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RESEARCH REPORT

Temporal control of Wnt signaling is required for habenular neuron diversity and brain asymmetry

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ABSTRACT

Precise temporal coordination of signaling processes is pivotal for cellular differentiation during embryonic development. A vast number of secreted molecules are produced and released by cells and tissues, and travel in the extracellular space. Whether they induce a signaling pathway and instruct cell fate, however, depends on a complex network of regulatory mechanisms, which are often not well understood. The conserved bilateral left-right asymmetrically formed habenulae of the zebrafish are an excellent model for investigating how signaling control facilitates the generation of defined neuronal populations. Wnt signaling is required for habenular neuron type specification, asymmetry and axonal connectivity. The temporal regulation of this pathway and the players involved have, however, remained unclear. We find that tightly regulated temporal restriction of Wnt signaling activity in habenular precursor cells is crucial for the diversity and asymmetry of habenular neuron populations. We suggest a feedback mechanism whereby the tumor suppressor Wnt inhibitory factor Wif1 controls the Wnt dynamics in the environment of habenular precursor cells. This mechanism might be common to other cell types, including tumor cells.

KEY WORDS: Habenula, Wnt, Asymmetry, Zebrafish, Brain, Wif1

INTRODUCTION

Following gastrulation, the developing vertebrate embryo is fine patterned into functional organs and domains. In the brain, this is mainly achieved through organizing centers, such as the midbrain-hindbrain boundary (MHB) and the mid-diencephalic organizer (MDO) (Cavodeassi and Houart, 2012; Kiecker and Lumsden, 2012). Here, secreted molecules, including Wnt ligands, form morphogen gradients to establish fore-, mid- and hindbrain, and the neurons within (Crossley et al., 1996; Picker et al., 1999; Peukert et al., 2011; Hagemann and Scholpp, 2012; Mattes et al., 2012; Chatterjee et al., 2014). Hence, during this period developing brain cells are exposed to multiple signals but have differential requirements for pathways to be activated. Although, for example, the prethalamus anterior to the MDO develops only when Wnt activity is low, the thalamus posterior to the MDO requires active Wnt signaling to develop (Hagemann and Scholpp, 2012). Thus, Wnt ligands secreted from the MDO selectively activate Wnt signaling in some MDO adjacent cells, while other cells must prevent this from happening. These latter cells either simply do not express Wnt signaling components, as in the prethalamus (Jones and Rubenstein, 2004; Shimogori et al., 2004; Quinlan et al., 2009; Peukert et al., 2011; Hagemann and Scholpp, 2012), or they need to inhibit Wnt signaling activation.

In the dorso-posterior vicinity of the MDO, Wnt signaling influences neurogenesis of the habenulae (Carl et al., 2007; Beretta et al., 2013; Hüsken and Carl, 2013; Hüsken et al., 2014). The habenulae are the central part of an evolutionarily conserved neurotransmitter system connected to the interpeduncular nucleus (IPN) and the median raphe in the ventral mid- and hindbrain (Sutherland, 1982; Aizawa et al., 2011; Beretta et al., 2012). The circuit has been linked to various behaviors and pathophysiological syndromes, and is used as a model for studying anatomical and functional left-right brain asymmetries (Okamoto et al., 2012; Dreosti et al., 2014; Benekareddy et al., 2018; Cui et al., 2018; Yang et al., 2018). Habenular neuron types can grossly be divided into lateral and medial populations, which in teleosts correspond to ventral and dorsal habenular neurons, respectively (Amo et al., 2010). The dorsal habenular neuronal clusters (dHb) consist of lateral (dHbl) and medial (dHbm) sub-nuclei, which differ in size between the left and the right side of the brain in many vertebrates, including zebrafish (Concha and Wilson, 2001).

