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Neuronal expression patterns of the PlexinA family during zebrafish development

Sarah E. Emerson, Sarah E. Light, Alicia M. Ebert*

Department of Biology, University of Vermont, Burlington, VT 05405, USA

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ABSTRACT

Plexins (Plxns) and Semaphorins (Semas) are key signaling molecules that regulate many aspects of development. Plxns are a family of transmembrane protein receptors that are activated upon extracellular binding by Semas. Activated Plxns trigger intracellular signaling cascades, which regulate a range of developmental processes, including axon guidance, neuronal positioning and vasculogenesis. Semas are a large family of both transmembrane and secreted signaling molecules, and show subtype specific binding to different Plxn family members. Each Plxn can play different roles in development, and so tightly regulated temporal and spatial expression of receptor subtypes is critical to ensure appropriate signaling. Here we elucidate the expression profiles of the *plxnA* family, *plxnA1a, A1b, A2, A3 and A4* at 18, 24, 36, 48, 60 and 72 h post fertilization in the developing zebrafish. We show that PlxnA family members are expressed in neuronal tissues during zebrafish development, but exhibit key differences in expression within these tissues. We also highlight that *plxnA1* has two genes in zebrafish, *A1a* and *A1b*, which show divergences in expression patterns during early development.

1. Introduction

Plexins (Plxns) and Semaphorins (Semas) are essential developmental signaling proteins. They were initially identified as axon guidance cues, mediating actin dynamics at developing axonal growth cones (Luo et al., 1993). It is now appreciated that Plxns and Semas play additional roles in development across multiple systems, including vasculogenesis (Serini et al., 2003), early eye development (Ebert et al., 2014; Emerson et al., 2017), immunity (Shi et al., 2000) and bone development (Behar et al., 1996), among others. Plxns are a large family of transmembrane protein receptors, which signal upon binding by specific members of the Sema family. There are 2 invertebrate Plxns (PlxnA and B) and 9 vertebrate Plxns (PlxnA1-A4, B1-3, C1 and D1) (Tamagnone et al., 1999). Due to a whole genome duplication event in zebrafish, many genes have two copies (Glasauer and Neuhauss, 2014). An in-depth search for all plxnA family members revealed that zebrafish have two *plxnA1* genes (*plxnA1a* and *plxnA1b*). There are 8 subclasses of Semas, Sema1 and 2 are invertebrate forms, 3–7 are vertebrate forms and V is virally expressed (Neufeld and Kessler, 2008). There are transmembrane (Sema classes 4, 5 & 6), GPI anchored (Class 7), and secreted Semas (Class 3 and V) (Kolodkin et al., 1993). Secreted Semas require additional neuropilin co-receptors in a complex with Plxns in order to signal (He and Tessier-Lavigne, 1997; Janssen et al., 2012; Kolodkin et al., 1997).

All Plxns share a common 500 amino acid extracellular SEMA domain that mediates Sema and Plexin binding. The SEMA domain has an atypical 7-blade propeller structure, and binds to other SEMA domains in a head to head conformation (Love et al., 2003). The SEMA domain is highly conserved, yet varies subtly between the different family members (Koppel et al., 1997), leading to restricted combinations of possible Plxn/Sema complexes. Extracellular Plxn domains include 3 repeating PSI (plexin, semaphorin, integrin) domains and multiple IPT (Ig-like, plexins, transcription factor) domains (Bork et al., 1999). Inside the cell, Plxns have a characteristic split GTPase activating protein (GAP) domain (C1 and C2) (Rohm et al., 2000), with a Rho binding domain (RBD) in-between (Oinuma et al., 2004). Once activated, folded GAP domains initiate multiple intracellular signaling cascades to ultimately regulate many cellular events, including but not limited to: cell migration via mediating cytoskeletal dynamics (Curreli et al., 2016; Deo et al., 2004; Rosslenbroich et al., 2005; Schmidt and Strittmatter, 2007; Serini et al., 2003), integrin-mediated adhesion (Basile et al., 2007; Choi et al., 2014; Walzer et al., 2005), transcriptional regulation (Emerson et al., 2017), cell proliferation (Ebert et al., 2014; Emerson et al., 2017) and apoptosis (Bagnard et al., 2001; Gagliardini and Fankhauser, 1999; Wehner et al., 2016).

The same Sema classes can activate multiple different Plxn family members. In this paper, we focus on the PlxnA family, which can be activated by both class 3 and class 6 Semas (Renaud et al., 2008; Suto

* Corresponding author.
E-mail address: amebert@uvm.edu (A.M. Ebert).

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Abbreviations used

CG	cranial ganglia
CMZ	cilliary marginal zone
DT	dorsal thalamus
FB	fin bud
Fbr	forebrain
GAP	GTPase activating protein
HB	hindbrain
HG	hatching gland
Hi	intermediate hypothalamus
Hpf	hours post fertilization
INL	inner nuclear layer
IPL	inner plexiform layer
L	lens
LF	lateral forebrain
Lfb	lateral forebrain bundle
lTeO	lateral optic tectum
MO	medulla oblongata
NR	neural retina
OpV	optic vesicle
OV	otic vesicle
P	pons
Plxn	Plexin
Po	pre-optic region
PTd	dorsal part of posterior tuberculum
PTv	ventral part of posterior tuberculum
R	retina
RBD	Rho binding domain
RGC	retinal ganglion cell layer
RPC	retinal precursor cell
SC	spinal cord
Sema	Semaphorin
T	thalamus
TeO	optic tectum
V	ventricle
VEGFR2	vascular endothelial growth factor receptor 2
V3	3rd ventricle

et al., 2005, 2007). Despite the high level of cross talk within the PlxnA family, each member has distinct roles in vertebrate development. PlxnA1 plays roles in axon guidance for retinal axons crossing the optic chiasm midline with Sema6D and Nr-CAM (Kuwajima et al., 2012). Additionally, PlxnA1 with Sema6D controls endothelial cell migration during heart development in combination with VEGFR2 (Toyofuku et al., 2004). PlxnA2/Sema6A signaling has been shown to be important for axon migration in the cerebellum (Renaud and Chedotal, 2014; Renaud et al., 2008), guidance of the corticospinal tract (Rünker et al., 2008), patterning of the retina (Sun et al., 2013) and the maintenance of cohesion and proliferation of retinal precursor cells (Ebert et al., 2014; Emerson et al., 2017), among others. PlxnA3 (also referred to as *sidetracked* in zebrafish) is important for the guidance of pioneer intraspinal motor neurons as they exit the spinal cord (Palaisa and

