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Expression of Robo/Slit and Semaphorin/Plexin/Neuropilin family members in the developing hypothalamic paraventricular and supraoptic nuclei

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Abstract

The hypothalamic paraventricular nucleus (PVN) and supraoptic nucleus (SON) contain neuroendocrine cells that modulate pituitary secretion to maintain homeostasis. These two nuclei have a common developmental origin but they eventually form at locations distant from each other. Little is known about the molecular cues that direct the segregation of PVN and SON. As a means to identify potential factors, we have documented expression patterns of genes with known guidance roles in neural migration. Here, we focus on two groups of ligand/receptor families classified to mediate chemo-repulsion of neurons and their axons: the Slit/Robo and the Semaphorin/Plexin/Neuropilin families. Their dynamic expression patterns within and around the common PVN/SON progenitor as well as the mature PVN and SON may provide a framework for understanding the formation of these two important nuclei.

1. Results and Discussion

Birth dating experiments in rodents have assigned the origin of the PVN and SON to the anterior ventral lobes of the diencephalon (Altman and Bayer, 1978). They are derived from a common progenitor origin (i.e. the PVN/SON progenitor), but segregate into two distinct nuclei distant from each other. The mature PVN contains both magnocellular (large-body) and parvocellular (small-body) hormone-producing neurons, while the mature SON contains only the magnocellular neurons (Swanson, 1986; Sawchenko et al., 1992). All magnocellular neurons are born by embryonic day 12.5 (E12.5) in the mouse (Karim and Sloper, 1980; Okamura et al., 1983; Jing et al., 1998). Thereafter, a portion of these neurons migrates to the lateral ventral surface above the optic nerve to form the SON. The remaining magnocellular neurons stay near the ventricle with latter born parvocellular neurons to form the PVN (Altman and Bayer, 1978). Their distinct locations diversify their efferents and afferents for monitoring physiological parameters and regulating homeostasis (Swanson and Sawchenko, 1983). Despite the importance of these two nuclei, the molecular cues that control their formation and axonal projections are not well studied.

Sim1, encoding a bHLH-PAS domain transcription factor, is expressed in the PVN/SON progenitor as well as the mature PVN and SON (Michaud et al., 1998). It is essential for

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endocrine neuron differentiation (Michaud et al., 1998) and for the formation of PVN and SON (Michaud et al, 1998; Xu and Fan, 2007). To determine the expression patterns of genes of interest relative to that of *Sim1*, we compared in situ hybridizations of adjacent sections. Three prominent groups of ligand/receptor pairs that mediate neuronal migration and axon guidance have been intensely studied in recently years: the Netrin/DCC pairs (Livesey, 1999; Culotti and Merz, 1998), the Slit/Robo pairs (Andrews et al, 2007; Dickson and Gilestro 2006), and the Semaphorin/Plexin pairs (Tamagnone et al., 1999; Puschel, 2002; Bagri and Tessier-Lavigne, 2002). For Class III Semaphorin ligands, selective members of the Plexin and Neuropilin families together (Plexin-Neuropilin) are required for reception. Each of the ligand and receptor families has multiple members. The expression patterns of Netrin1 and DCC have already been determined in the developing PVN and SON (Deiner and Stretavan, 1999). Here, we focus on the expression patterns of Slit/Robo and Semaphorin/Plexins-Neuropilin families using *in situ* hybridization on brain sections. The section planes and positions are diagrammed in Fig. 1 A1-A5. We present data from E12.5 to E15.5 at daily intervals. We started at E12.5 when the PVN/ SON progenitor is still in a coherent group as marked by *Sim1* expression (Fig. 1 B1) and ended at E15.5 when the PVN and SON have clearly segregated into distinct domains (also can be visualized by *Sim1* expression domains, Fig. 1 B4). Genes examined are listed in Table 1. Only genes that show overlapping expression with that of *Sim1* in at least one stage are shown in figures.

