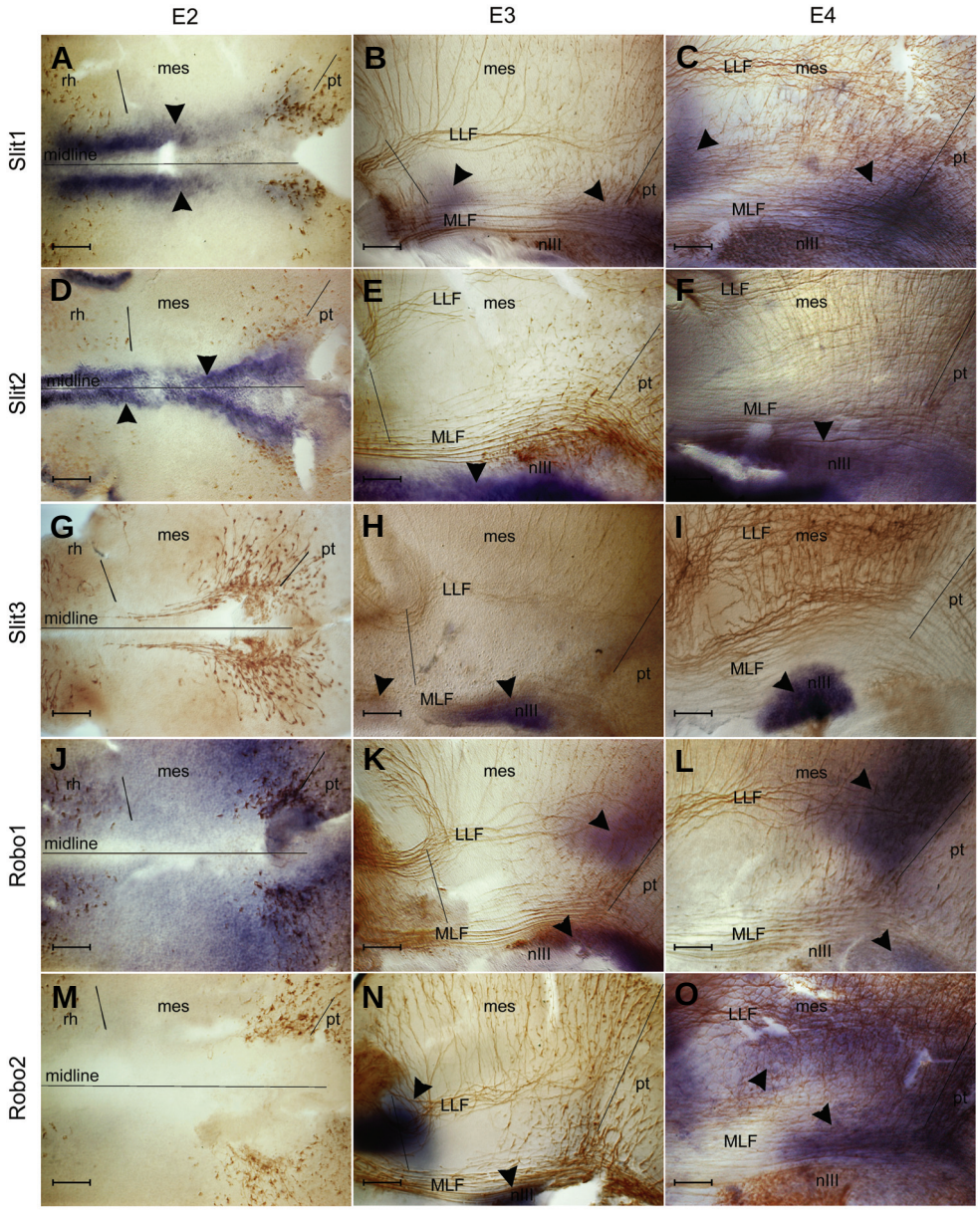

Fig. 3. Expression of Slits and their receptors. *The expression of* Slit1, Slit2, Slit3, Robo1 *and* Robo2 *was visualised using RNA* in situ *hybridisation (blue staining, black arrowheads). The neurons have been stained using immunohistochemistry with an anti-neurofilament antibody. In photographs of E2 embryos, rostral is orientated to the right and the ventral midline (line) runs through the middle of the photograph. The images of later stages focus on the ventral midbrain, with rostral to the right and dorsal orientated towards the top. The positions of MHB and MFB are indicated by lines. The scale bar is 100 μm.* **(A-C)** Slit1 *expression.* **(A)** *At HH13,* Slit1 *is expressed in a narrow stripe of the mesencephalic, ventral basal plate, running along the ventral midline.* **(B,C)** *At later stages,* Slit1 *expression is restricted to two domains, one broadly overlying the nMLF and the second at the ventral MHB.* **(D-F)** Slit2 *expression is seen throughout the ventral floor plate, extending up to but not overlapping the MLF.* **(G-I)** Slit3 *expression.* **(G)** *At HH14, no* Slit3 *signals are detectable in the brain.* **(H,I)** *At later stages,* Slit3 *is expressed in the oculomotor nucleus.* **(J-L)** Robo1 *expression.* **(J)** *Weak* Robo1 *expression is visible throughout basal and alar plates at HH13, with stronger staining located in the rostral mesencephalon.* **(K,L)** Robo1 *expression later becomes restricted to the rostral mesencephalon; one domain is located within the tectal region of the mesencephalon whilst a second domain overlaps the nMLF.* **(M-O)** Robo2 *expression.* **(M)** *No* Robo2 *expression is seen at HH13.* **(N)** *At HH18,* Robo2 *expression overlies the oculomotor nucleus and is located within the basal plate region of the caudal mesencephalon.* **(O)** *In the HH21 brain, the* Robo2 *expression domain has expanded in size, spanning the dorsal mesencephalon, and ventrally labelling the rostral mesencephalon including nMLF and oculomotor nucleus. Key: LLF (lateral longitudinal fascicle), MLF (medial longitudinal fascicle), mes (mesencephalon), nIII (oculomotor nucleus), pt (pretectum), rh (rhombencephalon).*

