WNT5A is transported via lipoprotein particles in the cerebrospinal fluid to regulate hindbrain morphogenesis

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WNTs are lipid-modified proteins that control multiple functions in development and disease via short- and long-range signaling. However, it is unclear how these hydrophobic molecules spread over long distances in the mammalian brain. Here we show that WNT5A is produced by the choroid plexus (ChP) of the developing hindbrain, but not the telencephalon, in both mouse and human. Since the ChP produces and secretes the cerebrospinal fluid (CSF), we examine the presence of WNT5A in the CSF and find that it is associated with lipoprotein particles rather than exosomes. Moreover, since the CSF flows along the apical surface of hindbrain progenitors not expressing Wnt5a, we examined whether deletion of Wnt5a in the ChP controls their function and find that cerebellar morphogenesis is impaired. Our study thus identifies the CSF as a route and lipoprotein particles as a vehicle for long-range transport of biologically active WNT in the central nervous system.

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nt proteins (Wnts) are key players in development and in adult organisms. A crucial feature underlying their function is the ability to spread over long distances leading to formation of concentration gradients, which elicit and orchestrate diverse patterning decisions during development. Post-translational modification, addition of lipid moieties in the endoplasmic reticulum by the acyltransferase Porcupine, plays a central role in the proper maturation of Wnt ligands and their secretion. The presence of essential lipid modifications in fully processed Wnts represents a challenge for unhindered transport of Wnts in the water-based extracellular space. Several mechanisms for long-range transport of Wnts have been proposed in Drosophila, such as lipoprotein particles, incorporation into exosomes, and direct binding to the transport protein Swim. Other proposed modalities include transport of Wnts via specialized filopodia such as cytonemes in Drosophila or zebrafish, or via migrating cells, such as neural crest cells in chicken. In mammals, Wnts have been found to be transported via exosomes in the epididymal fluid of mice and in vitro, via binding to the transport protein Aflamin. However, the relevance of some of these mechanisms to the mammalian physiology remains unclear.

A previous study has reported that deletion of Otx2 in the hindbrain choroid plexus (HbChP) increases Wnt4 expression in this structure as well as the levels of WNT4 in the cerebrospinal fluid (CSF). This study proposed a role of the ChP in regulation of WNT4 secretion into the CSF and WNT signaling at a distant site. However, it is unclear whether WNT4 does really control proliferation in a direct manner at a site distant to where it is produced in vivo. Moreover, it remains to be determined how can a lipophilic molecule such as WNT be transported via the CSF. We thus decided to examine the capacity of the ChP to secrete WNT proteins into the CSF and investigated the mechanism of transport of WNT proteins in the CSF.

Our results show that the embryonic HbChP, but not the telencephalic choroid plexus (TelChP), specifically expresses and secretes high levels of WNT5A into the CSF. Mechanistically, our data indicate that WNT5A preferentially associates to lipoprotein particles, rather than exosomes. Moreover, analysis of hindbrain progenitors that do not express Wnt5a and do not have access to WNT5A protein from neighboring cells revealed a morphogenetic defect upon Wnt5a deletion. Thus, our result identifies WNT5A as a key regulator of morphogenetic behavior of dorsal hindbrain progenitors near, but not adjacent, to the ChP and identify lipoprotein particles as the mechanism of transport of biologically active WNT proteins in the CSF.

Results

Distinct expression of Wnt5a in the choroid plexuses. To identify the Wnt family members expressed in the various ChPs, we first analysed expression profiles of all Wnt ligands by means of in situ hybridization at mouse embryonic day (E) 13.5 (Supplementary Fig. 1a). Wnt5a was the Wnt with the strongest expression in the HbChP (located in the fourth ventricle) (Fig. 1a, b and Supplementary Fig. 1a). Wnt5a expression was maintained from E12.5 to E17.5, as assessed by qPCR (Fig. 1c). These results were further corroborated by in situ analysis at E13.5 and E17.5 (Fig. 1d). Notably, Wnt5a was not detected in the TelChP (located in the lateral ventricle) (Fig. 1b, c), a result in line with previous findings. On the other hand, Wnt5a was found in the adjacent cortical hem (CH) (Fig. 1b and Supplementary Fig. 1a, asterisks), where Wnt2b, 3a, 7a, 7b, 8b, and 9a are also expressed (Supplementary Fig. 1b). Interestingly, high Wnt5a expression was found in the epithelium of the HbChP (Fig. 1b, d and Supplementary Fig. 2a), while Wnt5b expression was very low at E13.5 (Supplementary Fig. 1a) or nearly undetectable at E14.5 (Supplementary Fig. 2b–d), suggesting that a possible redundancy between these two Wnts is unlikely.

Using a specific WNT5A antibody, validated in the Wnt5a−/− (Wnt5aKO) mice (Supplementary Fig. 3a, b), we found that WNT5A protein localizes to the HbChP from E12.5 to E17.5, but it is absent in the TelChP (Fig. 1e, f). At later embryonic and early postnatal stages, the expression and protein levels of WNT5A in the HbChP progressively decreased (Fig. 1d and Supplementary Fig. 3c, d), suggesting a role of WNT5A in the HbChP during embryonic development. WNT5A was typically found in the apical part of the cyttoplasm of secretory epithelial cells, and on occasion, in punctate structures close to or above the apical cell membrane stained with Aquaporin-1 (AQP1) (Fig. 1g).

Analysis of human fetal brains (week 9 post conception) confirmed that WNT5A protein is found in the HbChP, but not in the TelChP, also in humans (Fig. 2a). Moreover, WNT5A was also identified in the apical part of the cyttoplasm of the HbChP epithelium (Fig. 2b) and in punctate structures close to or above the AQP1l apical membrane of epithelial cells, in direct contact with CSF (Fig. 2c, arrowheads), a result suggestive of WNT5A being secreted from the HbChP to the CSF.