We first examined genes encoding the receptor molecules, as they likely act cell-autonomously in the PVN/SON cells to mediate migration or axonal projections. Among the three Robo members (receptors for Slit members), only *Robo3* is expressed at the E12.5 PNV/SON progenitor region (Fig. 1 C1). It is expressed non-uniformly in a stripe of cells (Fig. 1 C5) in the middle of the *Sim1* domain. The expression level of *Robo3* gradually decreases with time (Fig. 1 C2–C4). At E15.5, *Robo3* is barely detectable in the PVN and SON, but is expressed at a low level above and below the PVN (Fig. 1 C4). Of the Plexin family members (receptors for Semaphorins), we found *PlexinA1* (*Plxna1*), *PlexinA4* (*Plxna4*), *PlexinB1* (*Plxnb1*), and *PlexinC1* (*Plxnc1*) expression overlapping with that of *Sim1*. At E12.5, *Plxna1* is generally expressed but highly up-regulated uniformly within the *Sim1* domain (Fig. 1 D1, D5), with a contiguous extension crossing the ventral midline (Fig. 1 D1). *Plxna1* expression becomes reciprocally regulated in the PVN/SON versus their surrounding regions at E13.5 and E14.5 (Fig. 1 D2, D3), and eventually emerges as a pattern at E15.5 with its lowest level of expression at the medial half of the PVN and at the SON, and its highest level of expression just ventral to the PVN, i.e. the ventro-medial nucleus (Fig. 1 D4). *Plxna4* expression is restricted to the dorsal-lateral portion of the progenitor region (Fig. 1 E1, E5). The expression level of *Plxna4* decreases from E12.5 to E15.5 (Fig. 1 E1–E4). *Plxna4* expression is medially concentrated in the mature PVN (though at low levels) but is not found at the SON (Fig. 1 E4). *Plxnb1* is expressed in the anterior diencephalon without a particular increase in the PVN/SON progenitor (Fig. 1 F1), but it is expressed at high levels in a small group of ventricular cells next to the progenitor. It is detected at low levels generally in the E13.5–E15.5 hypothalamus (Fig. 1 F2–F4). During this period, it becomes highly expressed in cells lining the third ventricle (Fig. 1 F2–F5). At E12.5, *Plxnc1* is also generally expressed but selectively up-regulated within the *Sim1* domain (Fig. 2 A1). Its high levels of expression persist in the *Sim1* domain at E13.5 and E14.5 (Fig. 2 A2, A3) and finally localized to the medial part of the PVN and the lateral portion of the SON at E15.5 (Fig. 2 A4) (Xu and Fan, 2007). We note that cells at the medial PVN expressing high levels of *Plxnc1* are not uniformly distributed (Fig. 2 A5).

We also examined the *Neuropilin* family, *Neuropilin 1* (*Nrp1*) and *Neuropilin 2* (*Nrp2*), which encode co-receptors (together with Plexins) for Class III Semaphorins. Both are expressed within the PVN/SON progenitor. At E12.5 *Nrp1* is expressed at the lateral edge of the *Sim1* domain (Fig. 2 B1) similar to that of *Plxna4*, whereas *Nrp2* is highly expressed in the *Sim1* domain (Fig. 2 C1). Both *Nrp1* and *Nrp2* also have extensive expression in the thalamus (dorsal

to the *Sim1* domain). Between E13.5 and E15.5, expression of *Nrp1* and *Nrp2* changes dynamically (Fig. 2 B2–B4, C2–C4). At E15.5, *Nrp1* and *Nrp2* are broadly expressed in the hypothalamus at varying levels within their perspective positive domains. *Nrp1* has low levels of expression at the mature PVN, but high levels of expression in bands of cells just above and below the PVN (Fig. 2 B4, B5). A group of strongly *Nrp2*-positive cells are found in the PVN (Fig. 2 C3–C5). Cells above the PVN also express *Nrp2*. There is also a ventral domain of *Nrp2* expression that likely encompasses the suprachiasmatic nucleus (SCN) (Fig. 2 C3, C4).