the receptors, *Robo1* and *Robo2* (Fig. 3) in the embryonic chick midbrain. Slit1 and Slit2 ligands were found to be expressed around the ventral midline already at HH13. At this early stage, *Slit1* is expressed in a narrow domain adjacent to the floor plate throughout the whole mesencephalon, extending into the hindbrain (Fig. 3A). *Slit1* signals were stronger in the caudal mesencephalon, but also covered the nMLF rostrally. At HH18 and HH21, the expression is separated into two ventral expression domains, a rostral area broadly overlying the nMLF at the MFB, and a caudal area around the MHB (Fig. 3 B,C). While the rostral expression domain is confined to ventral aspects of the basal plate, the caudal domain extends dorsally from the MLF, reaching the LLF by HH21. In contrast to the dynamic *Slit1* expression, *Slit2* expression between HH13 and HH21 is invariably limited to a ventral stripe (Fig. 3 D-F). Starting at the floor plate, at later stages *Slit2* signals later spread into the ventral basal plate, dorsally limited by the MLF. *Slit3* expression was not detectable at the early stage (Fig. 3G), but at HH18 is present in the oculomotor nucleus (Fig. 3H). At HH21, *Slit3* expression persists in the oculomotor nucleus (Fig. 3I), and could also be detected in the trochlear nucleus (not shown).

At HH13, *Robo1* is expressed throughout the embryonic midbrain except the floor plate (Fig. 3J), with strongest expression rostrally around the MFB. At later stages, *Robo1* expression becomes more confined. At HH18 and HH21, *Robo1* signals were observed in two distinct domains in the rostral mesencephalon (Fig. 3 K,L): a ventral expression domain of *Robo1* extends from the oculomotor nucleus up to and across the nMLF into the