We next examined whether the HbChP expresses mouse Wntless (Wls; also known as Gpr177), a gene encoding a protein indispensable for Wnt secretion. qPCR analysis revealed that Wls is expressed in a similar pattern as Wnt5a in the HbChP from E12.5 to E17.5 (Fig. 3a), with its expression being restricted to HbChP epithelium at E13.5 (Fig. 3b), while in TelChP Wls transcripts can be detected only in the adjacent CH region (Fig. 3b, asterisk). At a protein level, WLS was readily detected in the HbChP, but not in the TelChP, as assessed by western blot at E14.5 and E17.5 using a validated WLS antibody (Fig. 3c and Supplementary Fig. 4a). Immunofluorescence analysis showed that while WLS was absent from the TelChP at E14.5 (Fig. 3d), high WLS levels were detected in the HbChP epithelium. WLS was also detected in the CH adjacent to the TelChP (Fig. 3d, asterisk). Notably, both Wls expression and its protein abundance were highest in HbChP epithelial cells that were most positive for Wnt5a (Fig. 3e, f, arrowheads). Levels of WLS and WNT5A showed similar dynamics with high levels at E12.5 and E14.5, by E17.5 signal intensity decreased and became undetectable by postnatal day 23 (Fig. 3d and Supplementary Fig. 4b). Thus, our results are compatible with a possible role of the HbChP in secreting WNT5A into the CSF during embryonic development.

Active WNT5A is secreted by HbChP cells. To address this possibility, we first examined whether WNT5A is present in the embryonic CSF at E11.5, before the formation of the HbChP, and after that, at E14.5. Western blot analysis revealed the presence of WNT5A in the CSF at E14.5, but not at E11.5 (Fig. 4a). We next established a primary culture of E14.5 ChP epithelial cells and examined their capacity to secrete WNT5A to the media. Both TelChP and HbChP cells were positive for the ChP epithelial markers, ZO1 and AQP1 (Fig. 4b). The analyses of media conditioned by these cells showed the presence of WNT5A in media from the HbChP, but not by the TelChP primary cells (Fig. 4c). These results indicate that WNT5A is indeed produced and secreted from primary HbChP epithelial cells. Moreover, we found that the conditioned medium (CM) obtained from the HbChP, but not from the TelChP, was able to activate Wnt signaling, as shown by the capacity of HbChP-derived CM to induce phosphorylation of Dishevelled-3 (DVL3) at a level comparable to that of recombinant WNT5A (Fig. 4d). Thus combined, our data indicate that the WNT5A secreted by epithelial cells from the HbChP is biologically active. To determine whether the
**Fig. 1** Wnt5a expression is restricted to HbChP. 

a | Localization of TelChP and HbChP within the embryonic brain and illustrative pictures of in situ hybridization for ChP marker Transthyretin (Ttr) in sagittal sections of TelChP and HbChP and coronal section of HbChP. Image credit: Allen Institute. A: anterior, P: posterior. 

b | Sagittal sections showing Wnt5a expression being restricted to HbChP epithelium and absent from the stromal cells at E13.5. Wnt5a expression is absent from the TelChP, but present in CH (asterisk). Image credit: Allen Institute. Scale bar: 100 μm. 

c | Real-time qPCR of Wnt5a expression in TelChP and HbChP at E12.5, E14.5 and E17.5. The expression was normalized against expression level of β-actin in each condition. Graph shows n = 3 biologically independent samples; error bars represent mean ± s.d.; P-values (two-tailed Student’s t-test with unequal variance): * P < 0.05, ** P < 0.01. TelChP vs HbChP: E12.5 P = 0.012; E14.5 P = 0.0011; E17.5 P = 0.0023. Biological replicates are indicated in the graph. 

d | In situ hybridization and immunostaining analysis of HbChP coronal sections shows Wnt5a expression level differences between E13.5 and E17.5, n = 3. Immunostaining of Aquaporin-1 (AQP1) was used as a marker of HbChP epithelial monolayer highlighting the apical membrane of the tissue. Inset: Magnified view of fluorescent signal of Wnt5a transcripts detected in HbChP epithelium. Scale bar: 50 μm, inset scale bar: 10 μm. 

f | Western blot analysis of WNT5A protein in lysates of TelChP and HbChP at E12.5, E14.5 and E17.5, n = 3. β-actin serves as a loading control. 

g | Immunofluorescence analyses of WNT5A in TelChP and HbChP at E14.5, n = 4. Comparison with the AQP1 shows absence of WNT5A in the TelChP. In contrast, WNT5A can be detected in the HbChP epithelial layer. Scale bar: 100 μm. 

High magnification view of HbChP epithelium apical membrane highlighted by AQP1 showing WNT5A in punctate structures in the extracellular domain of the plasmatic membrane (arrowheads); n = 4. Scale bar: 5 μm. Source data are provided as a Source Data file.
induced by recombinant WNT3A or Wnt3a CM in the same cells (Fig. 4k and Supplementary Fig. 5a, b). Altogether these results indicate that WNT5A and no other WNTs are the source of the WNT signaling activity in the media produced by HbChP primary cells and that such HbChP-produced WNT5A activates Wnt/β-catenin independent signaling.

**WNT5A associates with lipoproteins in the HbChP epithelium.**

Previous studies have described the association of WNTs to either exosomes or lipoproteins in different cellular models. Extracellular vesicles, such as exosomes, and lipoprotein particles can both be separated from cell supernatants by differential centrifugation. We thus fractionated mouse HbChP supernatants by ultracentrifugation and characterized the different fractions (Fig. 5a and Supplementary Fig. 6a). Immunoblot analysis showed that WNT5A is not present in the fraction containing exosomes and positive for various exosomal markers, such as CD63, HSP70, tumor susceptibility gene 101 (TSG101), and Flotillin-2 (FLOT2). Instead, WNT5A was enriched in the supernatant, a fraction positive for Apolipoprotein E (APOE) and Apolipoprotein J (APOJ), also known as Clusterin, which are integral structural components of lipoprotein complexes (Fig. 5b).

In support of this finding, immunofluorescence analysis of E14.5 mouse embryos revealed that WNT5A does not colocalize with any exosomal marker (Fig. 5c, arrowheads).

