Plexin-A2 and *neuropilin-2* in the axonal guidance of cranial nerves in avian embryos

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Abbreviations

AP	Alkaline Phosphatase
bp	base pair
cm	centimeter
C	Cervical
CN	Cranial Nerve
CNS	Central Nervous System
DNA	Deoxyribonucleic Acid
DIG	Digoxigenin
DRG	Dorsal Root Ganglia
DSHB	Developmental Studies Hybridoma Bank
ECM	Extra Cellular Matrix
e. g.	exemplu gratii (Latin): for example
ETS	E-Twenty-Six
EGFP	Enhanced Green Fluorescent Protein
Fig.	Figure
FP	Floor Flate
g	gram
Gam-Cy2/Cy3	Goat anti-mouse-cyanine 2/3
Gar-Cy2/Cy3	Goat anti-rabbit-cyanine 2/3
HH	Hamilton and Hamburger
h	hour
IgG	Immunoglobulin G
kb	kilo base pair
kDa	kilo dalton
l	liter
Lab.	Laboratory
M	Molar weight
min	minute
mg	milligram
mm	millimeter
ml	milliliter

mM	millimolar
MNs	Motor Neurons
mRNA	messenger RNA
ms	millisecond
μg	microgram
μl	microliter
μm	micrometer
n	number
ng	nanogram
Npn-1	Neuropilin-1
Npn-2	Neuropilin-2
O	Occipital
P	Probability value (Student's t test)
PBS	Phosphate Buffered Saline
PFA	Paraformaldehyde
PNS	Peripheral Nervous System
r	rhombomere
rpm	rounds per minute
RT	Room Temperature
RNA	Ribonucleic Acid
RNAse	Ribonuclease
Sema	Semaphorin
shRNA	short hairpin RNA
SD	Standard Deviation
tRNA	transfer Ribonucleic Acid
Tab.	Table
TF	Transcription Factor
UV	Ultraviolet light
V	Volt

Summary

Secreted class-III semaphorins exert their effects in axon guidance and neuronal migration by binding with receptors, such as plexins and neuropilins. Neuropilins are insufficient to convey signals of their own; rather, they form complexes with plexins to propagate signals of semaphorins into the cells. Though the role of class-III semaphorins in governing fasciculation, axon growth and cell migration has been studied previously, it is far away from our understanding how their receptors (plexin-As and neuropilins) take part in the axonal guidance of cranial and spinal motor neurons. It has been demonstrated that *plexin-A2* and *neuropilin-2* (*Npn-2*) control the motor somal positioning in the chick spinal cord. However, it is still unknown whether they are involved in the regulation of cranial motor neurons. For this purpose, we first analyzed the expression of *plexin-A1, plexin-A2, plexin-A4* and *Npn-1* and *Npn-2* in the motor neuronal groups within the chick hindbrain. Our results demonstrated that all analyzed *plexins* and *neuropilins* were selectively expressed by hindbrain motor neurons. For instance, *plexin-A1, plexin-A2* and *Npn-1* were expressed by both dorsal and ventral exiting cranial motor neurons, whereas *plexin-A4* and *Npn-2* only by dorsal exiting cranial motor neurons. Based on the expression data, we selected *plexin-A2* and *Npn-2* genes for knockdown experiments by *in ovo*-electroporation of *short hairpin RNA* (*shRNA)* constructs into the ventral neural tube at the post-otic hindbrain level, from which motor neurons of the vagus (nX), accessory (nXI) and hypoglossal (nXII) nerves originated. Unlike the spinal cord, where loss of function of either *plexin-A2* or *Npn-2* induced ectopic migration of motor neuron somata along the ventral root, only *Npn-2* in the hindbrain induced ectopic migration of motor somata but along the dorsal root. In addition, inhibition of function of *Npn-2* resulted in misrouting and severe defasciculation of dorsal exiting (vagus and accessory) motor axons. Furthermore, knockdown of *plexin-A2* led to the significant ($P<0.001$) reduction of motor neuron population in the ventral neural tube and impaired fasciculation of ventral exiting (hypoglossal) motor axons. These results indicate that *plexin-A2* and *Npn-2* act independently in the axonal guidance of cranial nerves in chick embryos.

1. Introduction

Developing neurons form a complex network in the central nervous system (CNS) and peripheral nervous system (PNS) to function properly. Formation of this network includes many steps: neuronal migration to proper regions, neurite outgrowth, formation of polarity, guidance of axons and dendrites to proper targets, dendritic maturation and synapse formation with appropriate partners. The migration of neurons is a key process in the development of the nervous system since sites of neurogenesis are often separated by long distances from final destinations. Neuron migration is complex, requiring synchronization of multiple stepwise processes that differ in important respects from other types of migrating cells. It is initiated independently of the cell soma by the extension of long processes preceded by an exploratory growth cone (Ridley et al., 2003). Somal translocation occurs only after the leading process becomes consolidated by sustained movement in one direction (Lambert et al., 2001; Ayala et al., 2007). On reaching its destination, the cell body stops and somal migration and axonal extension become irreversibly disengaged by unknown mechanisms. Evidence suggests that this may be achieved by the column- and pool-specific expression of receptors for guidance cues.

A central issue in neurobiology is determining how axons find their targets. Developing axons navigate to their targets by the combined influence of guidance receptors expressed on axon surfaces and the distributions of relevant cues axons encounter in the environment (Fig. 1). Their interactions activate intracellular signaling cascades which are followed by dynamic changes in the cytoskeleton. These result in directional axon extension and target recognition (Goodman, 1996; Guan & Rao, 2003; Huber et al., 2003). Thus, accurate pathfinding depends critically on the establishment of reproducible and precise patterns of expression for both receptors and guidance cues. Subpopulations of neurons whose axons make divergent decisions at choice points express different sets of receptors for guidance cues. These cues are expressed in a spatially and temporally discontinuous manner along axon pathways. Transcription factors control receptor expression in the growing neurons and guidance cue expression in the surrounding pathways (Raper and Mason, 2010).

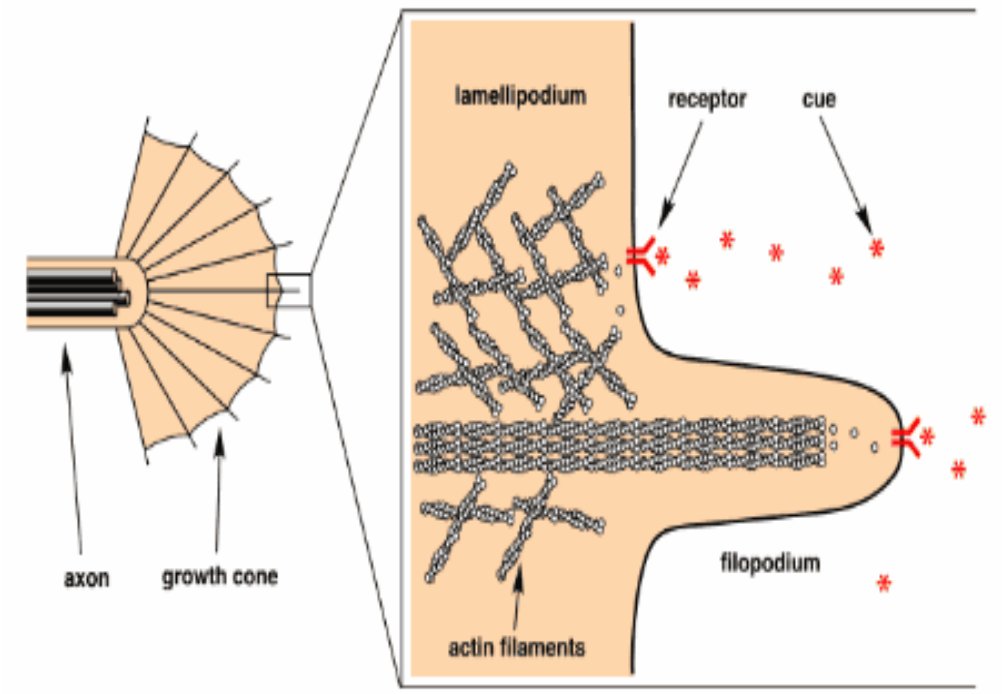
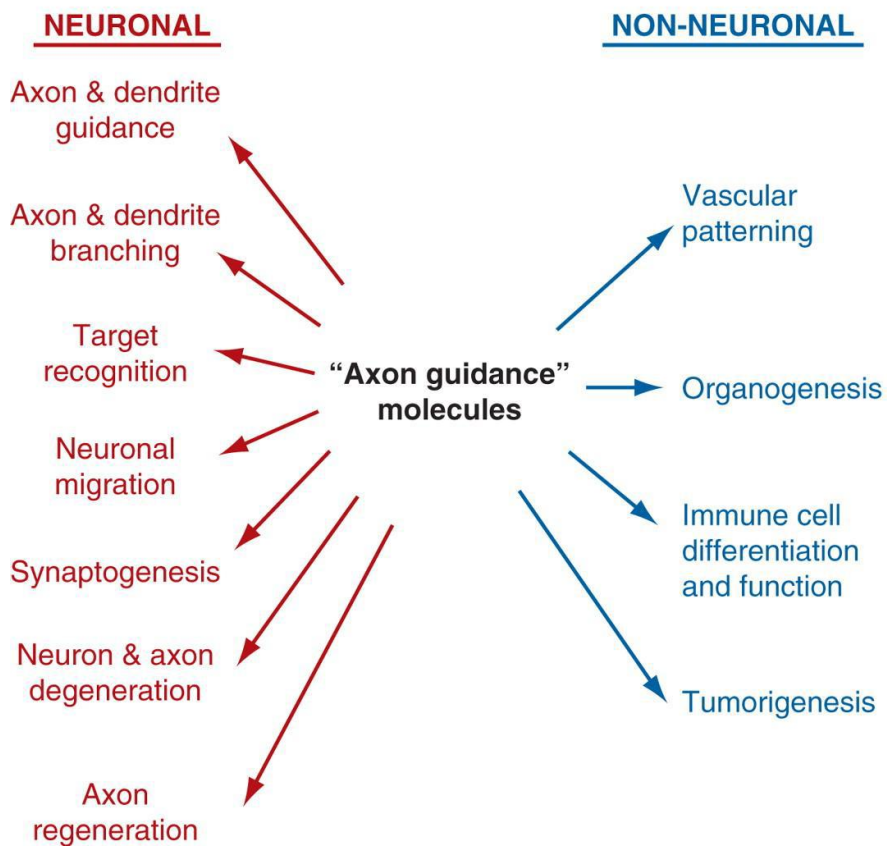


Fig. 1: Interactions of axon guidance cues with the cell surface receptors (adapted from Huber et al., 2003).

1.1 Axon Guidance Molecules

Growing axons rely on a variety of guidance molecules in deciding upon a growth pathway. Biochemical and genetic studies have revealed a variety of families of axon guidance molecules, including netrins, slits, semaphorins and ephrins (Dickson B. J., 2002). Netrins, slits and some semaphorins are secreted and associate with cells or the ECM. Ephrins and some semaphorins are membrane bound. Based on the direction of response, axon guidance molecules are categorized into two groups, attractive (axons move toward the source) and repulsive cues (axons avoid the source). Netrins are attractive guidance molecules, while slits, semaphorins and ephrins belong to repulsive cues. The receptors for these guidance molecules are identified as DCC, UNC-5, robos, plexins, neuropilins, and Ephs.

The axon guidance molecules are involved in a variety of cellular (neuronal and non-neuronal) processes in the living systems which are outlined as follows:



We discuss below the most characterized guidance cues and their receptors that influence axon guidance:

1.1.1 Netrins

Netrins are a small family of highly conserved guidance molecules (~70-80 kDa). One found in c.elegans (UNC6), two in Drosophila (Netrin-A and -B), two in chick (netrin-1 and -2). In addition to netrin-1 and -2, a third netrin identified as netrin-3 (mouse and human). Netrin-1 is produced by the floor plate and Netrin-2 by the ventral spinal cord except the floor plate. Both netrins become associated with the ECM and the receptor DCC. Netrins are bifunctional molecules, attracting some axons and repelling others. The repulsive activity of netrin first had been shown in vertebrates for populations of motor axons that project away from the midline.

The receptors that mediate the attractive and repulsive effects of netrins are also highly conserved. Growth cone attraction involves the transmembrane receptors of the DCC family and repulsion involves the transmembrane receptors of the UNC-5 family (Fig. 2; Dickson B.J., 2002; Chilton J.K., 2006).

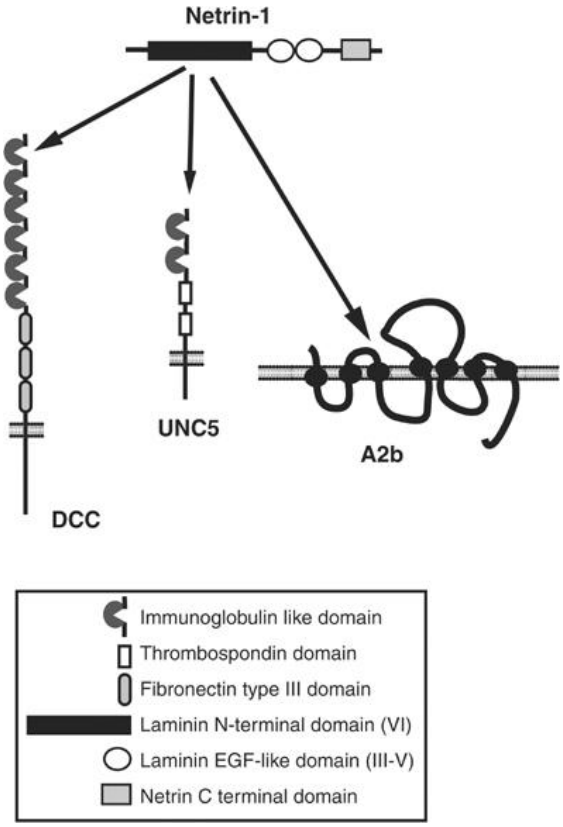


Fig. 2: Netrin-1 and its receptors. Netrin-1 is a laminin related protein, containing a laminin N-terminal domain, two laminin EGF-like domains and a netrin C terminal domain. Several transmembrane netrin-1 receptors are known. Deleted in Colorectal Cancer (DCC) contains six Ig-like and three fibronectin type III (FNIII) repeats. UNC5A−UNC5D are composed of two Ig-like and two thrombospondin domains. Netrin also binds the adenosine receptor A2b, a seven membrane domain receptor (adapted from Chédotal et al., 2005).

1.1.2 Slits

Slit proteins are large (~190 kDa) extracellular matrix proteins containing leucine-rich repeats and epidermal growth factor-like repeats (Fig. 3). Slit is a secreted protein which is most widely known as a repulsive axon guidance cue. Its receptor robo is a transmembrane protein. There are four different robos and three slits in vertebrates: robo1, robo2, robo3/rig-1, and robo4, and slit1, slit2, slit3 (Yuan et al., 1999). Slit-robo interactions regulate axon guidance at the midline for commissural (Sabatier et al., 2004), retinal (Hussain et al., 2006), olfactory (Nguyen-Ba-Charvet et al., 2002), cortical (Shu et al., 2003) and precerebellar axons (Marillat et al., 2004).

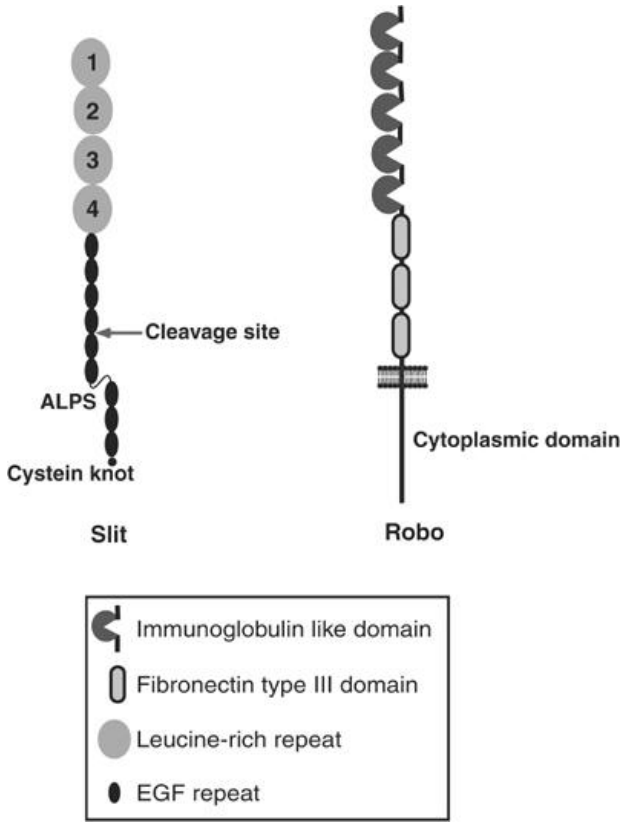


Fig. 3: Slits and their receptors. Slit are large ECM glycoproteins comprising, from their N terminus to their C terminus, a long stretch of four leucine rich repeats, seven to nine EGF repeats, and an LG module. Slits are proteolytically processed into a large N-terminal and shorter C-terminal fragments. Roundabout (robo) are slit receptors and define a small subgroup within the immunoglobulin superfamily characterized by the presence of five Ig-like followed by three fibronectin type III (FNIII) repeats, a transmembrane portion and a long cytoplasmic tail containing robo-specific motifs (adapted from Chédotal et al., 2005).