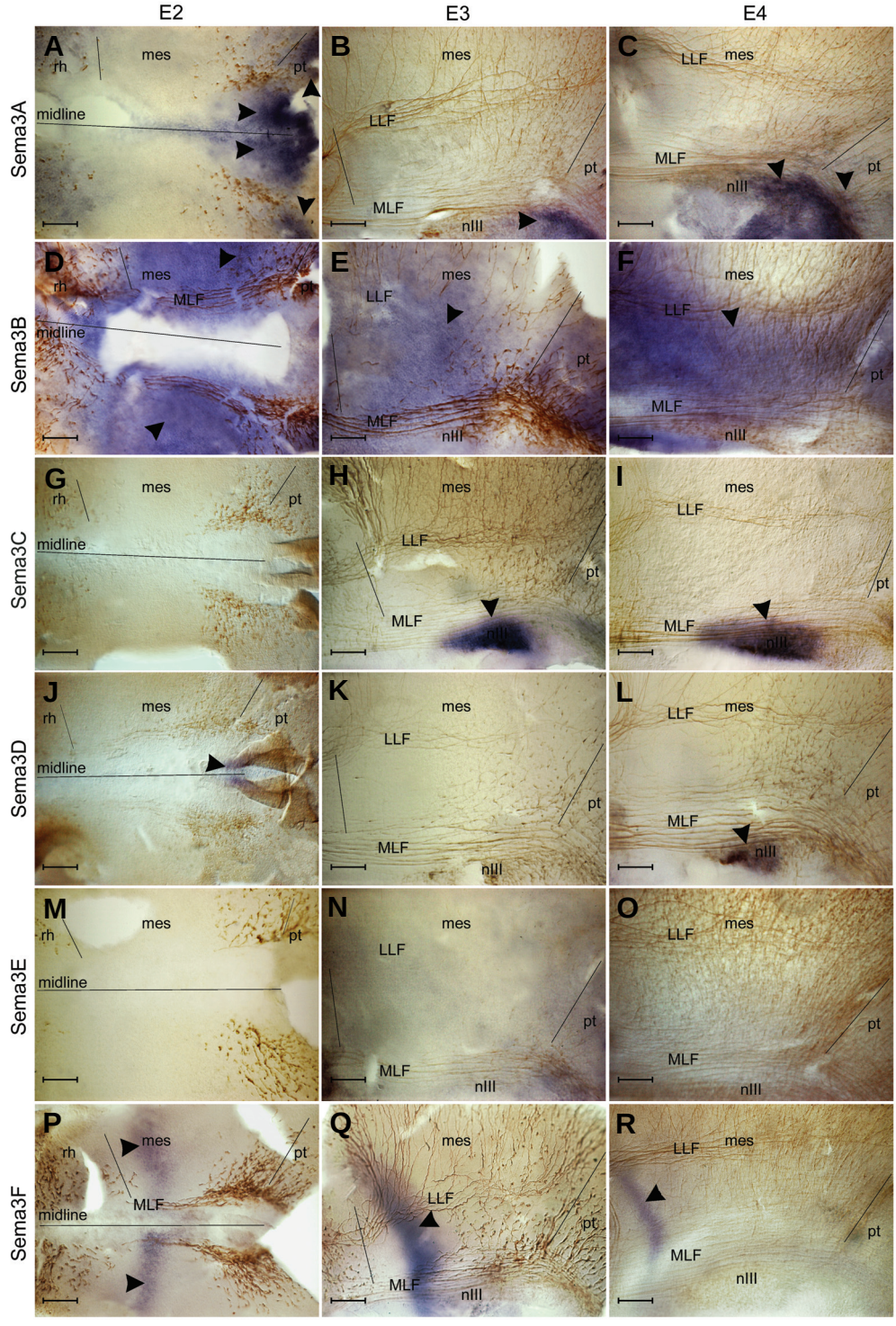

Fig. 4. Expression of Class3 Semaphorins. *The expression patterns of the Class3 Semaphorins in the early chick brain have been analysed using in RNA situ hybridisation (blue staining). This was coupled with immunohistochemistry using an anti-neurofilament antibody, allowing the visualisation of neurons (brown staining). In photographs of E2 embryos, rostral is orientated to the right and the ventral midline (line) runs through the middle of the photograph. The images of later stages focus on the ventral midbrain, with rostral to the right and dorsal orientated towards the top. The positions of MHB and MFB are indicated by lines. The scale bar is 100 μm.* **(A-C)** Sema3A *expression lies ventrally and rostrally to the nMLF at all stages analysed.* **(A)** *At HH13, Sema3A is also observed in a domain lying within the alar plate of the rostral mesencephalon and dorsal pretectum.* **(D-F)** Sema3B *expression.* **(D)** *At HH14,* Sema3B *expression is observed in the basal and alar plates throughout the midbrain.* **(E,F)** *In the older stages,* Sema3B *expression is increasingly restricted to the basal plate of the mesencephalon.* **(G-I)** Sema3C *expression.* **(G)** *No* Sema3C *expression is observed at HH13.* **(H,I)** *In HH18 and HH20 embryos,* Sema3C *expression overlaps with the oculomotor nucleus.* **(J-L)** Sema3D *expression.* **(J)** *Early* Sema3D *expression occupies a small patch of floor plate in the pretectum.* **(K)** *At HH17, no* Sema3D *expression is seen within the mesencephalon.* **(L)** *From HH20,* Sema3D *expression overlaps with the caudal part of the oculomotor nucleus.* **(M-O)** *No* Sema3E *expression is observed in the embryonic chick mesencephalon.* **(P-R)** *In all the developmental stages investigated, the* Sema3F *expression domain forms a stripe in the caudal mesencephalon, close to the MHB. Key: LLF (lateral longitudinal fascicle), MLF (medial longitudinal fascicle), mes (mesencephalon), nIII (oculomotor nucleus), pt (pretectum), rh (rhombencephalon).*

pretectum, while a dorsal domain spans the rostral mesencephalon adjoining the MFB. In contrast to the early, broad expression of *Robo1*, no *Robo2* signal was observed at HH13 (Fig. 3M). However, at HH18 *Robo2* is expressed in two distinct domains, a caudal domain at the MHB between MLF and LLF, and a rostral domain overlying the oculomotor nucleus (Fig. 3N). At HH21, *Robo2* signals were seen in a much larger area, covering the mesencephalon dorsal to the MLF. Rostrally, *Robo2* is additionally expressed in a ventral area that covers a broad region around the nMLF (Fig. 3O). The two expression domains appear to merge at the MFB.

Class3 semaphorins and their receptors

Previous studies have investigated Semaphorin expression in the hindbrain (Chilton and Guthrie, 2003) and the expression of selected Semaphorins and the Neuropilin receptors in the rostral

