

Distribution of *plexin* and *neuropilin mRNAs* in the cranium and brain stem of chick embryos

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Abstract

Semaphorins exert their effects in neuronal and non-neuronal processes by binding with receptors. Both *in vitro* and *in vivo* studies have shown that *plexin-As* (*plexin-A1*, *plexin-A2* and *plexin-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) are the binding moieties for class-III *semaphorins*. These receptors are expressed in a spatially and temporally discontinuous manner during organogenesis and central nervous system (CNS) development in vertebrates. However, the signaling receptors that accompany with the class-III *semaphorins* to mediate their actions are still poorly characterized. To assess their diverse roles in neural circuit formation, we analyzed the expression patterns of *plexin-A1*, *plexin-A2*, *plexin-A4*, *Npn-1* and *Npn-2* in the head and brain stem of chick embryos. In developing eye, *plexin-A1* expressed in the lens, whereas *plexin-A2* and *Npn-1* in the periorbital mesenchyme. *Plexin-A1* also expressed throughout the otic vesicle but *Npn-1* expressed only in the dorsal part. Among the analyzed *plexins* and *neuropilins*, only *Npn-2* expression was detected in the branchial arches. *Npn-2* and *plexin-A4* were also expressed in the trigeminal and vago-accessory ganglia. In the hindbrain, all analyzed *plexins* and *neuropilins* were expressed by the selective sets of dorsal- (dMNs) and ventral exiting motor neurons (vMNs) except *plexin-A4* (only dMNs). Interestingly, *plexin-A2* and *Npn-1* were co-expressed by the similar sets of dMNs and vMNs (trochlear, trigeminal, abducens, glossopharyngeal, vago-accessory and hypoglossal nuclei). These expression findings suggest that *plexins* and *neuropilins* are involved in neurodevelopment of the cranial structures in chick embryos.

Introduction

The development of the cranium requires deployment of a variety of cell types to specific locations in the embryonic head. The migration of cells to these positions and their subsequent patterning give rise to specific structures, e.g. brain, branchial arches, eye, ear etc. The cranial motor neurons arise from the basal plate of neural tube. One subtype of motor neurons extends their axons through the dorsal root, hence, termed dorsal-exiting motor neurons (dMNs). The axons of the other subtype called ventral-exiting motor neurons (vMNs) follow the ventral trajectory [1]. 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. While sm neurons (vMNs) avoid sensory ganglia, bm/vm neurons (dMNs) exit close to the incoming sensory fibers and typically invade nearby cranial sensory ganglia [2].

Developing axons are navigated to their targets by binding of guidance molecules to receptors on the growth cone plasma membrane leads to the activation of intracellular signaling cascades. This results in dynamic change in the cytoskeleton and subsequent directional axon extension and target recognition [3]. Biochemical and genetic studies have revealed a variety of families of axon guidance molecules, including *netrins*, *slits*, *semaphorins* and *ephrins* [4]. The *semaphorins* exert their effects by binding with signal transducing *plexins* and ligand binding *neuropilins* (*Npn-1* and *Npn-2*). *Plexins* are a family of transmembrane proteins that are distributed widely in both vertebrates and invertebrates [5]. *Neuropilins* are cell surface glycoproteins, initially described by Fujisawa and colleagues as an orphan receptor expressed in the tadpole *neuropilin* [6]. During a search for other *semaphorin* receptors, a *neuropilin-1* related molecule, *Npn-2*, was identified [7,8].

Neither *Npn-1* nor *Npn-2* is able to convey *semaphorin* signals on their own [9] rather they form complexes with *plexins* [8,10-12]. *Plexins* are subdivided into 4 subclasses (A, B, C & D) based on the structural criteria. The largest subfamily is the *plexin-A* with four members (A1, A2, A3 & A4), followed by the *plexin-B* subfamily with three members (B1, B2 & B3). Subfamilies C and D contain only one member each (C1 and D1). In the chicken genome only seven *plexins* (*plexin-A1*, -A2, -A4, -B1, -B2, -C1 & -D1) are found in contrast to mammals, where nine *plexins* have been identified [13].

