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Habenular commissure formation in zebrafish is regulated by the pineal gland specific gene *unc119c*

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Abstract

Background—The zebrafish pineal gland (epiphysis) is a site of melatonin production, contains photoreceptor cells, and functions as a circadian clock pacemaker. Since it is located on the surface of the forebrain, it is accessible for manipulation and therefore is a useful model system to analyze pineal gland function and development. We previously analyzed the pineal transcriptome during development and showed that many genes exhibit a highly dynamic expression pattern in the pineal gland.

Results—Among genes preferentially expressed in the zebrafish pineal gland, we identified a tissue-specific form of the *unc119* gene family, *unc119c*, which is highly preferentially expressed in the pineal gland during day and night at all stages examined from embryo to adult. When expression of *unc119c* was inhibited, the formation of the habenular commissure (HC) was specifically compromised. The *Unc119c* interacting factors *Arl311* and *Arl312* as well as *Wnt4a* also proved indispensable for HC formation.

Conclusions—We suggest that *Unc119c*, together with *Arl311/2*, plays an important role in modulating *Wnt4a* production and secretion during HC formation in the forebrain of the zebrafish embryo.

Keywords

Zebrafish; pineal gland; epiphysis; habenular commissure; *unc119*; *wnt4*

Introduction

The *unc119* gene represents a novel neural gene that shares conserved sequences in all metazoans examined. Although this gene is highly conserved from worms to human, its expression shows two distinct patterns. In invertebrates (*C. elegans* and *Drosophila*), the single *unc119* gene is expressed throughout the nervous system (Maduro et al., 2000), and a *C. elegans* mutant for the *unc119* gene exhibits movement, sensory and behavioral abnormalities (Maduro et al., 2000). It has been suggested that *Unc119* protein is located in neuron cell bodies and axons, and acts cell-autonomously to inhibit axon branching (Knobel et al., 2001). In mammals, a related gene was originally identified as *HRG4* (human retinal gene 4), a gene expressed predominantly in the photoreceptors of the retina (Higashide et al., 1996), while rat *Unc119* is highly expressed in the retina and pineal gland (Bailey et al., 2009). Consistent with its retinal expression pattern, a mutation in *HRG4* was found in a late-onset cone-rod dystrophy patient (Kobayashi et al., 2000). Further, mutant mice

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carrying mutations in the *unc119* gene developed retinal degeneration (Ishiba et al., 2007; Kobayashi et al., 2000; Mori et al., 2006). A truncated mutant HRG4 protein showed increased affinity to its target Arl2, which likely resulted in the sequestration of Arl2 and delayed inactivation of Arl2's downstream target, mitochondrial ANT1, which mediates photoreceptor synaptic and retinal degeneration by apoptosis (Ishiba et al., 2007; Mori et al., 2006). More recently, Unc119 has been implicated in the transport of transducin (T), the key visual G protein, to the outer segment of rod photoreceptors in the dark through its interaction with the N-acetylated GTP-bound form of Gat1 α (Gopalakrishna et al., 2011; Zhang et al., 2011). In zebrafish, one *unc119* homolog has been described (*unc119*) that is expressed throughout the central nervous system, as in invertebrates; its knock down results in disorganized neural architecture (Manning et al., 2004). A second *unc119* homolog (*unc119b*) has been identified in the zebrafish genome, but its expression pattern and function have not been characterized (Manning et al., 2004). In an analysis of the zebrafish pineal transcriptome we identified a third *unc119* gene, which we named *unc119c* (Toyama et al., 2009). The present work describes a functional analysis of this gene.

Several proteins have been identified as Unc119 interacting partners. ADP-ribosylation factor-like protein2 (Arl2) was first identified to interact with Unc119 (Kobayashi et al., 2003). Later, the Arl2 homologue Arl3 was shown to form a ternary complex with its GTPase Activating Protein (GAP), Retinitis Pigmentosa 2 (RP2), and Unc119 (Veltel et al., 2008b). Furthermore, the mouse Unc119 protein interacts with the synaptic ribbon-specific protein RIBEYE at photoreceptor ribbon synapses (Alpadi et al., 2008), and *ribeye a*, one of two homologs in zebrafish, is expressed in the pineal gland (Wan et al., 2005).