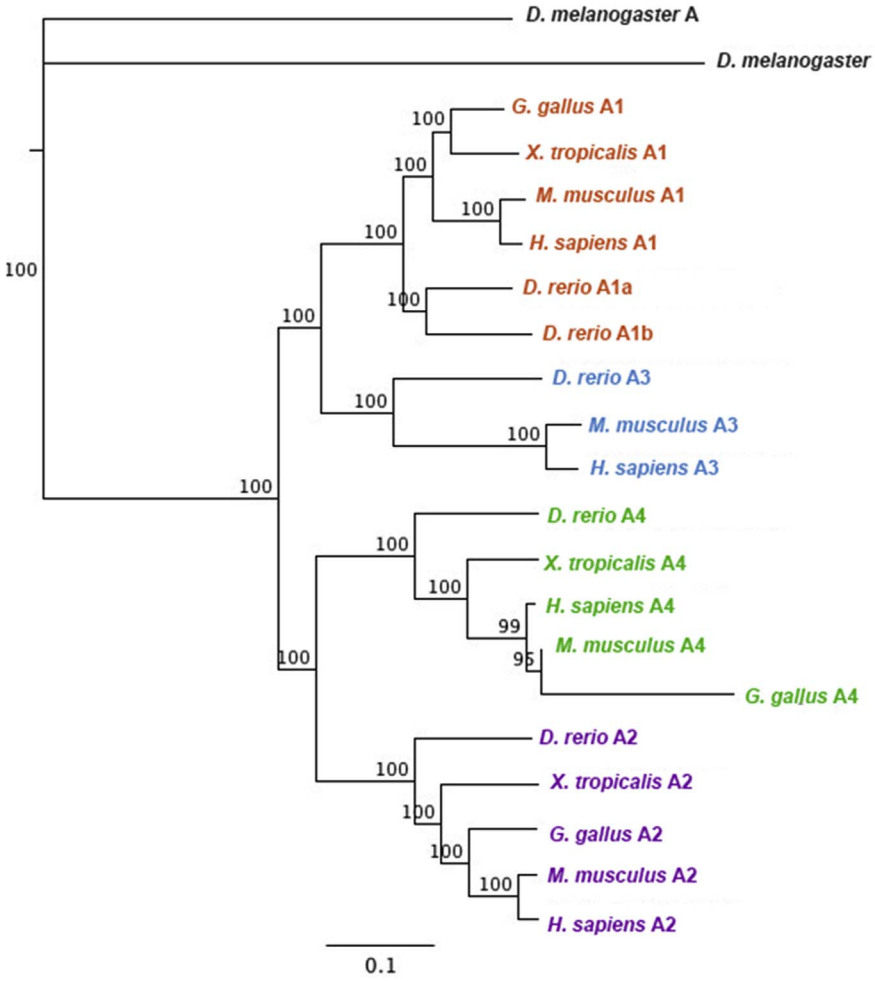

Fig. 1. Phylogeny of PlxnA family members.
Phylogenetic tree displaying the relationships of PlxnA family member full-length amino acid sequences. Numbers indicate maximum likelihood bootstrap values.

Granato, 2007), facial branchiomotor nerve axon guidance in conjunction with PlxnA4 (Schwarz et al., 2008), and pruning of hippocampal axon branches during brain development (Bagri et al., 2003). PlxnA4 is important for axon guidance during development. It guides mossy fibers of the hippocampus (Suto et al., 2007), and both sympathetic and sensory neurons in dorsal root ganglia (Haklai-Topper et al., 2010). Furthermore, in retinal development, PlxnA4 is important for guiding axons to their correct sub-laminar targets to correctly pattern the inner plexiform layer (Matsuoka et al., 2011).

It is clear that PlxnA/Sema signaling is essential in development, and although some of the functions of the PlxnA family members are known, limited data exists for their expression. Here we elucidate the specific expression patterns of each PlxnA across the early developmental stages of the zebrafish. We show that there are distinct temporal and spatial differences in expression patterns of each family member, including the two novel *plxnA1* paralogs in zebrafish, *A1a* and *A1b*. This data will enable a platform from which the developmental roles of specific plxnA genes can be determined.