We next decided to investigate the possible association of WNT5A to lipoprotein particles. Lipoproteins represent a heterogeneous family of macromolecule complexes classified by their size, lipid-to-protein ratio and presence of specific apolipoproteins. Immunofluorescence showed a high degree of colocalization between extracellular WNT5A puncta and different species of apolipoproteins including Apolipoprotein A-I (APOA1), Apolipoprotein B-100 (APOB) and APOE in HbChP epithelium (Fig. 5d). Quantitative analysis of the colocalization of WNT5A and apolipoproteins or exosomal markers in the apical portion of HbChP epithelium (see methods and Supplementary Fig. 7a) revealed that 20–30% of WNT5A colocalized with apolipoproteins: 33.25% with APOJ, 20.77% with APOA1, 31.28% with APOB, 27.19% with APOE, while only 3-7% co-localized with exosomal markers—CD63 and TSG101 (Fig. 5e). The colocalization of WNT5A and apolipoproteins was specific as shown by the near complete absence of colocalization of WNT5A and APOA1+ puncta in the Wnt5aKO compared to Wnt5aWT HbChP epithelium (Supplementary Fig. 7b) as well as by additional controls of antibody specificity (Supplementary Fig. 7c and 8a–i). Furthermore, quantitative analysis of the colocalization of WNT5A with APOJ, an apolipoprotein highly enriched in the central nervous system (CNS), and other apolipoproteins revealed extensive triple co-localization with APOA1 (71.93%), APOB (48.72%), or APOE (49%) (Fig. 5f). In contrast, there was almost no triple colocalization of APOJ, WNT5A, and the exosome marker CD63 (Fig. 5g). From these data we conclude that WNT5A is mainly found in association with apolipoproteins in epithelial cells of the HbChP.

**Extracellular WNT5A associates with lipoproteins.** To study the association of WNT5A and different classes of lipoprotein particles outside of the ChP cells, we performed discontinuous gradient ultracentrifugation of HbChP-derived primary culture supernatants. WNT5A was found to segregate both with the low-density lipoprotein (LDL) fraction and the high-density lipoprotein (HDL) fraction, (Fig. 6a and Supplementary Fig. 9a). In addition, mouse V5-tagged WNT5A (WNT5A-V5) co-immunoprecipitated HA-tagged APOJ or APOE (Fig. 6b, c, asterisk) in HEK293T cells. Likewise, APOE or APOJ were able to
**Fig. 3** Wls is specifically expressed and produced in HbChP. 

**a** Gene expression analysis by real-time qPCR of Wntless (Wls) expression in TelChP and HbChP at E12.5 and E17.5. Wls gene expression was normalized against expression level of β-actin in each condition. Graph shows n = 3 biologically independent samples; error bars represent mean ± s.d.; P-values (two-tailed Student’s t-test with unequal variance) for differences between analysed ChPs: * P < 0.05. TelChP vs HbChP: E12.5 P = 0.0261; E17.5 P = 0.0237. Biological replicates are indicated in the graph. 

**b** In situ hybridization analysis of HbChP coronal sections confirms Wls expression levels difference between E13.5 TelChP and HbChP, n = 6. Wls and Wnt5a expression in TelChP region is restricted mostly to CH (asterisk) and not to TelChP epithelium. Scale bar: 50 μm. Inset images show higher magnification view of fluorescent signal corresponding to Wls and Wnt5a transcripts in corresponding TelChP and HbChP epithelium. Scale bar: 10 μm. 

**c** Western blot analysis of WLS levels in tissue lysates of TelChP and HbChP isolated at E14.5 and E17.5, n = 3. β-actin serves as a loading control. 

**d** Representative images from immunofluorescent analysis of TelChP and HbChP at E14.5 displaying presence of WLS only in the HbChP, n = 4. WLS is absent from the TelChP, but present in CH (asterisk). Scale bar: 100 μm. 

**e** Detailed view of Wnt5a and Wls transcripts distribution highlights correlation between levels of Wls and Wnt5a in individual HbChP epithelial cells at E13.5 (arrowhead - Wnt5a\textsuperscript{high}/Wls\textsuperscript{high}; empty arrowhead - Wnt5a\textsuperscript{low}/Wls\textsuperscript{low}), n = 6. Scale bar: 5 μm. 

**f** Magnified image of immunofluorescent analysis of HbChP epithelium at E14.5 shows high degree of correlation between WNT5A and WLS levels observed within HbChP epithelial cells (arrowheads), n = 4. Scale bar: 5 μm. Source data are provided as a Source Data file.
pull-down WNT5A-V5 indicating that WNT5A and APOE or APOJ can form a complex (Fig. 6d, e). In addition, WNT5A-V5 was able to pull-down endogenous APOJ from the lipoprotein fraction isolated from the media of TR-CSFB cells, a ChP-derived cell line29, (Supplementary Fig. 9b). Moreover, mass spectrometry analysis of a WNT5A-V5 pull-down from TR-CSFB cells CM identified enrichment in additional proteins commonly associated with the HDL-specific proteome, such as Apolipoprotein A-I, Apolipoprotein A-II or Vitamin D-binding protein (Supplementary Fig. 9c). Strengthening these observations, we also...
demonstrate direct in vitro interaction of WNT5A with human APOJ as shown via co-immunoprecipitation analysis (Fig. 6f).

To further investigate the necessity of lipoprotein particles for WNT5A transport, we used a lipid removal agent (LRA) to delipidate the serum used in primary HbChP epithelial cell cultures (Fig. 6g). Upon lipid removal, WNT5A was not detected in the supernatants of primary HbChP epithelial cultures, while it slightly increased in cell lysates, suggesting that the ligand was
retained inside the cells (Fig. 6h). On the other hand, when cells grown in delipidated serum for 3 days, were treated with serum (which does not contain WNT5A), WNT5A was again found in the media (Fig. 6h). Moreover, treatment of delipidated cultures with HDL or LDL fractions isolated from mouse serum rescued the presence of WNT5A in the media (Fig. 6i), indicating that lipoproteins are sufficient to restore the presence of soluble WNT5A in the media of HbChP epithelium primary cultures. In addition, in vitro experiments showed that recombinant WNT5A physically binds to LDL and forms a high-molecular weight complex (Fig. 6, k) that retains capacity to activate Wnt signal transduction (Fig. 6i, m). Thus, our results indicate that apolipoproteins are required for the secretion of WNT5A by HbChP cells and that the WNT5A-lipoprotein complexes are functional.

