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Neuropilin asymmetry mediates a left-right difference in habenular connectivity

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The medial habenular nuclei of the zebrafish diencephalon, which lie bilateral to the pineal complex, exhibit left-right differences in their neuroanatomy, gene expression profiles and axonal projections to the unpaired midbrain target – the interpeduncular nucleus (IPN). Efferents from the left habenula terminate along the entire dorsoventral extent of the IPN, whereas axons from the right habenula project only to the ventral IPN. How this left-right difference in connectivity is established and the factors involved in differential target recognition are unknown. Prior to IPN innervation, we find that only the left habenula expresses the zebrafish homologue of Neuropilin1a (Nrp1a), a receptor for class III Semaphorins (Sema3s). Directional asymmetry of *nrp1a* expression relies on Nodal signaling and the presence of the left-sided parapineal organ. Loss of Nrp1a, through parapineal ablation or depletion by antisense morpholinos, prevents left habenular neurons from projecting to the dorsal IPN. Selective depletion of Sema3D, but not of other Sema family members, similarly disrupts innervation of the dorsal IPN. Conversely, Sema3D overexpression results in left habenular projections that extend to the dorsal IPN, as well as beyond the target. The results indicate that Sema3D acts in concert with Nrp1a to guide neurons on the left side of the brain to innervate the target nucleus differently than those on the right side.

KEY WORDS: Brain asymmetry, Diencephalon, Epithalamus, Interpeduncular nucleus, Axon guidance, Semaphorin, Neuropilin, Zebrafish

INTRODUCTION

Development of correct connections between neurons is crucial for proper functioning of the central nervous system (CNS). Aberrant wiring of neuronal circuits can lead to abnormal behavior and neurological disease (Jen et al., 2004; Ponnio and Conneely, 2004; ten Donkelaar et al., 2004). Left-right (L-R) differences in fiber tracts have been correlated with hemispheric specialization of the brain (Nucifora et al., 2005), and presumably underlie asymmetries in axonal connections. How differences in connectivity could arise between the left and right sides of the brain is a question that remains largely unexplored.

The zebrafish provides a simple model to study the molecular and developmental basis of anatomical asymmetry in the developing brain. The epithalamus of the dorsal diencephalon consists of the habenular nuclei and the pineal complex. The habenulae exhibit L-R differences in their size, volume of dense neuropil and patterns of gene expression (Concha et al., 2000; Concha et al., 2003; Gamse et al., 2003). For example, *leftover* (*lov*) and *right-on* (*ron*), two members (*kctd12.1* and *kctd12.2*, respectively – Zebrafish Information Network) of the KCTD (potassium channel tetramerisation domain containing) gene family, are expressed more extensively by the left and right habenula, respectively (Gamse et al., 2003; Gamse et al., 2005). Previous work has shown that habenular asymmetry is mediated by the parapineal, an accessory organ to the pineal that is situated on the left side of the brain in over 95% of zebrafish larvae. Even earlier in development, lateralized Nodal signaling influences the directional asymmetry of the parapineal (Concha et al., 2000; Concha et al., 2003; Gamse et al., 2003) and, in the absence of this signaling, the location of the parapineal becomes randomized (i.e. approximately 50% of larvae develop the parapineal on the left side of the brain and 50% on the right).

The majority of habenular efferents project to the interpeduncular nucleus (IPN) in the midbrain tegmentum through the prominent fasciculus retroflexus (FR) fiber bundle, as part of a highly conserved conduction system in the vertebrate brain (Cajal, 1995; Herrick, 1948; Sutherland, 1982). Differential labeling of the zebrafish habenulae with fluorescent dyes or immunodetection of the Lov and Ron proteins reveals that efferents from the left habenula terminate throughout the dorsoventral (D-V) extent of the IPN, whereas right habenular neurons only project to the more ventral region (Aizawa et al., 2005; Gamse et al., 2005). In this manner, a distinct D-V pattern of innervation is established by neurons from the left and right sides of the brain. How differential target recognition is accomplished by habenular neurons is unknown, but a likely mechanism is through differences in the distribution of, or receptivity to, growth cone guidance cues.

Previously, class III Semaphorins (Sema3s) were shown to be important signals for axonal tract formation in the vertebrate forebrain, and notably for the habenulointerpeduncular connection. The FR is defasiculated in mice with targeted mutations in *sema3F* or *nrp2* (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). Sema3F and Nrp2, along with netrin1 and Dcc (deleted in colorectal cancer) may also guide growing habenular axons toward the IPN (Funato et al., 2000).

Class III Sema3s are highly conserved secreted molecules that bind specifically to receptors from the Neuropilin (Nrp) protein family, which require Plexin family members to transduce guidance signals (He et al., 2002; Kolodkin et al., 1993; Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000; Yu and Kolodkin, 1999). Growth cones can interpret Semaphorins as repulsive or attractive cues, depending on the dynamics of the receptor complex expressed on the membrane of the responding neuronal processes. Both attraction and repulsion by Sema3B are crucial for the development of the forebrain anterior commissure (Falk et al., 2005). Sema3A attracts the Nrp1-expressing apical dendrites of

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pyramidal neurons to grow towards the pial surface of cortical slices (Polleux et al., 2000). By contrast, high levels of Sema3A prevent pontocerebellar axons from innervating the hemispheric lobules (Solowska et al., 2002).

In zebrafish, four Nrp-related proteins (Nrp1a, Nrp1b, Nrp2a, Nrp2b) and six Sema3s (Sema3Aa, Sema3Ab, Sema3D, Sema3Fa, Sema3Ga and Sema3Gb) have been described so far (Bovenkamp et al., 2004; Martyn and Schulte-Merker, 2004; Yu et al., 2004; Yu and Moens, 2005). Consistent with studies from other organisms, these proteins play diverse roles during zebrafish embryonic development, in axon guidance, cell migration and angiogenesis. Sema3D inhibits ventral retinal ganglion cells from extending into the ventral tectum (Liu et al., 2004; Sakai and Halloran, 2006), and prevents medial longitudinal fascicles from growing rostrally (Wolman et al., 2004). Zebrafish Nrp1a is involved in formation of the anterior commissure and in motor axon outgrowth from the spinal cord (Feldner et al., 2005; Wolman et al., 2004). Other Sema and Nrp family members regulate cranial neural crest cell migration (Yu and Moens, 2005) and have been implicated in vascular development (Lee et al., 2002; Martyn and Schulte-Merker, 2004; Shoji et al., 2003; Torres-Vazquez et al., 2004).