To investigate the function of the pineal gland-specific *unc119c* gene in the zebrafish, we used morpholinos (MOs) to knock down its expression. We observed that injection of *unc119c* MO affected specifically the formation of the habenular commissure (HC). The HC arises in the forebrain adjacent to the pineal gland during early neurogenesis of the zebrafish embryo. The HC neurons are initiated from the eminentia thalami, posterior tuberculum, and pallium (Hendricks and Jesuthasan, 2007; Taylor et al., 2010). Here we report that the pineal gland specific *unc119c* gene is involved in HC formation in the zebrafish.

Results

Unc119c is involved in HC formation

To analyze the function of *unc119c* in the zebrafish, we injected *unc119c* ATG morpholino (MO) into 1-2 cell-stage zebrafish embryos. The MO did not affect overall development of the embryo. However, when we visualized neurons of MO-injected embryos with anti-acetylated tubulin antibody, we noticed that HC formation was specifically compromised (Fig. 1A-D). The HC forms across the midline of the developing diencephalon beneath the pineal gland (Fig. 1A, B). In *unc119c* MO injected embryos the HC was often centrally interrupted (Fig. 1C), while in other cases the axon bundle that did cross the midline was very thin. This phenotype was gene-specific as control MO injection had no effect on the HC in more than 80 embryos tested (Fig. 1D). Even more importantly, the defect was rescued by co-injecting *in vitro*-synthesized MO-resistant *unc119c* RNA (Fig. 1E, F). Furthermore we observed similar dose-dependent HC defects after injecting two different *unc119c* splice MOs into the embryo (Fig. 1G). The splice MOs greatly reduced the production of mature *unc119c* mRNA in the embryo (Fig. 1H). These results indicate that *unc119c* plays a role in HC formation in the zebrafish forebrain. It is notable that the posterior commissure (PC), which forms slightly posterior to the HC, was not affected by *unc119c* MO injection, suggesting that this phenotype is specific to the formation of the HC (Fig. 1C).

Unc119 interacting proteins cooperate in HC formation

In a previous study we demonstrated the co-expression of *unc119c* and ADP-ribosylation factor-like protein 3 like 2 (*arl3l2*; two homologues of *arl3*, *arl3l1* and *arl3l2*, occur in zebrafish) in the zebrafish pineal gland (Toyama et al., 2009). We also showed that *Unc119c* physically interacts with *Arl3l2* proteins (Toyama et al., 2009). Zebrafish *arl3l1* is also expressed in the pineal gland (B. Thisse, C. Thisse, 2004; retrieved from the Zebrafish Information Network (ZFIN), University of Oregon, Eugene, OR 97403-5274; World Wide Web URL: http://zfin.org/cgi-bin/webdriver?Mlval=aa-fxallfigures.apg&OID=ZDB-PUB-040907-1&fxallfig_probe_zdb_id=ZDB-CDNA-040425-2589). This finding suggested that *unc119c* and *arl3l2* might be involved in common biological functions. Therefore, we analyzed the function of *arl3l1* and *arl3l2* in HC formation. In both cases, MO-injected embryos showed dose-dependent disruptions of HC formation, similar to the effects of *unc119c* MO (Fig. 2A-I). *Arl3l2* MO was slightly more efficient in disrupting HC formation than *arl3l1* MO (Fig. 2J). To test the specificity of the knock-down effect we used a second pair of MOs targeting the two genes. Again, effective disruption of HC formation was observed in a dose-dependent manner (Fig. 2K). These observations suggest that the specific HC phenotype is strongly associated with *Unc119c* and *Arl3*, its known physical and functional interacting partner, and is not due to non-specific effects of general interference with gene expression in the pineal gland.

Disruption of *wnt4a* affects HC formation

We hypothesized that *Unc119c* and *Arl3l1/2* may regulate secretion of molecule(s) which act as positive guidance cues for HC axons. As a candidate molecule we focused on *Wnt4a*, because it is expressed in the zebrafish pineal gland (Hendricks et al., 2008), acts as an attractant in axon guidance under certain conditions (Bovolenta et al., 2006), and its MO-mediated knock down caused HC abnormalities (Hendricks et al., 2008). We therefore repeated the injection of *wnt4a* MO into zebrafish embryos, and observed a dose-dependent disruption of the HC (Fig. 3). The appearance of the disrupted HC in these experiments closely mimicked that obtained by knock down of *Unc119c* or *Arl3l2*. At the same time, development of the pineal gland itself was not impaired by *Wnt4a* knock down (Fig. 3).