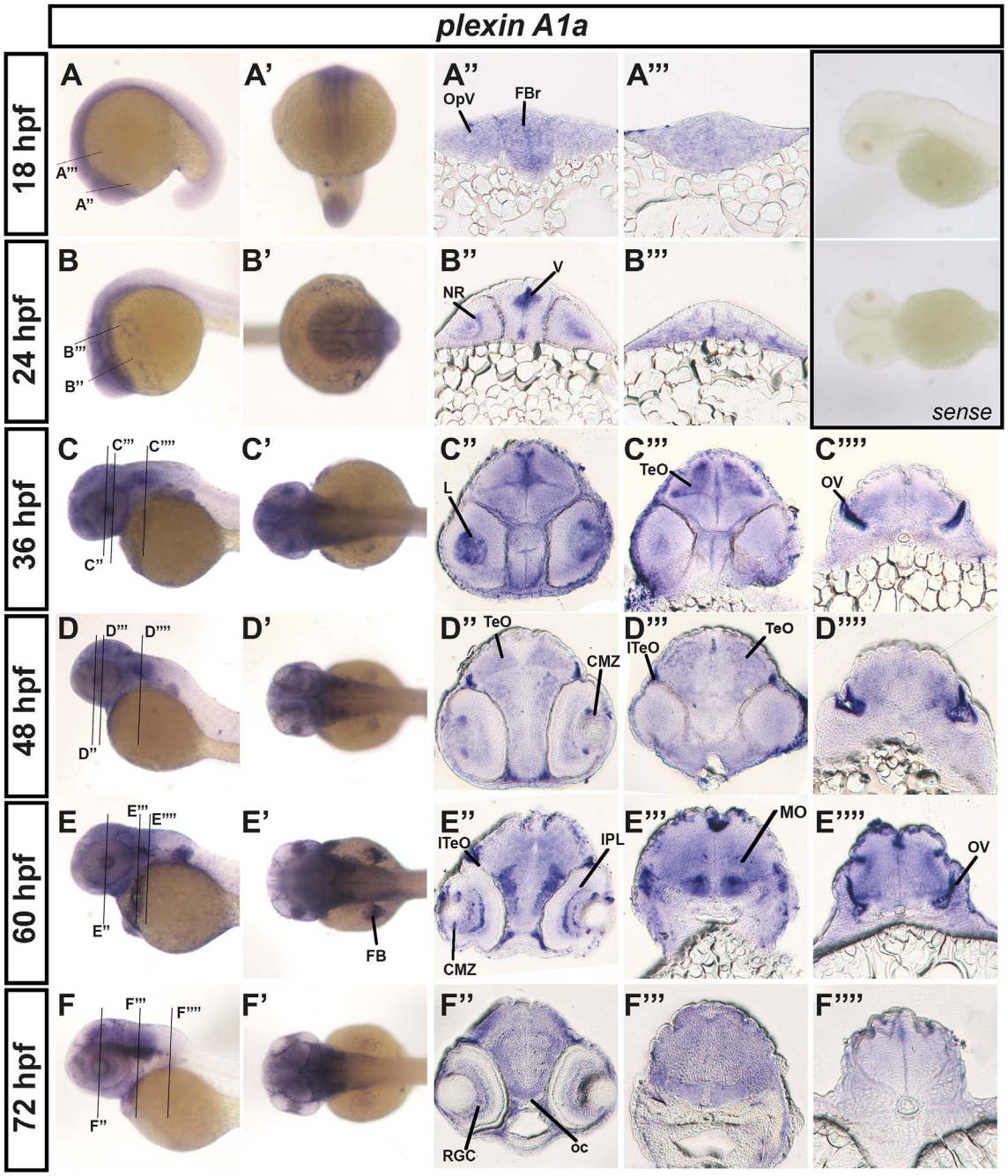

Fig. 2. *plxnA1a* expression in the developing zebrafish.
Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicles, FBr-forebrain, V-ventricle, NR-neural retina, L-lens, TeO- optic tectum, OV- otic vesicle, CMZ-cilliary marginal zone, lTeO- lateral optic tectum, MO- medulla oblongata, IPL-inner plexiform layer, RGC-retinal ganglion cell layer, FB- fin bud.

2. Results

2.1. PlxnA family phylogenetic tree

A neighbor–joining consensus phylogenetic tree of full-length amino acid sequences was generated in Geneious R10 (Kearse et al., 2012). Analysis shows that each PlxnA family member clusters in the same clade as expected across species (Fig. 1). Zebrafish (*D. rerio*) have two *plxnA1* genes, *plxnA1a* and *A1b,* due to a whole genome duplication (Glasauer and Neuhauss, 2014), resulting in two protein products. These group with orthologous PlxnA1s of other species. Protein alignments for the SEMA and Ras-GAP domains of the plxnA family show that although family members are overall well conserved, the majority of differences occur in the SEMA binding domains (Supplemental Fig. 1).

2.2. plxnA1a expression

plxnA1a is expressed in the undifferentiated retinal precursor cells of the optic vesicles (OpV), and the forebrain (FBr) at 18 hpf (Fig. 2A-

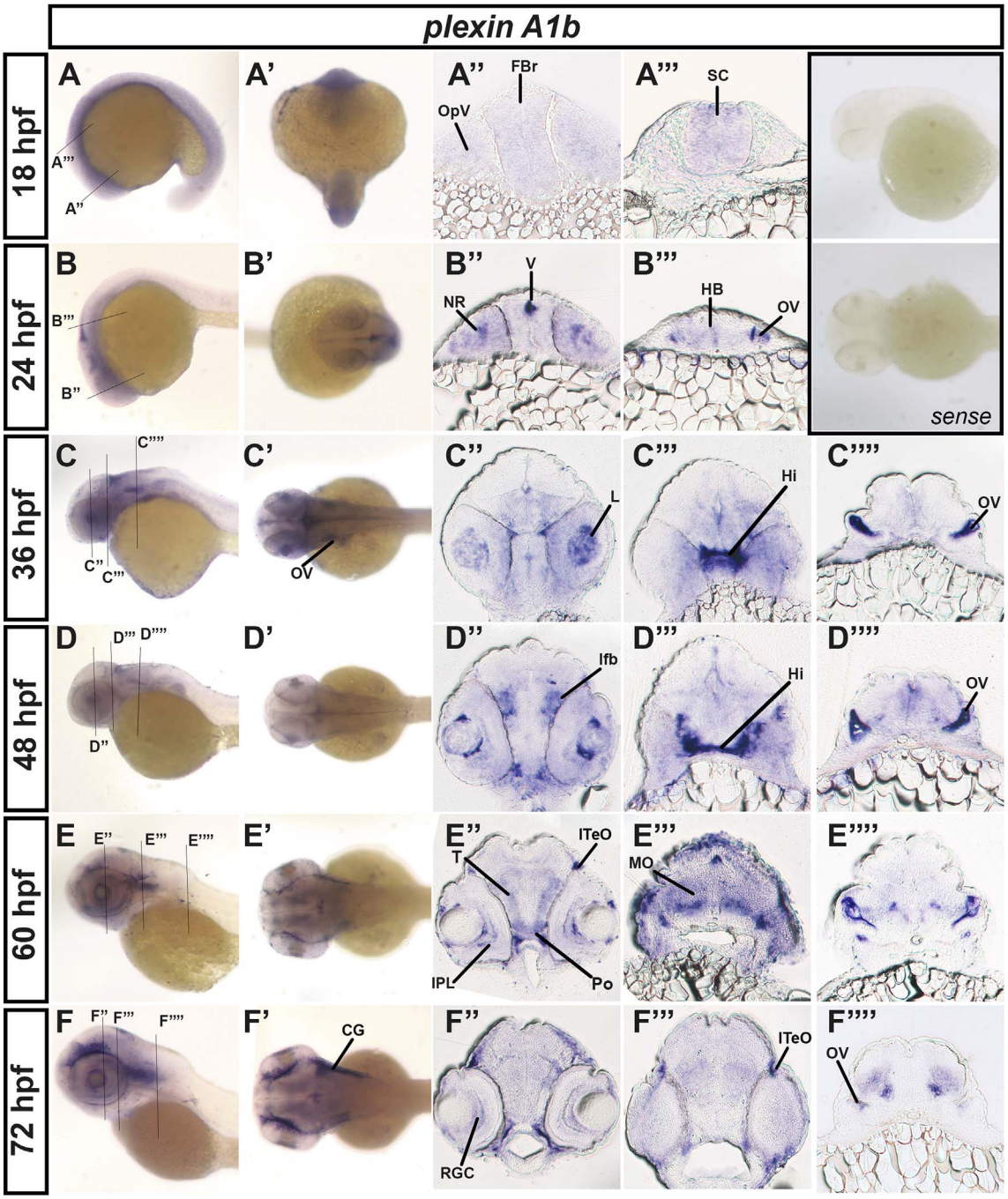

Fig. 3. *plxnA1b* expression in the developing zebrafish.
Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicle, Fbr, forebrain, SC-spinal cord, NR-neural retina, V- ventricle, HB- hindbrain, OV- otic vesicle, L-lens, Hi-intermediate hypothalamus, lfb-lateral forebrain bundle, Po-pre-optic region, T-thalamus, lTeO-lateral optic tectum, MO- medulla oblongata, RGC- retinal ganglion cell layer, IPL-inner plexiform layer, CG-cranial ganglia.