We report here that *nrp1a* is selectively expressed in the left habenula of zebrafish larvae at the time when left and right habenular neurons are establishing different D-V projections onto the IPN. As with other habenular asymmetries, Nodal signaling and the left-sided parapineal mediate this L-R difference in *nrp1a* expression. Loss of Nrp1a prevents dorsal innervation of the IPN by left habenular neurons, as does depletion of a specific Semaphorin, Sema3D. Conversely, Sema3D overexpression results in ectopic axonal projections from the left habenula that extend beyond the IPN. These results indicate that Sema3D signaling through the asymmetrically distributed Nrp1a receptor in left and right habenular neurons mediates the differential D-V innervation of their shared synaptic target.

MATERIALS AND METHODS

Zebrafish

Zebrafish were raised, mated and embryos staged as previously described (Gamse et al., 2005). The AB wild-type strain (Walker, 1999), and the transgenic lines Tg(*flh*:GFP)^{c161}, Tg(*flh*:GFP)^{c162} (Gamse et al., 2003) and Tg(*foxd3:*GFP)^{fkg17} (Gilmour et al., 2002) were used in this study.

In situ hybridization and *sema3D* mRNA production

The *nrp1a*, *nrp1b*, *nrp2a*, *nrp2b*, *sema3Aa*, *sema3Ab*, *sema3D*, *sema3Fa*, *sema3Ga* and *sema3Gb* cDNA clones used for synthesis of antisense digoxigenin or fluorescein RNA probes were as previously described (Yu and Moens, 2005). RNA in situ hybridization was performed as described (Gamse et al., 2002). Bright-field images were captured by an Axiocam digital camera mounted on an Axioskop (Carl Zeiss). The *nrp1a*+3′UTR construct for mRNA production was generated by cloning the PCR-amplified open reading frame (ORF) from the *nrp1a* clone into the pCRIITOPO vector (Invitrogen), followed by the addition of the reverse transcription-PCR (RT-PCR) -generated 3′UTR fragment. The *sema3D*+3′UTR construct for mRNA production was generated by RT-PCR from the ORF, followed by addition of the 3′UTR from the *sema3D* clone described above. The *sema3Gb*+3′UTR construct for mRNA production was generated by PCR from the s*ema3Gb* clone, followed by subcloning into the pCRIITOPO vector. Sense mRNAs were generated utilizing the mMESSAGE mMachine Kit (Ambion); approximately 0.25-0.5 ng of mRNA was injected into 1- to 2-cell stage embryos.

Morpholino injections

Sequences of antisense morpholino oligonucleotides (MOs; Gene Tools, Philomath, OR) targeting the *nrp1a* translation initiation site were reported previously (Wolman et al., 2004; Yu and Moens, 2005). MOs designed in both studies were effective at perturbing IPN innervation at similar concentrations; however, less non-specific toxicity at high doses was observed using the *nrp1a* MO sequence of Wolman et al. (Wolman et al., 2004). Antisense MOs targeting *sema3Aa*, *sema3Ab*, *sema3D*, *sema3Fa*, *sema3Ga* and *sema3Gb* were synthesized as described (Yu and Moens, 2005). Optimal concentrations of *nrp1a* and *sema3D* MOs were determined by assaying titrations from 3.0 to 0.3 ng. Optimal concentrations for *sema3Aa*, *sema3Ab*, *sema3Fa*, *sema3Ga* and *sema3Gb* were determined by assaying titrations from 8 to 3 ng. Desired concentrations were achieved by diluting stock solutions of MO (10-20 mg/ml) in RNase-free water containing Phenol Red (0.2%). Approximately 1 nl was injected into embryos at the 1- to 2-cell stage. The specificity of each MO was tested previously (Wolman et al., 2004; Yu and Moens, 2005) and, in the present study, in rescue experiments in which 0.5 ng of in vitro-translated *nrp1a* or *sema3D* mRNA was combined with 0.6-0.8 ng of Nrp1a or Sema3D MO, respectively. For mock-injected controls, 1 nl of Phenol Red (0.2%) was injected into sibling embryos. Injection of the *southpaw* (*spaw*) MO was performed as previously described (Gamse et al., 2005). Details of MO injection experiments are shown in Table 1.

Immunofluorescence

Embryos and larvae were fixed in 4% paraformaldehyde at 4°C overnight. Anti-Lov, anti-Ron and anti-acetylated tubulin immunofluorescence was performed as previously described (Gamse et al., 2005; Gamse et al., 2003), with a minor modification. For Lov and Ron antibody staining only, embryos were permeabilized in 100% acetone at –20°C for 20 minutes before rehydration and blocking. Immunolabeled embryos and larvae were mounted between bridged coverslips in glycerol and images were collected on a Leica SP2 confocal microscope. Double labeling to detect protein and *nrp1a* RNA localization was performed as described (Gamse et al., 2005).

Laser-mediated cell ablation

Parapineal ablation was performed on Tg(*flh*:GFP)^{c161} or Tg(*flh*:GFP)^{c162} embryos at 28-32 hours post-fertilization as previously described (Gamse et al., 2003).

Dye labeling

Individual larvae were anesthetized with tricaine (4 mg/ml) at 4 days post-fertilization and mounted dorsal side up in 1.2% low-melting (LM) agarose (Cambrex) on a glass slide. The lipophilic dyes DiI and DiO (Molecular Probes) were dissolved in dimethylformamide (DMF) at 5 mg/ml by heating at 50°C for 5-10 minutes, and the solutions back-loaded into glass needles. Each dye was pressure-injected into the left or right habenula of larvae after the habenular commissure had been severed with a tungsten needle. Progression of the dye from the habenulae to the IPN was monitored under a Leica MZFLIII stereomicroscope. Once the dye had reached the IPN (typically within 1-3 hours), the larva was remounted in 1.2% LM agarose with the left side facing upward. Lateral view images of axonal endings on the IPN were collected using a Leica SP2 confocal microscope.