Plexin-As are the best characterized among *plexins*. They are shown to mediate homophilic cell-cell adhesion in a calcium-dependent manner [14]. Furthermore, they are shown to mediate effects of membrane-bound class-6 *semaphorins* in a *neuropilin*-independent manner [15,16]. An interaction of *plexin-B1* with *Sema4D* has been described, but little is known about the role of *plexin-Bs* *in vivo* [17,18]. *Plexin-C1* is demonstrated to interact with *Sema7A* [19], although the only functional study available to date indicates Integrins rather than *plexin-C1* as the function-mediating receptor for *Sema7A* [20]. *Plexin-D1* finally is linked to the development of the heart and the vascular system consistent with its predominant expression in endothelial cells [21-23].

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Despite the fact that *plexin*-As and *neuropilins* are involved in the formation of neuronal circuits, the temporal changes of their expression patterns during central nervous system development have not been analyzed in detail. For a better understanding of the diverse roles of *plexin*-As and *neuropilins*, we decided to assess their expression patterns in chick embryos. Here, we have analyzed the expression of *plexin*-As (*plexin-A1*, *-A2*, and *-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) in the embryonic head. We have also focused on their expression by cranial motor neurons in the brain stem, which settle down into certain cranial nuclei, innervating the extraocular muscles, branchial arch derived muscles and tongue muscles.

Materials and methods

Chick 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. Eggs were incubated in a humidified (80%) atmosphere at 37.8°C for the desired length of time and staged [24]. The embryos were fixed by 4% PFA in PBS or Dent's fixative for four hours to overnight at 4°C. The hindbrains were opened dorsally along the roof plates and dissected free from the rest of the brain and the surrounding mesenchyme.

Digoxigenin-labelled RNA probe synthesis

Digoxigenin labeled sense and anti-sense RNA probes for the chick *plexin*-As (*plexin-A1*, *plexin-A2* and *plexin-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) were synthesized from the template plasmids provided by Esther T Stoeckli, Institute of Zoology, University of Zurich, Switzerland and Gera Neufeld, Israel Institute of Technology, Israel, respectively. In brief, 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 50-100 ng/µl of probe was used for *in situ* hybridization.

In situ hybridization

In situ hybridization was performed by modified protocol as described by [25]. Briefly, the embryos were washed 2 times (5 min each) in PBT at room temperature (RT) with gentle rocking. They were subjected to bleach with 6% hydrogen peroxide in PBT for 1 hr, followed by washing twice for 5 min with PBT. The embryos were treated with proteinase-K in PBT (10 µg/ml) for up to 10 min at RT. After rinsing carefully with PBT they were immediately post-fixed in 4% buffered formaldehyde for 30 min at RT. Then washed twice for 5 min with PBT at RT and placed the embryos in a mix of PBT: hybridization buffer (1:1) at RT and allowed them to be settled down at the bottom of the vials. This step was repeated by using hybridization buffer. The hybridization buffer was replaced with fresh buffer and incubated at 65°C for 1 hr. It was followed by hybridization with hybridization buffer-probe mix

(0.05–0.1 µg/ml). The hybridization mix was pre-warmed and the embryos were incubated with hybridization mix overnight at 65°C. They were allowed for post-hybridization washes (2X SSC/CHAPS and 0.2X SSC/CHAPS, 20 min each at 65°C) followed by KTBT buffer at RT for 10 min. The embryos were blocked by 10% FCS in KTBT for 4 hrs at RT and incubated with Anti-DIG-AP/ KTBT (1: 2000) overnight at 4°C. Then washed 6 times in KTBT (1 hour each) followed by two washes in AP buffer (20 min each) at RT. Finally, the antibody was detected by using NBT/BCIP in AP (20 µl/ml).

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.

Results and discussion

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. We analyzed the expression of *plexin*-As (*plexin-A1*, *plexin-A2* and *plexin-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) found in the chicken genome during embryonic development of head and brain stem. We analyzed the expression patterns of *plexin*-As (*plexin-A1*, *plexin-A2* and *plexin-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) in chick embryos by *in-situ* hybridization (whole-mount and flat-mount) using relevant mRNA probes. Our results showed expression in orbit, otic vesicle, branchial arches, cranial ganglia and hindbrain.

Orbital expression

Periorbital mesenchyme of neural crest origin accumulates and later gives rise to the iris, sclera, and cornea [26]. We observed that both *plexin-A2* and *Npn-1* expressed in the periorbital mesenchyme (Figure), supporting their co-operative roles in eyeball development. Besides, *plexin-A1* expression was found in the lens (Figure). The lens has been shown to be the source of a chemorepellent activity toward retina [27,28]. *Npn-1* and *Npn-2* signaling are required for proper innervations of the mouse cornea [29]. Knockdown of *plexin-A1* or *plexin-A2* result in striking and reproducible axon guidance defects of oculomotor nerve [30]. These observations together with our data suggest the comprehensive role of *plexin-A1*, *plexin-A2* and *Npn-1* in development and innervations of eye.