A''). At 24 hpf, staining is seen adjacent to the lens in the neural retina (NR) and in the forebrain ventricle (V) (Fig. 2B–B''). *plxnA1a* is expressed in the optic tectum (TeO) at 36 and 48 hpf (Fig. 2C''', 2D'', 2D'''). In the developing eye, *plxnA1a* is expressed in the retinal ganglion cell layer (RCG) and the inner plexiform layer (IPL) of the retina at 60 and 72 hpf (Fig. 2E'', F''). Staining is seen in the lateral forebrain at 60hpf and around the optic chiasm (oc) at 72 hpf (Fig. 2F''). *plxnA1a* is expressed in the otic vesicle (OV) of the developing ears during all stages of development shown (Fig. 2C''''-F'''').

2.3. plxnA1b expression

plxnA1b is expressed in the retinal precursor cells of the optic vesicles (OpV) and the forebrain (FBr) at 18 hpf (Fig. 3A–A''). Staining persists in the neural retina (NR) close to the developing lens at 24 h post fertilization (hpf) (Fig. 3B–B''). *plxnA1b* is expressed in the inner plexiform layer (IPL) at 60 hpf (Fig. 3E'') and retinal ganglion cell layer (RGC) at 72 hpf (Fig. 3F''). As the forebrain develops, *plxnA1b* is expressed first dorsally at 36 hpf (Fig. 3C'') and can be distinctly seen in the lateral forebrain bundle (lfb) by 48 hpf (Fig. 3D''). At 60 hpf,

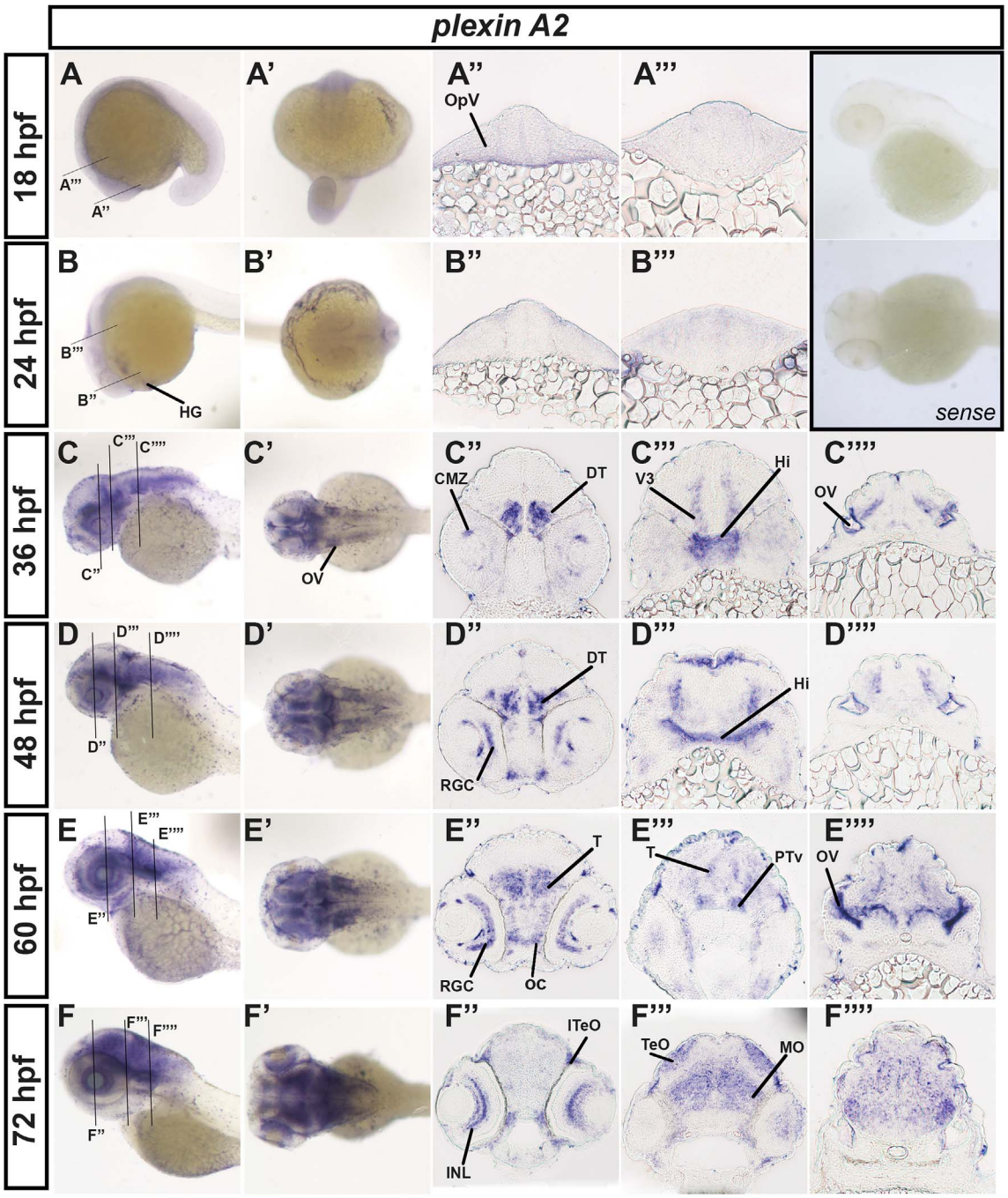

Fig. 4. *plxnA2* expression in the developing zebrafish.
Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OpV- optic vesicle, HG-hatching gland, CMZ-cilliary marginal zone, DT-dorsal thalamus, V3- 3rd ventricle, Hi-intermediate hypothalamus, OV- otic vesicle, oc-optic chiasm, RGC- retinal ganglion cell layer, INL-inner nuclear layer, T-thalamus, PTv-ventral part of posterior tuberculum, lTeO- lateral optic tectum, MO- medulla oblongata.

staining can be seen more ventrally around the pre-optic region (Po) (Fig. 3E”). Additionally, staining is seen in the lateral optic tectum (lTeO) at 60 and 72 hpf (Fig. 3E”, F”-F”’). During development *plxnA1b* is distinctly expressed in the developing ear and in the otic vesicles (OV) (Fig. 3B”, 3C””-F””). At 36 and 48 hpf *plxnA1b* is expressed in the hypothalamus (H) (Fig. 3C”’, D”’) and in the medulla (MO) at 60 hpf (Fig. 3E”’). The cranial ganglia (CG) also express *plxnA1b* at 60 and 72 hpf (Fig. 3E, E’, F, F’).