RESULTS

Left-right habenulae form divergent projections onto the IPN between day 3 and 4

Previous studies demonstrated that differential labeling of the habenular nuclei with lipophilic dyes or by immunofluorescent detection of Lov and Ron proteins reveals their stereotypic dorsoventral projections onto the target IPN (Aizawa et al., 2005; Gamse et al., 2005). We examined when this stereotypic D-V pattern is established in the larval CNS by comparing the two methods for labeling L-R habenular efferents. As in the adult, DiI-labeled axons from the left habenula innervate both the dorsal and ventral IPN (Gamse et al., 2005); whereas DiO-labeled axons from the right habenula were found to innervate only the ventral IPN of 4-day larvae (Fig. 1A-D). This D-V innervation pattern of the IPN is reproduced by anti-Lov and anti-Ron immunofluorescence at 4 days (Gamse et al., 2005), suggesting that labeling earlier stages with these antibodies would accurately reflect the developmental

Table 1. Cumulative data for MO-injection experiments

	Nrp1A MO	Sema3Aa MO	Sema3Ab MO	Sema3D MO	Sema3Fa MO	Sema3Ga MO	Sema3Gb MO	Nrp1A Sema3D MO	Nrp1A MO + mRNA	Sema3D MO + mRNA
High conc.										
ng/nl	1.0-1.5	6-7	9-10	1.0-1.6	7-8	7-8	7-8	NA	NA	NA
# of larvae	25	41	50	34	40	55	37	NA	NA	NA
Abnormal IPN (%)	85±4	5±1	10±5	79±8	8±6	6±1	5±1	NA	NA	NA
Optimal conc.										
ng/nl	0.6-0.8	4-5	7-8	0.6-0.8	5-6	5-6	5-6	0.3+0.3	0.8+0.5	0.8+0.5
# of larvae	150	140	121	117	133	103	128	71	42	41
Abnormal IPN (%)	75±5	3±2	8±3	66±5	6±2	7±2	6±1	68±6	41±9	33±9
Low conc.										
ng/nl	0.3	2	5	0.3	3	3	3	NA	NA	NA
# of larvae	114	59	25	63	31	37	36	NA	NA	NA
Abnormal IPN (%)	25±6	3±0.6	8±0.5	27±3	7±0.5	5±0.5	6±0.5	NA	NA	NA
Mock injection										
# of larvae	202									
Abnormal IPN (%)	4±0.4									
No injection										
# of larvae	257									
Abnormal IPN (%)	2±1.5									

The total number (#) of larvae examined for each MO condition and the concentrations (conc.) injected in ng per nl are indicated. The average percentage±s.d. of larvae lacking dorsal IPN innervation (Abnormal IPN %) was calculated from at least three repeated trials.
NA, not applicable.

progression of the habenulointerpeduncular connection. Although Lov$^+$ and Ron$^+$ axons were detected at 2 days within the FR (Fig. 1E-H), the first evidence for differences in growth cone morphology was not observed until 3 days, at which time Lov$^+$ filopodial processes begin to extend dorsally (Fig. 1I-L). Between 3 and 4 days, left and right habenular axons established their differential D-V projections onto the IPN (Fig. 1M-P). Thus, if asymmetric guidance cues underlie the L-R difference in IPN connectivity, then these cues should be present during this time period.

nrp1a is asymmetrically expressed in the habenular nuclei

We took a candidate approach to search for molecules involved in differential target recognition by L-R habenular neurons by examining the gene expression patterns of several known axon-guidance molecules in the zebrafish larval brain. As a starting point, expression of zebrafish genes encoding Neuropilin receptors and their Semaphorin binding partners was re-evaluated because of their known role in FR formation in the mouse (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003). We discovered that *nrp1a*, which encodes a known receptor for secreted class III Semaphorins (He et al., 2002; Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000; Yu and Kolodkin, 1999), is expressed asymmetrically in the diencephalon (Fig. 2A,B); specifically, within the left habenular nucleus (Fig. 2C). Many, but not all, left habenular neurons were found to express *nrp1a*, as revealed by double labeling with Lov and Ron antisera (Fig. 2D,E). Asymmetry in *nrp1a* expression could be distinguished as early as 2 days (Fig. 2A), which precedes D-V innervation of the IPN by habenular neurons. In contrast to *nrp1a*, none of the other known Nrp genes (*nrp1b*, *nrp2a* and *nrp2b*) was expressed asymmetrically in the habenulae at corresponding stages (Fig. 2F-H).

L-R differences in habenular gene expression are under the influence of the left-sided parapineal organ. Nodal signaling biases the position of the parapineal, which, in turn, directs laterality of the habenulae, including their asymmetric expression of Lov and Ron and D-V axonal projections to the IPN (Concha et al., 2003; Gamse et al., 2005; Gamse et al., 2003). Perturbation of the zebrafish Nodal signal Southpaw by injection of an antisense MO (Gamse et al., 2005; Long et al., 2003) also resulted in L-R randomization of *nrp1a* expression, with the direction of asymmetry corresponding to parapineal laterality (Fig. 2I,J). Approximately half of Spaw MO-injected populations (~56%, *n*=96) expressed *nrp1a* in the left habenula, as compared with 95% of mock-injected controls (*n*=89). Moreover, parapineal ablation resulted in the loss of habenular *nrp1a* expression (Fig. 3G,H). The data support the hypothesis that, under the influence of Nodal signaling and the left-sided parapineal, asymmetric Nrp1a selectively guides left habenular axons.

Depletion of Nrp1a perturbs innervation of the dorsal IPN by left habenular axons

To test the role of asymmetric *nrp1a* expression in habenular axon guidance, embryos were injected with an antisense MO that inhibits Nrp1a translation. Habenular asymmetry was not affected (Fig. 3A,B); however, depletion of Nrp1a resulted in a reduction of left habenular projections to the dorsal IPN, as demonstrated by a decrease in Lov-immunoreactive (Fig. 3C,D) or DiI-labeled (Fig. 3E,F) axonal endings. Approximately 75% of MO-injected larvae (*n*=150) exhibited a loss in dorsal IPN that was only rarely observed in mock-injected controls (~3.5% of larvae, *n*=202; see Table 1). Similarly, the absence of *nrp1a* expression in the left habenula following ablation of the parapineal (Fig. 3G,H) correlated with a decrease in projections to the dorsal IPN from the left habenula