Expression in the otic vesicle

The otic vesicle is an intermediate step in inner ear development between the initial formation of the otic placode and the differentiation into vestibular and auditory structures. The dorsolateral otic vesicle gives rise to the vestibular organ, whereas the ventromedial half forms the cochlear. Many genes that determine this differentiation are already localized to specific areas of the otic vesicle before any obvious morphologic changes are apparent [31,32]. We found expression of *plexin-A1* throughout the otic vesicle (Figure 1a), while restricted expression (dorsal) was evident for *Npn-1* (Figure 1c). But *plexin-A2*, *plexin-A4* and *Npn-2* did not express in the otic vesicle. The region of the otic vesicle expressing *Npn-1* (Figure 1c) correlates with that expressing *Dlx5* (Merlo *et al.*, 2002). Mice lacking this transcription factor have dysmorphogenesis of the vestibular organ (Merlo *et al.*, 2002). All these observations in the developing otic vesicle suggest that *plexin-A1* and *Npn-1* play role in controlling the subsequent morphology of the

vestibular and auditory derivatives or in their innervations.

Branchial arch expression

The branchial arches consist of a mesodermal core containing muscle precursor cells surrounded by a peripheral population of neural crest cells, which will give rise to skeletal and connective tissue [33,34]. We found expression of *Npn-2* in the branchial arches (Figure 1e) supporting innervations of arch derived muscles. The bm/vm (dMNs) motor neurons in hindbrain innervate muscles differentiating within the branchial arches that derive from myogenic cells emigrating from the ventral edge of the cranial paraxial mesoderm [35,36]. A parallel situation exists in the case of precursors of the limb musculature that migrate from the ventrolateral edge of the trunk somites [37], 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* [38], 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 [35,36].

Expression in the cranial ganglia

Neural crest cells (NCCs) that do not invade the branchial arches generate cranial sensory ganglia [39]. *Npn-2* is preferentially expressed in NCCs emigrating from r2 and giving rise to trigeminal ganglia [40] and its signaling is essential for trigeminal ganglion condensation (Gammill *et al.*, 2007). *Neuropilins* control cranial gangliogenesis and axon guidance through NCCs [41]. We found expression of *plexin-A4* and *Npn-2* in both ganglia and motor neurons (trigeminal and vago-accessory, Figure 1c and 1e) supports their roles in trajectory of respective cranial nerves. This pairing could be responsible for the signal produced by the ganglion to direct the migration and outgrowth of the motor neuron cell bodies and their axons [42,43].

Hindbrain expression

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 [44-47]. 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.

Subpopulations of cranial motor neurons express different *semaphorin* receptors and make divergent decisions along their axonal trajectories. We analyzed the expression of chick *plexin-As* (*plexin-A1*, *-A2* and *-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) in the flat-mounted hindbrains of developing chick embryos (Figure 2a-2i). At 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 (Figure 2a). The expression patterns of *plexin-A2* and *plexin-A4* were found almost similar in the hindbrain (Figure 2d and 2g). Both expressed into two longitudinal domains medially which were expanded gradually lateral from r2-8. *Plexin-A4* expression was much stronger at the level of r7-8 (Figure 2d) but r6-8 in case of *plexin-A4* (Figure 2g), both of which became narrower close to

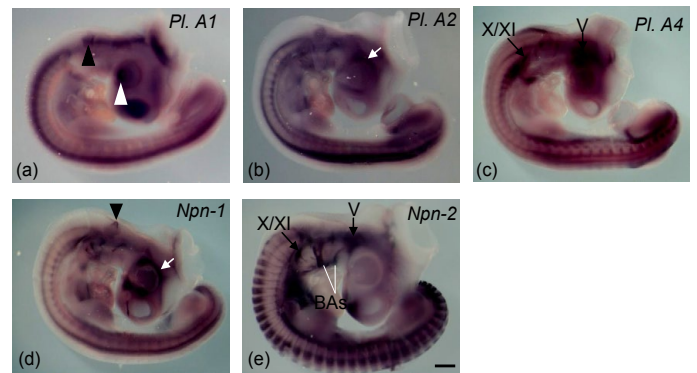


Figure 1. *Plexin-As* (*-A1*, *-A2*, and *-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) expression in the embryonic chick head and brain stem at stage 21 (a-e). Black and white arrow heads indicate expression in the otic vesicle (a and c) and lens (a), respectively. White arrows show expression in the periorbital mesenchyme (b and d) and black arrows in the cranial ganglia (c and e). V= trigeminal ganglia, X/XI= vago-accessory ganglia and BAS= branchial arches. Scale bar in all images= 250 μ m.