1.1.3 Ephrins

Ephrins also known as Eph family receptor interacting proteins are a family of membrane-bound proteins that serve as the ligands of the ephrin receptor (Fig. 4). Ephrins are divided into two subclasses of ephrin-A and ephrin-B based on their structure and linkage to the cell membrane. Eph receptors in turn are classified as either EphAs or EphBs based on their binding affinity for either the ephrin-A or ephrin-B ligands. The binding and activation of Eph/epherin intracellular signaling pathways can only occur via direct cell-cell interaction. During the development of the central nervous system Eph/ephrin signaling plays a critical role in the cell-cell mediated migration of several types of neuronal axons to their target destinations. Eph/ephrin signaling controls the guidance of neuronal axons through their ability to inhibit the survival of axonal growth cones, which repels the migrating axon away from the site of Eph/ephrin activation (Marquardt et al., 2005). The growth cones of migrating axons do not simply respond to absolute levels of Ephs or ephrins in cells that they contact, but rather respond to relative levels of Eph and ephrin expression (Reber et al., 2004), which allows migrating axons that express either Ephs or ephrins to be directed along gradients of Eph or ephrin expressing cells towards a destination where axonal growth cone survival is no longer completely inhibited (Marquardt et al., 2005). Although Eph-ephrin activation is usually associated with decreased growth cone survival and the repellence of migrating axons, it has recently been demonstrated that growth cone survival does not depend just on Eph-ephrin activation, but rather on the differential effects of "forward" signaling by the Eph receptor or "reverse" signaling by the ephrin ligand on growth cone survival (Marquardt et al., 2005; Petros et al., 2010).

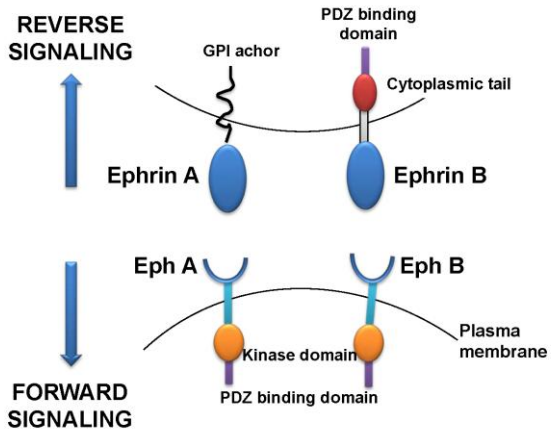


Fig. 4: Ephrin/Eph forward (ligand to receptor) and reverse (receptor to ligand) signaling (adapted from Wikipedia).

1.1.4 Semaphorins

A distinctive protein module of about 500 amino acids, called the Sema domain, characterizes all semaphorins, and this domain is located in N-terminal regions (Fig. 5). Semaphorins comprise a large family of secreted or transmembrane proteins which have been shown to regulate axonal pathfinding during the development of the nervous system (Kolodkin et al., 1993, Luo et al., 1993). To date, more than 20 semaphorins have been identified, and they are now classified into eight subclasses on the basis of sequence similarity and distinctive features (Fig. 5; Nakamura et al., 2000). Classes I and II are invertebrate semaphorins, classes III to VII are vertebrate semaphorins, and class V is viral-encoded semaphorins, found in the genome of non-neurotrophic DNA viruses. Among them, classes I, IV, V and VI are transmembrane molecules, and class VII is a membrane-associated form. On the other hand, classes II, III and V are secreted proteins.

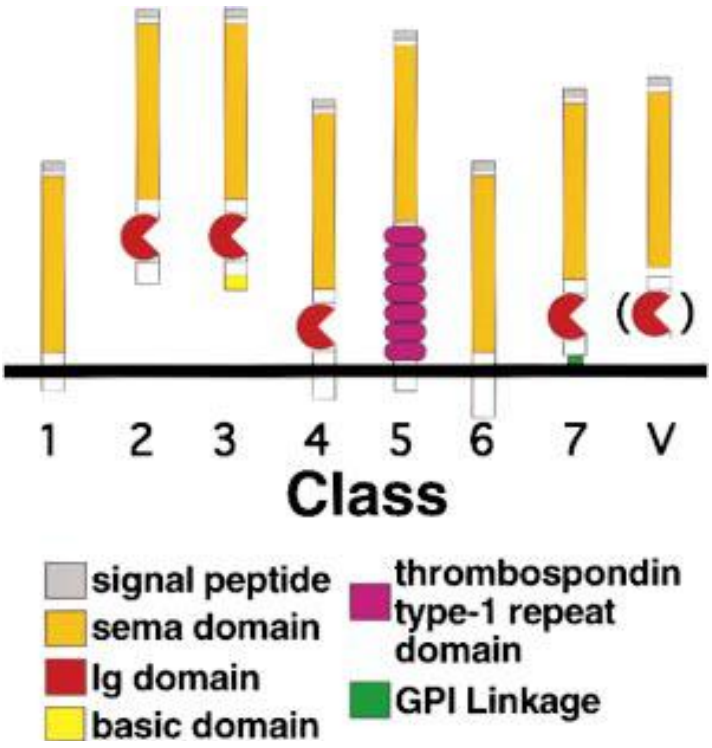


Fig. 5: Semaphorin family (adapted from Unified nomenclature of semaphorins, Cell 1999).

The secreted class III semaphorins are the best characterized among the semaphorins. They exert their effects by binding with ligand binding neuropilins (Npn-1 and Npn-2) and signal transducing plexins (Fig. 6). However, neither Npn-1 nor Npn-2 is able to convey semaphorin signals on their own (Feiner et al., 1997). They form complexes with plexins and act as co-receptors for semaphorins (Fig. 7).

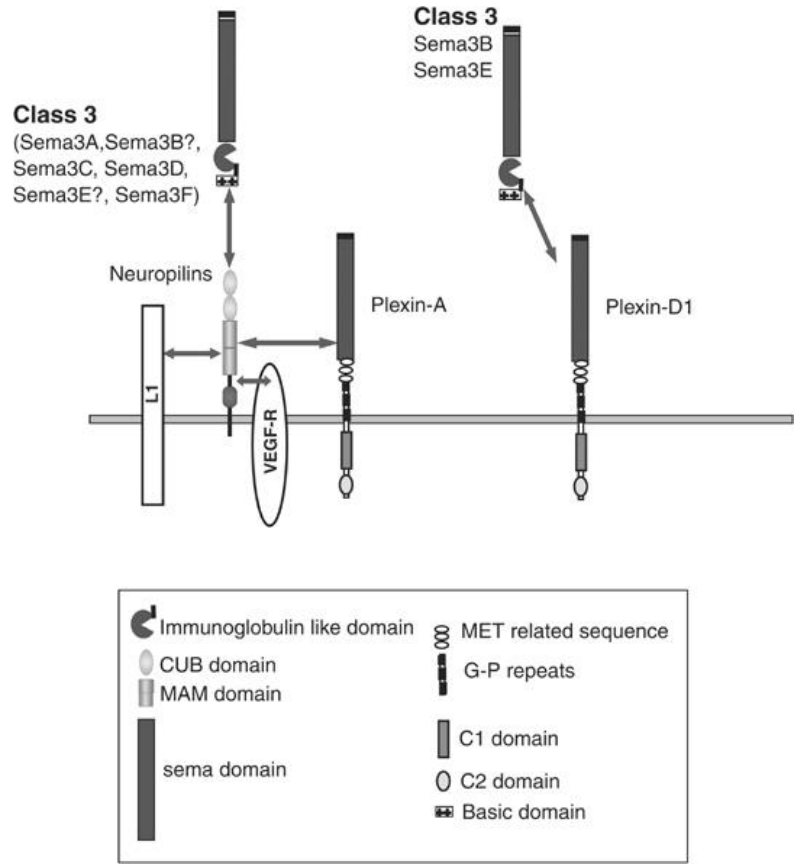


Fig. 6: Secreted semaphorins and their receptors (plexins and neuropilins). All class 3 semaphorins identified to date were initially found to bind to neuropilins and use plexin-As as signaling subunits (adapted from Chédotal et al., 2005).

The members of the Class III semaphorins (Sema 3A-F) have been shown to function in axon guidance. Sema 3A acts as a repulsive factor on chicken DRG neurons by inducing the collapse and retraction of their growth cones (Luo et al., 1993). Further studies showed a repulsive effect of Sema 3A on other neuronal cells, such as sensory, sympathetic and cortical neurons (Raper JA, 2000).

Some members have a growth promoting effect on specific neuronal subpopulations. This is the case of Sema 3C that promotes the growth of cortical axons (Bagnard et al., 1998) and Sema 3F that promotes the growth of olfactory bulb axons (de Castro et al., 1999). *In-vivo* experiments conducted in the zebrafish showed that Sema 3D triggered attraction or repulsion depending on a differential recruitment of receptor subunits (Wolman et al., 2004). Npn-2-expressing growth cones are repelled by the Sema 3F-containing dorsal limb region (Huber et al., 2005).

The genetic analysis of semaphorin function revealed several defects such as abnormal projections of sensory axons, abnormal cortical neurites orientation in Sema 3A-deficient mice (Taniguchi et al., 2003). In many cases, the most severe phenotype was the defasciculation of axonal tracts in absence of Sema 3A signaling (Kitsukawa et al., 1997; Taniguchi et al., 1997). Several defects in projections in the hippocampus, mid brain, forebrain and in the PNS of Sema 3F deficient-mice have also been described (Sahay et al., 2003). In the cortex, a combination of Sema 3A (acting as a repellent for axons and attractant for dendrites) and Sema 3C (acting as a chemoattractant) is thought to control the establishment of the cortical efferent projections (Polleux et al., 1998). Multiple combinations of semaphorins participate in the construction of axonal projections in the hippocampus (Chèdotal et al., 1998; Steup et al., 1999), the olfactory bulb (de Castro et al., 1999), the thalamus (Bagnard et al., 2001), the spinal cord (Huber et al., 2005; Cohen et al., 2005) or in the peripheral system (Kitsukawa et al., 1997). Recently, it has been shown that not only neurons are sensitive to semaphorins in the nervous system, but also glial cells and particularly oligodendrocytes, which express semaphorin receptors. In vitro experiments showed that class III semaphorins control oligodendrocytes outgrowth (Ricard et al., 2001) and are able to induce the collapse of their growth cones (Cohen et al., 2003). Oligodendrocytes migration is also controlled by class III semaphorin (Spassky et al., 2002) and a comparable function in cell migration has been described for Sema 3A and Sema 3F (Marín et al., 2001). Indeed, some of the semaphorins have been shown to induce cell death of dopaminergic and sensory neurons (Shirvan et al., 1999) as well as neural precursors (Bagnard et al., 2001). Hence, there is also increasing evidence for a potential role of semaphorin signaling in different pathologies of the nervous system. For example, Sema 3A is over-expressed in the cerebellum of schizophrenic patients (Eastwood et al., 2003). Sema 3A is also accumulated in the hippocampus during Alzheimer disease (Good et al., 2004).

In a rat model of temporal lobe epilepsy, Sema 3A is down-regulated thereby permitting mossy fibers sprouting and subsequent hyper excitability of the hippocampal formation (Holtmaat et al, 2003). Finally, the role of class III semaphorins in the context of nerve lesion has also been largely documented (Niclou et al., 2006).

From these observations, it appears that semaphorins have multiple roles ranging from axon guidance (attraction or repulsion) to cell migration or cell death. This functional diversity must be ensured by a complex signaling mechanism recruiting various receptors and co receptors coupled to specific intracellular pathways. Several studies have demonstrated that both plexins and neuropilins are essential components of the receptors for the secreted class III semaphorins (Chen et al., 1997, 1998; He and Tessier-Lavigne, 1997; Kitsukawa et al., 1997; Kolodkin et al., 1997; Giger et al., 1998).

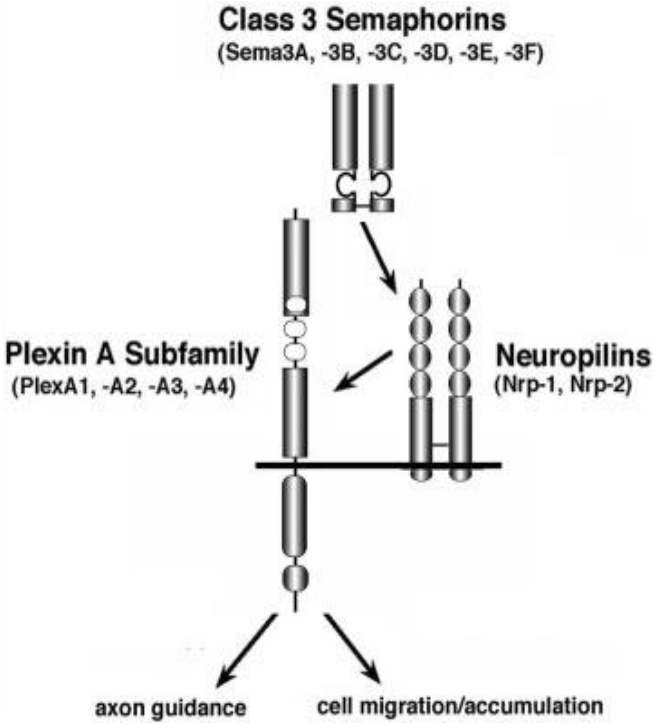


Fig. 7: Signaling of class III semaphorins. In neuronal cells, two neuropilins (Npn-1 and Npn-2) make receptor complexes with four members of plexin-A subfamily (plexin-A1, -A2, -A3, -A4) to propagate signals of secreted class 3 semaphorins (Sema-3A, -3B, -3C, -3D, -3E, -3F), and regulate directional guidance of axons and neuronal cell migration and accumulation (adapted from Fujisawa H, 2004).

1.1.5 Plexins

Plexins are single-membrane-spanning protein of approximately 240 kDa that possess a sema domain near the N-terminal part, followed by cysteine-rich motifs, Met-related sequences (MRS) and glycine-proline-rich repeats (Fig. 6). Plexins are considered to be the primary binding sites for semaphorins that do not bind to neuropilins. Hence, with the exception of Sema3E (Gu et al., 2005), class III semaphorins are unable to bind directly to plexins (Potiron et al., 2005). Rather, plexin-neuropilin complexes are required as high-affinity receptors for secreted semaphorins, neuropilin acting as the ligand binding subunit while the plexin subunit ensures signal transduction (Takahashi et al., 1999).

To date, nine plexins divided into four subfamilies (A-D) have been identified (Tamagone et al., 1999). The largest and best-characterized plexins are plexin-A subfamily members (Tamagone et al., 2000; Fiore et al., 2003; Mauti et al., 2006). In human and mouse, three members of the plexin-A subfamily (plexin-A1, -A2 and –A3) have been isolated (Maestrini et al., 1996; Tamagone et al., 1999). In chick, the *plexin-A* subfamily members are identified as plexin-A1, -A2 & -A4 (Mauti et al., 2006). The functions plexin-As have been studied predominantly as co-receptors with neuropilins for secreted class-III semaphorins (Fiore et al., 2003; Huber et al., 2003). Furthermore, plexin-As were shown to mediate effects of membrane-bound class-6 semaphorins in a neuropilin-independent manner (Toyofuku et al., 2004; Suto et al., 2005). Recently, it has been shown that knockdown of plexin-A2 causes motor neuron somata streaming out of the spinal cord along the ventral roots (Bron et al., 2007). Specific combinations of plexins, such as plexin-A4 and plexin-A3, are involved in the patterning of Sema 3A responsive sensory and sympathetic axons, whilst plexin-A3, but not plexin-A4, is essential for the guidance of Sema-3F responsive trochlear axons (Cheng et al., 2001; Suto et al., 2005; Yaron et al., 2005). Plexin-A3 and plexin-A4 convey semaphorin signals during facial nerve development (Schwarz et al., 2008). These findings suggest that they can propagate a wide variety of semaphorin signals into cells or neurons, dependent or independent of neuropilins.

1.1.6 Neuropilins

Neuropilins are cell surface glycoproteins of about 130 kDa (Fig. 6). Npn-1 was initially described by Fujisawa and colleagues as an orphan receptor expressed in the tadpole neuropil (Takagi et al., 1987; Fujisawa, 2002). During a search for other semaphorin receptors, a neuropilin-1-related molecule, Npn-2, was identified (Kolodkin et al., 1997; Chen et al., 1997). A model for the semaphorin/neuropilin complex has been proposed by several groups (He and Tessier-Lavigne, 1997; Feiner et al., 1997; Gu et al., 2002). Npn-1 is essential for the patterning of the facial nerve in the mouse, as it binds Sema 3A to guide facial branchiomotor axons in the second branchial arch and the vascular endothelial growth factor isoform VEGF164 to control the position of facial branchiomotor neuron cell bodies within the hindbrain (Kitsukawa et al., 1997; Schwarz et al., 2004; Taniguchi et al., 1997). Mouse embryos lacking Npn-1 show defasciculation of cranial nerves, including the trigeminal, glossopharyngeal and vagus nerves (Kitsukawa et al., 1997; Taniguchi et al., 1997). Loss of Npn-2 or sema 3F causes partial defasciculation of the facial branchiomotor and ophthalmic trigeminal nerves and severe defasciculation of the oculomotor nerve; in addition, the trochlear nerve fails to project to its target in these mutants (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). It has been shown that boundary cap (BC) cells confine vMN cell bodies to the vertebrate spinal cord through semaphorin-plexin mechanism (Bron et al., 2007; Mauti et al., 2007; Chauvet et al., 2007). The role of Npn-1 and Npn-2 for semaphorin signaling suggests that the semaphorin/neuropilin interaction is the initial step for the assembly of a receptor complex recruiting transducing elements.