WNT5A derived from HbChP regulates hindbrain morphogenesis. After demonstrating that active WNT5A is secreted into the CSF by epithelial cells of the HbChP, we focused our attention on the possible effect of HbChP-derived WNT5A on target cells in vivo. For our analysis we selected the ventricular zone of the developing cerebellum since it is in the proximity of the HbChP, and it is exposed to high local concentrations of HbChP-derived factors, as previously described for Shh.\(^{31}\) Dorsal hindbrain progenitor cells lining the ventricle, anterior to the HbChP, devoid of Wnt5a expression at E13.5 (empty arrowheads in Fig. 7a) were found to express WNT receptors, such as Fzd3 and Fzd10, and WNT/PCP signaling components, such as Vangl2 or Celsr2 (Supplementary Fig. 10a, arrowheads).\(^{32}\) In addition, lipoprotein receptors such as Scarb1,\(^{33}\) Lrp2,\(^{34}\) and Lrp4,\(^{35}\) receptors previously shown to be involved in Wnt signaling,\(^{36}\) are also expressed in this region (Supplementary Fig. 10b). Moreover, while WNT5A protein was found in the apical surface of WT hindbrain progenitors, colocalizing with APOE and APOJ, it was not found in the Wnt5a\(^{KO}\) embryos (Fig. 7b, c). These findings support the hypothesis that WNT5A/apolipoprotein complexes target hindbrain progenitor cells lining the ventricular cavity. We thus decided to investigate the developing cerebellum and examine whether WNT5A produced by HbChP cells can control developmental processes typically regulated by WNT5A, such as morphogenesis or the balance between cell proliferation and differentiation.\(^{37,38}\) Analysis of either complete or conditional HbChP Wnt5a knock-out (Wnt5a\(^{KO}\) or Wnt5ac\(^{KO}\)) embryos revealed a robust reduction of the size (area) of the developing cerebellum (Fig. 7d–h). While the dorso-ventral axis in the midline decreased (Fig. 7i), no major difference was found in the latero-medial axis (Fig. 7j). Diminished tissue size can be thus described as a disproportionate shortening of the medial dorso-ventral axis and an increase in the width-to-length ratio (Fig. 7k).

Analysis of the proliferative markers in this region showed a significant increase in the number of KI67\(^{+}\) but not EdU\(^{+}\) cells in Wnt5a\(^{KO}\) embryos at E16.5 (Supplementary Fig. 11a–d). However, no change in KI67\(^{+}\) cells was observed in Wnt5a\(^{KO}\) embryos at E16.5 (Supplementary Fig. 11e, f). Additionally, we did not observe any change in the number of activated caspase 3-positive cells in either Wnt5a\(^{KO}\) or Wnt5ac\(^{KO}\) embryos (Supplementary Fig. 11g, h). Altogether this indicates that changes in cerebellum morphology in the Wnt5a\(^{KO}\) and Wnt5ac\(^{KO}\) are not due to an alteration of proliferation or survival, but rather can be attributed to an aberrant morphogenesis.

Discussion
In the present study, we demonstrate that WNT5A is produced in the epithelial cells of the HbChP in a very specific temporal developmental window, from E12.5 to early postnatal age in mouse. Likewise, analysis of human fetal brains at week 9 confirmed the presence of WNT5A in the HbChP. Our results show that WNT5A colocalizes with lipoproteins in the apical part of both the HbChP epithelium, a cell type that expresses Wnt5a, and in target hindbrain progenitors lining the ventricle, that express Wnt receptors, signaling components as well as receptors for lipoprotein particles, but do not express Wnt5a. Moreover, we found that the HbChP secretes WNT5A, which associates with lipoprotein particles in the CSF. We thus suggest that lipoprotein particles provide a vehicle for the transport of WNT5A through the CSF, from the HbChP to target cells lining the ventricle. In agreement with this possibility, we found that Wnt5a is required for morphogenesis of dorsal hindbrain. This observation is in line with previous reports highlighting the pivotal role of Wnt5a in morphogenesis of various regions of the developing CNS, including the ventral midbrain\(^{39}\) and the cerebellum.\(^{40}\) However, unlike the developing ventral midbrain\(^{41}\), we did not observe an effect on proliferation in the Wnt5a\(^{KO}\), or on cell survival, suggesting a selective alteration of morphogenesis.

With regards to the mechanism of the transport of WNT5A in the CSF, several lines of evidence support WNT5A being transported via lipoproteins rather than exosomes. Firstly, immunofluorescence analysis indicates that WNT5A mainly colocalizes with apolipoproteins rather than with exosomal markers in the apical part of the HbChP epithelium. Second, biochemical fractionation of media from HbChP cultures showed that WNT5A segregated with apolipoproteins, but not with exosomal markers. Third, WNT5A was also found to co-localize with apolipoproteins in target cells lining the ventricle, highlighting the role of lipoproteins in the transport of WNT5A from its source to target cells. Notably, we found that WNT5A interacts with several apolipoproteins, such as APOA1, APOB, APOE, and APOJ, a result in agreement with the previous identification of all major
classes of lipoprotein particles in CSF by proteomic analyses. This is also in agreement with the putative role of the ChP as the main entry point for lipoprotein particles into the CSF for their distribution into the CNS.

Our study also identifies WNT5A as an additional member of a growing family of biologically active signaling molecules produced in the ChP that are present in the CSF, and are transported in lipoprotein particles. Moreover, WNT5A shares with another lipid-modified morphogen, SHH, the fact that it is produced in the HbChP, distributed in a transventricular fashion, transported via lipoproteins and capable of regulating neural development in progenitor cells lining the ventricular cavity. On the other hand, lipoproteins such as LDL, have been found to contribute to the capacity of the CSF to control neural development and promote neuronal differentiation. Interestingly, we found expression of various receptors for lipoprotein particles within the ventricular zone of developing brain, which combined with the previously reported accumulation of lipoprotein particles in the ventricular zone of the brain, suggests a possible role for lipoproteins complexes to act as a universal vehicle for distribution of lipids and lipid-modified signaling molecules within the CNS. Our data suggest that one of the mechanisms by which lipoproteins may control neural development is by associating to and transporting signaling...
molecules such as WNT5A. Indeed, deletion of Wnt5a eliminated the presence of WNT5A associated to lipoprotein particles in the apical part of hindbrain progenitor cells lining the ventricle, indicating the necessity of WNT5A for lipoprotein particles to gain such localization. Notably, our results also show overlapping expression domains of lipoprotein receptors and Wnt ligands receptors such as Frizzled receptors in ventricular zone of developing cerebellum. Moreover, Syndecans, a class of heparan sulfate proteoglycans that bind Wnts and are implicated in regulating the formation of Wnt ligand gradients and Wnt signaling, can interact with lipoprotein particles. Combined, our results and data in the literature support the notion that lipoproteins, WNT5A and their receptors are required for proper neural development.