We next examined the expression patterns of potential ligands for the above documented receptors. For Robo ligands (Slit1-3), *Slit1* (Fig. 2 D1) and *Slit2* (Fig. 2 E1) are expressed in the PVN/SON progenitor whereas *Slit3* is not expressed (not shown). Intriguingly, *Slit1* is expressed non-uniformly at the fringes of the medial, dorsal, and ventral borders of the progenitor. Its expression continues to be at the borders above and below the PVN at E13.5–E15.5 (Fig. 2 D2–D4). Eventually at E15.5, strongly *Slit1*-positive cells are found outside of the PVN dorsal boundary (Fig. 2 D5). *Slit2* is expressed in a punctated pattern within the ventricular zone next to the PVN/SON progenitor (Fig. 2E1, E5). Within the progenitor, *Slit2* is also expressed in a small group of cells at the dorsal-medial portion (Fig. 2 E5) above the *Robo3*-positive domain (compared Fig. 2 E1 to Fig. 1 C1). There is a persistent expression of *Slit2* in the ventricular cells next to the PVN at E13.5–E15.5 (Fig. 2 E2–E4).

Of the potential ligands for *Plexins* and *Neuropilins*, we examined 12 *Semaphorin* genes of different classes. We found only 4 of them to be expressed near or within the PVN/SON progenitor or in the PVN. Two of them are Class III Semaphorins (*Sema3c* and *Sema3f*), which require both a Neuropilin and a Plexin for function. *Sema3c* is not initially expressed in the PVN/SON progenitor (Fig. 3 A1), but starts to be expressed from E13.5 onward (Fig. 3 A2–A4). We note that cells at the medial PVN expressing high levels of *Sema3c* are not uniformly distributed (Fig. 3 A5 for E13.5). At E15.5 *Sema3c* is expressed in cells at the medial and most lateral PVN (Fig. 3 A4). *Sema3f* is expressed in the lateral portion of the PVN/SON progenitor (Fig. 3 B1) with variable intensity (Fig. 3 B5) and in a domain smaller than that of *Plxna1*. *Sema3f* expression becomes reciprocally regulated in the PVN/SON at E13.5 and E14.5 (Fig. 3 B2, B3), similar to that of *Plxna1* (compared Fig. 3 B2 to Fig. 1 D2, and Fig. 3 B3 to Fig. 1 D3). At E15.5, a low level of *Sema3f* expression in the medial-ventral and lateral PVN is noted (Fig. 3 B4). The uneven high-level of expression in cells ventral and dorsal to the PVN appears to be overlapping with the high level *Plxna1*-expressing cells. *Sema5b* is evenly expressed in the ventricular lining (Fig. 3 C1–C5) as that of *Plxnb1*. Although some cells next to the ventricle express low levels of *Sema5b* (Fig.3 C5), its most persistently strong expression is in the ventricular cells. In addition, it is expressed in cells just below the progenitor domain, juxtaposing to the high-level *Plxna1* domain (compare Fig. 3 C1 to Fig. 1 D1). At E15.5, we found diffusely distributed weak *Sema5b*-positive cells in the medial PVN. *Sema6d* is expressed in a similar pattern to that of *Sema5b*, except that it is expressed at a lower level at E12.5 (Fig. 3 D1) than at later stages (Fig. 3 D2–D4) and that its ventricular expression is uneven with the lowest level next to the dorsal-most portion of the PVN (Fig. 3 D3–D5). In summary, expression of all the above 4 *Semaphorin* genes in the PVN are detected but in different patterns and at different signal intensities.