1.1.7 Summary of the Instructive Guidance Molecules

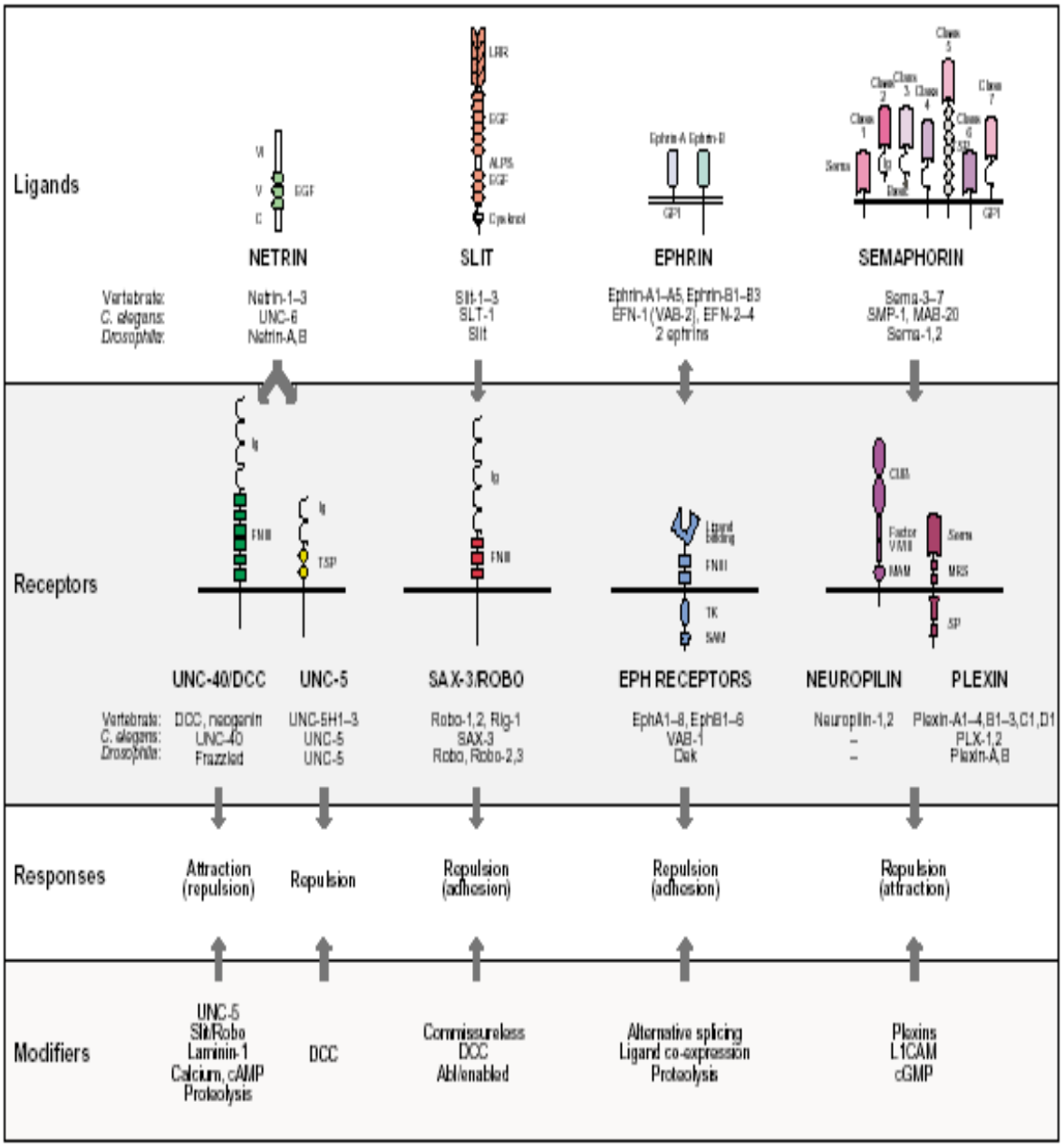


Fig. 8: Schematic representation of the four families of axon guidance cues and their receptors (adapted from Adams and Eichmann, 2010).

1.2 Axon Guidance Molecules in the Pathfinding of Motor Neurons

The motor neurons within the CNS represent one of the better understood model systems for exploring the molecular mechanisms that specify axonal pathfinding. Motor neuron subtypes arise from a common source of ventricular zone progenitors that migrate into the ventral neural tube where they are initially intermingled. Later, motor neurons with similar muscle targets and sensory afferent inputs cluster together into columns and sort out into distinct pools (Dilon et al., 2005). Expression of guidance molecules by the motor neurons and/or their exit points regulates both the distinct settling positions of motor neuron soma within the ventral spinal cord and the pathfinding of their axons in the periphery. For instance, in mouse, Slits and Netrin-1 guide cranial motor axons along a dorsally-directed trajectory away from the ventral midline and toward their dorsal exit points (Hammond at al., 2005; Burgess et al., 2006). In chick, Ephrin-A and Eph-A4 interactions regulate axon guidance along the dorsoventral axis of limbs but appear to have no influence on motor neuron settling positions (Kania et al., 2003). In zebrafish neuropilin (Npn)-1a mutants, motor axons have abnormal branching and exit the spinal cord at inappropriate levels whilst at the same time the somata of some motor neurons migrate to ectopic positions (Feldner et al., 2005). In chick and mouse, Sema- 6A and Npn-2 are required for the confinement of spinal motor somata within the CNS (Bron et al., 2007). In chick, plexin-A1 and plexin–A2 prevent spinal motor somata from inappropriately migrating out of the CNS (Bron et al., 2007; Mauti et al., 2007). Sema-3ab and plexin-A3 are required for proper positioning of spinal motor exit points in zebrafish (Palaisa et al., 2007; Sato-Maeda et al., 2008). These evidences suggest that axon guidance cues are involved in the migration, guidance, and fasciculation of cranial and spinal motor neurons. However, it is still unknown whether and how plexin-A2 and Npn-2 interact with the hindbrain motor neurons and regulate their pathfinding.

1.3 Hindbrain Motor Neurons and their Axonal Trajectories

In the hindbrain, distinct transverse domains or rhombomeres are separated by discrete boundaries along the rostro-caudal axis which have different morphogenic potentials during brain development (Lumsden, 1990; Rubenstein et al., 1994; Shimamura et al., 1997; Martinez and Puelles, 2000; Garcia-Lopez et al., 2004). In addition, the rhombomeres are further divided into longitudinal subdivisions called alar, basal, roof and floor plates (Rubenstein et al., 1994; Shimamura et al., 1997). The cranial motor neurons differentiate ventrally, on either side of the floor plate and settle down to their final positions into a series of nuclei. They extend their axons towards the exit points (dorsal and ventral), cross the basal lamina and finally come out of the CNS (Ericson et al., 1997; Osumi et al., 1997; Sharma et al., 1998; Briscoe et al., 1999; Pattyn et al., 2000; Hirsch et al., 2006). Though all motor neurons in the hindbrain arise commonly from the basal plate of neural tube, they differentiate into two subtypes (dMNs and vMNs) (Fig. 9; reviewed by Bravo-Ambrosio and Kaprielian, 2011). Motor neuron subtypes can be distinguished by the path that their axons take to exit the neural tube (Jacob et al., 2001; Shirasaki et al., 2002; Schneider et al., 2003). Somata of dMNs (bm/vm) migrate dorsally into the alar plate to reach the vicinity of their nerve exit point and they extend their axons through the dorsal root and vMNs (sm) follow the ventral trajectory (Verela-Echavarria et al., 1996).

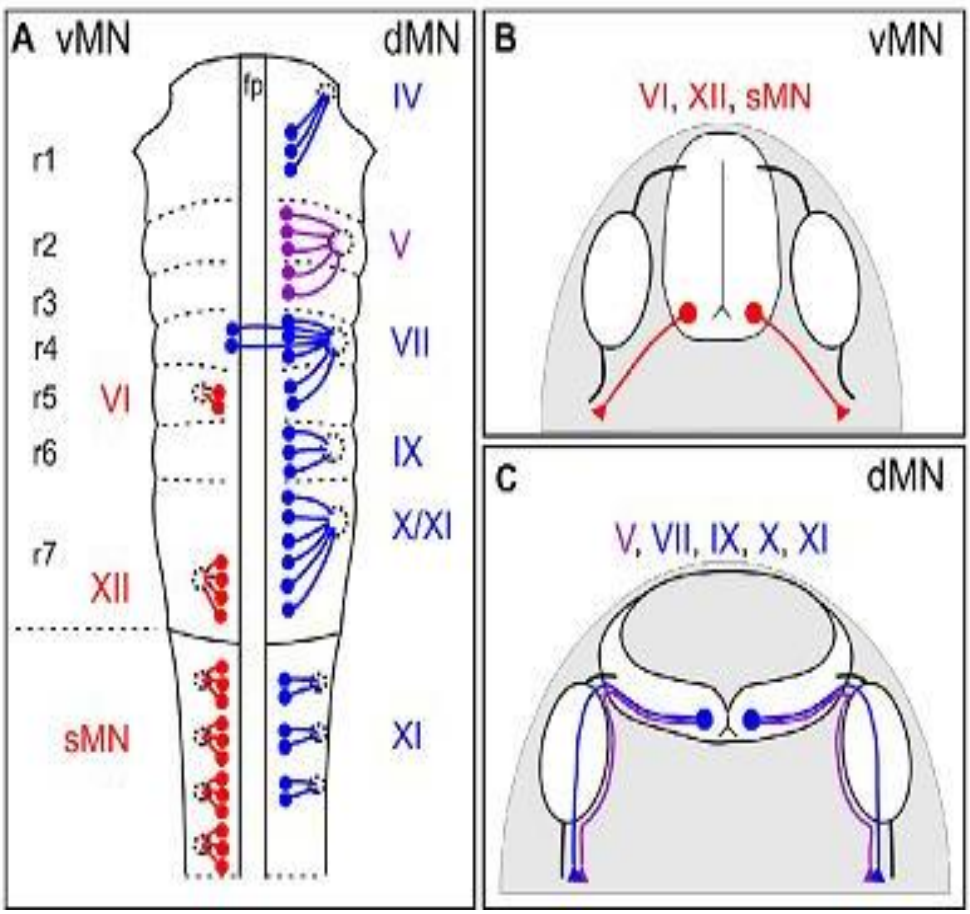


Fig. 9: Motor neuron subtypes and the projections of their axons in the hindbrain. (**A**) Schematic of motor neuron nuclei in the developing brainstem (rhombomere, r1 to r7). vMNs are indicated in red on the left, whereas dMNs are indicated in blue on the right of the schematic. Trigeminal (V) motor nuclei are shown in purple. Each cranial motor nucleus is numbered in Roman numerals, e.g., CN XI. Abbreviations: fp, floor plate; sMN, spinal motor neuron; (**B**) Axonal projections of vMNs in the hindbrain (VI, XII) and spinal cord (sMN) are shown in red; (**C**) Axonal projections of dMNs (VII, IX, X, XI) and trigeminal (V) dMNs are shown in blue and purple, respectively. Axons extending from trigeminal dMNs avoid sensory ganglia (white ovals), while axons of other dMN invade these ganglia (adapted from Bravo-Ambrosio and Kaprielian, 2011).

Once in the periphery, their axons follow a well-defined trajectory to navigate to their final target tissues and hence, are classified as branchiomotor (bm), visceromotor (vm) and somatomotor (sm) neurons (Fig. 10). While bm/vm (dMNs) neurons exit close to the incoming sensory fibers and typically invade nearby cranial sensory ganglia, sm (vMNs) neurons avoid sensory ganglia (Jacob et al., 2001; Moody and Heaton, 1983).

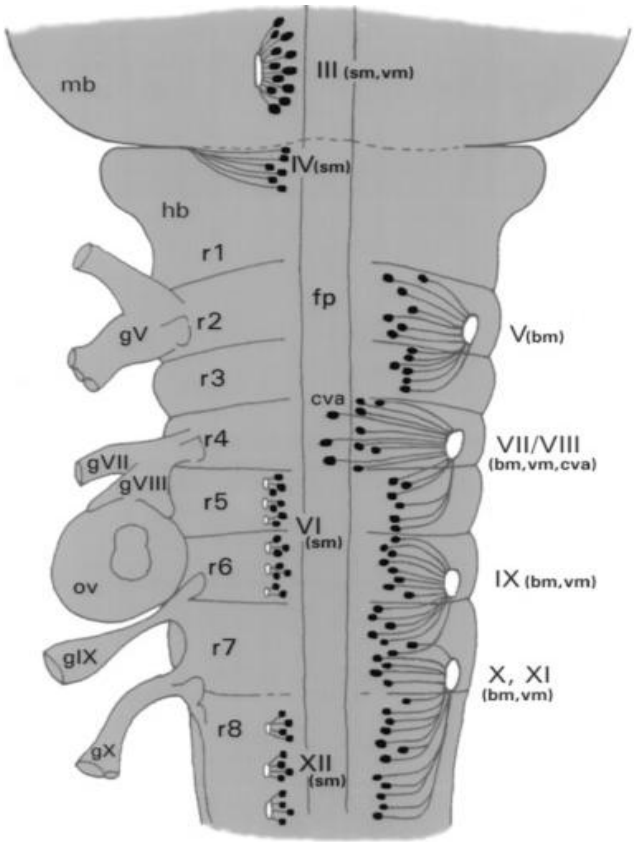


Fig. 10: Diagram of a flat-mounted hindbrain of chick embryo (pial side). The diagram shows the rhombomeres (r1–r8), the cranial motor nuclei, and the sensory ganglia. The motor nuclei are III (oculomotor), IV (trochlear), V (trigeminal), VI (abducens), VII (facial), IX (glossopharyngeal), X (vagus), XI (accessory), and XII (hypoglossal). Sensory ganglia are gV trigeminal, gVII geniculate, gVIII vestibuloacoustic, gIX glossopharyngeal, and gX vagus. The motor neuron classes are sm, somatic; bm, branchiomeric; vm, visceral; and cva, contralateral vestibuloacoustic efferents. mb, midbrain; hb, hindbrain; fp, floor plate; ov, otic vesicle. The nerve exit points are shown as white ellipses (adapted from Lumsden and Keynes, 1989).

1.3 Aim of the project

The central aim of this thesis was to understand how semaphorin pathways play role in the axonal guidance of cranial nerves. Although much information has been gathered with regards to semaphorins involved in the axonal guidance of cranial and spinal nerves, little is known how their receptors behave during patterning mechanisms that produce a diversity of motor neuron subpopulations in the hindbrain and determine their pathfindings. The chick *plexin-As* (*plexin-A1, -A2* and *–A4*) and *neuropilins* (*Npn-1* and *Npn-2*) have been reported to be expressed in the spinal motor neurons (Mauti et al., 2006; Bron et al., 2007) and contributed in the confinement of motor neuron somata within the CNS (Bron et al., 2007). However, their functional role in the hindbrain especially in the posterior hindbrain is mostly unknown. In this context, our major focus was to study the functions of *plexin-A2* and *Npn-2* in the axonal guidance cranial nerves at the post-otic hindbrain level (r7-8) of chick embryos from which originate two distinct motor neuron populations, d-MNs (vagus and accessory) and v-MNs (hypoglossal) in chick embryos. To gain insight into it, we first addressed the dynamic expression patterns of *plexin-A1*, *plexin-A2*, *plexin–A4* and *Npn-1* and *Npn-2* in the developing hindbrain of chick embryos. Finally, we knocked down the function of *plexin-A2* and *Npn-2* at the desired level of chick embryos by *in-ovo* electroporation of *shRNA* constructs.

2. Materials and Methods

2.1 Materials

2.1.1 List of Laboratory Equipments

Name	Supplier
Cover slips (24 X 60 mm)	Labomedic
Culture dishes (94 X 16 mm)	Greiner Bio-one
Disposable syringes (2 ml)	BD Biosciences
Egg Incubator	Grumbach
EP21 Current Amplifier	Intracel
Fiber Illuminator	Nikon
Fluorescence Microscope (SM21500)	Nikon
Forceps (microsurgical)	Fine Science Tools
Glass Pasteur pipettes (150 mm)	Brand
Light Microscope (SMZ1000)	Nikon
Nitrocellulose membranes	Roche
Ovodyne TSS20 Electroporator	Intracel
pH-Meter	Hanna Instruments
PCR strip tubes (0.2 ml)	Fermantas
Round bottom tubes (12 x75 mm)	Greiner Bio-one
Scissors (microsurgical)	Fine Science Tools
Serological pipettes (5 ml, 10 ml, 25 ml)	Falcon
Superfrost plus glass slides (25 X 75 X 1 mm)	Thermo Scientific
Tubes (0.5 ml, 1.5 ml, 2 ml, 15 ml, 50 ml)	Nerbeplus

2.1.2 Preparation of Micro-manipulating Tools

Tungsten needle

Tungsten needle is the main tool for dissecting the embryo during isolation of hindbrain from the surrounding mesenchyme. To make the tungsten needle, 20 mm tungsten wire was inserted into the anterior opening of a glass Pasteur pipette whose thin nozzle had been removed with a diamond knife. The glass around the wire was heated over the flame of a Bunsen burner and removed off when it began to melt. It was then pressed with the forceps and the tungsten wire was fixed in the anterior end of the Pasteur pipette. The tip of the tungsten wire was sharpened by electrolysis in saturated $NaNO_3$ solution.

Dye tip

Since staining of the transluscent embryonic tissue helps to improve its visibility, dye tip is one of the necessary prelimary to microsurgery. At the middle of the narrow end of Pasteur pipette was melted over the flame of a Bunsen burner until it began to melt. Then the anterior end of the pipette was removed from the flame and quickly pulled off to form a long thread. The sharp end of glass thread again heated over the flame shortly until it forms a small bulb (0.5-1 mm). Dye solution with 2.5% agarose and 1% nile blue sulfate was placed on a heating block and stirred repeatedly to keep in the liquid state. The small bulb of the glass pipette was dipped into the dye solution several times to coat with the blue dye.

Glass capillary

The glass Pasteur pipettes were used to prepare glass capillaries. The narrow end of the glass pipette was flame heated at the middle over the Bunsen burner until it starts to melt. Immediately, the tip was pulled off with the forceps to form a very fine capillary. Then the bilnd end at the tip of the capillary was opened with the fine forceps.

Agar dish

Agar dish provide support for the embryo during micromanipulation. 2% agarose in distilled water was heated on a heating block and kept stirring with a magnetic fish until the agarose was completely dissolved and the solution became sticky. Pteri dishes were half-filled with the hot solution, allowed to be cooled down at room temperature and stored at 4° C.