In sum, our study provides key evidence supporting the association of Wnts with lipoprotein particles, not only in WNT-producing cells of the ChP, but also after their secretion to the CSF and in target cerebellar progenitor cells lining the ventricular cavity. Our results thus support the hypothesis that the incorporation of Wnts, e.g., WNT5A, to lipoprotein particles, provides a mean for their secretion, extracellular dispersal and cell targeting. It remains to be determined whether the association of WNT5A to lipoprotein particles is a specific mechanism restricted to the HbChP or part of a more general strategy used by different cells to transport Wnts.

**Methods**

**Mouse strains.** Embryos were obtained from female mice of C57BL/6J (Charles River Laboratories, Germany), Wnt5afl/fl (referred to as Wnt5aKO in the article)56 or newly generated conditional knock-out mouse strain described below (referred to as Wnt5alox/lox in the article).

Mouse strain Wnt5afl/fl (referred to as Wnt5alox/lox in this article)56 was purchased from Jackson laboratories; Foxj1creERT2/GFPPh (referred to as Foxj1creERT2 in this article)57 and Gt(Rosa)26Sor14CAG-tdTomatoHze (referred to as tdTomato in this article)58 were shared with Karolinska Institutet, Sweden on collaboration agreement. Induction of conditional knock-out or tdTomato reporter was induced by single dose tamoxifen (Sigma) intra-peritoneal injection of pregnant female mice in concentration of 4.5 mg of tamoxifen dissolved in sterile sunflower oil per 20 g weight of mouse. Embryos were harvested at E14.5 or E16.5. All mouse strains were housed, bred, and treated in accordance with protocols approved by the local ethical committees (Stockholm’s Norra Djurförsöksätskommitté, Nämnd - N158/15, N326/12 or Czech Centre for Phenogenomics (Institute of Molecular Genetics, CAS) and Central Commission for Animal Welfare of Ministry of Agriculture Czech Republic - PP-90-2015).

**Embryonic CSF isolation.** Embryonic CSF was isolated from embryos of pregnant CD1 IGS mice. Embryonic CSF was obtained by microaspiration from E11.5 embryos, using pulled tip glass microcapillary pipette glass. Needle was inserted into the fourth brain ventricle with embryo being placed on its side. CSF collected for the analysis was obtained from 2 or 3 entire litters depending on the embryonic stage (20–25 embryos). Samples were microscopically controlled for presence of blood contamination and samples showing signs of the contamination were discarded.

**Choroid plexus epithelial cells primary culture.** ChP tissue was collected from E14.5 embryos isolated from sacrificed pregnant CD1 mice and choroid plexus epithelial cells (CPEC) were isolated from TelChP and HbChP. During isolation, extracted tissue was kept at room temperature (RT) in HBSS solution (Gibco). After isolation, extracted tissue was briefly centrifuged (200 g, 10 s at RT). Following aspiration of supernatant, 500 µl of 2 mg/ml solution of Pronase (Sigma–Aldrich) was added to the extracted tissue and incubated for 5 min at 37 °C. Solution was then transferred to DMEM/F-12 medium containing 10% FBS (Gibco) and cultured (300 g, 2 min at RT). Tissue was transferred to complete culture medium consisting of DMEM/F-12 supplemented with 10% FBS, 10 ng/ml EGF (Invitrogen), 20 µg/ml bovine serum albumin (Sigma) 50 U/ml penicillin, and 50 U/ml streptomycin. Cells were mechanically dissociated by 6–8 times forced passage through a 21-gauge needle, followed by gentle repeated resuspension with 200-µl pipette. Finally, cells were seeded onto laminin (Invitrogen) coated 24-well plates (2–3×10⁵ cells per well). To achieve higher purity of epithelial cells, adhering-off method has been applied to reduce fibroblast contamination. After the initial seeding, supernatant containing unadsorbed cells was transferred to new laminin coated well thus removing from culture fibroblasts characterized by higher adherence affinity.

In order to produce CM, CPEC primary cultures were maintained in complete culture medium. CM was collected every 48 h up to 10 days after seeding. Supernatant was subjected to sequential centrifugation steps of 200 g for 5 min (to remove viable cells), 1500 g for 10 min (to remove dead cells), and 6000 g for 15 min (to remove cell debris). Used reagents are listed in the Supplementary Table 1.

**Fetal tissue section.** Ethical approval allowing human fetal tissue acquisition and analysis was provided by the National Research Ethics Service Committee East of England—Cambridge Central, UK (ethics number 96/085).

**Cell culture and transfection.** HEK293T cells were seeded in complete DMEM medium containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin (Gibco) on 10 cm dishes 24 h prior transfection. The cells were transfected with total of 5 µg of DNA at ~40% confluence in DMEM medium only. The transfection reaction mixture was prepared using OptiMEM (Gibco) and Lipofectamine 2000 (Invitrogen), with ratio of 1 µg DNA: 2 µl Lipofectamine 2000, followed by incubation with cells for 4–6 h. Afterwards the transfection medium was exchanged for the complete medium. Used constructs are listed in the Supplementary Table 2.

**TR-CSFB cells** were seeded in DMEM containing 10% FBS, 2 mM L-glutamine, 50 U/ml penicillin, and 50 U/ml streptomycin (Gibco) on 10 cm dishes 24 h prior transfection. The cells were transfected with total of 8 µg of DNA in ~70% density in DMEM medium only. The transfection reaction mixture was prepared using PEI (Sigma) with ratio of 1 µg DNA: 2.5 µl PEI, followed by incubation of cells for 6 h. Afterwards the transfection medium was exchanged for the complete DMEM medium. CM was collected after 48 h and same pre-clearing protocol as mentioned for primary culture medium has been applied to remove any cell contamination. Used constructs are listed in the Supplementary Table 2.