In total, we have provided expression data for 13 (out of 26 examined) genes with dynamic patterns in the developing PVN and SON. For those expressed within the PVN and SON, we use diagrams to summarize their expression domains as groups of potential ligand and receptor pairs: *Slit2* and *Robo3* in Figs. 3 E1 and E2; *Sema5b, Sema6d, Plxna1, Plxna4*, and *Plxnc1* in Figs. 3 E3 and E4; *Sema3c, Sema3f, Nrp1*, and *Nrp2* in Fig. 3 E5 and E6. These diagrams are based on expression relative to *Sim1* as well as between these genes on adjacent sections. Together, they provide a foundation for directed investigations of gene function in the formation of PVN and SON. Our expression data support a laminar organization of the early

PVN/SON progenitor region (Caqueret et al., 2006): Striped expression domains of *Slit2*, *Robo3*, *Nrp1*, *Nrp2*, and *Plnxa4*, roughly perpendicular to the direction of radial migration. The differences in their domain sizes suggest that complex interactions between various guidance cues are utilized for various subpopulations of cells. Intriguingly, several possible ligand/receptor pairs are expressed in overlapping patterns. For example, in the progenitor region *Sema3f* expression overlaps with that of *Nrp1* and *Nrp2*, as well as with that of *Plxna1*, *Plxna4*, and *Plxnc1*. On the other hand, the *Robo3* ligand is expressed near, but not overlapping with, the *Slit1* and *Slit2* domains. The progressive changes of gene expression patterns from E12.5 to E15.5 for many of the genes examined likely result from re-arranged cell position by migration as well as gradually altered regulation. As there is yet no definitive marker for the future SON neurons only, where they are located during the migratory phase is not known. Curiously, no genes in this survey group display SON-only expression, either. The sub-domain and scattered expression of many genes in the mature PVN likely reflects its composition of diverse endocrine neuron types. Whether any of them is selectively associated with one specific hormone-expressing neuronal type requires further investigation. In addition to their possible involvement in the migration of PVN and SON neurons, these genes may also serve to orchestrate the endocrine neurons' efferent and afferent projections.

2. Materials and Methods

2.1 Experimental Animals

Mice of BL6/129sv mixed genetic background were used for mating. The vaginal plug date is assigned as E0.5. The anatomical nomenclatures are based on the rat brain atlas by Altman and Bayer (1995). All animal experimentation described herein was conducted in accordance with accepted standards of humane animal care outlined in Ethical Guidelines and approved by IACUC.

2.2 In situ hybridization (ISH)

Whole embryo heads were fixed in Methancarn (60% methanol, 30% chloroform and 10% acetic acid), dehydrated in methanol, embedded in paraffin, and then sectioned at 10 μm thickness. ISH was performed as described in Schaeren-Wiemers and Gerfin-Moser (1993). DIG-labeled anti-sense RNA probes were generated by T7, T3 or SP6 RNA polymerases using templates containing cDNA fragments of specified genes. The description of the 26 probes used and their providers are in Table 1. All probes were sequence-confirmed prior to use. We re-made some plasmids to reduce background. Detailed information for each plasmid is available upon request. Alkaline phosphatase conjugated anti-DIG antibodies (Roche) were used to detect hybridized probes, followed by color reaction using the NBT/BCIP (Roche) substrates to reveal signals. The images were taken using the Zeiss Axiocam digital camera under a Zeiss Axioskop compound microscope.