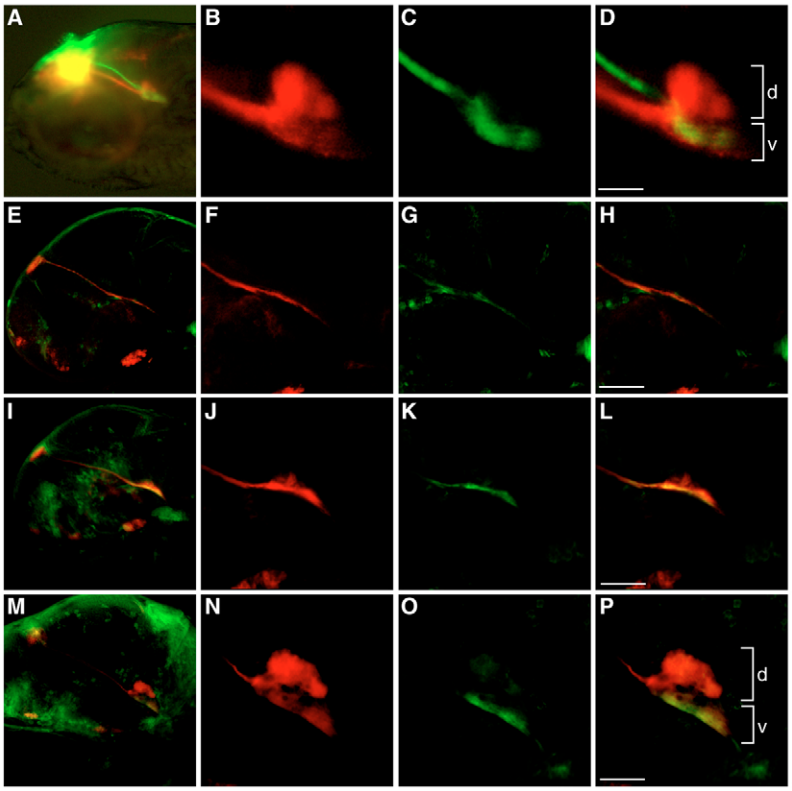

Fig. 1. Differential dorsoventral innervation of the IPN by the left and right habenulae. (**A**) Dorsolateral view of the habenulo-IPN projections in a 4-day-old zebrafish larva. DiI (red) and DiO (green) were injected into the left and right habenula, respectively. The yellow signal is a visual artifact owing to the superimposition of the differentially-labeled L-R habenulae in this orientation. (**B-D**) Higher magnification of A confirms that the left habenula innervates dorsal (d) and ventral (v) IPN, whereas the right habenula only projects ventrally. (**E-P**) Lateral views of (E-H) 2-, (I-L) 3- and (M-P) 4-day-old larvae labeled with anti-Lov (red) and anti-Ron (green) antibodies. F-H, J-L and N-P are higher magnifications of E, I and M, respectively. Scale bars: 50 μm.

(~76% of larvae, *n*=24; Fig. 3I). These observations indicate that the asymmetric distribution of the Nrp1a receptor accounts for the L-R differences in habenular connectivity with the IPN.

Depletion of Sema3D phenocopies the loss of Nrp1a

Nrp receptors specifically bind the secreted class III Semaphorins. Several potential ligands to Nrp1a are produced in the zebrafish CNS, including Sema3Aa, Sema3Ab, Sema3D, Sema3Fa, Sema3Ga and Sema3Gb (Wolman et al., 2004; Yu and Moens, 2005), and some sema genes are expressed in cells along the trajectory of the FR and in the vicinity of the IPN (see Fig. S1 in the supplementary material). This precludes identification of the specific signal that most likely guides Nrp1a-expressing axons from the left habenula.

To address this issue, we examined whether the IPN projection pattern was perturbed following injection of antisense MOs specific for each of the Sema3 transcripts. Because Neuropilins and Semaphorins are involved in many different contexts in axon guidance and cell migration (Sakai and Halloran, 2006; Wolman et al., 2004; Yu and Moens, 2005), we first assessed whether the overall pattern of axonal projections and, more specifically, the asymmetric habenular projection to the IPN was disrupted by MO treatments. Brain morphology and organization, as assayed by immunolabeling for acetylated tubulin (Fig. 4A-H), appeared grossly normal at the concentrations of Nrp1a and Sema MOs determined to be optimal (see Materials and methods; Fig. 5). Expression of *nrp1a* and *sema3D* in the brain was also unaffected by Sema3D or Nrp1a MO injection (data not shown). Habenular projections along the FR, as demonstrated by Lov immunofluorescence, were also intact (Fig. 4A-H). However, this analysis revealed that injection of a MO directed against one Sema, Sema3D, resulted in a phenotype at the IPN resembling that generated by the Nrp1a MO (Fig. 4, compare B with E).

Double labeling with Ron and Lov antisera or direct DiI labeling of the left habenula further confirmed that depletion of Sema3D disrupted innervation of the dorsal IPN by left habenular neurons. Although habenular L-R asymmetry was maintained (Fig. 4I,J), the majority of Lov^+ axons were coextensive with Ron^+ axons (Fig. 4, compare L with M) and predominantly projected to the ventral IPN (Fig. 4, compare O with P) in larvae that had received Sema3D or Nrp1a MOs. By contrast, MO-mediated depletion of Sema3Aa, Sema3Ab, Sema3Fa, Sema3Ga or Sema3Gb did not affect axonal projections from the left habenula to the dorsal IPN, which were indistinguishable from mock-injected controls (Fig. 4R-V). Taken together, the data suggest that Sema3D is the key ligand responsible for guidance of Nrp1a-expressing left habenular axons to the dorsal IPN.

Synergistic action of Nrp1a and Sema3D

The effect of Nrp1a and Sema3D MO injections on the formation of left habenular connections with the dorsal IPN was determined to be concentration-dependent and could be rescued by co-injection of the corresponding mRNA (Fig. 5 and see Table 1). Moreover, the combined injection of both MOs at sub-threshold doses proved significantly more effective at disrupting innervation of the dorsal IPN than either MO alone (Fig. 4N,Q, Fig. 5; Table 1). The synergistic effect of the Nrp1a and Sema3D MOs provides additional support for the role of this receptor-ligand pair in guiding left habenular axons to the dorsal IPN.