**Western blotting.** For western blot, samples were subjected to SDS-PAGE, electrotransferred onto Hybond-P membrane (GE Healthcare), immuno detected using appropriate primary and secondary antibodies and visualized by ECL.
Western blot signal intensities were calculated using ImageJ. Briefly, area of the peak intensity for the protein of interest was divided by corresponding values of peaks intensity obtained for control protein. Uncropped images for all the western blots presented in the article can be found in Supplementary Fig. 12. Used reagents are listed in Supplementary Table 1. Used antibodies are listed in Supplementary Table 3.

Co-Immunoprecipitation. Dishes with transiently transfected HEK293T cells were placed on ice, washed twice with ice-cold PBS and lysed in 1 ml of lysis buffer for 20 min. Lysis buffer contained 50 mM Tris pH 7.6, 200 mM NaCl, 1 mM EDTA, 0.5% NP40, fresh 0.1 mM DTT (Sigma), and protease inhibitor cocktail (Roche). The lysis was centrifuged at 4 °C at 20,000 g for 20 min. After the centrifugation, an aliquot of the input sample representing 4% of the total cell lysate was taken from each condition. The lysates were then pre-cleared using 20 µl/sample DynaBeads.
Fig. 7 WNT5A secreted by HbChP controls morphogenesis of embryonic cerebellum. a In situ analysis of Wnt5a and Wf5 expression in the dorsal neuroepithelium adjacent to HbChP at E13.5 shows transcripts restriction to the HbChP epithelium, n = 4. Lower panel highlights absence of Wnt5a and low Wf5 expression in cerebellar ventricular zone (cVZ, empty arrowheads) compared to the HbChP (arrowheads). Scale bar: top 50 μm, bottom 10 μm. b WNT5A observed in the apical cVZ at E14.5 (arrowheads) is absent in Wnt5a KO embryos (empty arrowheads), n = 3. Scale bar: top 5 μm, inset 2 μm. c WNT5A and APOE (arrowheads) or APOE with APOJ (empty arrowhead) immunostaining in the apical cVZ (dotted line) at E14.5, n = 4. Scale bar: top 5 μm, bottom 2 μm. d Scheme of analyzed cerebellar parameters: total area (dash line), width (lateralo-medial, L-M, horizontal arrows) and length (dorsoproventral, D-V, vertical arrows). Scale bar: 200 μm. e Coronal sections of cerebellum in Wnt5a WT and Wnt5a KO embryos. Scale bar: 200 μm. f Tomato staining in E14.5 Foxi-CreERT2 embryo demonstrates recombination in HbChP epithelium, which is absent in the cVZ (inset, empty arrowhead) compared to HbChP (arrowheads), n = 3. Scale bar: top 50 μm, inset 20 μm. g Coronal sections of cerebellum in Wnt5a WT vs Wnt5a KO embryos. Scale bar: 200 μm.

h-k Analysis of the h, i total area, j width, and k width/length ratio of cerebellum in Wnt5a WT, Wnt5a KO and Wnt5a KO embryos. Graphs show individual data points (dots) from n = 3 biologically independent samples; error bars represent mean ± s.d.; P-values (two-tailed Student’s t-test with unequal variance): * P < 0.05, ** P < 0.01, *** P < 0.001. Corresponding P-values for differences between Wnt5a WT (WT), Wnt5a KO (KO) and Wnt5a KO (kKO) - WT vs. KO: 0.0004, WT vs. cKO: 0.0001; WT vs KO: 0.0353, WT vs cKO: 0.0098; WT vs KO or cKO - not significant; k WT vs KO: 0.016, WT vs cKO: 0.0115. l Schematic depiction of the model for WNT5 secretion by HbChP and its transventricular delivery to recipient regions. Source data are provided as a Source Data file.

In vitro protein binding assay. To assess physical interaction of WNT5A and apolipoproteins, 2 μg of human native APOE (RDI6203-025, Biovendor) or human native APOJ (cat.no. SP6303, Sigma) were incubated with 150 μg of recombinant WNT5A (P22725, R&D Systems) in 200 μl of binding buffer (50 mM Tris pH 7.6, 100 mM NaCl, 1 mM EDTA) overnight at 4°C. The samples were washed five times in 800 μl of lysis buffer (DTT- and protease inhibitors-free) and twice in detergent-free lysis buffer. Protein complexes were eluted with 50 μl of 1X Laemmli buffer in 95°C for 5 min and were loaded directly onto a 10% acrylamide gel for western blot analysis.

In case of co-immunoprecipitation from supernatant, CM was collected after 48 h and same protocol as mentioned for primary culture medium was applied to remove any cell contamination. CM was next pre-cleaned using 0.25% volume of G-protein Sepharose beads (GE Healthcare) and then incubated overnight with 0.3 μg/ml of anti-V5 antibody (cat. no. 96025, Invitrogen). Afterwards, the supernatant has been incubated with 50 μg of G-protein Sepharose beads. Resulting immunoprecipitates were washed several times with lysis buffer (DTT- and protease inhibitors-free) and twice in detergent-free lysis buffer. Protein complexes were eluted with 50 μl of 1X Laemmli buffer in 95°C for 5 min and were loaded directly onto a 10% acrylamide gel for western blot analysis. Used reagents are listed in the Supplementary Table 1. Used antibodies are listed in the Supplementary Table 3.