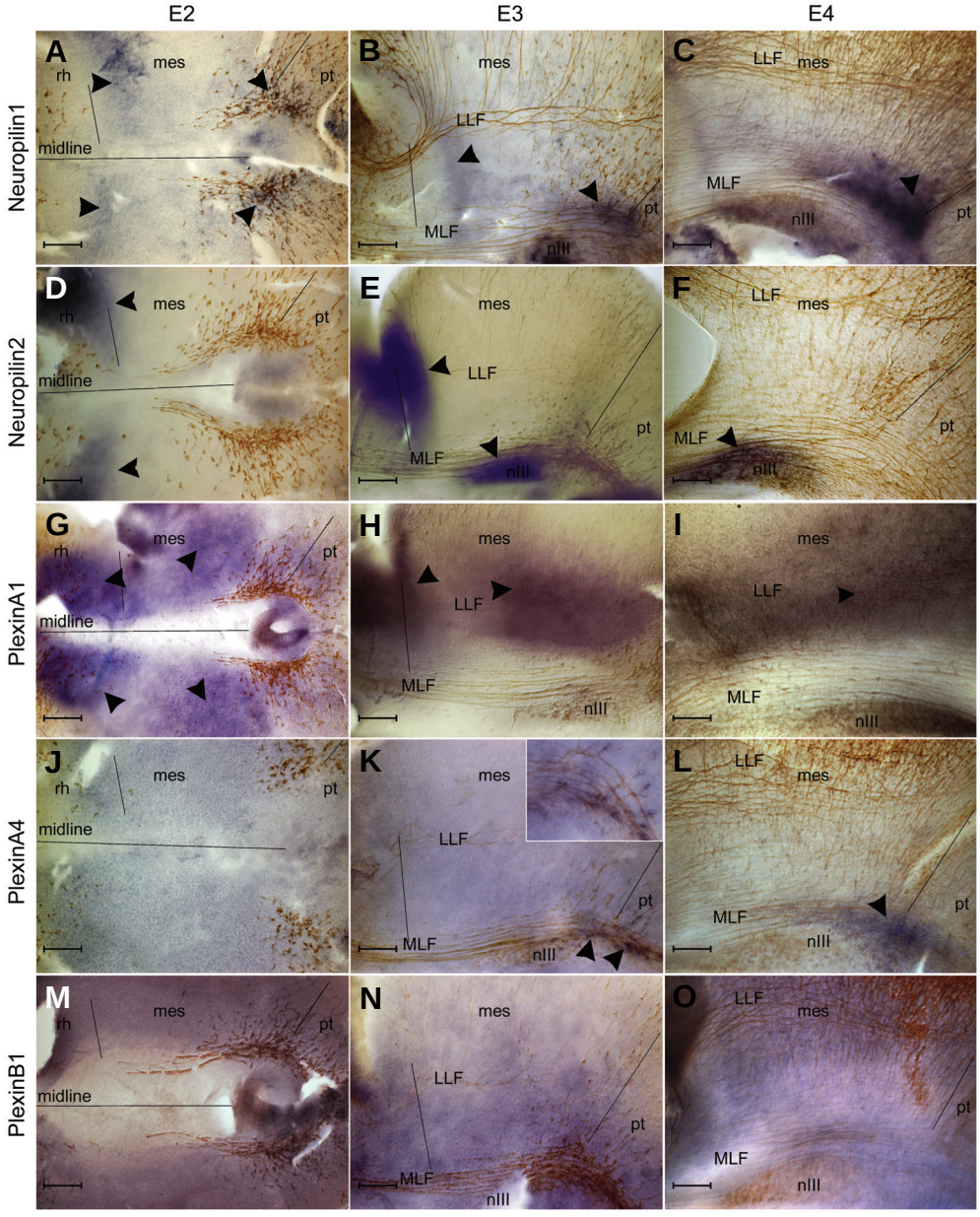

Fig. 5. Expression of Class3 Semaphorin receptors. *The expression of* Neuropilins *and* Plexins *(blue staining, black arrowheads) in the chick brain at E2, E3 and E4 was analysed in relation to the developing axon tracts (anti-neurofilament antibody, brown staining). In photographs of E2 embryos, rostral is orientated to the right and the ventral midline (line) runs through the middle of the photograph. The images of later stages focus on the ventral midbrain, with rostral to the right and dorsal orientated towards the top. The positions of MHB and MFB are indicated by lines. The scale bar is 100 μm.* **(A-C)** *Throughout the developmental stages analysed,* Neuropilin1 *expression overlies the nMLF and the caudal mesencephalon along the MHB.* **(D-F)** Neuropilin2 *is expressed in a domain spanning across the dorsal part of the MHB, extending into the rostral rhombencephalon and caudal mesencephalon.* **(E,F)** *From HH17 onwards,* Neuropilin2 *expression also overlaps with the oculomotor nucleus.* **(G-I)** PlexinA1 *expression.* **(G)** PlexinA1 *expression spans the entire length mesencephalon at HH13, ventrally delimited by the MLF. Expression levels in the caudal mesencephalon are lower than in the MHB and the rostral mesencephalon.* **(H,I)** *From HH17 onwards,* PlexinA1 *expression overlies the LLF but does not extend as far ventrally as the MLF. At HH21, PlexinA1 signals are additionally visible in the rostral, ventral basal plate of the mesencephalon* **(I)**. **(J-L)** PlexinA4 *expression overlaps with the nMLF (black arrowheads). Background staining throughout the mesencephalon partly obscures the signal, but the inset in* **(K)** *demonstrates PlexinA4 expression in individual MLF neurons at high magnification.* **(M-O)** PlexinB1 *expression.* **(M)** *At HH14,* PlexinB1 *is expressed widely in the mesencephalon exept the floor plate.* **(N)** *By HH17,* PlexinB1 *expression is stronger in the basal plate of the mesencephalon. Key: LLF (lateral longitudinal fascicle), MLF (medial longitudinal fascicle), mes (mesencephalon), nIII (oculomotor nucleus), pt (pretectum), rh (rhombencephalon).*

brain (Melendez-Herrera and Varela-Echavarria, 2006). We have characterised the expression of several Class3 Semaphorins (Fig. 4) and the Neuropilin and Plexin receptors (Fig. 5) within the developing chick mesencephalon and pretectum in relation to the developing axon tracts.

The different Class3 Semaphorins showed distinct expression patterns during early brain development. *Sema3A* is expressed in a distinct domain in the ventral MFB and pretectum, located rostral and ventral to the nMLF (Fig. 4 A-C). At HH13, a second domain of *Sema3A* expression lies in the alar plate of the rostral mesencephalon and dorsal pretectum, dorsal to the MLF neurons (Fig. 4A). From HH18 onwards, this dorsal expression of *Sema3A* was no longer detectable, but *Sema3A* signals were still observed in the MFB and pretectum, rostral and ventral to the nMLF (Fig. 4 B,C). At stage HH14, *Sema3B* is expressed throughout mesencephalon and pretectum apart from the floor plate (Fig. 4D). *Sema3B* signals in the alar plate are reduced by HH17 (Fig. 4E), and by HH21 the mesencephalic expression is restricted to the basal plate between LLF and floor plate (Fig. 4F). The expression of *Sema3C* is restricted to the oculomotor nucleus. While no staining was detectable at HH13 (Fig. 4G), strong *Sema3C* signals were seen at the older stages (Fig. 4 H,I). *Sema3D* was found to be expressed in two different domains, depending on the developmental stage. At HH14, *Sema3D* is weakly expressed within the floor plate of the pretectum (Fig. 4J). At HH17, no *Sema3D* expression was seen throughout the mesencephalon and pretectum (Fig. 4K). At stage HH20, *Sema3D* signals overlay the caudal region of the oculomotor nucleus (Fig. 4L). No *Sema3E* mRNA could be detected within

the mesencephalon, throughout the three developmental stages investigated (Fig. 4 M-O). *Sema3F* expression at all stages analysed occupied a narrow region in the basal and alar plates of the caudal mesencephalon, aligning the MHB (Fig. 4 P-R).