Sema3D overexpression results in ectopic axonal projections

To test whether the Sema3D ligand can influence the axonal projections of Nrp1a-expressing neurons, global overexpression was performed by injecting *sema3D* mRNA into 1- to 2-cell stage zebrafish embryos. Habenular efferents at the IPN were examined

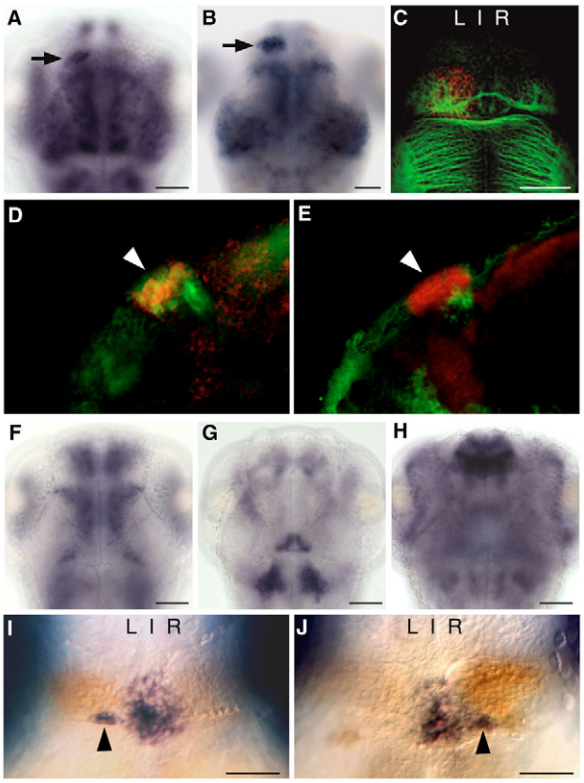

Fig. 2. Asymmetric expression of *nrp1a* in the dorsal diencephalon. (**A**,**B**) Left-sided expression of *nrp1a* (black arrow) is detected by 2 days (A) and increases by 4 days (B). (**C**) *nrp1a* expression (red) overlaps with the dense neuropil of the left habenula revealed by anti-acetylated tubulin immunolabeling (green) at 4 days. (**D**,**E**) The *nrp1a*-expressing neurons (red) are a subset of the Lov-immunoreactive population (D, green) and are also distinct from Ron-immunoreactive cells (E, green) of the left habenula (arrowheads). (**F-H**) Expression of (F) *nrp1b*, (G) *nrp2a* and (H) *nrp2b* was not detected in the habenular nuclei. (**I**,**J**) Laterality of *nrp1a* expression (orange) correlates with parapineal position (*otx5* expression, black arrowheads) in (I) mock-injected (94±2% left bias, *n*=89) and (J) spaw MO-injected 4-day-old larvae (56±2% sinistral and 44±2% dextral, *n*=96). A-C and F-J are dorsal views of the brain; C and D are lateral views of composite Z-stack confocal images through the left habenula. Left (L) and right (R) sides of the brain are indicated. Scale bars: 50 μm.

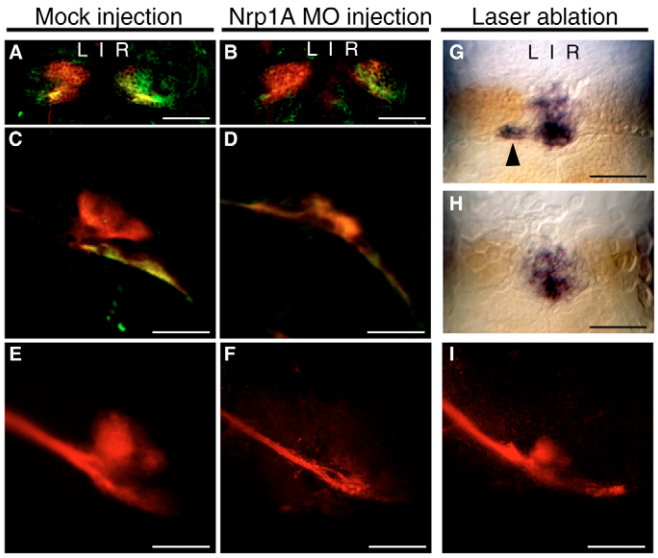

Fig. 3. Innervation of the dorsal IPN requires Nrp1a. Dorsal views of habenular nuclei (A,B,G,H) and lateral views of the IPN (C,D,E,F,I) of 4-day-old larvae. (**A-D**) Mock-injected (A,C) or Nrp1a MO-injected (B,D) larvae were labeled with anti-Lov (red) and anti-Ron antibody (green). (**E**,**F**,**I**) Mock-injected (E), Nrp1a MO-injected (F) or parapineal-ablated (I) larvae were labeled at 4 days with DiI (red) in the left habenula. (**G**) All control-ablated larvae (*n*=27) had an intact parapineal (black arrowhead) as confirmed by *otx5* expression (blue), and strong *nrp1a* expression (orange) in the left habenula. (**H**) In the majority of parapineal-ablated larvae, *nrp1a* expression (orange) was greatly reduced in the left habenula (~71%, *n*=39). Scale bars: 50 μm.

in the resultant 4-day-old larvae by Lov and Ron immunofluorescence or by direct dye labeling of the left habenula. Using either labeling method, axonal processes were sometimes visualized not only at the dorsal IPN, but extending abnormally beyond it (Fig. 6, compare A,D with B,E; 9% of larvae, *n*=64). The ectopic processes were directed toward the dorsal midbrain where endogenous *sema3D* is expressed (Fig. 6G,H).

In contrast to Sema3D, ectopic axonal projections from the left habenula were never observed following injection of mRNA encoding Sema3Gb, the other Sema produced in the midline of the brain (Fig. 6C,F, *n*=69). Increasing *sema3D* RNA, therefore, appears to have a specific effect on the guidance of left habenular axons that is not a general feature of Sema overexpression.

DISCUSSION

The left and right habenular nuclei of zebrafish develop molecular and anatomical differences under the influence of the asymmetrically-positioned parapineal organ (Gamse et al., 2005; Gamse et al., 2003). One notable difference is in the connections that habenular axons form with the midbrain interpeduncular nucleus: axon terminals from right habenular neurons are confined to the ventral IPN, whereas left habenular neurons innervate ventral as well as dorsal regions (Aizawa et al., 2005; Gamse et al., 2005). In this study, we have shown that asymmetric expression of the Nrp1a receptor mediates this difference in connectivity between left and right habenular efferents, through their differential response to the guidance cue Sema3D. Although it is known that axon-guidance molecules play essential roles in the establishment of neuronal connections in the developing nervous system, this is the first demonstration of their function in directing differences in target recognition between neurons on the left and right sides of the vertebrate brain.