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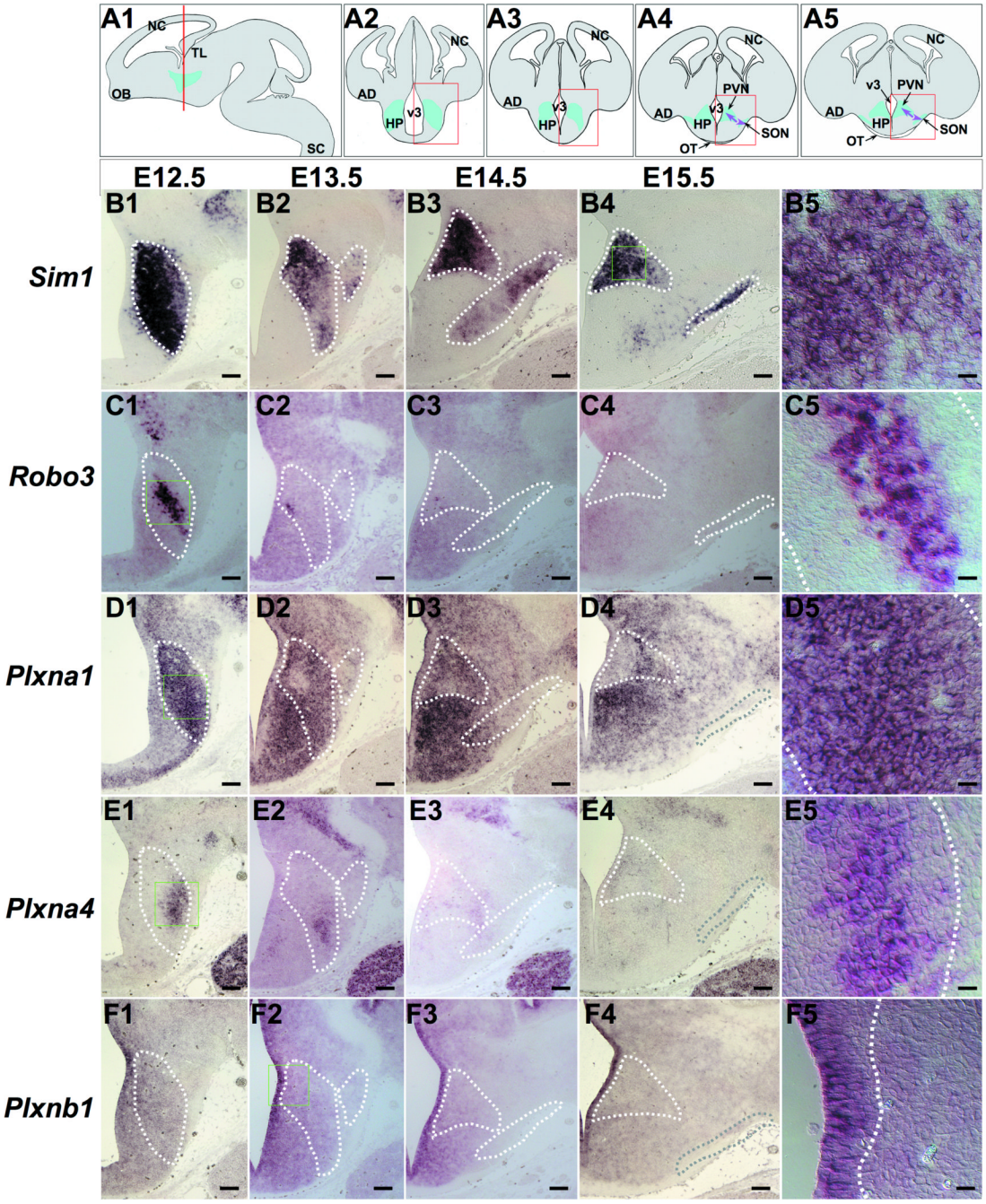

Figure 1.

Expression patterns of *Sim1*, *Robo3*, *Plxna1*, *Plxna4*, *and Plxnb1* in the developing PVN and SON. A schema of sagittal plate of the mouse brain is shown in A1; coronal planes of brains made through the red line are shown in A2 (E12.5), A3 (E13.5), A4 (E14.5) and A5 (E15.5). In A2–A5, the red rectangles indicate regions of focus; expression domain of *Sim1* is in blue; OB, olfactory bulb; NC, neocortex; TL, thalamus; AD, amygdale; HP, hypothalamus; v3, third ventricle; OT, optic tract; SC, spinal cord; the purple arrows indicate the orientation of PVN and SON neuronal migration route in the E14.5 and E15.5 brain. Coronal sections of E12.5, E13.5, E14.5 and E15.5 (labeled on top) brains were subjected to pair-wise comparative ISH using adjacent sections for *Sim1* (B1–B4) and a given gene of interest (as labeled to the left).