At all stages analysed, *Neuropilin1* was expressed in two distinct domains, a dorsoventral stripe in the caudal midbrain aligning the MHB, and a smaller area at the ventral MFB, overlying the nMLF (Fig. 5 A-C). The expression pattern of *Neuropilin2* is more dynamic. At HH14, *Neuropilin2* is expressed in a dorsal domain in the caudal mesencephalon and rostral rhombencephalon, extending across the isthmic region. This expression is stronger in the alar plate in both these regions (Fig. 5D). By HH17, this expression domain had broadened and extended ventrally, and a second *Neuropilin2* domain overlapping the nucleus of the oculomotor nerve had appeared (Fig. 5 E-F). *PlexinA1* was initially expressed widespread in the mesencephalon except the floor plate, though at lower levels along the MHB and in the rostral alar plate (Fig. 5G). From HH17 onwards the *PlexinA1* signal focussed to the dorsal basal plate and ventral alar plate, around the LLF (Fig. 5H). This domain extends across the pretectum, mesencephalon and rhombencephalon. By HH21, staining was also observed rostrally in a domain ventral to the MLF tract and rostral to the oculomotor nucleus (Fig. 5I). Similar to *Neuropilin1*, the expression pattern of *PlexinA4* overlaps with the nMLF (Fig. 5 J-L). Barely visible at HH13, this staining became more distinct at older stages, and at HH21, strong *PlexinA4* signals overlapped the nMLF (Fig. 5L). In contrast to the cell-specific expression of the other receptors, *PlexinB1* was expressed throughout the neural tube except the floor plate at all stages analysed (Fig. 5 M-O), although at later stages staining in the basal plate was much stronger than in the alar plate (Fig. 5N).

Discussion

Many of the early developing axon tracts in the vertebrate brain either originate in, or traverse through the midbrain and pretectum. Yet, little is known about the cues governing the outgrowth of these axons. We have investigated the expression patterns of the main secreted axon guidance molecules, Slits, Netrins and Class3 Semaphorins, and their receptors during early stages of brain development. Many of the guidance cues are expressed in distinct patterns during the growth of the pioneering axons, and several receptors correspondingly are expressed in specific groups of neurons. It is noteworthy that our analysis is limited to the mRNA level, and does not address the distribution of the secreted axon guidance proteins, or the actual presence of receptor molecules on the growth cones of the early axons.

The longitudinal guidance system

Two longitudinal axon tracts are formed in the early chick midbrain, the medial (MLF) and lateral (LLF) longitudinal fascicles. The MLF is formed by neurons located at the ventral MFB that project their axons caudally. The tract runs as a loose bundle adjacent to the floor plate through midbrain and hindbrain towards the spinal cord (Ahsan *et al.*, 2007). The LLF is made up of two groups of neurons: neurons in the mesencephalic nucleus of the trigeminus are found dorsally, close to the roof plate, and send their axons ipsilaterally to the trigeminal ganglion; neurons dispersed in the tectum mostly project contralaterally, growing their axons across the midline before joining the LLF to form the tectobulbar tract.

The expression patterns of several axon guidance molecules are correlated with the longitudinal tracts. Notably, Netrins and Slits are expressed in distinct domains in or around the floor plate. In the spinal cord, these guidance cues control commissure formation, with Netrin acting as attractant and Slit as repellent (reviewed in Chilton, 2006; reviewed in Dickson and Gilestro, 2006). Similar to earlier reports describing *Netrin1* to be expressed by the floor plate up to the caudal diencephalon (Kennedy *et al.*, 1994), we found strongest *Netrin1* expression in the floor plate of midbrain and pretectum, diminishing dorsally within the basal plate (Fig. 6 A,B). The expression domain includes the axons of the MLF, but the most intense *Netrin1* signal is located ventral to the MLF. In contrast to *Netrin1*, *Netrin2* expression is temporally and spatially associated with the appearance of the LLF, aligning the tract dorsally and ventrally (Fig. 6 A,B). Among the Netrin receptors, *Neogenin* is expressed broadly in the midbrain, while the expression domain of *Unc5H3*, the receptor mediating chemorepulsion by Netrins, correlates with the position of the MLF neurons (Fig. 6 C,D). We were unable to detect *DCC* in chick, probably due to deletion of the gene, which is surprising since the knockout of *DCC* in mice leads to neonatal death (Fazeli *et al.*, 1997), and *DCC* and *Neogenin* have distinctly different expression patterns in mice (Gad *et al.*, 1997). Slit expression at the isthmus has previously been shown to channel the MLF into a tight bundle (Molle *et al.*, 2004). Similar to the study by Molle *et al.*, we found *Slit2* to be expressed ventral to the MLF all along the tract, and *Slit1* expressed dorsal to the MLF at the isthmus (Fig. 6 E,F). The broad ventral expression domain of the *Robo1* receptor includes the area of the nMLF, indicating that it might constitute the receptor mediating Slit repulsion (Fig. 6 G,H). This expression pattern is consistent with Slits being involved in the fasciculation of the MLF, alike their role in the rostrally located tract of the postoptic commissure (Devine and Key, 2008).