2.1.3 List of Chemicals, Reagents and Supplements

Name	Supplier
Agarose	Sigma
Anti-Digoxigenin-AP, Fab fragments	Roche
Blocking reagent	Roche
$CaCl_2.2H_20$ (Calcium chloride dehydrate)	Sigma
DABCO (1,4-diazabicyclo 2.2.2 octane)	Carl Roth
DEPC (Diethylpyrocarbonate)	Sigma
DNA ladder (100 bp, 1 kd)	Fermantas
DMSO (Dimethylsulfoxide)	Merck
EDTA (Ethylenediaminetetraacetic acid)	Fluka
EGFP (Enhanced green fluorescent protein)	R&D systems
Ethanol	Merck
Ethidium bromide	Carl Roth
Fast Green FCF	Sigma
FCS (Fetal calf serum)	Invitrogen
Formamide	Sigma
Gluteraldehyde solution (25%)	Sigma
Glycerol	Sigma
Sucrose	Merck
HCl (Hydrochloric acid)	Merck
Heparin	Sigma
H_2O_2 (Hydrogen peroxide)	Merck
India ink	Pelikan
KCl (Potassium chloride)	Merck
KH_2PO_4 (Potassium dihydrogen phosphate)	Merck
LB Agar	Sigma
Maleic acid ($C_4H_4O_{4)}$	Merck
Methanol	Applichem
$MgCl_2$ (Magnesium chloride)	Merck
Mowiol	Merck
NaCl (Sodium chloride)	Merck

Na_2HPO_4 (Disodium hydrogen phosphate)	Merck
NaOH (Sodium hydroxide)	Merck
$NaHCO_3$ (Sodium bicarbonate)	Merck
Nile blue sulfate	Merck
Paraformaldehyde	Merck
Penicillin-G sodium salt	Sigma
Proteinase-K	Roche
Sodium azide	Merck
Sodium citrate	Merck
Tissue Tek (O.C.T.)	Sakura Fintek
Tris	Carl Roth
Tris-HCl (Tris hydrochloride)	Sigma
Triton-X100	Sigma
tRNA	Roche
Tryptone	Fluka
Tween-20	Dako
Yeast extract	Sigma

2.1.4 Buffers, Solutions and Media

Alakaline phosphatase (AP) buffer

5 ml of Tris 1M pH 9.5, 2.5 ml of 1M $MgCl_2$, 1 ml of 5M NaCl, and 2 ml of 25% Triton X-100 with the final volume adjusted to 50 ml with distilled water. For optimal results, this buffer was prepared immediately prior to use.

AP staining solution

NBT/BCIP was mixed well in AP buffer (20 µl/ml) to make staining solution.

1% blocking solution

1 g Blocking reagent in100 ml Maleic acid buffer (pH 7.5).

10% CHAPS

10g CHAPS powder was dissolved in 100 ml of DEPC-treated water. Solution was stored at 4°C.

Dent's bleach

5 ml H_2O_2 and 10 ml DMSO were added to a final volume of 50 ml with methanol and mixed well. The solution was always prepared fresh.

Dent's fixative

100 ml DMSO was added to 400 ml of methanol.

DEPC-treated water

0.5 ml DEPC was added to 500 ml distilled water and allowed to sit for a minimum of 2 hour before sterilization by autoclaving.

Howard Ringer's solution

7.2 g NaCl, 0.17 g $CaCl_2.2H_2O$ and 0.37 g KCl were dissolved to prepare 1000 ml of solution and sterilized by autoclaving.

Hybridization buffer

250 ml deionized formamide, 125 ml 20X SSC, 2.5 g CHAPS, 0.5ml Triton X-100, 5 ml 0.5M EDTA, 25 mg heparin powder, 500 mg tRNA, 10 g blocking reagent were added and made up to 500 ml with DEPC-treated water. Solution was heated at 65ºC to dissolve all ingredients. Hybridization buffer was stored at –20ºC in 50 ml tubes.

KTBT buffer

8.8 g NaCl, 1.5 g KCl, 10 ml Tween-20 and 25 ml 1M Tris pH 7.3 were dissolved in 965 ml of distilled water and the solution was sterilized by autoclaving.

LB medium

5 g Yeast extract, 10 g Tryptone and 10 g sodium chloride were added to prepare 1 liter of LB medium. The pH was adjusted to 7.0 by adding NaOH and the medium was sterilized by autoclaving.

Magnesium chloride (1 M $MgCl_2$*)*

101.5 g $MgCl_2$ was made up in a final volume of 500 ml of distilled water. The solution was sterilized by autoclaving.

Maleic acid buffer (pH 7.5)

100 mM Maleic acid, 150 mM NaCl, and 195 mM NaOH were mixed well and the pH of the solution was adjusted to 7.5.

Mounting medium (Mowiol)
6 ml H2O, 4.8 ml Glycerol and 2.4 g Mowiol were added and stirred overnight. 12 ml of 0.2M Tris (pH 8.5) was added and incubated at 50°C until dissolved followed by addition of 45 mg DABCO. The solution was cleared by centrifugation at 5000 rpm for 15 min.

4% paraformaldehyde in PBS
20 g of paraformaldehyde powder was dissolved in a final volume of 500 ml of DEPC-treated PBS, heated to 65ºC and shaken periodically to dissolve. Solution was stored in 50 ml tubes at -20°C.

Phosphate buffered saline (PBS)
8 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4 and 0.1 g KH_2PO_4 were dissolved in 1000 ml of distilled water and the pH was adjusted to 7.4. The solution was sterilized by autoclaving.

PBT
0.1% Tween-20 was added to PBS and stored at room temperature.

Proteinase-K
100 mg of Proteinase-K powder was dissolved in DEPC-treated water (20 μg/μl), vortexed and aliquoted into 50 μl. The aliquots were stored at -20°C and thawed on ice immediately prior to use.

Standard saline citrate buffer (SSC, 20X)
175 g Sodium chloride and 88 g Sodium citrate was made up to 1000 ml with distilled water. The pH was adjusted to 7.0 and the solution autoclaved and stored at room temperature.

Sucrose/PBS Solutions
2.5 g, 7.5 g and 15 g sucrose were dissolved in PBS to make 50 ml of 5%, 15% and 30% sucrose solutions, respectively. The solutions were made fresh every usage.

TAE buffer (50X)
2 M Tris, 50 mM EDTA (pH 8.0), and 1 M glacial acetic acid were mixed well and stored at room temperature.

Tris buffer (pH 9.5 and 7.3)

121.12 g Tris base was dissolved in 800 ml of distilled water. The pH was titrated to 9.5 or 7.3 with concentrated HCl. Distilled water was added to a final volume of 1 liter. The solution was autoclaved and stored at room temperature.

2.1.5 Plasmids and Constructs

The plasmid DNAs were used for the generation of *in situ* hybridization probes and constructs. The plasmids used for the probes were based on *Bluescript II SK+* (Stratagene), *pCR2 and pGEMTeasy* vectors. The constructs used for *in-ovo* electroporation were based on *pCAβ-shRNA-EGFP* vector.

Tab. 1: List of Plasmids and Constructs

Name of the gene/construct	**Vector backbone**	**Source**
Chick *plexin-A1*	*Bluescript II SK+*	Esther T Stoeckli, Switzerland
Chick *plexin-A2*	*Bluescript II SK+*	Esther T Stoeckli, Switzerland
Chick *plexin-A4*	*Bluescript II SK+*	Esther T Stoeckli, Switzerland
Chick *Npn-1*	*pGEMTeasy*	Jonathan A Raper, USA
Chick *Npn-2*	*pCR2*	Jonathan A Raper, USA
Chick *plexin-A2 shRNA*	*pCAβ-shRNA-EGFP*	Matthieu Vermeren, UK
Chick *Npn-2 shRNA*	*pCAβ-shRNA-EGFP*	Matthieu Vermeren, UK

2.1.6 Antibodies

Tab. 2: List of Primary and Secondary Antibodies

Name	Antigen	Species	Source	Dilution
3A10	Neurofilament-associated antigen	Mouse monoclonal	DSHB	1:100
39.4D5	Islet-1/2	Mouse monoclonal	DSHB	1:200
Anti-GFP	GFP	Rabbit polyclonal	Invitrogen	1:200
Gam-Cy2	Mouse IgGs	Goat polyclonal	Dianova	1:200
Gam-Cy3	Mouse IgGs	Goat polyclonal	Dianova	1:200
Gar-Cy2	Rabbit IgGs	Goat polyclonal	Dianova	1:200
Gar-Cy3	Rabbit IgGs	Goat polyclonal	Dianova	1:200

2.2 Methods

2.2.1 Collection and Processing of Embryos

Fertile White Leghorn chicken (*Gallus gallus domesticus*) eggs were obtained from the farm maintained by the Institute of Animal Science, University of Bonn, Germany. The eggs were incubated in a humidified (80%) atmosphere at 37.8°C for the desired length of time and staged according to Hamburger and Hamilton (HH), 1951. After the required period of incubation, the eggs were windowed; embryos were collected and prepared for *in situ* hybridisation or immunohistochemistry. The embryos were separated from the attached membranes using fine surgical scissors and transferred into Petri-dishes containing PBS or PBT. Fixing agents were chosen according to the subsequent procedures adopted. Embryos for whole-mount *in situ* hybridization were fixed in 4% paraformaldehyde made in PBS. For whole-mount immunoflourescence, the alcohol based Dent's fixative (DMSO/Methanol=1:4) was used as it generated less background compared to PFA fixed tissue. Embryos for cryosectioning were fixed in 4% PFA in PBS. The hindbrains of the embryos were opened dorsally along the roof plates and dissected free from the rest of the brain and the surrounding mesenchyme.

2.2.2 *In ovo*-Electroporation

To know the *in vivo* role of *plexin-A2* and *Npn-2*, we applied knockdown strategy by *in ovo*-eletroporation of plasmid construct against *plexin-A2* and *Npn-2* genes. The *pCAβ-shRNA-EGFP* vector, co-expressing *shRNA* and EGFP and the vector based constructs were used (Fig. 11a; Bron et al., 2004). Eggs were rinsed with 70% alcohol and 3 ml. of albumin was removed prior to cutting a window through the shell. A solution of 10% India ink (Pelikan Fount; PLK 51822A143) in Howard Ringer's solution was injected below the blastoderm to visualize the embryos. Plasmid DNA, resuspended in TE buffer at 1 mg/ml with 0.2% Fast Green FCF, was injected into the neural tube of HH stage 10–12 embryos by using glass capillaries. The embryos were electroporated ventrally of the neural tube at the level of r7-8. For this purpose, positive electrode was placed ventral to the embryo by inserting the vitelline membrane, while the negative electrode was placed dorsal to the embryo (3-4 mm apart). The electrodes were placed parallel to each other leaving the embryo in the middle (Fig. 11b). Subsequently, 3–5 square pulses, 200 ms and 12-15 V were applied by Intracel TSS20 Ovodyne Electroporator. The

unelctroporated contralateral side and the electroporation of *EGFP* vector alone were treated as controls. The effects of electroporation were assessed after 48–54 hours of reincubation (HH 22–24) by *in situ* hybridization and immnunohistochemistry. For each construct, at least five embryos (n=5) were analyzed.

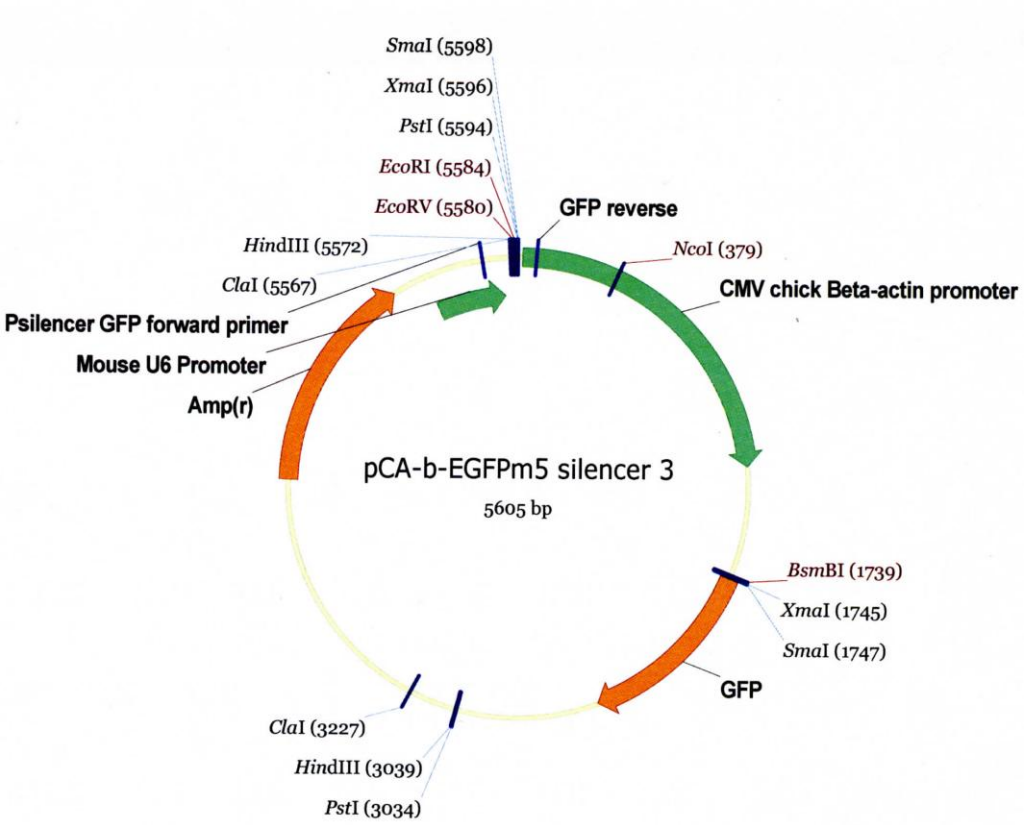
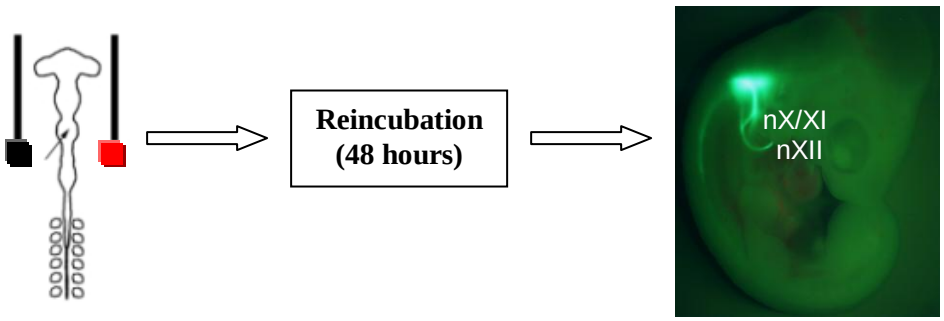


Fig. 11a: *pCAβ-shRNA-EGFP* vector backbone for the *shRNA* constructs.



(HH 10-12)

Fig. 11b: *In-ovo* electroporation of chick embryos **(**nX/nXI = vago-accessory nerve, nXII = hypoglossal nerve).

2.2.3 Immunohistochemistry

2.2.3.1 Whole-mount Fluorescence Immunohistochemistry

Prior to incubating with antibody, embryos destined for whole-mount fluorescence immunostaining were fixed in Dent's fixative for at least overnight. Embryos were then bleached in Dent's bleach for 3-5 hours (depending on the stage, HH 22– 24), rinsed for 10 min in PBS and then incubated with the primary antibody for two days. Thereafter, the embryos were washed thoroughly in PBS for 1 day (minimum of 6 change in PBS), before incubating with the secondary antibody (2 μg/ml) overnight. Embryos were washed in PBS for 1 day and examined under the Nikon SM21500 fluorescence microscope.

2.2.3.2 Fluorescence Immunohistochemistry on Cryosections

PFA fixed embryos determined for cryosectioning, were incubated in gradient solutions of sucrose in PBS (5%, 15%, and 30%). The embryos were kept in each solution until they had sunk to the bottom of the container. They were then placed in Tissue Tec and allowed them to be hardened by using liquid nitrogen. Finally, they were sectioned at 20 μm using the Bright cryostat and stored at -20°C. For immunohistochemical detection, the sections were washed 10 min in PBS followed by pre-blocking with 10% FCS/PBS for 30 min. Then they were incubated with primary antibody (2 hrs to overnight) followed by incubation with appropriate Cy2 or CY3 conjugated 2ndary antibody (1-2 hrs). Finally, the sections were examined under Nikon SM21500 fluorescence microscope.

2.2.4 Whole-mount *in situ* Hybridization

For *in situ* hybridization, all solutions were prepared with RNase free DEPC water, with autoclaved instruments and in autoclaved bottles. All steps before hybridization with the RNA probe were performed on ice or at 4ºC in the fridge if not indicated otherwise. Embryos determined for *in situ* hybridization were fixed in 4% PFA in PBT for at least overnight. Then the embryos were washed in PBT for 10 min followed by dehydration in ascending grades of methanol in PBT (25%, 50%, 75%, and 100%). Finally, the dehydrated embryos were preserved in 100% methanol at -20º C. The digoxigenin labeled antisense RNA probes for the *plexin-A1*, *plexin-A2*, *plexin-A4* and *Npn-1* & *Npn-2* genes were synthesized from the template plasmids. *In situ* hybridization was performed as described (Wilkinson, 1992) using

digoxigenin-labeled antisense *plexin-A1, plexin-A2, plexin-A4* and *Npn-1, Npn-2* riboprobes on whole-mounts and flat-mounted hindbrains of chick embryos.