Selective higher power images of each gene are shown in column 5; positions of these images are indicated by green rectangles of selected lower magnification images. The white dashed lines demarcate the PVN/SON progenitors at E12.5 and E13.5; and the PVNs (triangular shape) and SONs (slanted rod shape) at E14.5 and E15.5. The white/grey dashed lines in C-F are drawn according to the *Sim1* expression region in corresponding adjacent sections (not shown). Each gene expression was examined on different brain samples, and therefore the outlined regions vary. Although the entire PVN and SON were examined, shown here are representative sections for each gene. Scale bars, 70μm in E12.5; 75μm in E13.5; 80μm in E14.5; 90μm in E14.5; 15μm in B5, C5, D5, E5, F5.

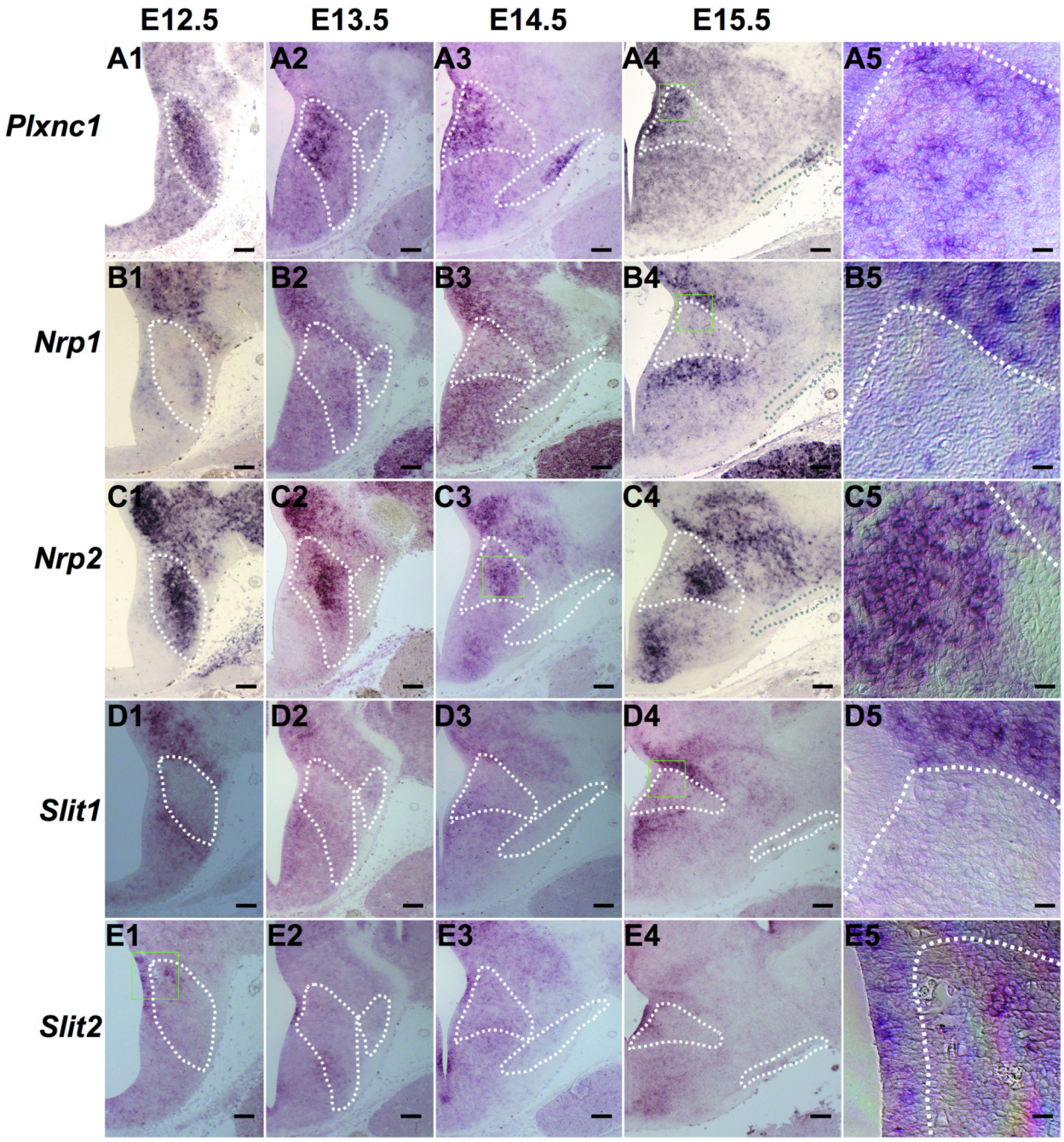

Figure 2.

Expression patterns of *Plxnc1*, *Nrp1*, *Nrp2*, *Slit1 and Slit2*. The gene names are as labeled. The stages of brain samples (labeled on top), the green rectangles, and the white/grey dashed lines are the same as in Fig. 1. Scale bars, 70μm in E12.5; 75μm in E13.5; 80μm in E14.5; 90μm in E14.5; 15μm in A5, B5, C5, D5, E5.