Previous studies in *Xenopus* have already shown that DCC (Anderson *et al.*, 2000a) and Neogenin (Wilson and Key, 2006) play a role in the guidance of early tracts in the forebrain, notably the longitudinally projecting tract of the postoptic commissure (TPOC). Our results indicate that Netrin signalling, in combination with Slits, likewise could be involved in guiding the longitudinal tracts in pretectum and midbrain. Consistent with this hypothesis, a recent study in mouse has implicated Slit-Robo signalling with the correct guidance of the early longitudinal tracts (Farmer *et al.*, 2008).

Expression of guidance molecules at choice points

In contrast to the extended expression domains associated with the longtitudinal fibre systems, several axon guidance molecules show localised expression domains that are correlated with choice points for specific tracts. For the MLF, the first decision is to project axons caudally, not rostrally. A distinct expression domain of *Sema3A* was found located rostral and ventral to the nMLF (Fig. 6 I,J), while the nMLF itself expressed *Neuropilin1* and *PlexinA4* already during initial axon outgrowth (Fig. 6 K,L). Interestingly, in zebrafish *Neuropilin1*a is expressed within cells lying along the pathways of the PC and the MLF (Yu *et al.*, 2004). However, in zebrafish *Sema3D* expression lies rostral to the nMLF, and in *Sema3D* deficient embryos, axons of the MLF extend rostrally into