2.4. *plxnA1a and plxnA1b comparisons*

At 18 somites and 24 hpf, *plxnA1a* and *plxnA1b* are expressed in similar areas. They both show staining throughout the developing optic vesicles (OpV) and developing forebrain (FBr) at 18 somites (Figs. 2A”, 2A”’, 3A” and 3A”’), in addition to the ventricle (V) and neural retina (NR) at 24 hpf (Figs. 2B”, 2B”’, 3B” and 3B”’). However, *plxnA1a* and *plxnA1b* differ in expression patterns by 36 hpf. *plxnA1a* is expressed in the optic tectum (TeO), whereas *plxnA1b* is seen in the intermediate hypothalamus (Hi) (Figs. 2C”’, 3C”’). At 48 hpf, *plxnA1a* and *plxnA1b* are both expressed in the developing lateral forebrain (lfb) (Figs. 2D”

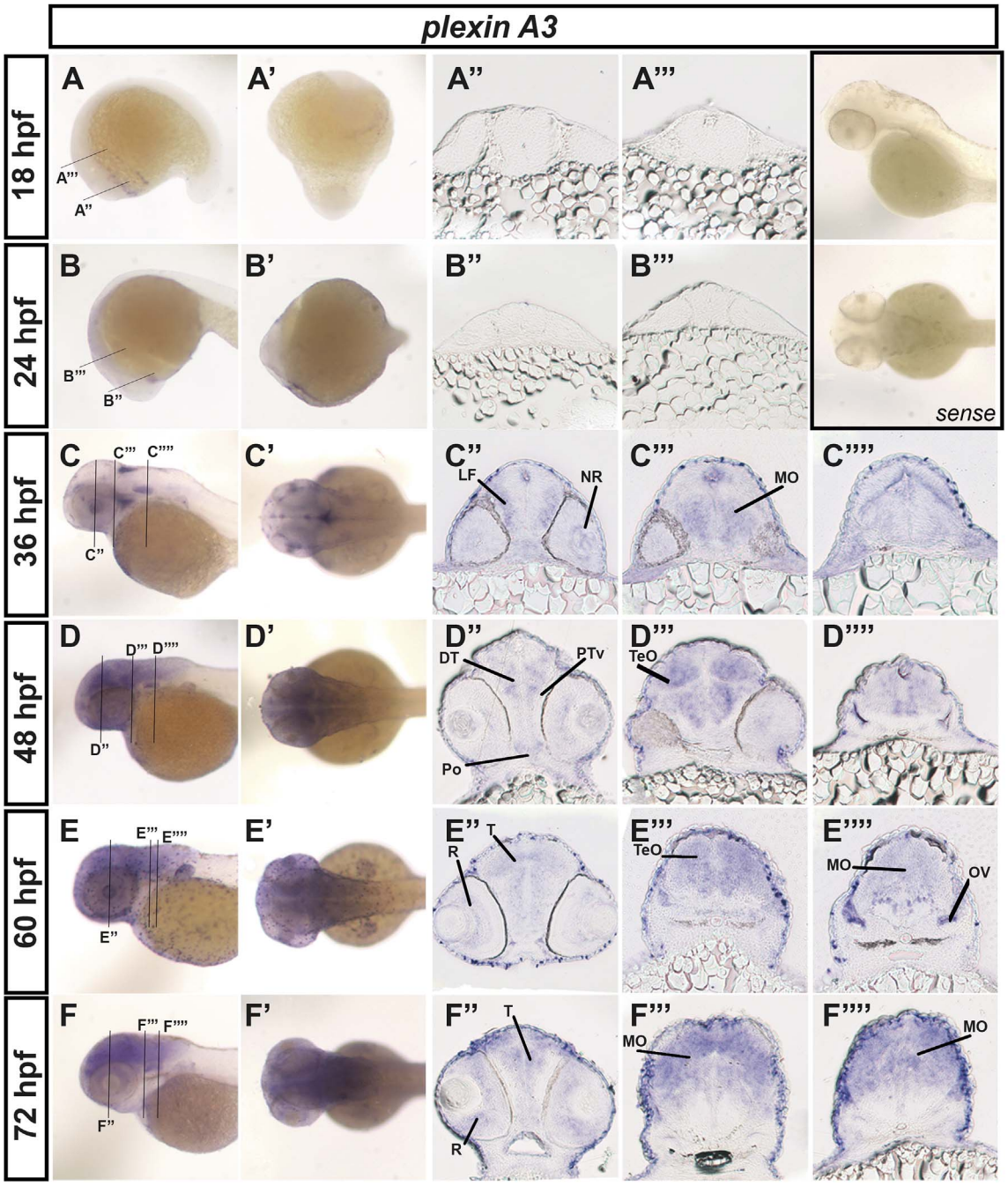

Fig. 5. *plxnA3* expression in the developing zebrafish.
Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A’-F’) whole-mount dorsal. Brightfield sections (A”-F”) forebrain, (A”’-F”’) midbrain and (C””-F””) hindbrain. Embryos were imaged at different developmental time points. (A-A”’) 18 hpf, (B-B”’) 24 hpf, (C-C””) 36 hpf, (D-D””) 48 hpf, (E-E””) 60 hpf, and (F-F””) 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, LF- lateral forebrain, NR-neural retina, MO- medulla oblongata, DT-dorsal thalamus, PTv-ventral part of posterior tuberculum, Po-pre-optic region, TeO- optic tectum, T-thalamus, OV- otic vesicle, R-retina.

and 3D''), although *plxnA1a* staining is more diffuse and can be seen more ventrally than *plxnA1b,* expanding into the ventral thalamus (T). At 60 hpf, *plxnA1a* and *plxnA1b* are both expressed in similar areas throughout the developing forebrain and midbrain (Figs. 2E'', 3E''), however they differ greatly in the developing ventral hindbrain where *plxnA1a* is ubiquitously expressed, and *plxnA1b* is absent (Figs. 2E'''', 3E''''). Both genes are expressed in the developing otic vesicles (OV) throughout the stages of development shown (Figs. 2C''''- 2F'''' and 3C''''- 3F''''). *PlxnA1a* is strongly expressed in the fin buds, especially at 60hpf (Fig. 2E'), whereas *plxnA1b* only shows very faint staining, if any, at all developmental stages shown (Fig. 3C' - F').