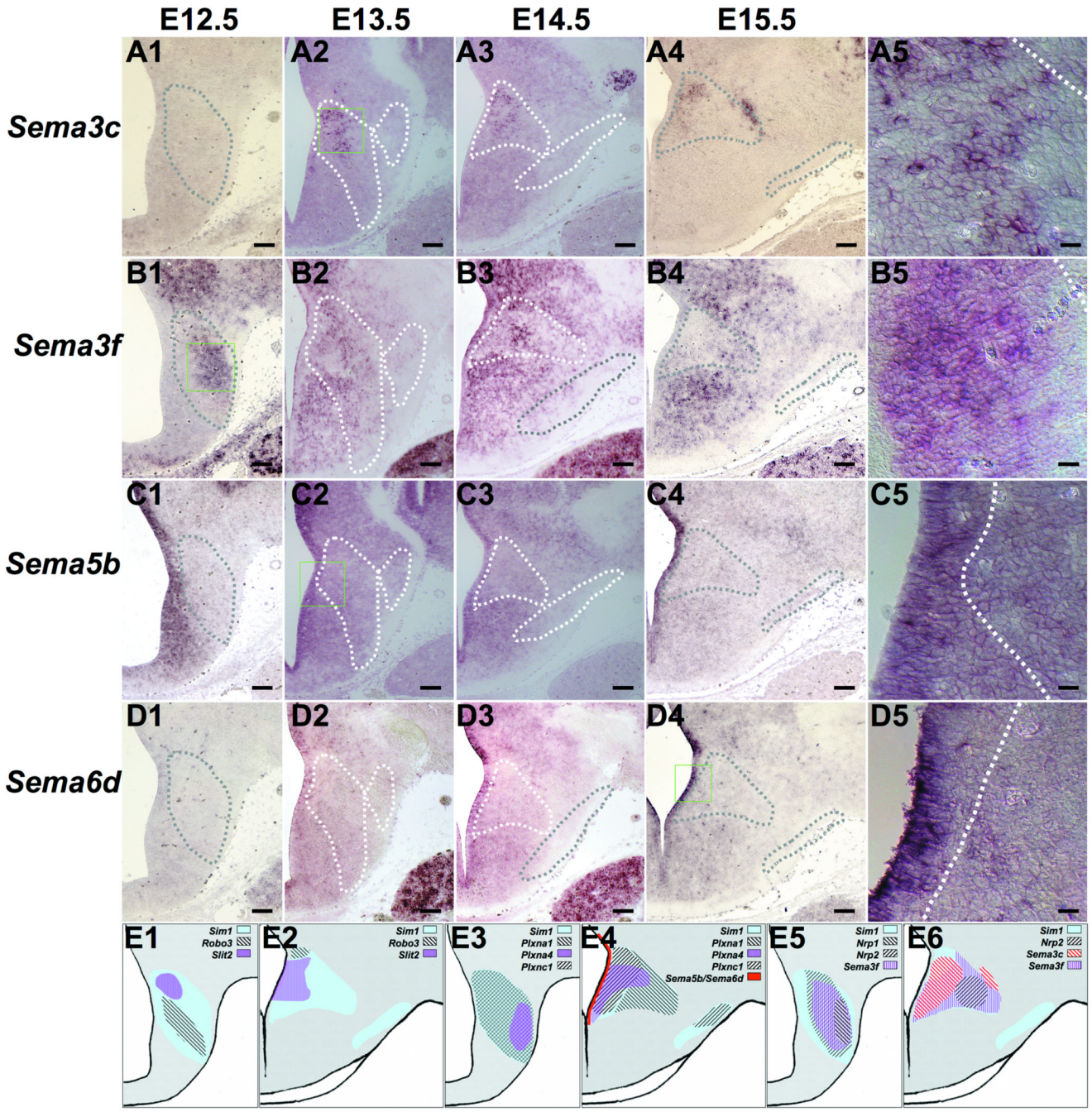

Figure 3.

Expression patterns of *Sema3c, Sema3f, Sema5b, and Sema6d*. Gene names are as labeled. The stages of brain samples (labeled on top), the green rectangles, and the white/grey dashed lines are the same as in Fig. 1. Scale bars, 70μm in E12.5; 75μm in E13.5; 80μm in E14.5; 90μm in E14.5; 15μm in A5, B5, C5, D5. E1–E6 are diagrams of documented expression patterns of ligand/receptor groups within the PVN/SON at E12.5 (E1, E3, E5) and E15.5 (E2, E4, E6): E1 and E2, *Robo3* and *Slit2*; E3 and E4, *Plxna1, Plxna4, Plxnc1, Sema5b*, and *Sema6d*; E5 and E6, *Nrp1, Nrp2*, and *Sema3c*, and *Sema3f*. The keys to the expression domain of each gene are at the upper-right corner of each figure. *Sim1* expression pattern is provided for comparison.

Table 1

Summary of probes used for ISH.

Gene	Sequence	Probe Size (bp)	Expressed in Hypothalamus[1]