Mass spectrometry. Beads with extracted proteins were washed three times with 50 mM ammonium bicarbonate buffer and subjected to digestion by trypsin (2h, 37°C; sequencing grade, Promega). Tryptic peptides were extracted into LC-MS vials by 2.5% formic acid (FA) in 50% acetonitrile with addition of polyethylene glycol (20,000; final concentration 0.01%) and concentrated in a SpeedVac concentrator (Thermo Scientific). LC-MS/MS analyses of tryptic peptide mixtures were done using RSLcnano system connected to Orbitrap Elite high mass spectrometer (Thermo Fisher Scientific). Prior to LC separation, tryptic digests were desalted using trapping column (100 μm × 30 mm, 3.5 μm X-Bridge BEH 130 C18 sorbent, Waters) and separated on Acaplam Pepmap100 C18 column (2 μm particles, 75 μm × 500 mm; Thermo Fisher Scientific) by 1 or 2 h gradient program (mobile phase: A: 0% FA in water; mobile phase B: 0.1% FA in acetonitrile). The analytical column outlet was directly connected to the Nanoflare Flex Ion Source. Up to top 10 precursors from the survey scan (350–2000 m/z; resolution 60,000) were selected for HCD fragmentation (target 50,000 charges; resolution 15,000, isolation window 2 m/z) with enabled dynamic exclusion (up to 45 s). Two independent LC-MS/MS analyses were done for on-beads digests. The analysis of the mass spectrometric RAW data files was carried out using the Proteome Discoverer software (Thermo Fisher Scientific; version 1.4) with in-house Mascot (Matrixscience; version 2.3.1) and Sequest search engines utilization. MS/MS ion scores obtained against the UniProtKB protein database for mouse downloaded from ftp://ftp.uniprot.org/pub/ database/uniprot/current_release/; version 20141001; 85,893 protein sequences with additional sequences from CRAP database (www.thegpm.org/crap/). Mass tolerances for peptides and MS/MS fragments were 10 ppm and 0.05 Da, respectively. Oxidation of methionine and deamidation (N, Q) as optional modifications and two enzymes miss cleavages were set for all searches.

Proteomisation of G as optional modification was set for database searches after in-gel digestion. Percolator was used for post-processing of Mascot search results. Peptides with false discovery rate (FDR; q-value) <1%, rank 1 and with at least six unique peptides labels were reported as protein groups. Protein groups/proteins were reported only if they had at least one unique peptide. Label-free quantification using protein area calculation in Proteome Discoverer was used (top 3 protein quantification). Two analyses of on-beads digests and analyses for all gel area within single gel line were searched as one dataset.

Wnt pathway activation readouts. To analyze shift in DVL1 or OR1 mobility upon treatment with conditioned medium, MEF cells were seeded on 24-well plates and grown to reach 70–80% confluency in DMEM medium supplemented with 10% FBS. Cells were treated overnight with recombinant human WNT3A (5036-WF-CN, R&D), WNT5A (645-WN-010, R&D), control-, Wnt3a- and Wnt5a-L cells-derived CM or CM obtained from TchP- and HbChP- primary culture derived from either Wnt5a KO or Wnt5a KO embryos. Twenty-four hour prior to the treatment cells were incubated with 1 μl/ml LGX794 inhibitor (974-02, StemRD). Used reagents are listed in the Supplementary Table 1. Used antibodies are listed in the Supplementary Table 3.

Molecular sieve assay. 100 ng of recombinant WNT5A were incubated with 0.6% CHAPS solution (C3023-5G, Sigma) or 50 μg of native LDL. Lipoproteins from mouse serum in 1 ml of the mixture was centrifuged at 150,000 g for 2 h using Optima L-90 Xp Centrifuge (Beckman Coulter). Sucrose density columns (Millipore) and washed several times in PBS. Finally, the samples was transferred to a 139 rotor for 2 h using Optima L-90 Xp Centrifuge (Beckman Coulter). Exosome purification. Exosomes were purified by differential centrifugation as described previously. In short, CM from E14.5 HbChP-derived primary culture was processed in series of centrifugation steps of 200 g, 1500 g, 6,000 g, and 14,000 g before pelleting exosomes at 100,000 g in SW55 swinging bucket (k factor = 29,700; Beckman Coulter). To discriminate exosomal protein concentration from its detergent-soluble part, the sucrose cushion step was included to increase the purity of exosomal fraction. The supernatant was discarded, and exosomes were resuspended in filtered PBS.

Lipoproteins isolation and fractionation. Lipoprotein isolation was performed using the protocol described previously. In brief, lipoproteins from mouse serum and CHF primary culture were separated from soluble proteins using KBr discontinuous gradient. To remove excessive KBr, fractions were filtered using concentration columns (Millipore) and washed several times in PBS. Finally, the ultrafiltrate of lipoproteins fractions was resuspended in PBS before further applications. Used reagents are listed in the Supplementary Table 1.

Exosilane purification. Exosomes were purified by differential centrifugation as described previously. In short, CM from E14.5 HbChP-derived primary culture was processed in series of centrifugation steps of 200 g, 1500 g, 6,000 g, and 14,000 g before pelleting exosomes at 100,000 g in SW55 swinging bucket (k factor = 29,700; Beckman Coulter). To discriminate exosomal protein concentration from its detergent-soluble part, the sucrose cushion step was included to increase the purity of exosomal fraction. The supernatant was discarded, and exosomes were resuspended in filtered PBS.
supernatant corresponding to delipidated serum was aliquoted and stored for further use at −80 °C. Used reagents are listed in the Supplementary Table 1.

**Immunofluorescence and EdU staining.** For mouse embryo immunofluorescent analysis and in situ hybridization, WT CD1 mice, Wnt5aKO or Wnt5aKO mice were dissected and isolated embryos were transferred into ice-cold PBS, fixed in 4% paraformaldehyde (PFA) in PBS for several hours followed by several washes in ice-cold PBS and finally cryoprotected by sequential incubation in 15% and then 30% sucrose solutions. Embryos were next frozen in Tissue-Tek optimum cutting temperature (OCT) compound (Sakura Fine-Tek) on dry ice. Serial 14 μm coronal sections were used for immunofluorescence analysis. Human fetal tissue for cryosectioning was immersion-fixed overnight in 4% PFA at 4 °C, then cryoprotected in sucrose before embedding in OCT compound and then 14 μm sections were cut using a Leica cryostat. Human fetal tissue samples were processed using an identical immunofluorescence protocol as indicated for the mouse samples.

For immunofluorescence analysis, all the sections underwent antigen retrieval by direct boiling for 10 min at 550 W in the microwave using antigen retrieval solution (DAKO). Sections were washed in PBT (PBS with 0.5% Tween-20) and blocked in PBT (PBS, 5% donkey serum, 0.3% Triton X-100, 1% BSA). Samples were incubated overnight at 4 °C with primary antibodies diluted in PBT. Following washes in PBT, samples were incubated with corresponding Alexa Fluor secondary antibodies (Invitrogen) for 1 h at RT, followed by 5 min incubation at RT with DAPI (1:5000). Finally, samples were mounted in DAKO mounting solution (DAKO). EdU (Life Technologies) was injected 72 h before the embryos were harvested at a concentration of 65 mg/g. Cells with incorporated EdU were visualized using a Click-iT EdU Alexa Fluor 555 Imaging Kit (C10338, Life Technologies).