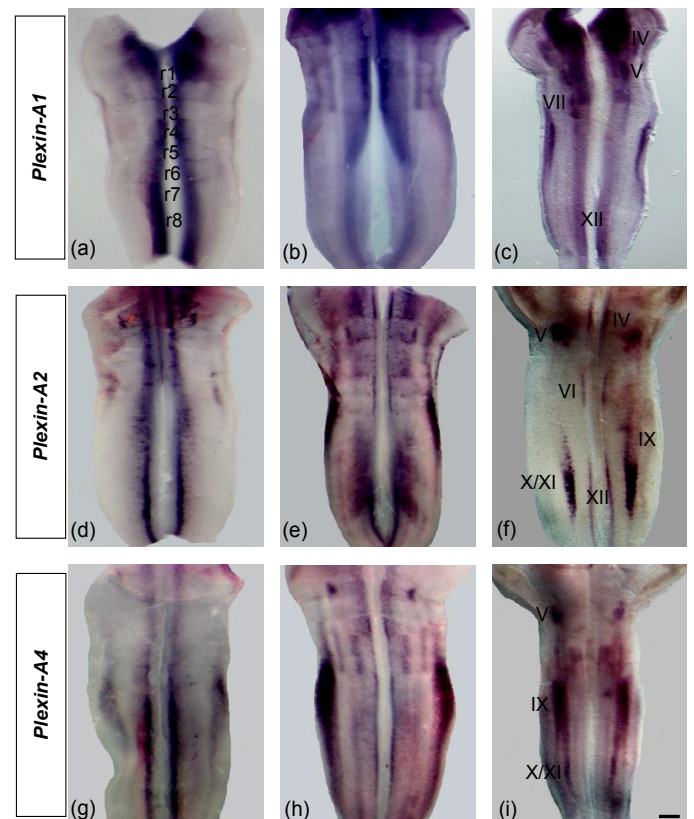


Figure 2. Expression of *plexin-As* (*-A1*, *-A2* and *-A4*) in the flat-mounted hindbrains (open book) of chick embryos (a-i). At HH 19, *plexin-A1*, *-A2* and *-A4* expressions appear as two longitudinal stripes throughout the ventral neural tube (a, d and g). At HH 23, the expressions expand toward the prospective nuclei within the rhombomeric boundaries (b, e and h). At HH 27, *plexin-A1*, *-A2* and *-A4* are expressed in the cranial nuclei (c, f and i) as marked by Roman numerical (III= oculomotor, IV= trochlear, V= trigeminal, VI= abducens, VII= facial, IX= glossopharyngeal, X/XI= vago-accessory, XII= hypoglossal). Scale bar in all images = 200 μ m.

the floor plate. At HH 23, *plexin-A1*, *-A2* and *-A4* expressions were expanded towards the prospective nuclei within the rhombomeres of hindbrain (Figure 2b, 2e and 2h). At HH 27, we compared the expression patterns of these genes with the expression of motor neuron marker *Islet-1* [48]. Our results showed that *plexin-A1* expressed in the