Influenced by Notch signaling (Aizawa et al., 2007), dHbl neurons are generated earlier with respect to dHbm neurons, which develop later and only in the presence of active Wnt/β-catenin signaling (Carl et al., 2007; Hüsken and Carl, 2013; Hüsken et al., 2014). Transient interference with the canonical Wnt signaling pathway has shown that the temporal requirement for Wnt signaling in this process is restricted to a narrow time window of about 2 h of development. Suppression of Wnt signaling around 36 h post-fertilization (hpf) causes postmitotic habenular precursor cells to develop only into dHbl neurons. At this time, the transcriptional co-factor of the Tcf/Lef family Tcf7l2 mediates Wnt signaling in developing habenular neurons (Hüsken et al., 2014). Intriguingly, although *tcf7l2* mRNA is widely expressed in the diencephalon also at earlier stages of development (Young et al., 2002), Tcf7l2 protein is expressed only at the time when Wnt signaling acts on habenular neuronal fate decisions. These findings led us to speculate that the activation of the pathway might be temporally controlled by as yet unknown players to prevent that premature Wnt signaling from interfering with the generation of neuronal diversity in the habenulae.

We report here that Wnt signaling can be prematurely activated within habenular precursor cells. This indicates that, unlike prethalamic cells, Wnt signaling components are readily present in habenular precursors but that signaling is initially suppressed. If precursor cells experience premature Wnt signaling activity, their differentiation is delayed, causing their predominant development into late-born dHbm neurons on both sides of the brain. We provide evidence suggesting that the secreted Wnt inhibitory factor 1 (Wif1) (Hsieh et al., 1999; Poggi et al., 2018) controls Wnt signaling in

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habenular precursor cells. *Wif1* is expressed in these cells before their differentiation, and functional downregulation of Wif1 mimics the phenotype caused by transient activation of premature Wnt signaling. Intriguingly, once initiated, *Wif1* expression in turn depends on Wnt signaling itself. These findings are consistent with the hypothesis that a Wif1-mediated regulatory feedback loop may dynamically buffer Wnt signaling within nascent habenular progenitors before they develop into neurons.

RESULTS AND DISCUSSION

Wnt/β-catenin signaling is suppressed in early habenular precursors

mRNAs encoding for multiple Wnt/β-catenin pathway components, including axins, Wnt ligands and Tcf gene family members, are already expressed in the developing diencephalon during segmentation of the embryo (Young et al., 2002; Thisse and Thisse, 2005, 2008; Carl et al., 2007; Beretta et al., 2011; Hüsken et al., 2014). However, Wnt signaling is required only 10-20 h later, during pharyngula stages (35-36 hpf), to facilitate the generation of dHbm neurons (Hüsken et al., 2014). This finding suggests that, during segmentation, Wnt/β-catenin signaling might play an early role in habenular precursors in addition to influencing their subsequent differentiation. Alternatively, the pathway may be suppressed in the course of habenular neuron differentiation until 36 hpf. To assess this issue, we first investigated whether Wnt signaling activity is detectable before 36 hpf in the developing dorsal diencephalon using triple transgenic embryos carrying the *tg(7xtcf-Xla.Siam:nlsmCherry)* Wnt reporter (Moro et al., 2012) and *tg(flh:GFP); tg(foxd3:GFP)* transgenes labeling the medially positioned pineal complex for orientation (Aizawa et al., 2005) (Fig. 1). Starting at 22 hpf, we find only a few Wnt active cells in the presumptive habenula region (1.9±1.5; n=11), the number of which increased by 28 hpf (7±3.73; n=19) (Fig. 1A,B,D). At this time, cells of the anteriorly adjacent MDO already show robust Wnt activity. The number of fluorescent nuclei in the developing habenulae multiplied by 36 hpf (29.3±7; n=10), indicating Wnt signaling activity in an increasing number of cells over time (Fig. 1A-D). This finding also suggested that Wnt signaling is normally inactive in the majority of habenular precursors before 36 hpf. To assess whether Wnt signaling can be prematurely activated in these cells, we interfered pharmacologically with the Wnt pathway inhibitor GSK3β at 26 hpf by exposing embryos for 30 min to lithium chloride (LiCl) (Stambolic et al., 1996). The treatment caused a significant increase in Wnt signaling active habenular precursors at 28 hpf (11.35±4.4, n=17, P=0.003) (Fig. 1E,F), thus supporting the idea that early habenular precursors are capable of responding to Wnt pathway activation already at 26 hpf. Similarly, treatments with the GSK3β inhibitor (2′Z,3′E)-6-bromo-indirubin-3′-oxime (BIO) (Meijer et al., 2003) caused an increase in the number of Wnt-responsive cells when compared with control DMSO-treated embryos (DMSO, 8.33±4.32, n=6; BIO, 14.29±4.96, n=7, P=0.04). However, the effect was less robust compared with LiCl treatments (Fig. 1F).