Nrp1[*]	NM_145098	1285	+
Nrp2[*]	NM_001077404	1256	+
Plxna1[**]	NM_008881	2911	+
Plxna2	NM_008882	1202	−
Plxna3	NM_008883	1998	−
Plxna4	NM_175750	1350	+
Plxnb1	NM_172775	1500	+
Plxnc1	NM_018797	996	+
Robo1[***]	NM_022188	902	−
Robo2[***]	AF182037	1696	−
Robo3	NM_011248	1500	+
Sema3a[**]	D85028	2328	−
Sema3b[**]	NM_009153	2244	−
Sema3c[**]	NM_013657	2476	+
Sema3d[**]	NM_028882	558	−
Sema3e[**]	BC057956	2335	−
Sema3f[**]	BC010976	845	+
Sema4d	NM_013660	1600	−
Sema5a	NM_009154	2789	−
Sema5b[****]	BC052397	851	+
Sema6a	NM_018744	1049	−
Sema6d	NM_172537	1205	+
Sema7a	NM_011352	1995	−
Sim1	NM_011376	2351	+
Slit1[***]	NM_022953	747	+
Slit2[***]	XM_001057837	1517	+
Slit3[***]	NM_031321	658	−

[*] Gifts form Dr. J. Rubenstein

[**] Gifts form Dr. A. Kolodkin

[***] Gifts form Dr. K. Brose

[****] Gift form Dr. L. Puelles

All other probes were generated by RT-PCR.

[1] +: positive; −: negative.