trochlear (vMNs), trigeminal (dMNs), facial (dMNs) and hypoglossal (vMNs) nuclei; *plexin-A2* in the trochlear (vMNs), trigeminal (dMNs), abducens (vMNs), glossopharyngeal (dMNs), vago-accessory (dMNs) and hypoglossal (vMNs) nuclei and *plexin-A4* only in dMNs-trigeminal, glossopharyngeal and vago-accessory nuclei (Figure 2c, 2f and 2i). Our expression data for *neuropilins* demonstrated that both *Npn-1* and *Npn-2* expressed strongly at the level of r1-2 and r7-8 (HH 19). However, *Npn-2* expression was much stronger and extensive than *Npn-1* (Figure 3a and 3d). The expressions were distributed towards the prospective nuclei within the rhombomeric boundaries of hindbrain at HH 23 (Figure 3b and 3e). By HH 27, most cranial motor neurons migrate to their final positions in hindbrain [49]. Here, we compared the expression of these two genes (*Npn-1* and *Npn-2*) with the expression of motor neuron marker *Islet-1* [48]. We found that *Npn-1* expressed in the trochlear (vMNs), trigeminal (dMNs), abducens (vMNs), glossopharyngeal (dMNs), vago-accessory (dMNs) and hypoglossal (vMNs) nuclei, whereas *Npn-2* in trochlear (vMNs), trigeminal (dMNs), facial (dMNs), glossopharyngeal (dMNs), vago-accessory (dMNs) and hypoglossal (vMNs) nuclei (Figure 3c and 3f). These results show that *plexin-A1* and *plexin-A2* are expressed by both dMNs and vMNs but *plexin-A4* only by dMNs. Besides, *Npn-1* and *Npn-2* are expressed by both sets of hindbrain motor neurons (dMNs and vMNs).

The expression data in mouse and chick embryos suggested that the multimeric surface receptors for secreted class-III *semaphorins* are *neuropilins* and/or *plexins* [13,50,51]. 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 [52]. Besides, *Npn-2* is expressed very strongly in dorsal commissural neurons, ventral populations of motor neurons, and in the floor plate [53,54]. 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 [13]. In the chick lumbo-sacral spinal cord, premigratory motor neurons expressed *plexin-A1*, *plexin-A2*, *Npn-1* but not *Npn-2* [13]. 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 [13]. [55], confirmed and extended the observation of [13]; showing dynamic patterns of expression of *plexin-A2* and *Npn-2*. They reported both expressions in spinal motor neurons or their progenitors at premigratory stage which expressed in medial and lateral subsets of motor neurons at the migratory stage. We observed that *plexins* and *neuropilins* expressed in the ventral neural tube at early migratory stage of hindbrain development. At the late migrating stage, the expressions appear dorso-lateral along with persistent expression medially. After most of the cranial motor neurons find their final positions, their expressions appear into two domains giving rise to medial (vMNs) and lateral nuclei (dMNs). The medial nuclei located close to the floor plate, while lateral close to the rhombomeric boundaries. All *plexins* (except *plexin-A4*) and *neuropilins* (*Npn-1* and *Npn-2*) were detected in both medial and lateral nuclei of chick hindbrain. *Plexin-A2* expression was similar to *Npn-1* in cranial nuclei. These combinatorial and differential expression patterns of *plexin-As* and *neuropilins* in the cranial nuclei suggest their roles in neural circuit formation for selective sets of cranial nerves during hindbrain development.

The differentiation of neuronal subtype (dMNs and vMNs) is controlled by the *Phox* and *LIM* homeodomain factors [56,57]. 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 [58]. Our expression data showed that these cranial motor neurons (dMNs) strongly express *plexin-A4* and *Npn-2*. This raises the possibilities that *Nkx2.9* acts to maintain the function of *plexin-A4* and *Npn-2* during axon guidance of dMNs in hindbrain. Loss of *Nkx6.1*, *Nkx6.2* and *Pax6* during somatic motor neuron development deletes abducens and hypoglossal motor neurons [59]. We found that *plexin-A2* and *Npn-1* co-expressed in abducens and hypoglossal (vMNs) motor neurons. From this observation, it is probable that the action of *plexin-A2* and *Npn-1* in 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 [56]. *Lhx3* and *Lhx4* are important for the vMN differentiation and the formation of the medial motor column [57]. These reports are also correlated with our expression data suggesting that *Phox* and *Lhx* genes might control the function of *plexins* and *neuropilins* in axon guidance of dMNs and vMNs. Considering the available reports mentioned above together with our observations, we can say 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 transcription factors involved in axon guidance of cranial nerves.

In conclusion, the expression patterns of the *plexins* and *neuropilins* presented in the cranium and hindbrain will undoubtedly reveal further, more refined combinations of receptors for ligand localizations. The current study highlights several possible roles for *plexins* and *neuropilins* in directing early cranial development, particularly the patterning of early axon tracts but also the guidance of cell migration and morphologic changes. Further research can be conducted to explore their functional roles during head and brain development.

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Conflict of interests

The authors declare that they have no competing interests.

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