Synergistic effects between *Unc119c* or *Arl3l2* and *Wnt4a* in HC formation

We speculate that *Unc119c* and *Arl3l1/2* may be involved in *Wnt4a* secretion from the pineal gland, and that *Wnt4a* plays a role as a positive guidance cue for axon migration during the formation of the HC. To test this idea, we first studied potential functional interactions among these genes. We were able to show that *unc119c* MO and *wnt4a* MO exerted synergistic effects on HC formation. Injection of a low dose of *unc119c* MO (1.5 ng) or *wnt4a* MO (5 ng) had a moderate effect on HC formation; only 25% of *unc119c* MO injected embryos and less than 10% of *wnt4a* MO injected embryos showed disrupted HC, respectively. However, when both MOs were combined, HC formation was almost completely abolished (Fig. 4A). We observed similar synergistic effects between *wnt4a* and *arl3l2* MOs (Fig. 4B). These results suggest that *Unc119c* and *Arl3l2* regulate *Wnt4a* function in HC formation in the zebrafish embryo.

Unc119c increases accumulation and secretion of *Wnt4a* protein in cultured cells

Based on evidence that implicates *Unc119* and *Arl* proteins in intracellular trafficking and in the regulation of lipid-modified proteins, as detailed in the Discussion, we hypothesized that *Unc119c* and its binding partners might be involved in *Wnt4a* trafficking and its secretion from the pineal gland. Secreted *Wnt4a* might then act as a guidance molecule in HC axon extension (Hendricks et al., 2008; Bovolenta et al., 2006). To test these notions we assayed *Wnt4a* secretion in a model system. In cultured HEK293T cells the expression of *Wnt4a* is

below the limit of detection, but after transfection with an expression plasmid we could detect Wnt4a protein in cell lysates and in the conditioned medium. Co-transfection of *wnt4a* with *unc119c* led to a substantial increase of Wnt4a protein in the cell lysates, and an even stronger increase in the medium (Fig. 5). A smaller increase was seen after co-transfection of *wnt4a* and *arl3l2*, while co-transfection of all three components matched the results seen with *wnt4a* plus *unc119c*; overexpression of Arl3l2 also increased the level of Unc119c protein. These results suggest that Unc119c, and to a lesser extent Arl3l2, stimulate Wnt4a secretion, and we surmise that the increased Wnt4a levels seen in the cell lysates may be due to its stabilization by Unc119 and Arl3l2. These observations are consistent with the interpretation that Wnt4a secretion from the pineal gland, stimulated by Unc119c, Arl3 isoforms, and possibly additional components, is a requirement for the extension of the HC across the midline.

Pineal gland ablation affects HC formation

Nitroreductase (NTR)-mediated cell/tissue ablation is widely used to achieve temporal and spatial regulation of cell ablation (Curado et al., 2007; Curado et al., 2008; Pisharath et al., 2007). We applied this technique to examine the effects of pineal gland ablation on HC formation. Since *unc119c*, *arl3l2*, and *wnt4a* are all expressed in the pineal gland, we anticipated that this manipulation would interfere with HC formation. For pineal ablation, we used transgenic line *Tg(tph2:NfsB-mCherry)y227* that expresses NTR in pineal photoreceptor neurons (Fernandes et al., 2012). In control embryos, the transgene is strongly expressed in the pineal gland as visualized by mCherry fluorescence (Fig. 6A). Upon exposure of transgenic embryos to 10 mM metronidazole (Mtz), we observed a substantial reduction of the mCherry signal in the pineal gland, indicative of a reduction in the number of pineal gland cells (Fig. 6B); we have previously shown that MTZ treatment results in a loss of the pineal photoreceptor neuron marker Ret-P1 (Fernandes et al 2012). Coincidentally, we observed disruption of the HC (Fig. 6D) in the Mtz-treated embryos. Of 56 embryos examined in 4 experiments, five showed disrupted HC, 16 thin HC, and 35 were normal. The defect in commissure formation was highly significant ($p < 0.01$) but not as strong as following knock-down by MOs (Figs. 1-4), possibly because the NTR-mediated ablation may not eliminate all pineal cell types. As shown in the next section, different cell types in the pineal express *unc119c*.