2.5. plxnA2 expression

plxnA2 is expressed in the ventral optic vesicles (OpV) and the neighboring mesenchyme at 18 hpf (Fig. 4A–A''), but is not strongly expressed at 24 hpf (Fig. 4B–B'''). Later in development, *plxnA2* is again expressed in the developing eye. It is expressed markedly in the cilliary marginal zone (CMZ) at 36 hpf (Fig. 4C'') and in the retinal ganglion cell layer (RGC) and inner nuclear layer (INL) from 48 hpf to 72 hpf

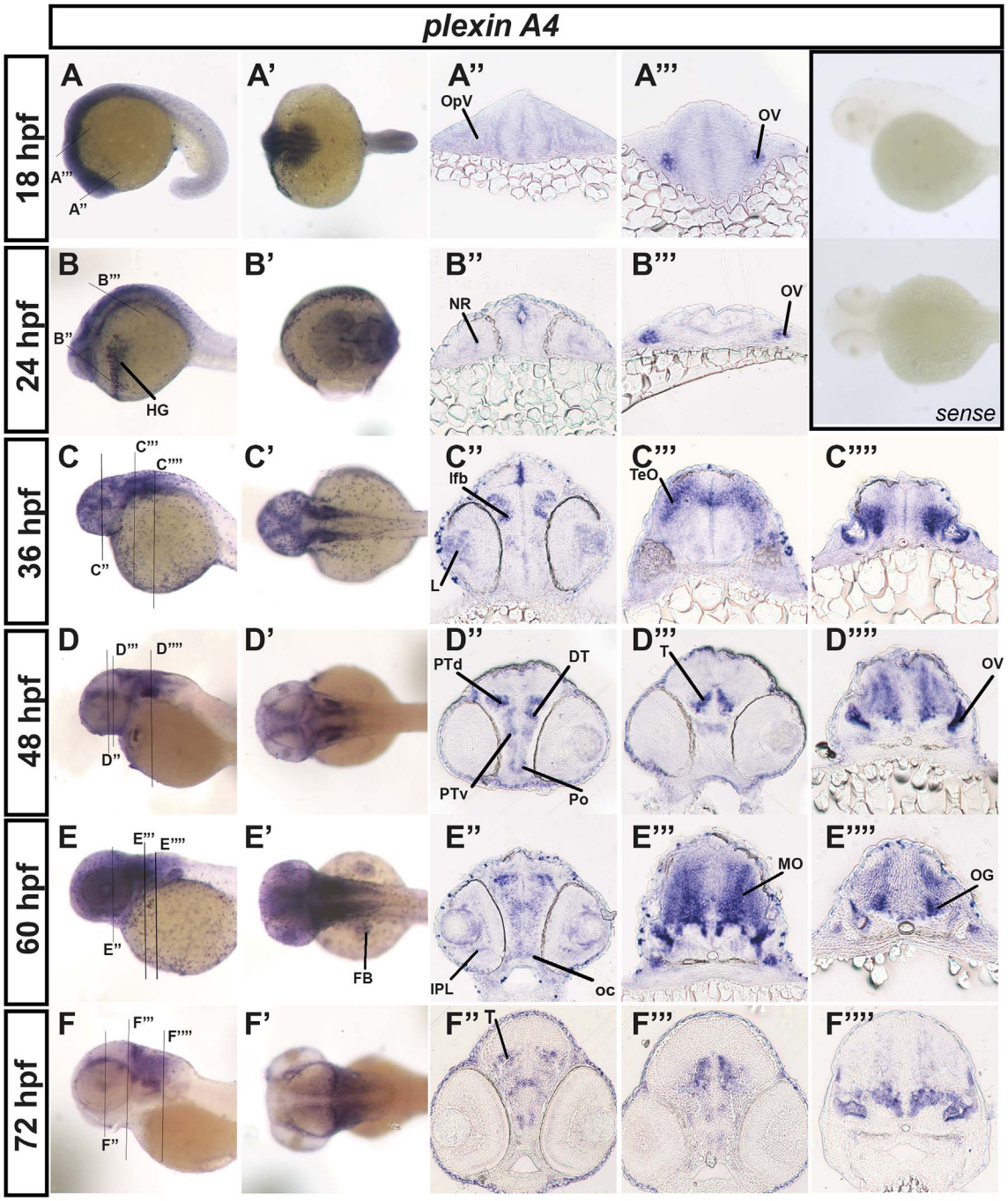

Fig. 6. *plxnA4* expression in the developing zebrafish.
Brightfield images of zebrafish embryos processed for *in situ* hybridization. (A-F) Whole-mount lateral, (A'-F') whole-mount dorsal. Brightfield sections (A''-F'') forebrain, (A'''-F''') midbrain and (C''''-F'''') hindbrain. Embryos were imaged at different developmental time points. (A-A''') 18 hpf, (B-B''') 24 hpf, (C-C'''') 36 hpf, (D-D'''') 48 hpf, (E-E'''') 60 hpf, and (F-F'''') 72 hpf. Lines in (A-F) indicate locations of the sections shown at that time-point. Inset shows sense probe control. Hpf-hours post fertilization, OV- otic vesicle, NR-neural retina, L-lens, lfb-lateral forebrain bundle, TeO- optic tectum, PTd-dorsal part of posterior tuberculum, IPL-inner plexiform layer, T-thalamus, DT-dorsal thalamus, PTv-ventral part of posterior tuberculum, Po-pre-optic region, and MO- medulla oblongata, oc-optic chiasm, OG-otic ganglion, FB- fin bud, HG-hatching gland.

(Fig. 4D”-F”). Staining can also be seen in the optic chiasm (oc) and optic tectum (TeO/lTeO) at 72 hpf (Fig. 4F”-F”’). At 36 hpf, staining can be seen in distinct nuclei of the thalamus (Fig. 4C”), moving ventrally as development proceeds (Fig. 4D”-F”). By 36 hpf, *plxnA2* is expressed in the intermediate hypothalamus (Hi) and the medulla (MO) (Fig. 4C”’, D”’, F”’).