2.2.4.1 Preparation of Template DNA for *in situ* Hybridization

The NucleoBond® Xtra Maxi DNA preparation kit was used to isolate DNA. A single colony from a streaked plate was used to inoculate 250 ml of LB medium with appropriate antibiotics. The culture was grown over night at 37°C with vigorous shaking. It was centrifuged for 10 min at 5000 x g. The pellet was re-suspended in 12 ml of buffer RES by pipetting up and down repeatedly. 12 ml of buffer LYS was added, mixed gently by inverting the tube (5 times) and the mixture was left at room temperature for 5 min. A NucleoBond® Xtra column was equilibrated by running through 25 ml of buffer EQU. Thereafter, 12 ml of buffer NEU was added and mixed by gently inverting the tube (10-15 times). The homogenous lysate was loaded directly to the equilibrated column and allowed the column to empty by gravity flow. The column and filter were washed by 15 ml of buffer EQU. After washing, the column filter was pulled out and discarded. Bound DNA in the column was washed by applying 25 ml of buffer WASH. The plasmid DNA was eluted by adding 15 ml of buffer ELU and collected in a 50 ml tube. The DNA was precipitated by adding 10.5 ml of isopropanol to the tube, vortexed well and allowed to sit for 2 min at room temperature. The precipitate mixture was loaded in 30 ml syringe and passed through the NucleoBond® Finalizer by pressing the plunger of syringe slowly. The flow was discarded and the finalizer was washed by 5 ml of 70% ethanol slowly. Then the air was passed strongly through the finalizer (6 times) to dry the filter membrane. The finalizer was removed and attached with the 1 ml syringe. The DNA was eluted by passing 800 µl of Buffer TRIS through the finalizer. The plasmid yield was determined by UV spectroscopy and the plasmid integrity was confirmed by agarose gel electrophoresis.

2.2.4.2 Linearization of Plasmid and Template Purification

20 µg of DNA was digested with 50 units of appropriate restriction enzyme in a volume of 100 µl at correct temperature (specific to enzyme) for 12 hours. The DNA was purified firstly by extracting with phenol/chloroform (1:1) to remove proteins. The top aqueous layer was then isolated and extracted with 100 µl of chloroform to remove the phenol carry-over. The top aqueous layer was then isolated. 10 ul of 3M sodium acetate (pH 5.2) was added, followed by 220 µl ethanol. The sample was mixed and placed on dry ice for 10 minutes before

centrifuging at 12,000 x g for 15 minutes at 4°C. The resulting pellet was washed in 100µl cold 70% ethanol which was again centrifuged at 12,000 x g for 10 minutes. The pellet was air dried and re-suspended in 20 µl water. The DNA concentration was determined using a spectrophotometer.

2.2.4.3 Antisense Digoxigenin-labelled RNA Probe Synthesis

1 µg/µl of linear DNA was suspended by RNAse free water. The followings were added to 20 µl of solution: 2 µl of 10X transcription buffer, 2 µl DIG-RNA labeling mix, 2 µl RNA polymerase, and 0.5 µl RNAse-Inhibitor. Sample was mixed and incubated at either 37°C (T3 or T7) or 42°C (Sp6) for at least 2 hours. The reaction was stopped by adding 0.5 µl of 0.5M EDTA (pH 8.0). Thereafter, 2.4 µl of 4 M LiCl and 75 µl of 100% cold ethanol were added and incubated overnight at -20 °C. The solution was centrifuged at 130000 rpm for 20 min. The fluid was removed carefully from the pellet and added 500 µl of 70% cold ethanol. Then the solution was centrifuged again at 13000 rpm for 10 min. The ethanol was removed from the pellet and dried at 37°C (3-5 min). 50 µl of RNAse free water and 0.5 µl of RNAse-Inhibitor were added to the pellet at 37°C for 20 min. The probe concentration was measured by Dot-blot technique. A concentration of 50 ng/µl of probe was used for *in situ* hybridization.

Tab. 3: Digoxigenin-labeled Anti-sense Probe Synthesis

Name of the gene	Size of insert	Linearization enzyme	RNA transcriptase
Chick *plexin-A1*	736 bp	Not I	T3
Chick *plexin-A2*	1037 bp	Not I	T3
Chick *plexin-A4*	863 bp	Not I	T3
Chick *Npn-1*	1031 bp	Sac II	Sp6
Chick *Npn-2*	1508 bp	Hind III	T7

Tab. 4: *In-situ* Hybridization Protocol

Process	Reagents	Time	Temperature
Wash	PBT	20 min	4°C
Dehydration	50% then 100% Methanol	20 min each	4°C
Rehydration	50% Methanol then PBT	20 min each	4°C
Enzymatic digestion	Proteinase- K (20 µg/µl)	1 min/ per stage	RT
Wash	PBT	20 min	4°C
Fix	Glutaldehyde in 4% PFA/PBS	20 min	4°C
Wash	PBT	20 min	4°C
Pre-hybridization	Hybridization buffer	Overnight	65°C
Hybridization	Probe in hybridization buffer	48 h	65°C
Post-hybridization wash	2 washes in 2 X SSC/CHAPS 2 washes in 0.2 X SSC/CHAPS	20 min each	65°C
Digoxygenin detection	KTBT buffer	20 min	RT
Antibody blocking	10% FCS in KTBT buffer	5 h	RT
Antibody	Anti-DIG-AP in KTBT buffer (1: 2000)	12 h	4°C
Antibody wash	6 washes in KTBT	1 h each	RT
Coloring preparation	2 washes in AP buffer	20 min each	RT
Color development	NBT / BCIP in AP buffer (20 µl/ml)	30 min-hrs	RT

Cease color development	2 washes in AP buffer	20 min each	RT
Preserve samples	4% PFA in PSB	30 min	4°C

2.2.5 Photographic Documentation and Data Analysis

Samples were photographed by using Nikon digital camera DXM1200C connected to a Nikon SM21500 fluorescence microscope. In all cases, images were assembled and annotated using Adobe Photoshop CS3. The data were analyzed by Student's *t*-test (two-tailed).

3. Results

To understand the function of semaphorins, a proper assessment of the expression of receptors in a given species or system is prerequisite. The chick embryo is an attractive model system to study the role of semaphorins and their receptors, given its accessibility and the potential for functional analysis using knockdown strategies. However, data on the expression of *plexin-As* and *neuropilins* in the developing chick hindbrain are not available. We analyzed the expression of *plexin-As (plexin-A1, -A2,* and *-A4)* and *neuropilins (Npn-1* and *Npn-2)* in the brainstem at various developmental stages of chick embryos (HH 19, HH 23 and HH 27). We next focused on the expression of *plexin-A2* and *Npn-2* by the cranial motor neurons at the post-otic hindbrain (r7-8) level. Finally, we knocked down the function of *plexin-A2* and *Npn-2* genes at this level by *in ovo*-electroporation.

3.1 *Plexin-As* are expressed in the developing hindbrain of chick embryos

The chick *plexin-As* (*plexin-A1, -A2, -A4*) have been reported to be expressed in the spinal cord (Mauti et al., 2006; Bron et al., 2007). Here, we asked whether they are also expressed in the hindbrain. For this purpose, we analyzed the expressions of chick *plexin-As* in the flat-mounted hindbrains of developing chick embryos (Fig. 12). At stage HH 19, *plexin-A1* was expressed longitudinally throughout the ventral region of neural tube. However, the expression was more prominent at the level of r1 and r7-8. The expression patterns of *plexin-A2* and *plexin-A4* were found almost similar in the hindbrain. Both expressed into two longitudinal domains medially which were expanded gradually lateral from r2-8. *Plexin-A2* expression was much stronger at the level of r7-8 but r6-8 in case of *plexin-A4*, both of which became narrower close to the floor plate. At HH 23, *plexin-A1, -A2* and *-A4* expressions were expanded towards the prospective nuclei within the rhombomeres of the hindbrain. At HH 27, we compared the expression patterns of these genes with the expression of motor neuron marker *Islet-1* (Verela-Echavarria et al., 1996). Our results showed that *plexin-A1, -A2* and *-A4* expressed in the trochlear (IV), trigeminal (V), facial (VII) and hypoglossal (XII); trochlear (IV), trigeminal (V), abducens (VI), glossopharyngeal (IX), vago-accessory (X/XI) and hypoglossal (XII); trigeminal (V), facial (VII), glossopharyngeal (IX) and vago-accessory (X/XI) nuclei; respectively (Fig. 12).

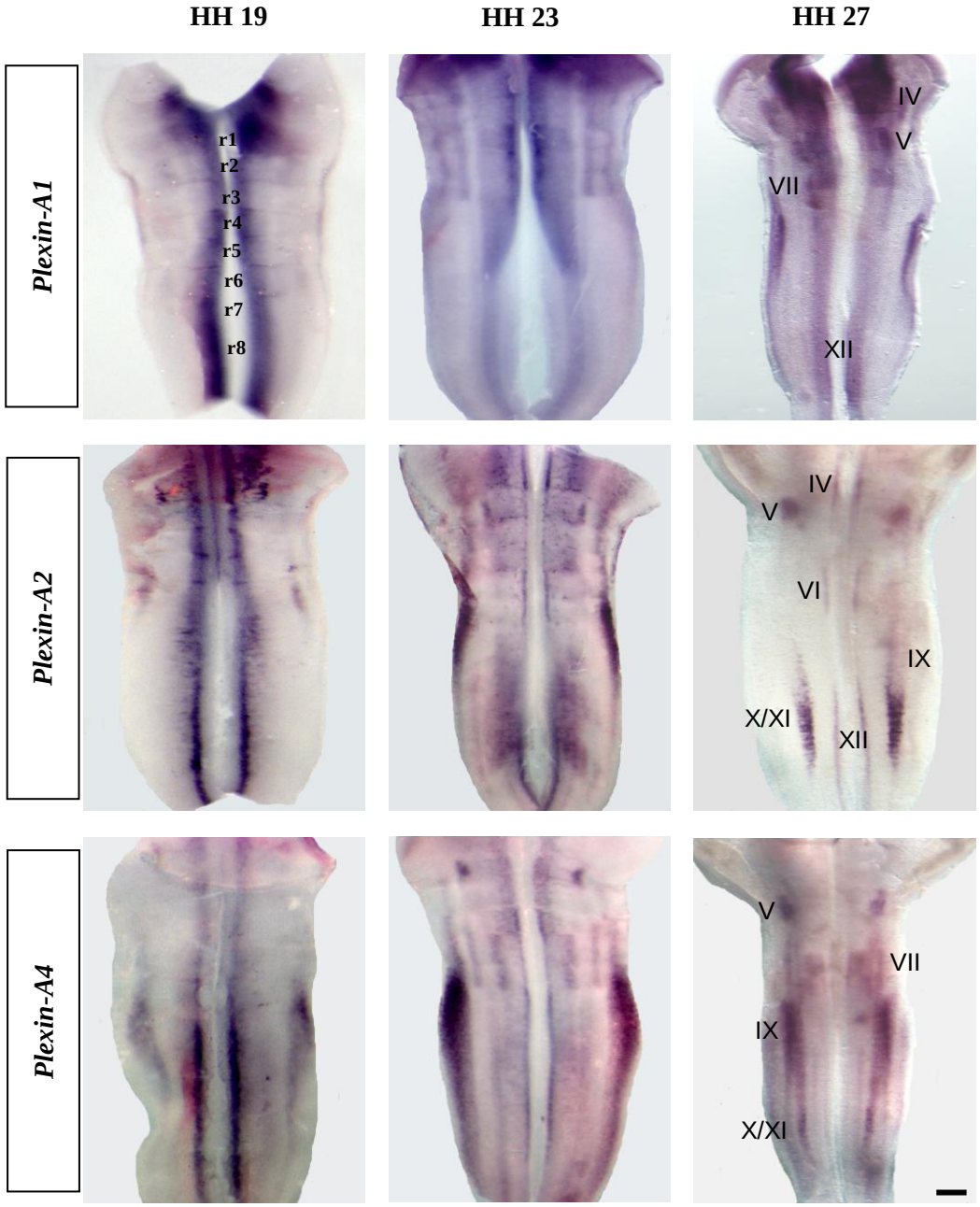


Fig. 12: Expression of *plexin-As* (*-A1*, *-A2* and *–A4*) in the flat-mounted hindbrains (r1-8) of chick embryos (HH 19, HH 23, HH 27). At HH 19, *plexin-A1*, *-A2* and *-A4* expression appears as two longitudinal stripes throughout the ventral neural tube which is relatively stronger in the posterior hindbrain. In the further developmetal stage (HH 23), their expression expands toward the prospective nuclei within the rhombomeres. At HH 27, *plexin-A1*, *-A2* and *-A4* are expressed in the cranial nuclei as marked by Roman numerical (III= occulomotor, IV= trochlear, V= trigeminal, VI= abducens, VII= facial, IX= glossopharyngeal, X= vagus, XI= accessory, XII= hypoglossal). Scale bar in all images = 200 μm.

3.2 *Neuropilins* are expressed in the hindbrain of chick embryos

We analyzed the expression of *neuropilins* (*Npn-1* and *Npn-2*) in the flat-mounted hindbrains of chick embryos (HH 19, HH 23, HH 27) by *in situ* hybridization with relevant mRNA probes. Our results demonstrated that both *Npn-1* and *Npn-2* were expressed throughout the ventral region of the hindbrain in all developmental stages (Fig. 13). At HH 19, both expressed strongly at the level of r1-2 and r7-8. However, *Npn-2* expression was much stronger and extensive than *Npn-1*. The expressions of *Npn-1* and *Npn-2* were distributed towards the prospective nuclei within the rhombomeric boundaries of the hindbrain at HH 23. By HH 27, most cranial motor neurons migrate to their final positions in the hindbrain (Simon et al., 1994). Here, we compared the expression (HH 27) of these two genes (*Npn-1* and *Npn-2*) with the expression of motor neuron marker *Islet-1* (Verela-Echavarria et al., 1996). We found that *Npn-1* was expressed in the trochlear (IV), trigeminal (V), abducens (VI), glossopharyngeal (IX), vago-accessory (X/XI) and hypoglossal (XII) nuclei whereas *Npn-2* in the trochlear (IV), trigeminal (V), facial (VII), glossopharyngeal (IX), vago-accessory (X/XI) and hypoglossal (XII) nuclei (Fig. 13). These expression patterns are suggestive of the involvement of *Npn-1* and *Npn-2* in the axonal guidance of hindbrain cranial nerves in chick embryos.

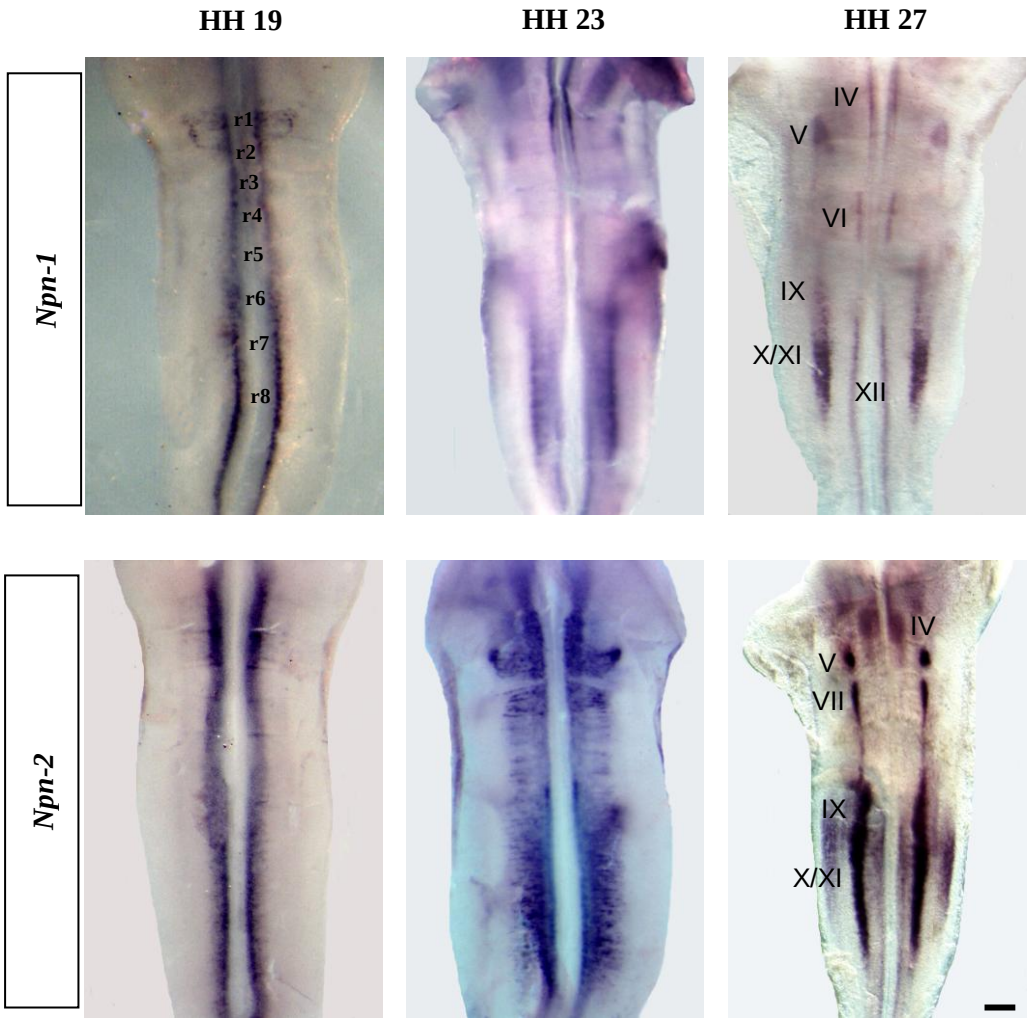


Fig. 13: Expression of *neuropilins* (*Npn-1* and *Npn-2*) in the flat-mounted hindbrains of chick embryos (HH 19, HH 23, HH 27). At HH 19, both *Npn-1* and *Npn-2* are expressed into two longitudinal domains in the ventral region of neural tube which appear relatively stronger at the level of r1-2 and r7-8. At HH 23, the expression is distributed towards the prospective nuclei within the rhombomeres and finally, expressed in the cranial nuclei at HH 27. *Npn-2* expression is more prominent and distinct than *Npn-1* in all stages of chick embryos. The cranial nuclei (HH 27) are marked by Roman numerical (III= occulomotor, IV= trochlear, V= trigeminal, VI= abducens, VII= facial, IX= glossopharyngeal, X/ XI= vago-accessory, XII= hypoglossal nuclei). Scale bar in all images = 200 μm.