Unc119c protein localization in the pineal gland

To map the cellular distribution of Unc119c within the pineal gland we generated a polyclonal antibody against this protein. The antibody readily stained cells in the zebrafish pineal gland, suggesting a cytoplasmic localization for the antigen (Fig. 7A, C, F). The pineal contains two main cell types, photoreceptor cells and projection neurons (Masai et al., 1997). To identify cells expressing Unc119c in the pineal gland, we used the *Tg(AANAT2:eGFP)* line which expresses eGFP in the photoreceptor cells (Gothilf et al., 2002) (Fig. 7B, D), and immuno-staining with anti-Isl-1, which marks projection neurons. We observed double staining of Unc119c antibody with GFP and Isl-1, suggesting that Unc119c is expressed in both photoreceptor and projection neuron cell bodies in the zebrafish pineal gland (Fig. 7D-G).

Discussion

We recently identified a tissue specific *unc119* gene, *unc119c*, in the zebrafish pineal gland in both embryonic and adult stages (Toyama et al., 2009). Unc119c is expressed in highly specific fashion in the pineal gland throughout all developmental stages we examined. In contrast, other zebrafish *unc119* homologues, *unc119a* and *b*, are expressed more widely (Manning et al., 2004). However in the rat, the single *unc119* gene found in the genome is

expressed predominantly in the pineal gland and retina (Bailey et al., 2009). This observation suggests that *unc119* genes play conserved roles in pineal gland function across vertebrate species.

To analyze Unc119c function, we took an MO-mediated loss-of-function approach. First, we injected MOs designed to knock down the expression of Unc119c and its binding partners Arl3l1 and Arl3l2. We reported previously that Unc119c and Arl3l2 physically interact *in vitro* (Toyama et al., 2009). All of these MOs yielded a very similar phenotype, a dose-dependent disruption of HC formation. The specificity of this phenotype was confirmed by the finding that MOs targeted to the ATG of *unc119c* or to splicing sites in this gene gave the same phenotype, which was partially rescued by coinjection of *unc119c* mRNA encoding an MO resistant version. Likewise, two MOs for each *arl3* gene resulted in the HC disruption phenotype. It is somewhat surprising that knock down of Unc119c, and Arl3l1/2, specifically expressed in the pineal gland, affected HC but not PC formation, even though both commissures are located at a similar distance from the pineal gland. This suggests that the HC and PC axons follow different guidance cues, or that the PC receives its cues from a different source than the HC in spite of their anatomical proximity.

Wnt proteins play major roles in multiple biological processes (Gordon and Nusse, 2006; van Amerongen and Nusse, 2009; Willert and Nusse, 2012) including axon guidance (Bovolenta et al., 2006; Killeen and Sybingco, 2008; Salinas, 2012). In axon guidance, Wnt factors can have both repulsive and attractive functions, depending on the combination of receptor and ligand (Bovolenta et al., 2006; Killeen and Sybingco, 2008); Wnt4 acts as an attractant via the frizzled 3 receptor in mice (Lyuksyutova et al., 2003). In the context of our work it is notable that zebrafish Wnt4a is expressed in the pineal gland, and that inhibition of Wnt4a expression causes malformation of the HC (Hendricks et al., 2008) (confirmed in this study). The Wnt4a loss-of-function phenotype is very similar to the one we observed upon injecting MOs against *unc119c* or *arl3l1/2*. Therefore, we hypothesized that Unc119c, Arl3 and Wnt4a act in a common pathway in regulating HC formation. This view was supported by the observation that co-injection of low doses of *unc119c* and *wnt4a* or *arl3l2* and *wnt4a* MOs had synergistic effects in impairing HC formation (Fig. 4). Our results imply the interpretation that Wnt4 acts as an axon guidance molecule in HC formation, and is required for the extension and midline crossing of HC axons.

A recent study showed that Unc119 can interact with the acylated N-terminus of the transducin α (T α) subunit (Gopalakrishna et al., 2011; Zhang et al., 2011). The crystal structure of Unc119 revealed that it adopts an immunoglobulin-like β -sandwich fold where the acylated N-terminal peptide of T α inserts into this hydrophobic cavity (Zhang et al., 2011). *Unc119* deletion in mice led to T α mislocalization (Zhang et al., 2011). Thus, Unc119 is a T α subunit cofactor essential for its trafficking, a functional role consistent with that we propose for Unc119c in the pineal gland. Another study showed that Unc119 binds the myristoylated ciliopathy protein nephrocystin-3 (NPHP3), and that Arl3-GTP acts to release myristoylated cargo from Unc119 (Wright et al., 2011). Members of the Arl family of small G proteins have previously been implicated in trafficking. Arl3 has a role in ciliary disease affecting the kidney, biliary tract, pancreas, and retina in mice (Schrack et al., 2006). Analysis of the *Drosophila arl3* mutant *dead end* revealed a role for Arl3 in trafficking of membrane components involved in fusion and luminal development in the tracheal system (Jiang et al., 2007). Furthermore, Arl3, Unc119 and Arl3 GAP (Retinitis Pigmentosa 2, RP2) are required for NPHP3 ciliary targeting, and this targeting requires Unc119 myristoyl-binding activity (Veltel et al., 2008a; Wright et al., 2011). Another study indicated that Arl3, but not Arl2, acts as a dissociating factor to release myristoylated cargo protein from Unc119 to promote membrane insertion (Ismail et al., 2011; Ismail et al., 2012). Thus Unc119 is a novel lipid-binding chaperone with specificity for a diverse subset of acylated