Antisera directed against the Leftover and Right-on proteins have proven to be valuable tools to determine when habenulointerpeduncular connections are first formed. In the adult (Gamse et al., 2005) and larval (this study) zebrafish brain, immunodetection of these related proteins closely reproduces what is observed following direct labeling of each habenula with lipophilic dyes. Lov is predominantly, but not exclusively, expressed in the left habenula and Lov^{+} immunoreactive axonal endings are found along the entire extent of the IPN, as are DiI-labeled projections from the left habenula. By contrast, more cells in the right habenula express Ron and all Ron-immunoreactive endings terminate in the ventral IPN, equivalent to dye-labeled efferents emanating from the right habenula. From labeling with these antisera, we find that the D-V pattern of target innervation is established between the third and fourth days of development, indicative of the window when guidance cues must be functioning.

The simplest model for generating L-R differences in neural connectivity is the asymmetric distribution of axon-guidance signals or the receptors they activate. However, prior reports on the expression of guidance molecules in the developing zebrafish brain provided no evidence for L-R asymmetry, at least at the level of transcription. We reinvestigated this issue by focusing on expression in the habenulointerpeduncular system, specifically between 2 and 4 days of development. Because Semaphorin signaling through Neuropilin receptors had been implicated previously in the fasciculation of the FR axon bundle (Chen et al., 2000; Funato et al., 2000; Giger et al., 2000; Sahay et al., 2003), and genes encoding zebrafish family members were known to be expressed in discrete forebrain and midbrain domains (Bovenkamp et al., 2004; Yu et al., 2004), these seemed to be logical candidates to re-examine. Only one, *nrp1a*, was found to fulfil the criteria of being expressed asymmetrically in the diencephalon during the relevant developmental period.

Zebrafish *nrp1a* is preferentially expressed in a subregion of the left habenula before differential target innervation by left and right habenular axons. Experimental manipulations indicate that the laterality in *nrp1a* expression is regulated by the same mechanisms previously implicated in mediating directional asymmetry of the epithalamus. Reversing laterality of the pineal complex through disruption of the Nodal-related signal Southpaw reverses the directionality of habenular asymmetry and the L-R origin of habenular connections onto the IPN (Gamse et al., 2005). Southpaw depletion also produces L-R reversals in the *nrp1a* habenular expression pattern. Laser-mediated ablation of the parapineal has been proposed to cause right isomerism, with both habenulae developing molecular properties of the right habenula (Gamse et al., 2005). Accordingly, following selective ablation of the parapineal, *nrp1a* expression is not detected in the left habenula and left habenular efferents fail to innervate the dorsal IPN. These results strongly suggest that *nrp1a* mediates the selective innervation of the