3.3 Hindbrain motor neurons express *plexin-A2* and *Npn-2*

Plexin-A2 and *Npn-2* were found to be most prominently and distinctly expressed at the level of r7-8 of the hindbrain from which two motor neuron populations originated; dorsal exiting vagus (nX), and accessory (nXI) and ventral exiting hypoglossal (nXII) nerves. For better correlation of their expression by the motor neurons in the ventral neural tube, *in situ* hybridization of chick embryos (HH 19, HH 23, and HH 27) was performed by using *plexin-A2* and *Npn-2 mRNA* probes, respectively. The embryos were transversely cryosectioned at the level of r7-8 and the comparable sections from the same stages of chick embryos were immunolabelled for Islet-1/2. Our results showed that both *plexin-A2* and *Npn-2* were expressed in the ventral motor neurons (Fig. 14). During early stage of development (HH 19), *plexin-A2* expression was restricted to the medially placed motor neurons. In the further developmental stage (HH 23), it was expressed by the motor neurons that were migrating dorso-laterally toward the dorsal exit point. At HH 27, *plexin-A2* expressing motor neurons found their final positions in the vago-accesory (X/XI) and hypoglossal (XII) nuclei (Fig. 14). *Npn-2* was found to be expressed by the laterally positioned motor neurons at HH 19. In the succeeding developmental stage (HH 23), it was expressed by the dorso-laterally migrating motor neurons of the neural tube. *Npn-2* expressing motor neurons migrated further toward the dorsal exit point and finally (HH 27), settled down in the vago-accessory (X/XI) nuclei (Fig. 14). These results suggest that *plexin-A2* and *Npn-2* are involved in the axonal guidance of hindbrain motor neurons in chick embryos.

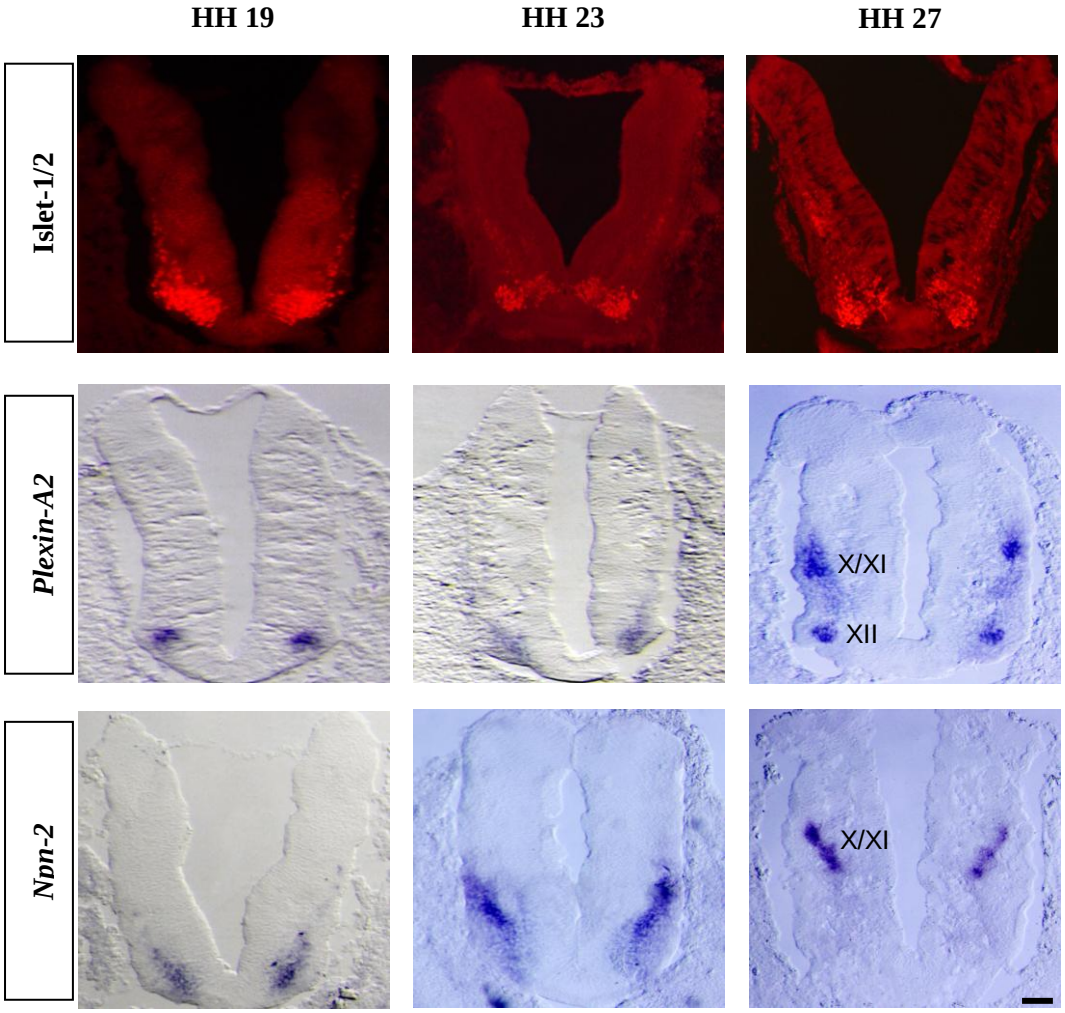


Fig. 14: Expression of *plexin-A2* and *Npn-2* in the hindbrain motor neurons of chick embryos (HH 19, HH 23, HH 27). Transverse cryosections (20 μm) of chick embryos at the level of r7/8 shows that *plexin-A2* expression confines in the medial motor pool of the ventral neural tube which extends dorso-laterally at HH 23 and finally, settles down in the vago-accessory (X/XI) and hypoglossal (XII) nuclei (HH 27). *Npn-2* is expressed in the dorso-laterally migrating motor neurons in the ventral neural tube at HH 19. The expression appears further dorso-lateral towards the dorsal exit point at HH 23 and finally, settles down in the vago-accessory nuclei (X/XI) at HH 27. Scale bar in all images = 125 μm.

3.4 *shRNA-EGFP* construct can down regulate *plexin-A2 mRNA* expression in the hindbrain

We adopted loss of function strategy by *in ovo*-electroporation of a plasmid construct, co-expressing *shRNA* and EGFP. For this purpose, *plexin-A2 shRNA* construct selective against *plexin-A2* gene was obtained (Bron et al., 2007). The chick embryos (HH 10-12) were successfully electroporated ventrally into the neural tube at the level of r7-8. To exclude the unspecific effects we also electroporated the *EGFP* vector alone. In each case, the unelectroporated (contralateral) side was considered as unaffected control. After 48 hours of reincubation, the eletroporated embryos were collected for *in situ* hybridization with *plexin-A2 mRNA* probe. The hindbrains were isolated, opened dorsally and flat-mounted. We analyzed the expression of *plexin-A2* at the electroporated level of hindbrain. Our results demonstrated that *EGFP* vector alone produce no remarkable change of *plexin-A2* expression between the electroporated and unelectroporated sides. To know the effect of the *plexin-A2 shRNA*, we then compared the expression of *plexin-A2* in the electroporated side with the unelectroporated and *EGFP* vector alone electroporated sides. Our results showed that *plexin-A2 shRNA* construct potentially down regulated the expression of *plexin-A2* at the electroporated level of hindbrain (Fig. 15).

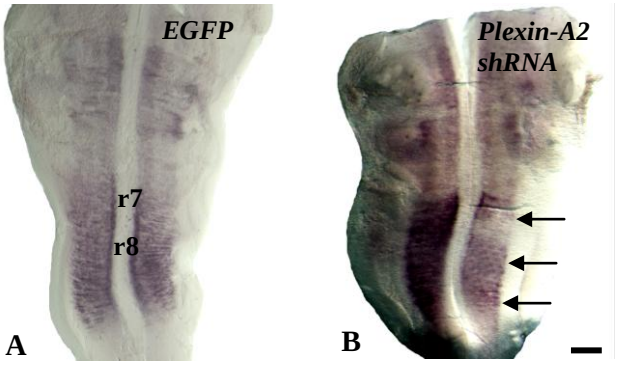


Fig. 15: Down regulation of *plexin-A2 mRNA* expression in the hindbrain of chick embryos (HH 24). **A**, electroporation of *EGFP* vector alone shows no change of *plexin-A2 mRNA* expression in the electroporated side (right) in comparison to the unelectroporated side (left). **B**, *plexin-A2 shRNA* down regulates the *plexin-A2 mRNA* expression at the electroporated level (arrows). Scale bar in all images (**A-B**) = 250 μm.

3.5 *Plexin-A2 shRNA* leads to the reduction of motor neurons in the hindbrain

To know the effect of *plexin-A2 shRNA* in the regulation of motor neurons, we electroporated the *EGFP* vector (alone) and *plexin-A2 shRNA* construct ventrally into the neural tube at the level of r7-8 of chick embryos (HH 10-12) followed by reincubation for 48 hrs (HH 22-24). The embryos were subjected to transverse cryosections at the electroporated level and immunostained for Islet-1/2 (39.4D5, DSHB). We observed that motor neuron population of the ventro-lateral domain was most severely affected after electroporation with the *plexin-A2 shRNA* construct. To confirm our observation, we counted the number of Islet-1/2 positive cells in the ventro-lateral domain close to the ventral exit point. In each case of electroporation (*EGFP* vector and *plexin-A2 shRNA*), we quantified 20 randomly selected cryosections of the electroporated embryos (n=5). The data were recorded (Tab. 5) and analyzed by Student's *t*-test (two-tailed). Our results demonstrated that *EGFP* alone produced no significant reduction in the quantity of motor neuron somata in the electroporated side comparing with the unelectroporated (contralateral control) side (Fig. 16). Besides, *plexin-A2 shRNA* significantly (***$P<0.001$) reduced the quantity of motor neuron population of the ventro-lateral domain in the elctroporated side in comparison to the *EGFP* alone electroporated and unelectroporated sides (Fig. 16). However, both *EGFP* vector and *plexin-A2 shRNA* construct caused no noticeable effect in the ectopic migration of motor neuron somata along the dorsal or ventral root of the neural tube. These results indicate that *plexin-A2 shRNA* regulate the quantity of motor neuron population in the ventral neural tube at the post-otic hindbrain level of chick embryos.

Tab. 5: Number of Islet-1/2 positive cells in the ventro-lateral domain of motor neuron population in the electroporated chick embryos (n=5).

No. of Embryos	Unelectroporated	*Plexin-A2 shRNA*	*EGFP*	*Plexin-A2 shRNA*
1	55	12	42	12
	51	8	45	8
	50	12	47	12
	54	11	44	11
2	60	10	43	10
	58	13	45	13
	49	15	46	15
	46	8	42	8
3	48	9	41	9
	53	7	39	7
	51	9	45	9
	57	11	50	11
4	47	13	51	13
	46	10	49	10
	43	9	38	9
	44	8	41	8
5	46	14	38	14
	55	11	52	11
	52	12	38	12
	51	10	42	10
Mean	**50.8**	**10.6**	**43.9**	**10.6**
SD	**4.7**	**2.1**	**4.3**	**2.1**
*****$P<0.001$ (Student's *t*-test)**				

(∗ indicates level of significance)

∗ = less significant ($P<0.05$)

∗∗ = moderately significant ($P<0.01$)

∗∗∗ = highly significant ($P<0.001$)

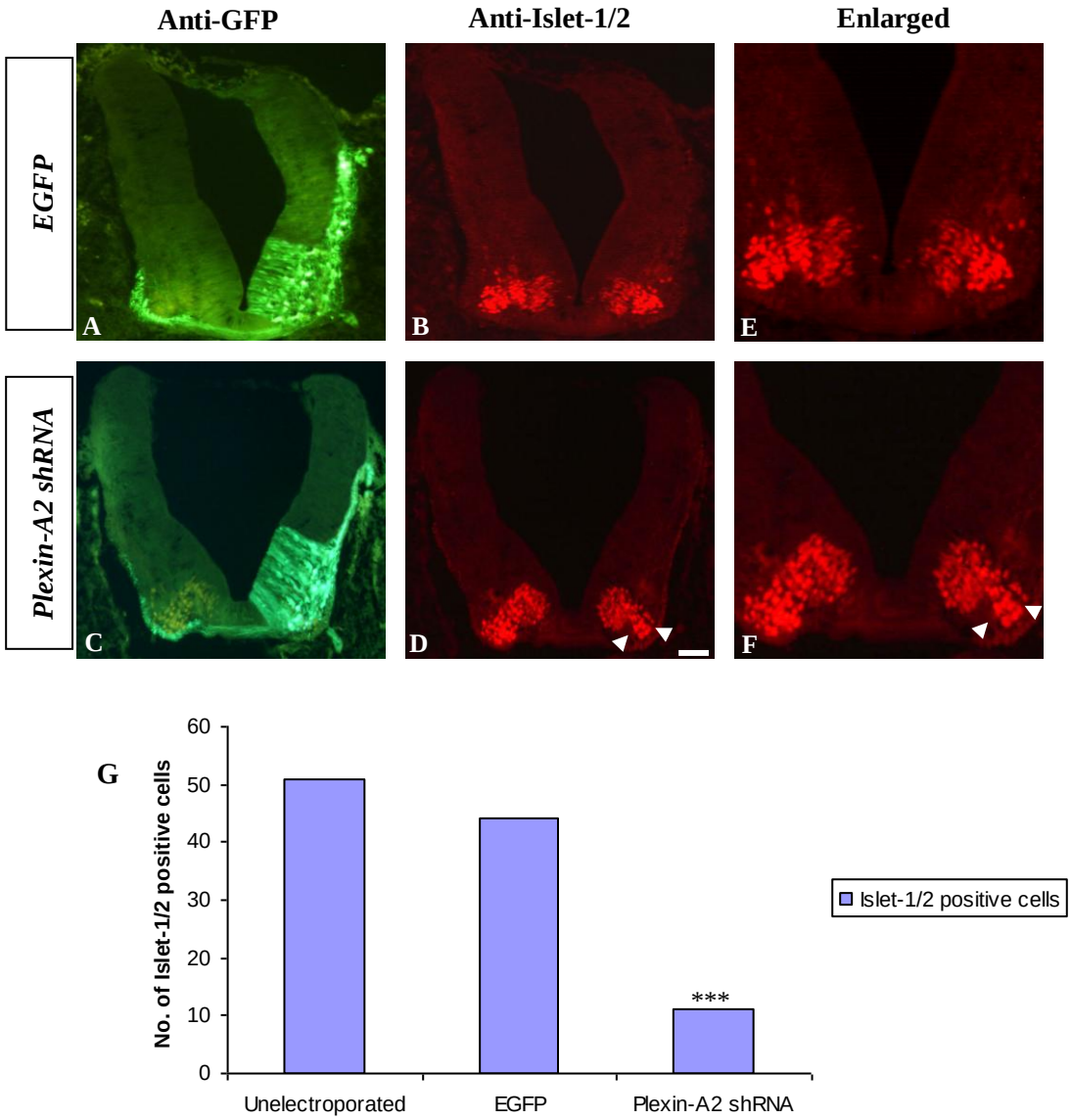


Fig. 16: Functions of *plexin-A2 shRNA* in the regulation of hindbrain motor neurons (HH 23). (**A-D**), tranaverse cryosections (20 μm) of electroporated chick embryos at the level of r7/8, immunostained with rabbit polyclonal anti-GFP (**A**, **C**) and anti-Islet-1/2 antibodies (B, D). White arrow heads in D indicates depletion of Islet-1/2 positive cells. **E** and **F** are enlarged views of **B** and **D**, respectively. **G**, quantitative analysis (Mean ± SD) of Islet-1/2 positive cells in the cryosections of chick embryos at the electroporated level (r7-8). *EGFP* vector alone shows no significant reduction in the quantity of Islet-1/2 positive cells in the unelectroporated (51 ± 5) and electroporated sides (44 ± 4). *Plexin-A2 shRNA* significantly reduces ventro-laterally placed Islet-1/2 positive cells (11 ± 2) in comparison to the *EGFP* alone electroporated side and unelectroporated side. ***$P < 0.001$; two-tailed *t*-test. Scale bar in all images (**A-D**) = 125 μm.

3.6 *Plexin-A2 shRNA* impairs assembly and fasciculation of hypoglossal nerve

Having found the effect of *plexin-A2 shRNA* in the developing hindbrain motor neurons, we next investigated the role in the axonal projection and guidance of r7-8 cranial nerves into the periphery. We followed the same electroporation procedures as before and the elelctroporated embryos were immunostained (whole-mount) with anti-neurofilament antibody (3A10, DSHB). We found that *EGFP* vector alone electroporated embryos showed no change in the peripheral trajectory of cranial nerves in the electroporated side in comparison to unelectroporated side. However, *plexin-A2 shRNA* severely affected the peripheral pathfinding of hypoglossal nerve (Fig. 17). The hypoglossal nerve is formed by the anastomosis of ventral rootlets of the axons at the occipital and upper cervical level. Four occipital (O1-O4) and upper cervical (C1-C3) nerves are involved into the formation of hypoglossal nerve (Kuratani et al., 1998). At the electroporated side of chick embryos, the roots of the hypoglossal (nXII) nerve were found to be weaker, failed to assemble into a tight bundle after exiting the neural tube in comparison to the unelectroporated (control) side (Fig. 17). The dorsal exiting vagus (nX) and cranial accessory (nXI) nerves were also partially defasciculated in the *plexin-A2 shRNA* electroporated embryos. Their nerve bundles were found to be loosely arranged, not so compact as those in the control sides.