proteins (Constantine et al., 2012), and Unc119 cooperates with Arl3 in at least some of its functions. These findings provide the background and context for our hypothesis that Unc119 and Arl311/12 are jointly involved in trafficking processes in the zebrafish pineal gland.

All aspects of the production of Wnt proteins are highly controlled, including modification and secretion of the proteins. Wnts are hydrophobic due to acylation at two positions, and this modification is essential for Wnt secretion and normal signaling function (Harterink and Korswagen, 2012; Takada et al., 2006; Willert et al., 2003). In addition, Wnt secretion depends on at least one factor, the conserved protein Wntless (Wls) (Banziger et al., 2006; Bartscherer et al., 2006; Goodman et al., 2006; Harterink and Korswagen, 2012). Our hypothesis that Unc119c and Arl3 are involved in Wnt4a secretion in the zebrafish pineal gland is consistent with the requirement of Wnt acylation for secretion, and with the ability of Unc119 proteins to bind acylated proteins and to act, together with Arl's, as acyl-protein trafficking factors. As Unc119c is specifically expressed in the pineal it could be involved in Wnt secretion in this tissue only. While other Unc119 proteins are more widely expressed there are many tissues that lack any Unc119 family member. Thus we do not suggest that Unc119 proteins are universal Wnt secretion factors, but rather that they might be involved in this process in certain specialized tissues and cells. The results we obtained in a heterologous reconstituted system suggest that Unc119c has the ability to stabilize Wnt4a and increase its secretion (Fig. 5), consistent with the hypothesis based on known properties of the participants. It will be interesting to learn whether additional examples can be found where Unc119 proteins are involved in modulating the secretion or more generally the function of Wnt factors.

Experimental Procedures

Zebrafish strains

Adults and larvae were kept under a 14-hr-light/10-hr-dark cycle. Wild type and the transgenic line *Tg(aanat2:EGFP)* (Gothilf et al., 2002) and *Tg(foxd3:GFP)* (Gilmour et al., 2002) were used for injection experiments. For nitroreductase-mediated cell/tissue ablation experiments, *Tg(tph2:NfsB-mCherry)y227* was used.

Morpholino oligonucleotide injection

MOs were obtained from Gene Tools, LLC. Sequences are:

control (MO), 5'-CCTCTTACCTCAGTTACAATTTATA-3';

unc119c ATG MO, 5'-GCTCCTTCACACCTTCACTATCCAT-3';

unc119c spMO E3I3, 5'-CTC TAA AAC AAG ACT TAC ATG GCTC

unc119c spMO I3E4, 5'-CAA GCC TGT TCA GAA GTT CAG TGTT

arl311 ATG MO, 5'-ACAAACCCTTTTGGACTTCTCCCAT-3';

arl312 ATG MO, 5'-GAGCTTCACCCATCTCGACTTTAGT-3';

arl311 spMO I1E2, 5'-AAACCCTTAGAAAACACACGTTTGT

arl312 spMO E2I2, 5'-TTGAGTGTTTTGCTCTGACCTGTGT

wnt4a MO, 5'-CGGGTAAGCTAAGTAAAGGTTGCTG-3';

MOs were injected into the yolk of one-to-two-cell stage embryos.

MO resistant *unc119c* mRNA was as follows: 5' ATG GAT AGT GAA GGT GTG AAG (wt); 5' ATG GAC AGC GAG GGA GTC AAA (resistant mutant; modified nucleotides bold).