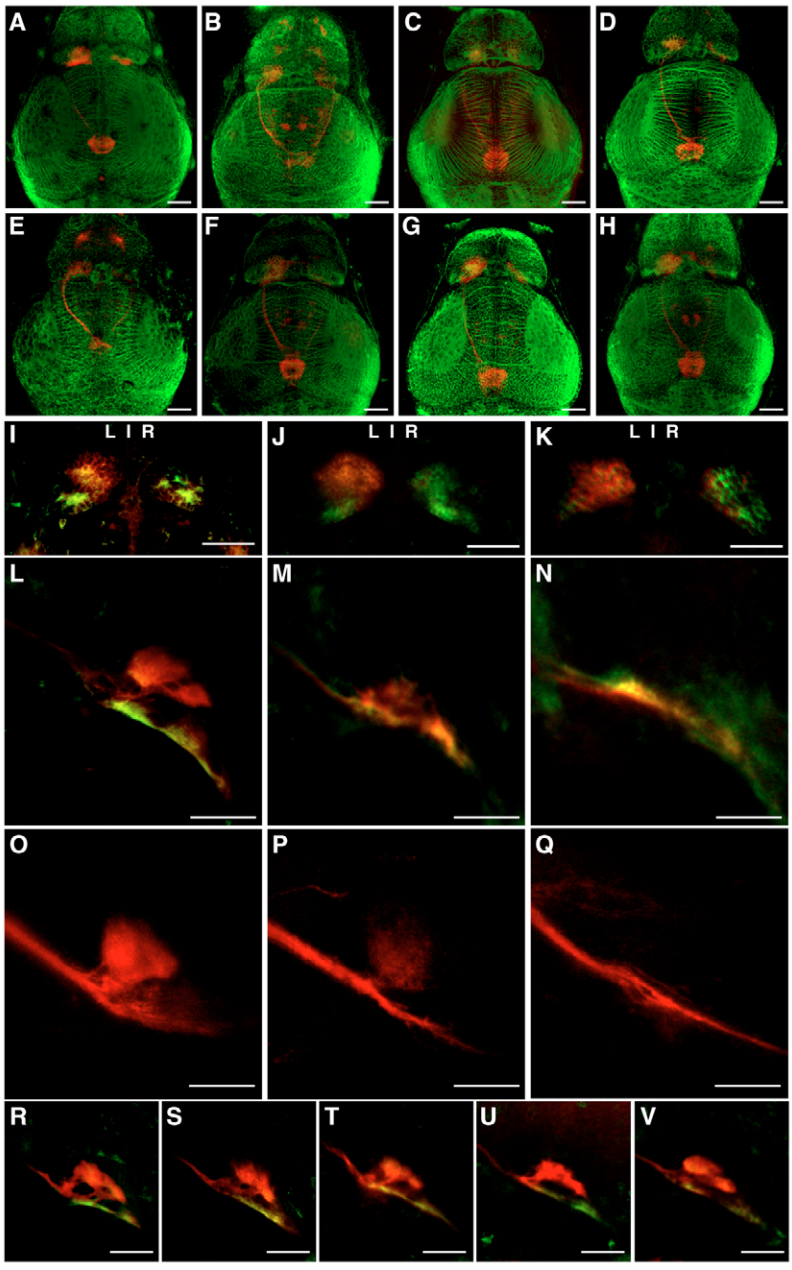

Fig. 4. Sema3D depletion disrupts innervation of the dorsal IPN. Larvae were stained with anti-Lov (red) and anti-acetylated tubulin (green) at 4 days. (**A-H**) Dorsal views of the brain in control (A), Nrp1a (B), Sema3Aa (C), Sema3Ab (D), Sema3D (E), Sema3Fa (F), Sema3Ga (G), or Sema3Gb (H) MO-injected embryos. Optimal concentrations of MOs (see Table 1) showed no effect on general brain morphology. (**I-V**) Dorsal views of the habenular nuclei (I-K) and lateral views of the IPN (L-V) of (I,L,O) mock-injected larvae or larvae injected with antisense MOs for (J,M,P) Sema3D, (K,N,Q) Sema3D and Nrp1a, (R) Sema3Aa, (S) Sema3Ab, (T) Sema3Fa, (U) Sema3Ga or (V) Sema3Gb. Scale bars: 50 μm.

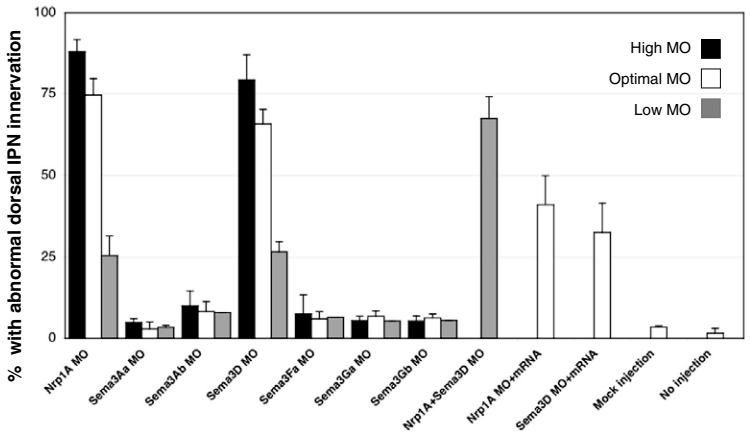

Fig. 5. Dose-dependence and synergism of Nrp1a and Sema3D MOs. Innervation of the IPN was assayed by anti-Lov immunolabeling and confocal microscopy and the number of larvae lacking dorsal projections (% abnormal dorsal IPN innervation) was determined for each experimental condition. Error bars represent the s.e.m. from at least three independent injection experiments. For a given MO, high MO refers to the concentration that caused morphological defects and lethality in a large proportion of larvae (above 50%); optimal MO concentrations did not produce morphological defects or lethality above mock-injected larvae; low MO concentrations were at least 25% less effective than the optimal MO concentration at causing defective IPN innervation. Co-injection of low MO amounts for Nrp1a and Sema3D had a significantly greater effect than injection of either MO alone. For the total number of larvae assayed and other experimental details see Table 1.

dorsal target by habenular neurons on the left side of the brain, direct support for which comes from the targeted disruption of Nrp1a protein synthesis by antisense morpholinos.

Nrp1a depletion by MOs results in the subsequent loss of left habenular projections to the dorsal IPN, similar to that observed following parapineal ablation. Although *nrp1a* transcripts are expressed far more broadly in the developing brain than just in the habenula, MO treatment under the described conditions did not appear to cause general disorganization in axonal tracts. Habenular asymmetry, including asymmetric expression of Lov and Ron, was also preserved. Thus, global depletion of Nrp1a and tissue-specific loss of *nrp1a* expression in the left habenula resulting from parapineal ablation have the same effect on limiting habenular projections to the ventral region of the target.

Further evidence for the role of the Nrp1a receptor in guiding left habenular neurons at the target was provided by the identification of the specific ligand that activates it. Nrp receptors bind a variety of secreted Class III Semaphorins with various affinities, and the ligands can operate at a considerable distance from the cells that produce and secrete them (Feiner et al., 1997; He et al., 2002; Kolodkin et al., 1993; Pasterkamp and Kolodkin, 2003; Tamagnone and Comoglio, 2000; Yu and Kolodkin, 1999). Therefore, it is important to determine experimentally the Sema ligand(s) that partners the Nrp receptor(s) in any given developmental context. Several lines of evidence support the hypothesis that Sema3D serves to guide Nrp1a-expressing axons from the left habenula towards the dorsal IPN. First, *sema3d* transcripts are localized in cells along the forebrain-to-midbrain trajectory of the FR, and dorsal to the IPN during the period when habenulointerpeduncular connections are established. Second, MO-mediated depletion of Sema3D phenocopies the loss of Nrp1a, causing a reduction in left habenular projections to the dorsal IPN. Targeted depletion of other Sema family members had no effect on the IPN innervation pattern. Third, the combination of sub-effective doses of both the Nrp1a and Sema3D MOs had a significantly more potent effect on perturbing left habenular innervation of the dorsal target than either MO alone, indicating that the two MOs function synergistically. Finally, abnormal projections from left habenular axons extending beyond the dorsal IPN were observed following overexpression of Sema3D.

On the basis of these results, we propose that Sema3D serves as an attractant to guide Nrp1a-expressing axons to innervate the dorsal IPN (Fig. 7). Normally, during development of the zebrafish brain, Sema3D is distributed dorsal to the IPN and diverts growth cones of some left habenular neurons towards the dorsal target (Fig. 7A). Upon loss of either Nrp1a or Sema3D, all left habenular axons project ventrally, as do *nrp1a*-deficient neurons of the left and right habenula (Fig. 7B,C). Conversely, Sema3D overproduction attracts left habenula neuronal processes toward the endogenous *sema3D*-expressing domain dorsal to the IPN (Fig. 7D). Surprisingly, these abnormal processes did not project randomly in all directions as expected from global overexpression of Sema3D. This finding may be explained by the timing of *nrp1a* expression. Growth cones of