2.6. plxnA3 expression

plxnA3 is not expressed in the developing zebrafish nervous system until 36 hpf (Fig. 5A–B”’). At 36 hpf, *plxnA3* is expressed in the early neural retina (NR) (Fig. 5C”), and persists to 72 hpf in the retina adjacent to the lens (R) (Fig. 5D”-F”). Expression is also seen in the developing optic system in the optic tectum (TeO) at 48 and 60 hpf (Fig. 5D”’, E”’), but is absent by 72 hpf (Fig. 5F”’). Furthermore, *plxnA3* is expressed throughout the developing lateral forebrain (LF), dorsal thalamus (DT), and the ventral tuberculum (PTv) from 36 hpf onwards (Fig. 5C”-F”). Staining can be seen in the medulla at 60 and 72 hpf (Fig. 5E””, F”’, F””), and the developing ear from 36 to 72 hpf (Fig. 5C””-F””). Prolonged staining shows expression in motor neurons along the tail, and in the cranial ganglia (Supplemental Fig. 2).

2.7. plxnA4 expression

At 18s *plxnA4* is expressed throughout the developing eye field in the optic vesicles (OpV) (Fig. 6A”). *plxnA4* has very distinct staining in the lateral forebrain (lfb) from 36 hpf (Fig. 6C”), spreading dorso-ventrally throughout the thalamus and the tuberculum (T/PTd/PTd) towards the pre-optic region (Po) at 48 hpf (Fig. 6D”), and is most ventrally in the optic chiasm (oc) at 60 and 72 hpf (Fig. 6E”-F”). *plxnA4* is expressed in the developing visual system from 24 hpf in the retinal precursor cells of the neural retina (NR) (Fig. 6B”), the lens (L) at 36 hpf (Fig. 6C”) and the inner plexiform layer (IPL) at 60 hpf (Fig. 6E”). *plxnA4* is strongly expressed in the hindbrain dorsal to the otic vesicles (OV) at 48 hpf (Fig. 6D””), in the medulla (MO) (Fig. 6E”’) and in the otic ganglia (OG) at 60 hpf (Fig. 6 E””). *plxnA4* is localized to the otic vesicles (OV) during the stages of early development shown (Fig. 6A”’, B”’, C””-F””).

3. Discussion

Our results show that the PlxnA family have some overlap in expression, but also have distinct differences, as summarized in (Fig. 7). It is not surprising that our results show areas of co-expression, as it is well documented that different Plxns can regulate each other. Sema6A can activate both PlxnA2 and A4, which often share spatial and temporal expression patterns (Haklai-Topper et al., 2010; Renaud et al., 2008; Suto et al., 2005, 2007). PlxnA2 and A4 have been shown to work together to guide migrating axons to their correct targets, via *cis* inhibition with Sema6A (Haklai-Topper et al., 2010; Suto et al., 2007).

Comparing the expression patterns of the PlxnAs to their ligands further supports our findings. As mentioned earlier, Sema3 and Sema6 are ligands for the PlxnA family, and here we are able to confirm that their expression domains overlap in certain areas during development. For example in the retina, *sema6A* and *plxnA2* are expressed in overlapping and neighboring neuronal layers at 72 hpf, *sema6A* in the RGC layer, and *plxnA2* in the RGC layer and the INL (Fig. 4E”-F”), as previously observed (Ebert et al., 2014). *sema3Aa, 3Fa, 3Fb, 3Gb*, *6A* and *6D* are expressed in the optic tectum at 50 hpf onwards (Callander et al., 2007; Ebert et al., 2012) which correlates with the expression of *plxnA1a, A2, A3* and *A4* later in development (Figs. 2 and 4-6). Additionally, *Sema3A, 3C 3Fa, 3Fb, 3Ga*, *3Gb, 6Ba* and *6D* are expressed in the dorso-lateral thalamus at 40–50 hpf (Callander et al., 2007; Ebert et al., 2012), matching the spatial and temporal expression of the *plxnAs* (Figs. 2–6). It is known that *sema3Aa* and *3E* are expressed in the optic chiasm, and that *3Fa* and *3GB* are expressed dorsally to the optic chiasm, forming a repulsive border to migrating axons, guiding RGC axons as they cross the midline (Callander et al., 2007). We show that *plxnAs* are also expressed in this area, *plxnA2 and A4* in the optic chiasm (Figs. 4 and 6), and *plxnA1a and A1b* in neighboring tissues (Figs. 2 and 3). Of the limited expression data in zebrafish, our findings corroborate published results. As mentioned, *plxnA2* is expressed in the ventral optic vesicles at 15 hpf, and the RGC layer and INL in zebrafish at 72 hpf (Ebert et al., 2014), matching our results (Fig. 4A”, D”-F”). We also show that *plxnA4* is expressed in the same neuronal areas in whole-mount at 48 hpf (Fig. 6D), as in Christie et al. (2006). *plxnA3* expression in 24–36 hpf zebrafish has been previously investigated, and expression is seen in developing motor neurons and in the cranial ganglia (Tanaka et al., 2007). We show similar staining patterns at the same ages (Supplemental Fig. 2). In order to observe distinct staining in specific brain regions, staining was stopped before the heads became too dark, this resulted in the staining in the tail to be faint, suggesting that *plxnA3* expression levels in the developing trunk are significantly lower compared to the dark staining observed in the brain, eye and ear. Staining was therefore prolonged to show that our probes do indeed show similar patterns to previously published results in the tail. (Supplemental Fig. 2). The expression of the *plxnA* family has been previously investigated in mice (Murakami et al., 2001; Perälä et al., 2005) and although precise timing is hard to compare, we see consistencies in neuronal expression patterns between these two species. For example, *PlxnA1-3* are expressed in the developing mouse hypothalamus (Murakami et al., 2001), and we show similar staining in that area in

	plxnA1a	*plxnA1b*	*plxnA2*	*plxnA3*	*plxnA4*
Optic vesicle	■	■	■		■
Early neural retina	■	■		■	■
Retinal ganglion cell layer	■	■	■		
Cilliary marginal zone	■		■		
Inner plexiform layer	■	■			■
Inner nuclear layer			■		
Otic vesicles	■	■	■	■	■
Hypothalamus		■	■		
Optic tectum	■		■	■	■
Lateral optic tectum	■	■	■		
Thalamus	■	■	■	■	■
Optic chiasm			■		■
Medulla oblongata	■	■	■	■	■

Fig. 7. Expression patterns of *plxnA1a, A1b, A2, A3* and *A4* in specific neuronal tissues in zebrafish.
Purple boxes indicate positive expression of the corresponding gene at any time-point between 18 hpf and 72 hpf in the developing zebrafish. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
Accession numbers for PlxnA family member amino acid sequences across different species, used for phylogenetic analysis. PlxnA family member denoted after species name.