Nitroreductase mediated cell/tissue ablation

Tg(tph2:NfsB-mCherry)y227 hemizygous embryos were treated starting from the shield stage with 10 mM metronidazole dissolved in DMSO, final concentration of DMSO was 0.2%. Embryos were fixed at 3dpf and stained with anti-acetylated tubulin antibody to visualize the HC.

Generation of zebrafish Unc119c antibody

A rabbit polyclonal antibody against 20 amino acids (N-ter-ERDEDEAEEDDEKEMADAQDR-C-ter) of the zebrafish Unc119c protein was generated by Covance, Inc.

Immuno staining

Three or 5 dpf zebrafish embryos were fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After fixation, embryos were stored in PBST0.1 (PBS with 0.1% TritonX-100 and 2% BSA) at 4°C. Samples were treated with blocking solution (PBST0.1 with 10% lamb serum and 1% DMSO) at room temperature for at least one hour prior to incubation with 1:100 diluted zebrafish anti-unc119c IgG fraction as primary antibody. For acetylated tubulin antibody staining, 3 dpf zebrafish embryos were fixed with 10% ice cold TCA for 30 min (or 4% paraformaldehyde at 4°C overnight), and treated as described above. Anti-acetylated tubulin (Sigma) was used at 1:400 dilution as the primary antibody. Anti-GFP antibody was obtained from Santa Cruz (sc-9996) and used at a 1:250 dilution. Anti-Isl antibody (39.4D) was obtained from Developmental Studies Hybridoma Bank and used at a 1:400 dilution. Secondary antibodies (Alexa Fluor 488 and 568; Molecular Probe/Invitrogen) were used at a dilution of 1:1000. Staining was observed with a Zeiss LSM510 confocal microscope. In some cases, contrast was adjusted in Photoshop for the entire image. Classification of HC disruption was done on confocal images. Disrupted HC are illustrated in Figures 1C, 2E,H, and 3E; in these cases approximately half of the length of the HC is missing centrally. Thin HC did cross the midline but were noticeable thinner than control in the central region. When no HC could be detected it was classified as "none."

Statistics

The significance of MO disruption of the HC and of rescue of the effect was evaluated by the Chi-squared test, considering normal HC as one category and all affected HC as the second category.

Cell culture, immunoprecipitation and blotting

For transfection, 5×10^5 293T cells were plated on 6-well plates for 24h prior to use, and 100 ng plasmid DNA encoding zebrafish Unc119c, Wnt4a and/or zebrafish Arl312 cloned in CS2⁺ was added. X-tremeGENE HP Transfection Reagent (Roche) was added and processed according to the manufacturer's protocol; GFP plasmid was added to equalize total DNA. After 24h, the cells were washed in cold PBS, and extracted with lysis buffer (50mM Tris, 150 mM NaCl, 10% Glycerol, 0.1% NP-40). Aliquots containing 20µg protein were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes (PVDF). The membranes were blocked in a TBS-Tween 20 (0.1%, V/V) solution containing 5% nonfat dry milk, incubated with anti-mouse Wnt4 antibody (R&D Systems, AF475) in

blocking solution overnight at 4°C, and washed with TBS-T three times for 10 min each. Horseradish peroxidase-conjugated secondary antibodies were used for chemiluminescence detection.

For Wnt4a immunoprecipitation, cells were transfected as above, the medium was collected after 24h, and centrifuged at 5,000 rpm to remove cells. The supernatant was incubated with Wnt4a antibody and protein G-sepharose beads overnight at 4°C. Beads were washed 10 times in lysis buffer, and proteins were recovered by boiling the beads in SDS sample buffer and analyzed using immunoblotting as described above.

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Bullet points

- Unc119c is involved in habenular commissure formation in the zebrafish forebrain
- Unc119c and Wnt4a have synergistic effects in habenular commissure formation
- We suggest that Unc119c acts in habenular commissure formation by affecting Wnt4a expression in the zebrafish pineal gland

Fig. 1.