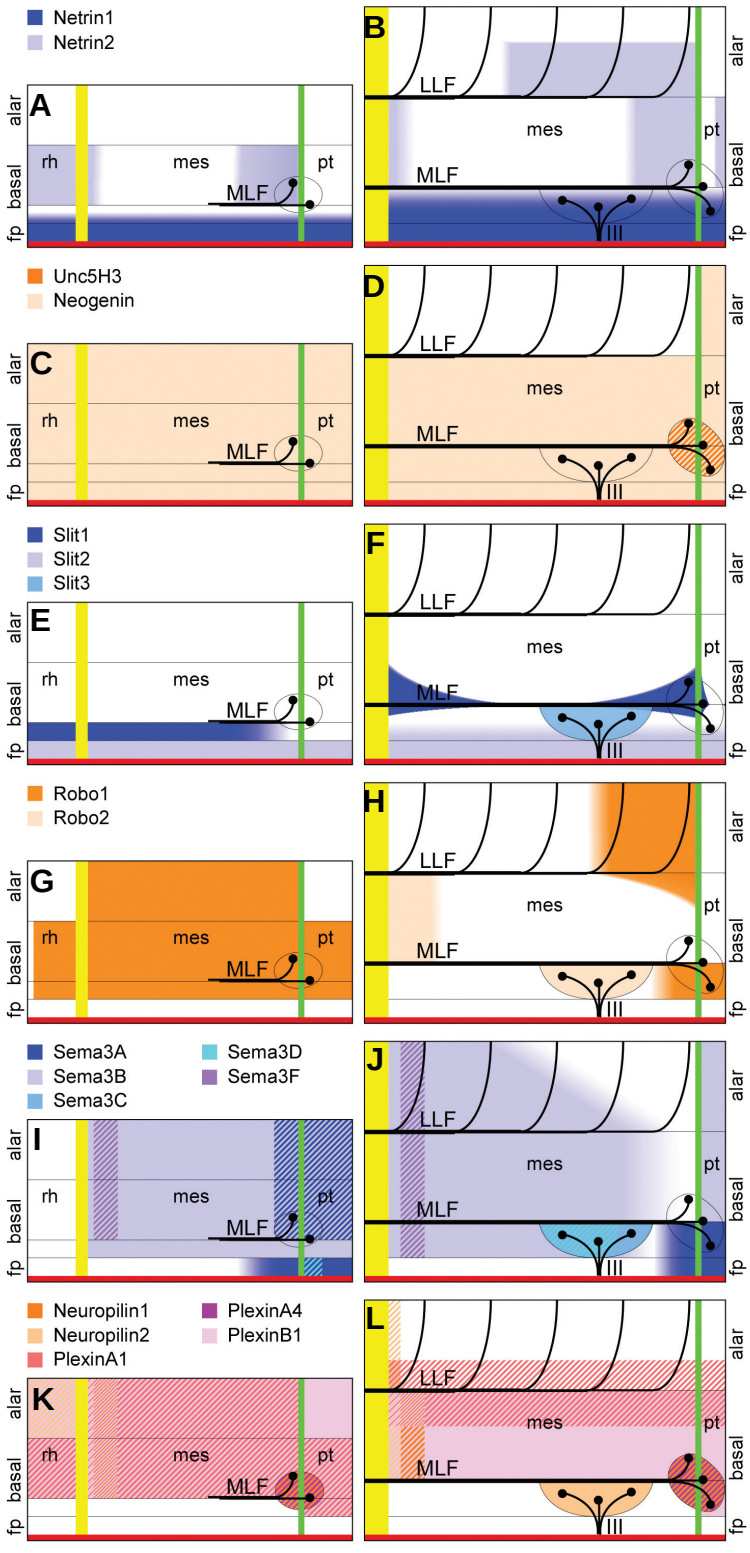

Fig. 6. Schematic representation of axon guidance molecule expression patterns in the mesencephalon. *This diagram represents the mesencephalic region analysed at E2* **(A,C,E, G,I,K)** *and E3* **(B,D,F,H,J,L)**. *The MFB is represented by a vertical green line, the MHB is shown by a vertical yellow line and the midline is shown as a horizontal red line. Thin horizontal lines separate floor plate, basal plate and alar plate, with a further horizontal line marking the course of the MLF in the basal plate, which forms an expression boundary within the basal plate for some of the genes. Neurons are shown as black circles, with axons representing the MLF, LLF and oculomotor nerve depicted as thick black lines. Gene expression domains are shown as coloured areas, hatched pattern indicating co-expression of genes.* **(A)** *At E2,* Netrin1 *(dark blue) expression in the floor plate extends across hindbrain, midbrain and pretectum, dorsally reaching into the basal plate.* Netrin2 *(light blue) expression is restricted to the basal plate around the MHB and a small area in the rostral mesencephalon.* **(B)** *At E3, the* Netrin1 *expression domain, in addition to the floor plate, includes the ventral basal plate up to the MLF.* Netrin2 *is still expressed in the basal plate of caudal and rostral mesencephalon. Further* Netrin2 *expression domains are the rostral alar plate of the mesencephalon and the basal plate of the rostral pretectum.* **(C)** Neogenin *(light orange) is expressed throughout the neural tube at E2, while no* Unc5H3 *(dark orange) signal is observed.* **(D)** *At E3,* Neogenin *expression remains expressed along the mesencephalon, but predominantly in the basal plate.* Unc5H3 *expression overlaps with the nMLF.* **(E)** *At E2,* Slit1 *(dark blue) is expressed in the ventral basal plate, weaker at the rostral end of the mesencephalon and more strongly towards the caudal mesencephalon and hindbrain.* Slit2 *(light blue) is expressed throughout the entire length of the floor plate. No* Slit3 *(mid blue) expression is observed at E2.* **(F)** Slit2 *expression remains throughout the floor plate and ventral basal plate at E3.* Slit1 *is expressed in two domains in the mesencephalic basal plate, the first rostrally around the MFB and the nMLF, and the second domain caudally.* Slit3 *expression overlies the oculomotor nucleus.* **(G)** *At E2,* Robo1 *(dark orange) is expressed throughout the basal and alar plates, whilst no* Robo2 *expression (light orange) is observed.* **(H)** *During later stages of development,* Robo1 *expression becomes restricted to two domains: the first domain is positioned ventral to the nMLF in the basal plate of pretectum and rostral mesencephalon; the second domain is located in the rostral alar plate of the mesencephalon, abutting the MFB.* Robo2 *is also expressed in two domains, the first overlapping with the oculomotor nucleus and the second in the basal plate of MHB and caudal mesencephalon.* **(I)** *At E2,* Sema3A *(dark blue) is expressed rostral and ventral to the nMLF.* Sema3B *(light blue) is expressed throughout the mesencephalon and pretectum except the floor plate.* Sema3C *(mid blue) is not yet expressed at E2.* Sema3D *(turquoise) is expressed in the floor plate of the pretectum.* Sema3F *(purple) expression forms a stripe in the caudal mesencephalon, just rostral the MHB.* **(J)** *At later stages,* Sema3A *expression remains both rostral and ventral to the nMLF.* Sema3B *expression in the mesencephalon is becoming more restricted to the basal plate.* Sema3C, *and and later also* Sema3D *expression overlies with the oculomotor nucleus.* Sema3C *spans the length of the oculomotor nucleus whilst* Sema3D *expression is more restricted to the caudal region of the oculomotor nucleus.* Sema3F *expression remains within the caudal mesencephalon.* **(K)** *At E2,* Neuropilin1 *(dark orange) and* PlexinA4 *(dark magenta) expression domains overlap with the nMLF.* Neuropilin1 *signals also form a stripe in the caudal mesencephalon, similar to Sema3F signals along the MHB.* Neuropilin2 *(light orange) is expressed in the alar plate of caudal mesencephalon and MHB.* PlexinA1 *(light red) and* PlexinB1 *(light magenta) are both expressed broadly in the neural tube, ventrally delimited by the MLF.* **(L)** *At E3, there is no change in* Neuropilin1 *and* PlexinA4 *expression.* PlexinB1 *signals become more restricted to the basal plate, while* PlexinA1 *expression is strongest around the LLF.* Neuropilin2 *expression is maintained at the MHB, and an additional expression domain is found in the oculomotor nucleus. Key: III (oculomotor nerve), fp (floor plate), LLF (lateral longitudinal fascicle), MLF (medial longitudinal fascicle), mes (mesencephalon), pt (pretectum), rh (rhombencephalon).*

the forebrain (Wolman *et al.*, 2004). In chick, no *Sema3D* expression is observed in this region, but the *Sema3A* expression pattern is comparable to that of *Sema3D* in zebrafish, and *Sema3A* may play a similar role in controlling the outgrowth of MLF axons. At later stages, when the MLF is already well established, *Sema3A* has been shown to be expressed within the mesencephalon, where it repels the axons of the tectobulbar tract, preventing these axons from crossing over the MLF and directing them in a caudal direction (Henke-Fahle *et al.*, 2001).