Species	Accession number
D. melanogaster A	NP_524637
D. melanogaster B	NP_524616.2
G. gallus A1	XP_015148852.1
X. tropicalis A1	NP_001090760.1
M. musculus A1	AA138024.1
H. sapiens A1	NP_115618.3
D. rerio A1a	XP_003201265.4
D. rerio A1b	NP_001103480.1
D. rerio A3	NP_001091959.1
M. musculus A3	NP_032909.2
H. sapiens A3	NP_059984.3
D. rerio A4	XP_005164747.1
X. tropicalis A4	XP_002931894.2
H. sapiens A4	NP_065962
M. musculus A4	NP_786926.2
G. gallus A4	XP_015147312.1
D. rerio A2	XP_689780.5
X. tropicalis A2	AA161575.1
G. gallus A2	XP_015154528.1
M. musculus A2	AAH68155.1
H. sapiens A2	EAW93457.1

zebrafish for *plxnA1b* and *A2* (Figs. 3C”’, 3D”’, 4C”’ and 4D”’). In the developing mouse retina, *A1* and *A3* are expressed in the RGC layer, and *A2* is expressed in the RGC and INL layers (Murakami et al., 2001), matching the expression patterns seen in zebrafish (Figs. 2F”, 3F”, 4D”-4F’, 3E” and 3F”). As a key model system for development, our work in zebrafish provides a much-needed data set for future studies in the field.

Our work is the first to document a distinction between the two *plxnA1* homologs *plxnA1a* and *A1b* in zebrafish. Due to whole genome duplication, many zebrafish genes have two transcripts. After a whole genome duplication event, copies of genes can either undergo neo-functionalization, sub-functionalization or a loss of function. Here we show that *plxnA1a* and *A1b* have some overlapping and some unique domains of expression during development, suggesting that the two genes may have evolved independent roles in development.

In this body of work, we comprehensively elucidate the spatial and temporal expression patterns of the *plxnA* family during zebrafish development. Each family member shows both overlapping and divergent expression domains in the developing nervous system, which correlates with the temporal and spatial patterns of their ligands, and expression patterns in mice. We have also uncovered novel expression patterns for two transcripts of *plxnA1*, *A1a* and *A1b*. Future work will address the functional relevance of the two *plxnA* transcripts, to determine if they display any level of redundancy or if they have evolved independent functions.

4. Experimental procedures

4.1. Zebrafish husbandry

TL embryos were raised at 28.5 °C and developmentally staged as previously described (Fishman et al., 1997; Kimmel et al., 1995). Embryos were raised in egg water and pigmentation was blocked by addition of 0.003% phenylthiourea (PTU) at 24 hpf. All procedures were approved by the University of Vermont Institutional Animal Care and Use Committee (IACUC), protocol number 15-031.

4.2. Phylogeny and protein alignments

Full-length *plxnA1a* and *A1b* nucleotide sequences were initially found by searching the zebrafish genome in the NCBI database. NCBI BLAST was used to establish that they had disparities in sequence homology. Geneious R10 software (http://www.geneious.com) (Kearse et al., 2012) was used to generate a neighbor-joining consensus tree based on alignments of the full-length amino acid sequences referenced in Table 1. ‘Majority greedy clustering’ was used, rooted to *D. melanogaster*. Numbers indicate bootstraps of percent consensus support. SEMA and Ras-GAP protein domain alignments were also generated using Geneious R10 software (http://www.geneious.com) (Kearse et al., 2012), after importing domain sequences identified using the NCBI Conserved Domain Finder tool (https://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

4.3. Whole-mount in situ hybridization

Zebrafish embryos were developmentally staged at 18, 24, 36, 48, 60 or 72 hpf, fixed in 4% paraformaldehyde overnight at 4 °C, and stored at −20 °C in 100% methanol until use. *In situ* hybridization was performed as described previously (Thisse and Thisse, 2008). Digoxygenin (DIG)-labeled antisense and sense (control) RNA probes were generated using primers listed in Table 2 (IDT Coralville, IA). A second probe was generated for *plxnA3* to confirm previously published expression data in the tail (Tanaka et al., 2007) (Supplemental Fig. 2). PCR products were sequenced at the UVM Cancer Center Advanced Genome Technologies Core, Burlington, VT prior to probe generation.

4.4. Embedding, sectioning, imaging and annotation

Post *in situ* hybridization, embryos were oriented in 4% methyl cellulose and imaged using a Nikon SMZ800 dissecting light microscope at 5× magnification. Embryos were dehydrated in 100% ETOH overnight prior to embedding using a JB4 Embedding Kit (Polysciences, Inc., Warrington, PA). Embedded embryos were sectioned on a Leica RM2265 microtome at 20μm and mounted on slides for imaging. Sections were imaged on an Olympus iX71 inverted light microscope at 20× magnification. Figures were cropped, adjusted for brightness and contrast and assembled using Adobe Photoshop CS6. Anatomical annotations were informed with the use of the zebrafish developmental atlases (Mueller, 2005) and (Bryson-Richardson et al., 2012).

Table 2
Primers and accession numbers for gene-specific antisense probe generation.

Name	Forward primer	Reverse primer	Accession #
plxna1a	GCAGCTGGATGAACCCCTGC	TGTAATACGACTCACTATAGGGGCTGATTGTGAGCAAGATCC	XM_003201217.4
plxna1b	CTCAGCCGGAAAACACATGG	TGTAATACGACTCACTATAGGGGAACTTCACCTCCGGGTTTC	NM_001110010.1
plxna2	ATGTGATACAA1qGGAGCCGAGG	AGAGTCAGAAGGCTGTCGGA	XM_684688.7
plxna3	ACCCGACCTTTGAACCTCTT	TGTCATTCGTCAGCTCTTGG	NM_001098489.1
plxna3 (2^0 *target)*	ATTTAGGTGACACTATAGCACCGAGAGTCCAGGAGAAG	TAATACGACTCACTATAGGGTTGCAAGAACTGCT	NM_001098489.1
plxna4	ATTTAGGTGACACTATAGGGAGACAAACCCTGTGCATT	TAATACGACTCACTATAGGGTTCTCCACCTGCTCCTGTCT	XM_005164690.3

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Author conflict of interest

None.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.gep.2017.10.007.

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