These data suggest that canonical Wnt pathway components are readily present in developing habenular neurons, but that Wnt signaling is suppressed in most of them until 36 hpf.

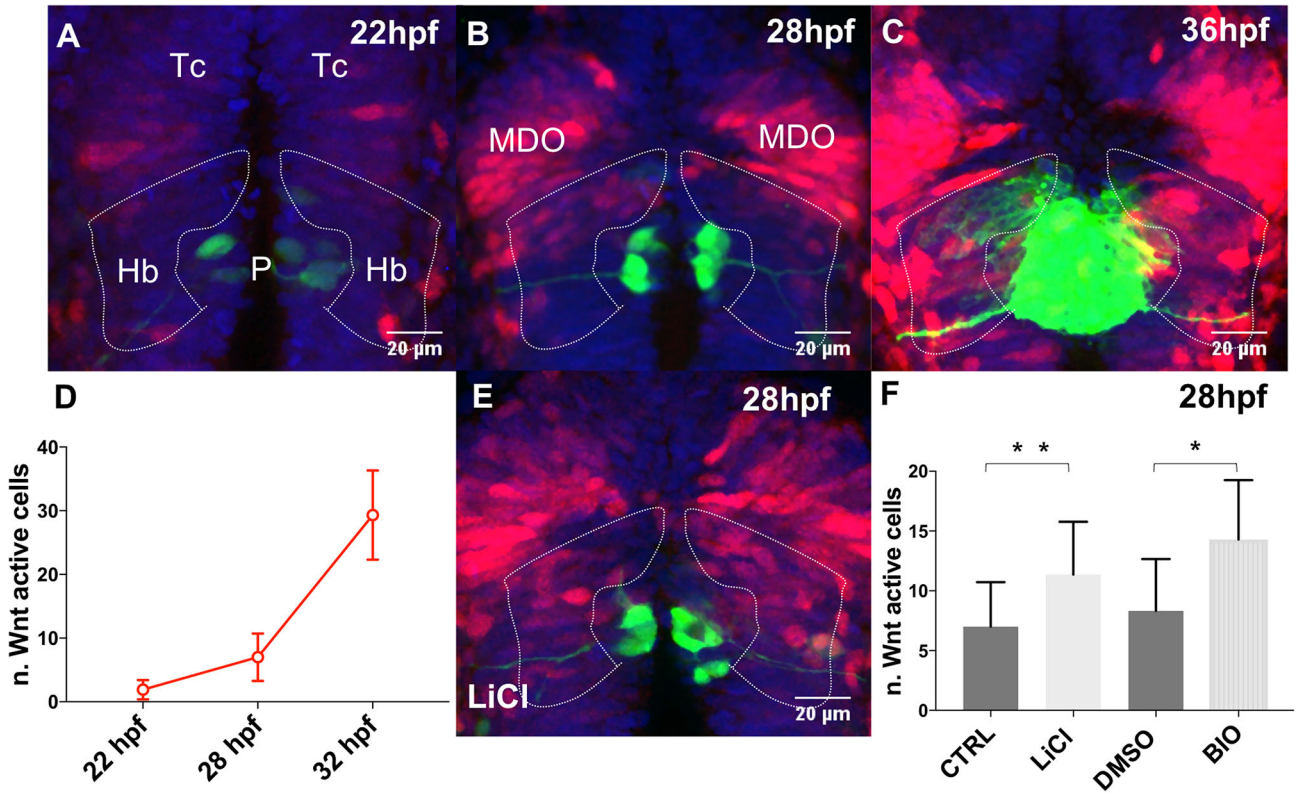

Fig. 1. Wnt signaling activity in and around the developing habenulae. (A-C,E) Projections of confocal *z*-stacks, dorsal views, anterior is towards the top focused onto the diencephalon of *tg(7xtcf-Xla.Siam:nls-mCherry); tg(flh:GFP); tg(foxD3:GFP)* transgenic embryos at stages indicated. Nuclei are blue, Wnt active cells are red and the pineal complex is green. Dotted lines highlight the region of the developing habenulae. (D) Graph shows the number of Wnt-active habenular precursors, which are (F) increased, when Wnt signaling is activated by drug treatments, as indicated. Hb, habenulae; MDO, mid-diencephalic organizer; P, pineal; Tc, telencephalon.