In *Xenopus*, NOC2-positive axons originating in the telencephalon project via the tract of the postoptic commissure (Anderson and Key, 1999). At the MFB, some of these axons cross the ventral midline to form the ventral commissure, while others join the MLF (Anderson *et al.*, 2000b). Sema3 signalling via Neuropilin1 is implicated in this decision in *Xenopus* (Anderson *et al.*, 2000b), and interestingly in the chick *Sema3D* is expressed in the ventral midline at the MFB at early stages (Fig. 6I).

The axons of the PC start to extend out later than the MLF axons, around HH17 (Chedotal *et al.*, 1995). The PC is limited to the caudal portion of the pretectum, where the PC axons initially project dorsally towards the roof plate. *Netrin2* is expressed in the ventral mesencephalon and pretectum, but notably excluding the path of the PC in the caudal pretectum (Fig. 6B). The expression of *Unc5H3* overlaps the nMLF (Fig. 6D), but the ventrally located neurons projecting into the tract of the PC are intermingled with the MLF neurons (Schubert and Lumsden, 2005), and thus might also express this receptor. Hence, repulsion by Netrin2 could be involved in the positioning of the PC to the caudal pretectum.

Guidance cues for the cranial nerves

Our analysis included two of the cranial nerves, the trochlear formed in the isthmus, and the oculomotor developing in the ventral midbrain. The receptors *Robo2* and *Neuropilin2* are prominently expressed in the oculomotor nucleus (Fig. 6 H,L), indicating that oculomotor neurons could be responsive to Slit and Sema3 signalling. At later stages, *Slit3* is present at the oculomotor and trochlear nuclei (Fig. 6F). Likewise, *Sema3C* and *Sema3D* expression are found in the oculomotor nucleus (Fig. 6J), as previously described by Chilton and Guthrie (Chilton and Guthrie, 2003; Chilton and Guthrie, 2004). While *Sema3C* expression has been observed in the nuclei of all cranial motorneurons (Chilton and Guthrie, 2003; Melendez-Herrera and Varela-Echavarria, 2006) the expression of *Sema3D* starts later than *Sema3C*, and is only observed in a subset of oculomotor neurons (Fig. 6J). The restricted expression of *Sema3D* could be significant with respect to the differential innervation of the eye muscles by specific subnuclei in the oculomotor complex (Chilton and Guthrie, 2004; Heaton and Wayne, 1983). In rat, *Sema3C* is additonally expressed within the pretectum and controls the outgrowth of axons of dopaminergic neurons (Hernandez-Montiel *et al.*, 2008). As previously described (Watanabe *et al.*, 2004), *Sema3F* expression aligns the rostral border of the isthmus (Fig. 6 I,J), where it has been shown to play a role in guiding trochlear axons along the MHB (Watanabe *et al.*, 2004).

Conclusions

By analysing the expression patterns of axon guidance molecules within mesencephalon and pretectum during early stages of chick brain development, we have shown that a number of guidance cues are present during the initial formation of the early axon scaffold. Some of the guidance molecules like Netrins and Slits could be involved in establishing the longitudinal tracts, while others like Class3 Semaphorins are more likely to guide specific axons at choice points.

Materials and Methods

Fertilised chicken eggs were obtained from Henry Stewart Ltd. and incubated at 38°C for 2, 3 or 4 days. Embryos were harvested in PBS, fixed in 4% paraformaldehyde, and staged according to Hamburger & Hamilton (1951).

In situ hybridisation and immunohistochemistry followed previously described procedures (Schubert and Lumsden, 2005). Briefly, following bleaching and detergent treatment the embryos were prehybridised in hybridisation buffer (50% formamide/5x SSC/2% SDS/2% Boehringer blocking reagent/250 $\mu g*ml^{-1}$ RNA/100 $\mu g*ml^{-1}$ heparin), before adding the Digoxigenin-labelled probe. After hybridisation, the embryos were washed in 50% formamide/2x SSC/1% SDS, before being transferred to maleic acid buffer. They were incubated with the alkaline phosphatase-conjugated anti-Digoxigenin antibody (Roche), and finally the signal was detected using NBT/BCIP (Roche) as substrate. Embryos were then re-fixed in 4% paraformaldehyde before proceeding to the immunohistochemistry. They were incubated in anti-Neurofilament antibody (Zymed RMO227, used 1:1000), followed by the secondary antibody (Jackson Laboratories Peroxidase-conjugated goat anti-mouse, used 1:100), finally detected with Diaminobenzidine (Vector Labs HRP kit) as substrate.

Probes used for the *in situ* hybridisations were either provided by colleagues or were EST clones obtained from MRC Geneservice. Probes for *Netrin1* and *Netrin2* (Serafini *et al.*, 1994) were kind gifts by M. Tessier-Lavigne, while for *Neogenin* (chEST741p10) and *Unc5H3* (chEST1023g17) we used EST clones. *Slit1* and *Slit2* were gifts from A. Chédotal, *Slit3* was a gift from L. Erskine and N. Vargesson, and *Robo1* and *Robo2* (Vargesson *et al.*, 2001) were gifts from E. Laufer. *Sema3C*, *Sema3D*, and *Sema3E* (Feiner *et al.*, 1997; Luo *et al.*, 1995) were gifts from J. Raper. *Sema3A* (chEST712j10), *Sema3B* (chEST771a21), and *Sema3F* (ROSO12B12) were EST's. *Neuropilin1* (Takagi *et al.*, 1995) was a gift from H. Fujisawa, and *Neuropilin2* (Watanabe *et al.*, 2004) was a gift from Y. Watanabe. Probes for *PlexinA1* (chEST21d16), *PlexinA4* (chEST 744O11), and *PlexinB1* (chEST1023G17) were all derived from EST's.

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