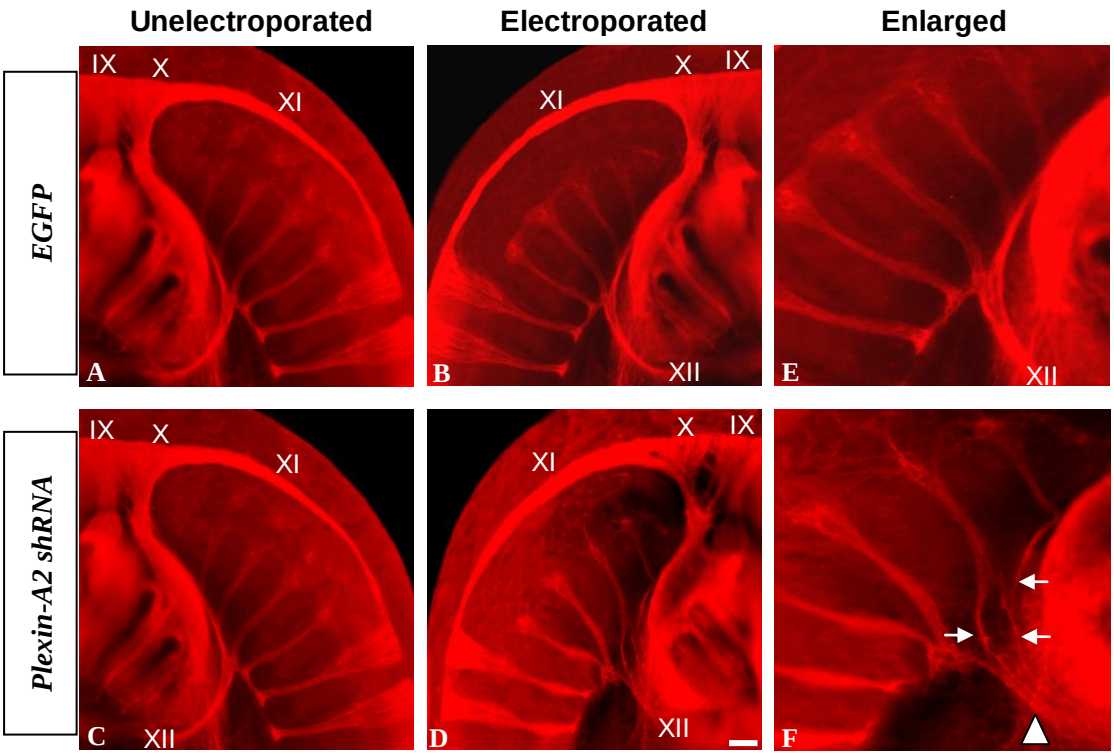


Fig. 17: Functions of *plexin-A2 shRNA* in the fasciculation of cranial nerves in the hindbrain (r7-8) of chick embryos (HH 23). (**A**, **C**), are representative images of *EGFP* vector and *plexin-A2 shRNA* unelectroporated sides (contralateral control) whereas (**B**, **D**), represent eletroporated sides of chick embryos. All of the embryos are immunostained with anti-neurofilament antibody. (**E**, **F**), are enlarged views of (**B**, **D**), respectively. Electroporation of *EGFP* vector alone shows no effect in the fasciculation of cranial nerves (X= vagus, XI= accessory and XII= hypoglossal nerves) in the electroporated side in comparison to the unelectroporated side. CI and C2 are cervical roots contribute in the formation of hypoglossal nerve (XII). In the electroporated side, *plexin-A2 shRNA* causes hypoglossal roots to become weaker (arrows in **F**), failed to assemble into tight bundle (arrow head in **F**) in comparison to the unelectroporated side (**C**) and *EGFP* alone electroporated side (**B**). Vagus (X) and accessory (XI) nerves are partially defasiculated (**D**). Scale bar in all images (**A-D**) = 200 µm.

3.7 *shRNA-EGFP* plasmid construct can down regulate *Npn-2 mRNA* expression in the hindbrain

To know the *in vivo* role of *Npn-2*, we used the plasmid construct (co-expressing EGFP and *Npn-2 shRNA*) selective against *Npn-2* gene (Bron et al., 2004). We electroporated the construct ventrally into the neural tube at the level of r7-8 of chick embryos (HH 10-12). To avoid the unspecific effects of electroporation we also electroporated the *EGFP* vector alone at this level. After 48 hours of reincubation, the eletroporated embryos were collected for *in situ* hybridization with *Npn-2 mRNA* probe. We found that *EGFP* alone produce no remarkable change of *Npn-2* expression in the electroporated side in comparison to the contralateral control (unelectroporated) side. But *Npn-2 shRNAs* knocked down the expression of *Npn-2* at the electroporated level of hindbrain in comparison to the contralateral and *EGFP* electroporated sides (Fig. 18).

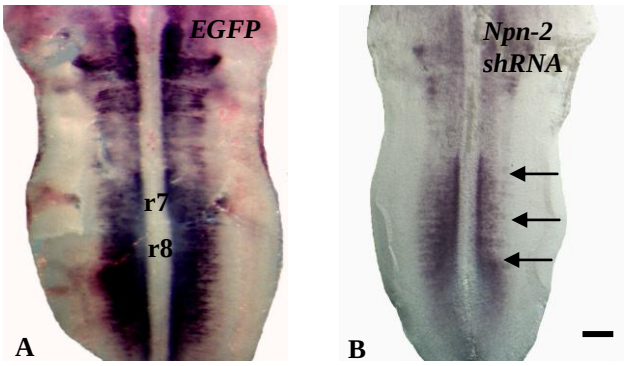


Fig. 18: Down regulation of *Npn-2 mRNA* in the hindbrain of chick embryos (HH 24). **A**, electroporation of *EGFP* vector alone shows no change of Npn-2 expression in the electroporated side (right) in comparison to the unelectroporated side (left). **B**, *Npn-2 shRNA* downregulates the *Npn-2* expression at the electroporated level (arrows). Scale bar in all images (**A-B**) = 250 μm.

3.8 *Npn-2 shRNA* causes ectopic migration of motor neuron somata and misprojection of the axons

To test the effect of *Npn-2 shRNA* in the hindbrain motor neurons, we electroporated the *Npn-2 shRNA* construct ventrally into the neural tube at the level of r7-8 of chick embryos (HH 10-12). We also electropoted the *EGFP* vector alone at this level as control. The electroporated embryos were allowed for reincubation (48 hrs). The embryos were collected and processed for transverse cryosectioning at the electroporated level. The sections were immunostained using anti-Islet-1/2 (mouse monoclonal) and anti-GFP antibodies (rabbit polyclonal) detected with appropriate Cy2/Cy3 conjugated secondary antibodies. In each case of electroporation, we examined 125 serial cryosections of 5 embryos at the electroporated level. Our results showed that *Npn-2 shRNA* induced significant (***$P<0.001$) translocation of motor neuron somata (20% of the sections) along the dorsal root in the electroporated side in comparison to the unelectroporated side (0%) and *EGFP* alone electroporated side (1%), (Fig. 19). In addition, *Npn-2 shRNA* caused misprojection of dorsal exiting motor axons within the CNS and into the periphery. However, no translocation of motor neuron somata was evident along the ventral root (Fig. 19).

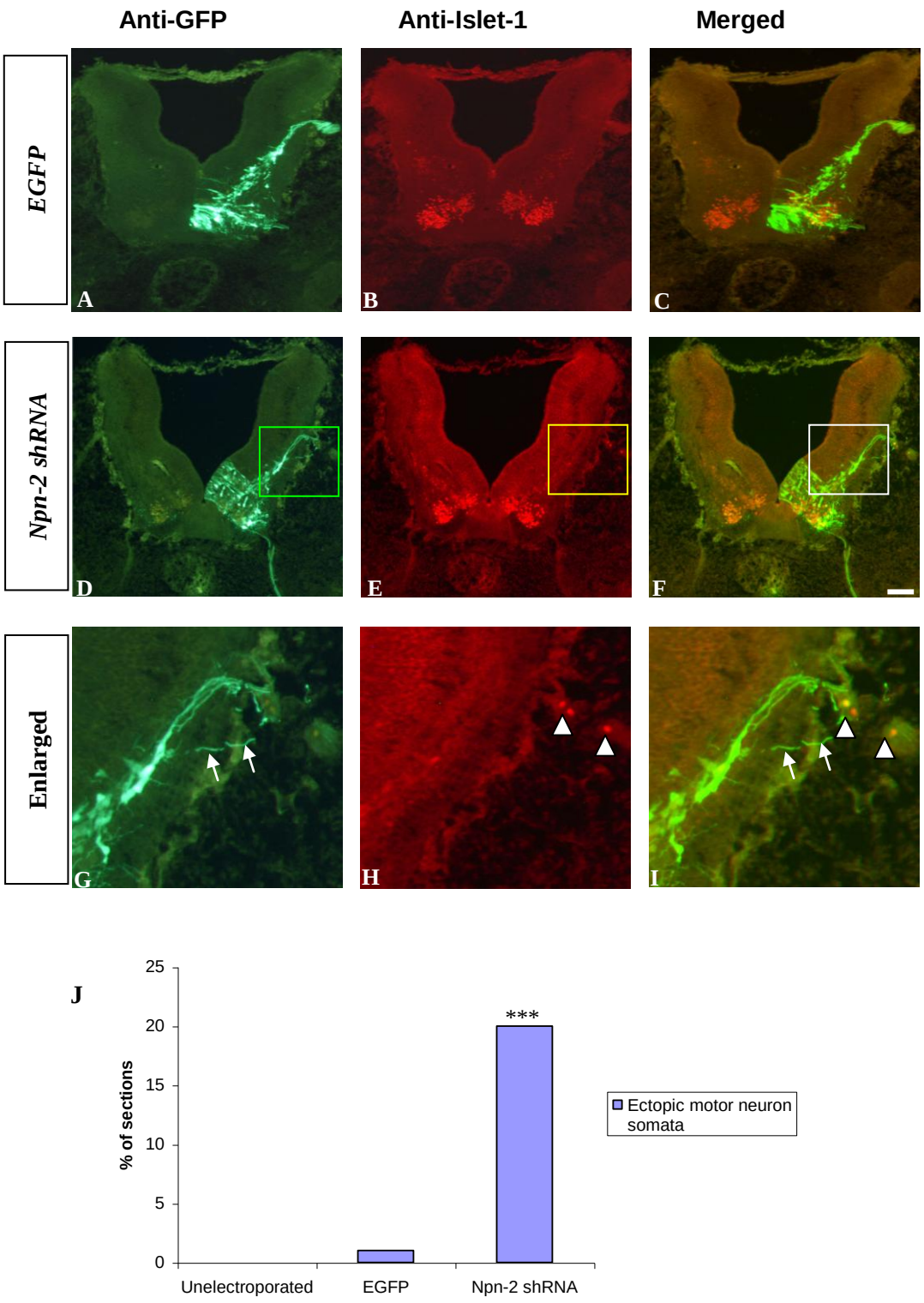


Fig. 19: Effects of *Npn-2 shRNA* in the positioning of motor neuron somata in the hindbrain of chick embryos (HH 23). Transverse cryosections (20 μm) of electroporated chick embryo with *EGFP* vector alone (**A**, **B, C**) and *Npn-2 shRNA* construct (**D**, **E, F**). **G, H,** and **I** are enlarged views of boxed areas in **D**, **E**, and **F**, respectively. The cryosections are immunostained with rabbit polyclonal anti-GFP (**A**, **D**) and anti-Islet-1/2 antibodies (**B**, **E**). Arrows indicates

misrouted axons (E) and arrow heads show ectopic motor neuron somata (**F**). **J**, quantitative analysis of ectopic motor neuron somata in the cryosections at the electroporated level (r7-8). In each case, we analyzed 125 serial cryosections of 5 chick embryos. Electroporation of *EGFP* vector alone produces no significant effect in the positioning of motor neuron somata in the electroporated and unelectroporated sides whereas *Npn-2 shRNA* induces significant ectopic positioning of motor neuron somata (20% of the sections) along the dorsal root (***$P < 0.001$; two-tailed *t*-test). Scale bar in all images (**A-F**) = 125 μm.

3.9 *Npn-2 shRNA* causes abnormal trajectory and fasciculation of vagus and accessory axons

To assess the effect of *Npn-2 shRNA* in the peripheral trajectory of cranial nerves at the level of r7-8, we followed the same electroporation and staining procedures (whole-mount) as described above. Our results showed that many rootlets of the vagus (nX) and accessory (nXI) nerves were misrouted ventrally immediately after exiting the neural tube in the *Npn-2 shRNA* electroporated side of the embryos (Fig. 20). This was accompanied by severe defasciculation of the vagus (X) and cranial accessory (XI) axons at the first somite level. The vago-accessory (X/XI) axons failed to bind together into a tight bundle as observed in the *EGFP* alone electroporated and unelectroporated sides of the chick embryos (Fig. 20). These results demonstrate that axonal guidance of vagus (nX) and accessory (nXI) nerves at the post-otic hindbrain level is regulated by *Npn-2*.

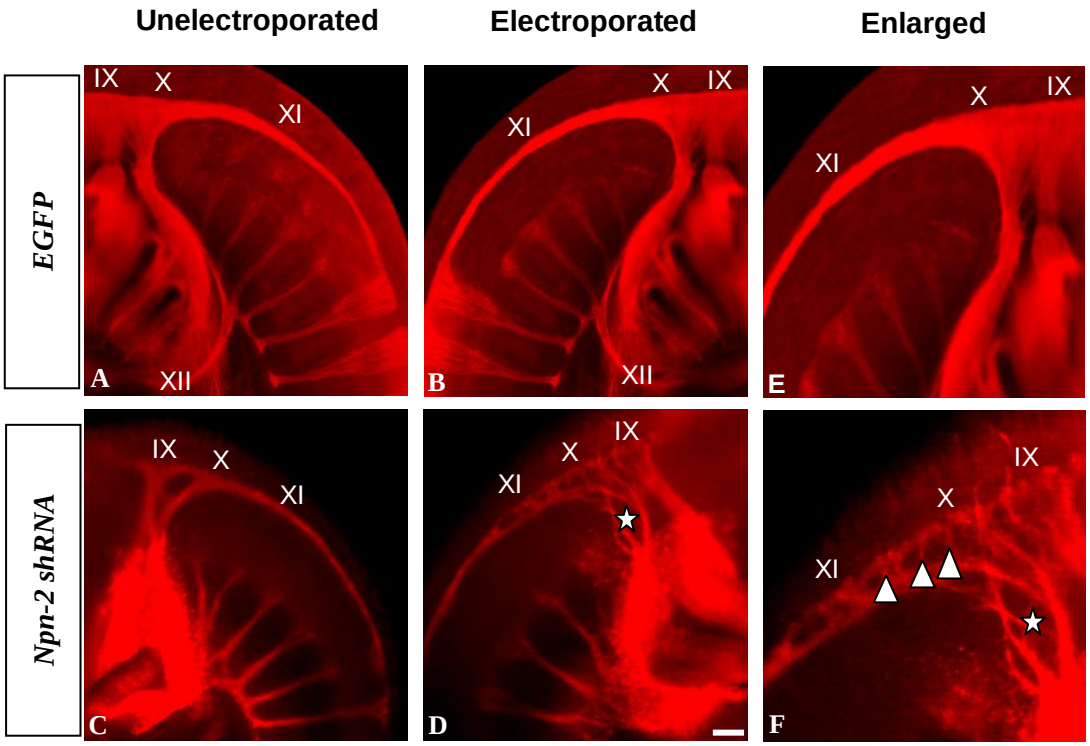


Fig. 20: Functions of *Npn-2 shRNA* in the fasciculation of cranial nerves in the hindbrain of chick embryos (HH 22). (**A**, **B**) and (**C**, **D**) are representative images of *EGFP* alone and *Npn-2 shRNA* electroporated chick embryos at the electroporated level (r7-8), respectively which are immunostained with anti-neurofilament antibody (3A10, DSHB). (**E**, **F**) are enlarged views of (**B**, **D**). *EGFP* alone shows no deffect in the projection and fasciculation of cranial nerves in the unelectroporated (**A**) and electroporated (**B**) sides. *Npn-2 shRNA* causes misprojection of vago-accessory axons (white arrow heads in F) and severe defasciculation of vago-accessory nerves (nX/XI) as indicated by asterisks in (**D**, **F**). Scale bar in all images (**A-D**) = 200 μm.

4. Discussion

Over the past two decades, the secreted class III *semaphorins* and their receptors (*plexins* and/or *neuropilins*), were shown to play a role in multiple features of axon guidance of spinal and cranial nerves, including regulation of the timing of growth, selective fasciculation, and mediation of sensory–motor axon interactions. Both *in vitro* and *in vivo* studies have shown that *plexins* and *neuropilins* are the binding moieties for class III *semaphorin*s. However, the signaling receptors that accompany with the class III *semaphorins* to mediate their actions are still poorly characterized. Our results showed that *plexin-A1*, *plexin-A2* and *Npn-1* were expressed by both the dorsal (dMNs) and ventral (vMNS) exiting motor neuronal groups within the rhombomeres of the hindbrain, while *plexin-A4* and *Npn-2* only by the dorsal (dMNs) exiting motor neuronal groups. Loss of function of *plexin-A2* reduced the motor neuron population at the post-otic hindbrain level whereas *Npn-2* induced translocation of motor neuron somata along the dorsal root. In the periphery, *plexin-A2* caused defasiculation of hypoglossal (XII) nerve, while *Npn-2* caused misrouting and defasiculation of vago-accessory (X/XI) nerve (Fig. 21).