Unc119c is involved in HC formation in zebrafish embryos. (**A, B**) Visualizing the HC. Transgenic zebrafish, *Tg(AANAT2:eGFP)* which express eGFP in the pineal gland, were used. The embryos were fixed at 54 hpf and stained with anti-GFP (green, pineal gland) and anti-acetylated tubulin (red, axonal tracts). The HC is located directly beneath the pineal gland. Dorsal view (**A**), lateral view (**B**). (**C-G**) Injection of *unc119c* MO disrupts HC formation. Wild type zebrafish embryos were injected with 1.5 ng *unc119c* ATG MO plus 100 pg GFP RNA (**C, F**), control MO (**D**), or *unc119c* ATG MO together with 100 pg *unc119c* RNA (**E, F**). Two different *unc119c* splice MOs, E3I3 and I3E4, also affected HC formation in a dose-dependent manner (**G**). Statistical significance is indicated as * signifying $p < 0.05$, and ** signifying $p < 0.01$. Experiments are compared to HC in control embryos (all of which were normal), except for the rescue data (**F**, second bar, MO+RNA), which are compared to MO-injected embryos (**F**, first bar). Total number of embryos examined is shown in parentheses above the bars. The splice MOs greatly reduced formation of mature mRNA (**H**). *dvdt*, dorsoventral diencephalic tract; *hc*, habenular commissure; *p*, pineal gland; *pc*, posterior commissure.

Fig. 2.

Injection of *arl3l1* and *arl3l2* MO disrupts HC formation. *Tg(AANAT2:eGFP)* embryos were injected with control (A-C), *arl3l1* (D-F, J), or *arl3l2* (G-I, J) ATG MO, fixed at 54 hpf and stained with anti-GFP (green, pineal gland) (A, D, and G) and anti-acetylated tubulin (red, neurons) (B, E, and H). Merged images (C, F, and I). Arrows indicate the HC (B, E, and H). Dose-dependence of the ATG MO effect (J). (K) Splice MOs were injected into wild type embryos which were stained for acetylated tubulin and classified as above. As the *arl3l2* E2I2 MO was somewhat toxic it was co-injected with 5 ng of p53 MO to suppress cell death (Robu et al., 2007). Experiments were compared to control HC, and statistical significance and number of embryos are indicated as specified in the legend to Figure 1.

Fig. 3.

Injection of *wnt4* MO affects HC formation in zebrafish embryos. *Tg(foxd3:GFP)* embryos were used to inject control (A-C) and *wnt4a* (D-F) MO, fixed at 54 hpf and stained with anti-GFP (green) (A and D) and anti-acetylated tubulin (red) (B and E). Merged image (C and F). Panels (D-F) are rotated counterclockwise by about 20 degrees. Dose-dependency of the MO effect (G). Experiments were compared to control HC, and statistical significance and number of embryos are indicated as specified in the legend to Figure 1.

Fig. 4.

wnt4a MO together with *unc119c* MO (**A**) or *arl3l2* MO (**B**) have synergistic effects on HC formation. *Tg(AANAT2:eGFP)* embryos were injected with MOs at the levels indicated (in ng); cont, control MO. Statistical significance and number of embryos are indicated as specified in the legend to Figure 1. HC in embryos injected with combined MOs (third bar) were compared to those in single MO-injected embryos (first and second bars). In **A** each comparison led to different significance, as indicated, while in **B** both comparisons were highly significant.

Fig. 5.

Cultured HEK293T cells were transiently transfected with Wnt4a, with or without HA-Unc119c and Flag-Arl3l2, as described in Experimental Procedures. Wnt4a, Unc119c and Arl3l2 in whole cell lysates were detected by immunoblotting, while secreted Wnt4a was recovered from the medium by immunoprecipitation before blotting (A). Quantification of the gel using ImageJ (B) shows the level of Wnt4a extracted from cells (open bars) or recovered from the medium (filled bars). Cells were transfected with Wnt4a (W), Wnt4a and Unc119c (W+U), Wnt4a and Arl3l2 (W+A), or all three (W+U+A). The level of expression in the Wnt4a lane was set as one.

Fig. 6. NTR-mediated pineal gland ablation caused HC disruption. *Tg(tph2:NfsB-mCherry)y227* embryos were treated with DMSO (control, **A**, and **C**), or 10 mM Mtz (**B** and **D**) for 70 hr, fixed at 78dpf, and stained with anti-acetylated tubulin (red). Arrows indicate the pineal gland (**A** and **B**), or the HC (**C** and **D**).

Fig. 7.

Unc119c antibody visualized Unc119c protein enriched in the pineal gland.

Tg(AANAT2:eGFP) embryos were double-stained with anti-GFP (green) to stain photoreceptor cells (**A**), and *unc119c* antibody (red, **B**). Wild type embryos were double-stained with anti-Isl-1 (green) to stain projection neurons (**D**), and *Unc119c* antibody (**E**). Merged images (**C** and **F**). The pineal gland is outlined in (**A** and **D**).