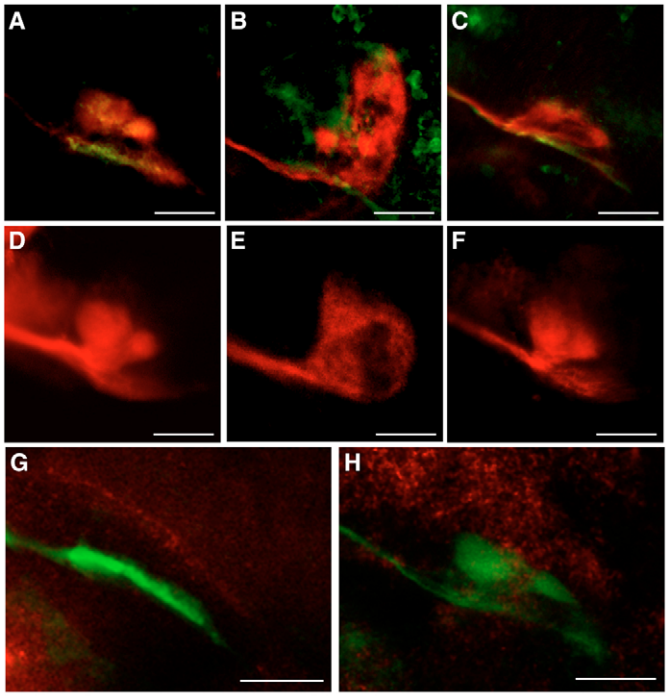

Fig. 6. Overexpression of *sema3D* causes ectopic left habenular projections. (**A-F**) Lateral views of the 4-day-old larval IPN from (A,D) mock-injected embryos (*n*=91) or embryos injected with (B,E) *sema3D* mRNA (*n*=64), or (C,F) *sema3Gb* mRNA (*n*=69). Larvae were stained with anti-Lov antibody (red) and anti-Ron antibody (green) in A-C, or labeled with DiI (red) in the left habenula in D-F. (**G**,**H**) Lateral views of (G) 2- and (H) 4-day-old larvae double-labeled with *sema3D* antisense RNA probe and anti-Lov antibody. *sema3d* transcripts (red) are abundant in the brain region dorsal to the IPN, as visualized by Lov$^+$ habenular projections (green). Scale bars: 50 μm.

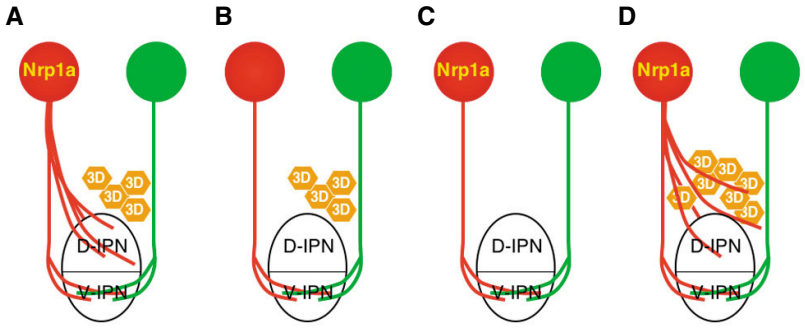

Fig. 7. Model of Nrp1a-Sema3D-mediated guidance of left habenular axons. (**A**) Sema3D attracts Nrp1a-expressing growth cones of left habenular axons toward the dorsal IPN. Following Nrp1a (**B**) or Sema3D (**C**) depletion, left habenular efferents innervate only the ventral IPN, similar to right habenular neurons that do not express *nrp1a*. (**D**) Sema3D overexpression can result in ectopic habenular projections that extend beyond the dorsal IPN.

habenular axons have already reached the midbrain target by day 2, the time when *nrp1a* transcripts are first detected asymmetrically in the habenulae. Left habenular neurons may only respond to Sema3D on day 3, when Nrp1a levels become high enough to influence growing axon tips just as they innervate the target IPN. Thus, when habenular axons are extending caudally through the diencephalon, we presume their growth cones are unable to respond to Sema3D distributed along the FR route to the midbrain owing to the lack of receptor. The low frequency (~9%) of larvae showing aberrant projections that extend beyond the dorsal IPN is likely to reflect variability in protein levels and distribution from injection of *sema3D* mRNA at the 1- to 2-cell stage. A more direct approach, such as expressing ectopic Sema3D in a spatially-restricted manner in the zebrafish brain or in cultures of habenular explants, will confirm whether Sema3D functions as an attractive cue in the context of dorsal IPN innervation.

The Nrp1a and Sema3D interaction plays a temporally-limited role in guiding left habenular growth cones at the choice point between the dorsal and ventral regions of the target IPN. Studies of mouse mutants have implicated several other guidance cues in outgrowth and fasciculation of habenular efferents within the FR. In vitro-cultured habenular neurons extend their axons towards netrin-expressing cells (Funato et al., 2000) and utilize membrane-associated Sema5A receptors as sensors of extrinsic proteoglycans that can either inhibit or attract their growth cones (Kantor et al., 2004). Disruption of either the diffusible signal Sema3F, or its receptor Nrp2, through targeted gene inactivation leads to defasciculation of the FR (Chen et al., 2000; Giger et al., 2000; Sahay et al., 2003), suggesting that this ligand-receptor pair normally repels axons to keep them from straying from the FR bundle as proposed from in vitro studies (Funato et al., 2000). In our experiments, however, MO-mediated depletion of Sema3Fa did not significantly alter the FR axonal bundles of zebrafish larvae. This apparent difference between vertebrate species might be due to the presence of duplicate Sema3F genes in zebrafish. The combined reduction of Sema3Fa and Sema3Fb activities will reveal whether they play redundant and conserved roles in maintaining the integrity of the zebrafish FR.

Although progress has been made in understanding how left-right asymmetry develops in the zebrafish limbic system, much remains unanswered concerning the basis of functional specialization of mammalian brain hemispheres. So far, there is little evidence to support extensive habenular asymmetry in mammals, nor does the habenulointerpeduncular projection appear to differ between the left and right sides of the brain (Y.-S.K. and M.E.H., unpublished). However, some recent reports have identified L-R differences in gene expression programs in the rodent cortex (Sun et al., 2006; Sun et al., 2005) and hippocampus (Moskal et al., 2006), and in the size of white-matter fiber tracts associated with language regions of the human brain (Nucifora et al., 2005). Our study suggests that the asymmetric distribution of axon-guidance cues could be an important mechanism to establish patterns of connectivity in the developing embryonic CNS that underlie functional specialization.

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Supplementary material

Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/134/5/857/DC1

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