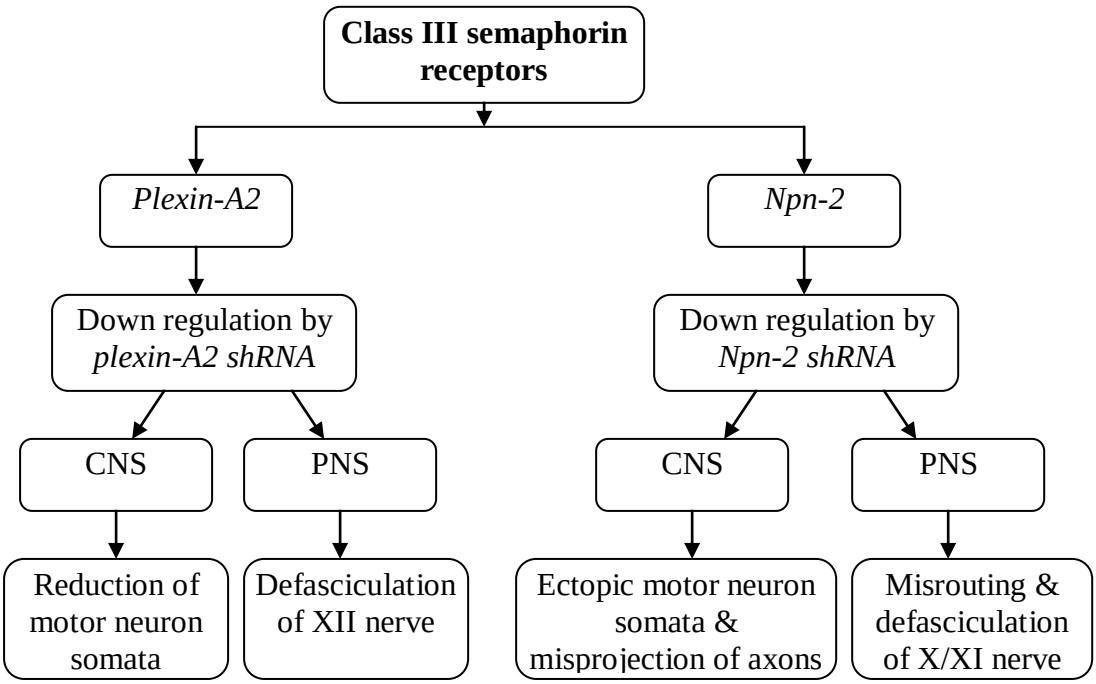


Fig. 21: Representative diagram of the *in-vivo* function of *plexin-A2* and *Npn-2* in the hindbrain.

4.2 Selective expression of *plexin-A2* and *Npn-2* in the hindbrain and spinal cord

We observed that both *plexin-A2* and *Npn-2* expressed in the ventral motor neurons at early migratory stage (HH 19). At the late migrating stage (HH 23), the expression appeared dorso-laterally which was correlated with the migration of the dMNs. After most of the cranial motor neurons find their final positions (Simon et al., 1994), the expression of *plexin-A2* was appeared in two domains, a ventral and an intermediate domain in the neural tube. The ventral domain was located close to the ventral exit point, while the intermediate domain was found close to dorsal exit point. *Npn-2* expression was restricted only to the intermediate domain. In our examined level of the hindbrain (r7-8), the ventral domain was correlated with the hypoglossal nucleus and the intermediate domain with the vago-accessory nucleus. The expression patterns of *plexin-A2* and *Npn-2* were found overlapping to the dorso-laterally migrated motor neurons and the motor neurons that settled down finally close to the dorsal exit point, supporting their cooperative function in the neural circuit formation of vago-accessory nerve.

Available expression data in mouse and chick embryos suggested that the multimeric surface receptors for secreted class-III *semaphorins* are *neuropilins* and/or *plexins* (Cohen et al., 2005; Huber et al., 2005; Mauti et al., 2006). In the mouse (E10.5-12.5), *plexin-A2* was found in the roof plate and in interneurons of the ventral spinal cord but not in motor neurons of the trunk region (Brown et al., 2001). Besides, *Npn-2* is expressed very strongly in dorsal commissural neurons, ventral populations of motor neurons, and in the floor plate (Kawakami et al., 1996; Chen et al., 2000). In chick, *Npn-2* is expressed in the motor neuron pools but never expressed in the embryonic chicken floor plate and dorsal spinal cord at comparable stages (Mauti et al., 2006). In the chick lumbo-sacral spinal cord, premigratory motor neurons expressed *plexin-A1*, *plexin-A2*, *Npn-1* but not *Npn-2* (Mauti et al., 2006). At the migratory stage, *Npn-2* appeared to be expressed predominantly in a dorso-lateral subset of Islet1-positive cells, while *plexin-A2* was scattered throughout the ventral horn (Mauti et al., 2006). Bron et al., 2007 confirm and extend the observation of Mauti *et al.*, 2006; showing dynamic patterns of expression of *plexin-A2* and *Npn-2*. They reported both expressions in the spinal motor neurons (or their progenitors) at premigratory stage which expressed in medial and lateral subsets of motor neurons at the migratory stage.

During embryonic development, motor exit points are prefigured by neural crest derivatives, referred to as boundary cap (BC) cells (Niederlander et al., 1996; Vermeren et al., 2003; Bron et al., 2007; Mauti et al., 2007). Boundary cap cells reside at the exit points of dMNs (Niederlander et al., 1996) and vMNs (Vermeren et al., 2003; Bron et al., 2007; Mauti et al., 2007). Notably, in *in vivo* model, the absence of BC cells failed to disrupt the pathfinding of vMN axons but, remarkably, their cell bodies ectopically migrated out of the CNS (Vermeren et al., 2003). BC cells expressing *semaphorins* (*Sema 3B*, *Sema 3G* and *Sema 6A*) signal through *plexin-A2* and/or *Npn-2* in spinal motor neurons (Bron et al., 2007). We observed that *plexin-A2* and *Npn-2* selectively expressed in the dMNs and vMNs of the post-otic hindbrain. From these observations, we assume that *plexin-A2* and *Npn-2* might bind to BC cells *semaphorins* at the exit points to regulate the pathfinding of cranial motor nerves.

4.1 *Plexins* and *neuropilins* expression in the hindbrain are regulated by transcription factors

Differential regulation of the receptors that mediate chemoattraction or chemorepulsion by axon guidance molecules such as members of the *semaphorin* family could result in the characteristic location of motor nuclei and connectivity patterns (Luo et al., 1993, 1995; Messersmith et al., 1995; Püschel et al., 1995; Adams et al., 1996). The migration of motor neurons to their final destinations is a key process in the development of the nervous system. This process must be coordinated with the establishment of connectivity and requires differential responses of distinct neuronal compartments to the multitude of guidance cues in the environment.

Our gene expression data showed that both dMNs and vMNs expressed all of the *plexins* and *neuropilins* in the chick hindbrain. Among the *plexins*, *plexin-A1* was expressed throughout the ventral neural tube in the early stage of development and finally expressed in the trochlear (IV), trigeminal (V), facial (VII) and hypoglossal (XII) nuclei. *Plexin-A2* expression was shifted medial to lateral of the neural tube in the early stage which was shown to be settled down in the trochlear (IV), trigeminal (V), abducens (VI), glossopharyngeal (IX), vago-accessory (X/XI), and hypoglossal (XII) nuclei. *Plexin-A4* expression appeared dorso-laterally of the neural tube in the early stage and finally established expression in the trigeminal (V), facial (VII), glossopharyngeal (IX) and vago-accessory (X/XI) nuclei. *Npn-1* expression was almost similar to that of *plexin-A2* in all developmental stages, while *plexin-A4* with the *Npn-*

2. These results showed that *plexin-A2* and *Npn-1* were co-expressed by both the dorsal (dMNs) and ventral (vMNs) subtypes of cranial motor neurons whereas *plexin-A4* and *Npn-2* only by the dorsal (dMNs) subtype. These observations support the idea that they form receptor complexes for specific ligands (*semaphorins*) during axonal guidance of cranial nerves in the hindbrain.

The differentiation of neuronal subtype is controlled by the Phox and LIM homeodomain factors (Sharma et al., 1998; Hirsch et al., 2007). In this regard, it is interesting to note that glossopharyngeal (nIX) and vagus (nX) nerves appear fused in Nkx2.9 null mice and the spinal accessory (nXI) motor neurons are unable to migrate to a dorso-lateral position and their axons are incapable of exiting the neural tube (Pabst et al., 2003). Our expression data showed that these cranial motor neurons (dMNs) selectively expressed *plexin-A4* and *Npn-2*. This raised the possibilities that *Nkx2.9* acts to maintain the function of *plexin-A4* and *Npn-2* during the axonal guidance of dMNs in the hindbrain. Loss of *Nkx6.1, Nkx6.2* and *Pax6* during somatic motor neuron development deletes abducens and hypoglossal motor neurons (Ericson et al., 1997). We found that *plexin-A2* and *Npn-1* were expressed by the abducens and hypoglossal (v-MNs) motor neurons. Here, it is probable that the action of *plexin-A2* and *Npn-1* in the axon guidance of abducens (nVI) and hypoglossal (nXII) nerves are regulated by *Nkx6* and *Pax6* genes.

The differentiation of the dMNs is required *Phox2a/b* expression (Hirsch et al., 2007). *Lhx3* and *Lhx4* are important for the vMN differentiation and the formation of the medial motor column (Sharma et al., 1998). These reports are also correlated with our expression data suggesting that *Phox* and *Lhx* genes might control the function of *plexins* and *neuropilins* in the axonal guidance of dMNs and vMNs. Motor neurons projecting to the body wall and limb muscles are not required *Lhx3* and *Lhx4* expression. These neurons form the lateral motor column at the limb level (Tada et al., 1979). The bm/vm motor neurons in the hindbrain express the gene Islet-1 only (Varela-Echavarri´a et al., 1996). These neurons innervate muscles differentiating within the branchial arches that derive from myogenic cells emigrating from the ventral edge of the cranial paraxial mesoderm (Noden, 1983; Couly et al., 1992). A parallel situation exists in the case of precursors of the limb musculature that migrate from the ventrolateral edge of the trunk somites (Christ et al., 1986), implying that there might be some similarities between the neurons innervating these two types of muscles. However, limb-

innervating motor neurons of the spinal cord expressed other LIM genes in addition to Islet-1. In particular, limb-innervating spinal motor neurons express Lim-1 (Tsuchida et al., 1994), expression of which was not detected in any cranial motor neuron subpopulation. The sm cranial motor neuron groups that express the same repertoire of LIM genes (Islet-1 and Islet-2) innervate muscles that are derived either from the cranial paraxial mesoderm or from the prechordal plate mesoderm (Noden, 1992; Couly et al., 1992). Considering the available reports mentioned above together with our observations, we can speculate that selective function of *plexins* and *Neuropilins* is likely to be the result of the differential expression by motor neurons that mediate responses to diffusible and contact-dependent signals. This expression might in turn be the consequence of the differential activation of developmentally regulated transcription factors involved in the axonal guidance of cranial nerves.

4.3 *Plexin-A2* in the pathfinding of cranial nerves

Our gene expression data showed that the expression of *plexin-A2* was expressed by the motor neuron subpopulations from which the vago-accessory (bm/vm) and hypoglossal (sm) motor neurons developed. Loss of *plexin-A2* significantly reduced the ventro-lateral domain of motor neuron population. In the chick r7/8, ventro-lateral domain was identified as bm/vm (Osumi et al., 1997; Tanabe et al., 1998). According to these observations, our knockdown study showed that *plexin-A2* significantly reduced the dorsal exiting (bm/vm) motor neuron somata at the post-otic hindbrain level of chick embryos. However, it caused no ectopic migration of motor neuron somata, translocated out of the CNS into the periphery as observed in the spinal cord (Bron et al., 2007). It has been shown that in addition to directing growth cone movements, *Sema 3A* regulates the migration and apoptosis of cells, including neural crest and neuronal progenitor cells (Song et al., 1998; Eickholt et al., 1999; Bagnard et al., 2001; Kawasaki et al., 2002). From these findings and our results, we supposed the idea that *plexin-A2* might control the generation of motor neurons in the ventral neural tube by binding with *semaphorins*.

In the periphery, loss of function of *plexin-A2* severely affected the hypoglossal rootlets and impaired the fasciculation of hypoglossal nerve (nXII). These results demonstrate that *plexin-A2* is required for the pathfinding of hypoglossal nerve in the post-otic hindbrain. The role of *plexin-A2* in the axonal guidance of hypoglossal nerve correlates with the *Npn-1* mutant mice (Huettl and Huber, 2011). *Npn-1* has been demonstrated to be a receptor for *sema-3A* (He and

Tessier-Lavigne, 1997; Kolodkin et al., 1997). *Neuropilins* must bind to their co-receptors, the *plexins*, to generate intracellular signal (Castellani and Rougon, 2002). Our expression data showed that *plexin-A2* and *Npn-1* were co-expressed by the hypoglossal motor neurons. From the above mentioned reports and our results, it is highly probable that both *plexin-A2* and *Npn-1* form a receptor complex for the ligand *(semaphorin)* during axon guidance of hypoglossal nerve.

The axons of vagal and accessory nerves originate from the C2 to O1 and turn cranial immediately after they exit the neuroepithelium (Kobayashi et al., 1997). The axons assemble to a bundle which takes a longitudinal course to approach the first somite (O1) level. Our previous studies show that the first somite acts like a gate through which the accessory motor axons pass to the periphery (Pu et al., 2013). Down regulation of the *plexin-A2* expression did not affect the longitudinal course of the axons. It caused only partial defasciculation of axons. In the dMNs, an unknown mechanism may be involved in the axonal guidance and fasciculation which could compensate the function of the *plexin-A2*.

4.4 *Npn-2* in the pathfinding of cranial nerves

Npn-2 was found to be expressed by the dMNs and their nuclei (vago-accessory) in the post-otic hindbrain but no remarkable expression was evident by the vMNs and nucleus (hypoglossal). In our study, loss of *Npn-2* induced ectopic migration of motor neuron somata along the dorsal root avoiding the ventral root. This result indicates that *Npn-2* is required for the confinement of dorsal exiting motor neuron somata within the CNS. This is not in accordance with the obsevation of Bron et al., 2007 who reported ectopic positioning along the ventral root of spinal cord. This may be due to differential patterning of motor neurons in the brain and spinal cord. In response to down signaling of *Nkx2.2* and *Nkx2.9*, d-MNs somata migrate to more dorsal positions in the neural tube (Briscoe and Ericson, 1999; Pabst et al., 2003) and their axons share dorsal exit points (reviewed by Guthrie, 2007). These observations suggest that the role of *Npn-2* in motor somal positioning in the hindbrain might be regulated by *Nkx2* genes. *Npn-2* was found to bind *Sema 3C* and *Sema 3F* (Chen et al., 1997, 1998; Giger et al., 1998). *Semaphorin-neuropilin* signaling influences the positioning of migratory neural crest cells in the hindbrain of chick embryo (Osborne et al., 2005). *Sema 3A* and *Sema 3F* are known to mediate their biological activity through their binding receptors,

Npn-1 and *Npn-2* that are expressed by cranial neural crest cells (Eickholt et al., 1999; Gammill and Bronner-Fraser, 2002; Osborne et al., 2005). However, analysis of *Npn-1* or *Npn-2* mutant mice failed to reveal obvious phenotypes indicative of effects in cranial neural crest cell migration (Kitsukawa et al., 1997; Giger et al., 2000; Gu et al., 2003).

In the *Sema 3A* mutant mouse, there is marked defasciculation with some aberrant projections of the trigeminal, facial, glossopharyngeal, vagal, and accessory nerves (Taniguchi et al., 1997). The same nerves are disorganised in mice lacking *Npn-1* (Kitsukawa et al., 1997), demonstrating the importance of this receptor-ligand interaction in the formation of these nerve pathways. In our study, loss of *Npn-2* caused misrouting and defasciculation of the vagus and accessory (dMNs) nerves but the hypoglossal (vMNs) nerve was unaffected. These results indicate that *Npn-2* is involved only in the axonal guidance of dMNs in the hindbrain. It has been shown that the cranial nerves having severe defasiculation phenotypes in *Npn-1* knockout mice (the ophthalmic branch and distal portions of the maxillary and mandibular portions of the trigeminal nerve [nV], the facial nerve [nVII], the glossopharyngeal nerve [IX], and the vagus nerve [nX]: Kitsukawa et al., 1997; Gu et al., 2003) are largely unaffected in the *Npn-2* mutant embryos, while those exhibiting no significant defects in *Npn-1* mutants (nIII and nIV) have severe defects in mice carrying the *Npn-2* mutant allele (Chen et al., 2000; Gammill et al., 2007). These reports together with our observations suggest that both *Npn-1* and *Npn-2* are required for the axonal guidance of selective sets of cranial nerves in the hindbrain.

4.5 Concluding remarks and future outlook

Our results demonstrated that *plexins* (*plexin-A1, -A2, -A4*) and *neuropilins* (*Npn-1* and *Npn-2*) are expressed in the specific neuronal groups within the hindbrain of chick embryos. While *plexin-A2* is required for pathfinding of both the dorsal exiting vagus (nX) and accessory (nXI) and ventral exiting hypoglossal (nXII) nerves, *Npn-2* only for the dorsal exiting vagus (nX) and accessory (nXI) nerves. These separate involvements of *plexin-A2* and *Npn-2* in axon guidance support the notion that both of them confer exquisite specificity of cranial nerves with respect to guidance responses. Future studies can be carried out to answer the following questions:

- How *plexin-A2* regulates the motor neuron population in the hindbrain?
- Which *semaphorin* interacts with *plexin-A2* and/or *Npn-2* in the axonal guidance of cranial nerves?

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7. Declaration

I hereby declare that the work in this thesis is original and has been carried out by myself at the Institute of Anatomy, Faculty of Medicine, University of Bonn. This thesis was prepared under the supervision of Prof. Dr. med. Ruijin Huang in fulfillment of the requirements of the doctoral degree in Natural Sciences of the University of Bonn. I further declare that this work has not been the basis for the awarding of any degree, diploma, fellowship, associateship or similar title at any university or institution.

Bonn, December 2013

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