For immunocytofluorescent analysis of ChP primary culture, cells grown on laminin-coated coverslips were fixed several times in ice-cold PFA, followed by fixation for 15 min in ice-cold 4% PFA. Later, cells were washed several times in PBT, blocked with PBT (PBS for 30 min and incubated overnight at 4 °C with primary antibodies. Following repeated washing in PBT cells have been incubated for 1 h at RT with appropriate secondary antibodies, DAPI for 5 min and mounted in DAKO mounting medium (DAKO). Used antibodies are listed in the Supplementary Table 1. Antibodies were used in the Supplementary Table 3.

Processing of immunofluorescence images and quantitative assessment of signal overlap for WNT5a puncta was performed using vesicle colocalization function as a part of microscope image analysis software Imaris® (Bitplane). Parameters used for image processing were as follows: 0.4 μm cut-off for analyzed particles size, 0.1 distance for the signal overlap analysis using vesicles-colocalization function. Quantification of Ki67+ and EdU staining was performed using ImageJ software.

**Transmission electron microscopy analysis.** For negative contrasted exosomes were adsorbed at activated formvar membrane coated with carbon EM grids (Pyser-SCI Limited), stained with 2% solution of ammonium molybdate, and visualized using transmission electron microscopy (TEM) Morgagni 268D (FEI) equipped with Mega ViewIII (Soft Imaging System) at 70 kV.

**In situ analysis.** In situ analysis of the gene expression was done on 14 μm cryosections of embryos at various stages of embryonic development isolated from CD1 mice. After isolation, embryos were immediately transferred and kept in fresh 4% PFA for 2 h, washed briefly in ice-cold PBS, incubated for 6 h in 30% sucrose solution at 4 °C, and frozen at −80 °C. Transcripts were detected using the RNAscope 2.0 assay for fresh frozen tissue (Advanced Cell Diagnostics). Staining was performed using the RNAscope Fluorescent Multiplex Reagent Kit (320850, Advanced Cell Diagnostics). Used reagents are listed in the Supplementary Table 1.

Indicated in situ images were adopted from Allen Institute for Brain Science: Allen Developing Mouse Brain Atlas61 (Available from: http://developingmousebrain.map.org) or from eurexpress.org62 (Available from: http://www.eurexpress.org/ee/).

**Real-time qPCR.** RNA was isolated from 3 different litters of WT CD1 embryos collected at indicated developmental stages. Samples were treated with DNase (M6101, Promega) to prevent contamination with genomic DNA. The specificity of primers was determined by BLAST run of the primer sequences. The sequences of primers are displayed in the Supplementary Table 4 and their annealing temperature is 57 °C for all used primers.

qPCR reactions were performed once for every gene/sample in triplicate. PCR was done according to the manufacturer’s protocol (Lightcycler 480 SYBR Green 1 Master Mix. Roche). The following thermo cycling program parameters were used for qPCR analysis: Incubation step at 95 °C for 5 min, then 45 cycles 95 °C for 10 s, 57 °C for 10 s, and 72 °C for 10 s. qPCR analysis was carried out using LightCycler® 480 Instrument II (Roche).

ΔCp values were calculated in every sample for each gene of interest with β-actin as the reporter gene. Relative change of expression level for analyzed gene (ΔCp) was performed by subtraction of the gene expression levels in TelChP or TeoChP to the gene expression level of housekeeping gene (β-actin). Next ratio of the gene expression level between β-actin and gene of interest in either HbChP or TeoChP was calculated using following formula: 2^ΔCp

**Statistics.** Gene expression data—Replicates are independent experiments. Data in Fig. 1c, Fig. 3a, Supplementary Fig. 5a and 5b are expressed as columns showing the mean with standard deviation (s.d.). Significance measured with paired two-tailed Student’s t-test with unequal sample variance. Biological replicates per condition are indicated in the corresponding graphs.

Confocal images—Images used for quantitative analyses reported in Fig. 5c and Fig. 5d are representative images chosen from the results obtained from six individual embryos. Confocal images reported in Supplementary Fig. 11c, e, g and h are representative images chosen from the results obtained from three individual embryos. Data in Fig. 5e, f, Fig. 7h-k and Supplementary Fig. 11b, d and f are expressed as columns showing the mean with s.d. Significance measured with unpaired or paired two-tailed Student’s t-test with unequal sample variance. Except for Fig. 5f, biological replicates or sections used per condition are indicated in the corresponding graphs.

Western blot images—Image used for quantitative analysis reported in Fig. 6i is a representative image chosen from the results obtained in three separate experiments. Significance measured with unpaired two-tailed Student’s t-test with unequal sample variance. Biological replicates per condition are indicated in the corresponding graph.

All the displayed immunostaining images and western blots are representative of at least three independent experiments.

**Reporting summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** The authors declare that all data supporting the findings of this study are available within this published article and its Supplementary Information files and from the corresponding author upon reasonable request. In situ hybridization data used in Fig. 1a, b and supplementary Figs 1a, 1b and 3c are available from the Allen Developing Mouse Brain Atlas (www.alleninstitute.org). In situ hybridization data used in Supplementary Figs 2a–d and 10a–b are available from the Eurexpress atlas (www.eurexpress.org). The mass spectrometry proteomics data have been deposited at the ProteomeXchange Consortium via the PRIDE partner repository63 with the dataset identifier PXD011918. Figures are deposited in figshare depository with the identifier [https://doi.org/10.6084/m9.figshare.7588481.v2]. A reporting summary for this article is available as a Supplementary Information file. The source data underlying Figs. 1c, 3a, 5e, 6f, 7a–k and Supplementary Figs. 5a–b, 7b and 11b, d and f are provided as Source Data File.

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Author contributions
K.K. designed and performed most of the experiments, analyzed data, prepared all fi-
ures, and wrote the manuscript; D.G. helped with the Wnt5a mouse strain and designed
schematic depictions; J.P., M.P., and R.S. produced Wnt5aKO strain; P.K. performed
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and R.A.B. contributed the human fetal samples; D.P. and Z.Z. performed the MS
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designed experiments, supervised the work, analyzed data, and wrote the manuscript.

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