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Premature induction of Wnt signaling delays habenular neuron differentiation

To assess the importance of early Wnt signaling suppression in nascent habenular neurons, we transiently activated the pathway for short periods between 22 hpf and 32 hpf. As a readout for habenular neuron development and innervation of the main target of habenular efferent axons, the interpeduncular nucleus (IPN), we used Et(-1.0otpa:mmGFP) transgenic embryos (Beretta et al., 2012, 2013, 2017). In these embryos, GFP expression in dHb neurons is initiated at 43 hpf. In contrast, the majority of embryos treated with LiCl at 24 or 26 hpf for 30 min showed no GFP expression in the dHb at 48 hpf, suggesting impaired habenular neuron differentiation (Fig. 2A,A′,C and Fig. S1A, A′ and Table S1). A similar but milder effect was observed when we treated embryos with BIO in the same interval (data not shown). In line with this observation, the number of HuC/D-positive differentiating habenular neurons was strongly reduced upon LiCl treatment (control, 19.5±4.4, n=12; LiCl, 10.6±5.8, n=12, P=0.0003), while the left-right ratio of HuC/D-positive neurons appeared unaltered (control, 0.67±0.22, n=12; LiCl, 0.55±0.30, n=12, P=0.29) (Fig. 2B,B′,D,E and Table S2). In contrast, pineal cell development adjacent to the habenulae was not affected by the treatment suggesting a specific effect on habenular neuron differentiation (control, 20.71±1.60, n=7; LiCl, 21.13±1.73, n=8, P=0.64) (Fig. S1A,A′). Similarly, *cxcr4b* expression in dHb precursor cells was not affected by the short activation of Wnt signaling, consistent with normal habenular precursor cell development (Fig. S1B,B′ and Table S2). Taken together, these results suggest that the suppression of early Wnt signaling in habenular precursor cells is required to facilitate the temporal sequence of habenular neuron differentiation.

Early suppression of Wnt signaling is important for habenular neuron diversity, brain asymmetry and axonal connectivity

At 3 dpf, treated Et(-1.0otpa:mmGFP) embryos showed a few GFP-expressing dHb cells, suggesting that premature Wnt signaling activity is not abrogating, but only delaying dHb differentiation (Fig. 3A,A′). Consequently, treated embryos exhibited a reduction of dHb markers. Notably, *kctd12.1* and the transgene *Et(gata2a:EGFP)* in the early born dHbl neurons were severely reduced or absent, while *kctd8* and *brn3a:GFP* in the subsequently generated dHbm neurons were less strongly reduced and expressed in most embryos (Gamse et al., 2005; Aizawa et al., 2007; Hüsken et al., 2014) (Fig. 3B-C′,E and Fig. S1C-D′ and Table S2). This implied that most dHb precursors become dHbm neurons as a consequence of their delayed differentiation. In strong support of this hypothesis, we find that dHb efferent axons from both sides target only the vIPN (Fig. 3D,D′,F and Table S3). This part of the IPN is the predominant target of dHbm efferent axons (Bianco et al., 2008). IPN development per se was largely unaffected, as judged by the IPN marker *somatostatin 1* (Thisse and Thisse, 2004) (Fig. S1E-F′).

Taken together, it appears that habenular precursors have temporally changing susceptibilities to Wnt signaling. At very early embryonic stages, Wnt signaling is required for the generation of precursors (Kuan et al., 2015). Subsequently during segmentation stages, Wnt signaling, when activated, can influence the timing of neuronal differentiation. 30 min of forced Wnt signaling activation between 24 hpf and 26 hpf is sufficient to cause a delay of dHb neuron differentiation and to compromise their development into dHbl neurons. The Tcf/Lef family member mediating Wnt signaling during these early phases is not known, although *lef1* may be a promising candidate judging from its spatio-temporal mRNA expression pattern (Bonkowsky et al., 2008). Previous work has shown that activation of Notch signaling slightly after this time causes a similar habenular phenotype, suggesting a potential link between Wnt and Notch signaling in this process (Aizawa et al., 2007). However, we could not detect a consistent effect on Notch target or reporter gene expression upon LiCl treatment at this early developmental stage (data not shown). Finally about 10 h later, when habenular precursors become post-mitotic,

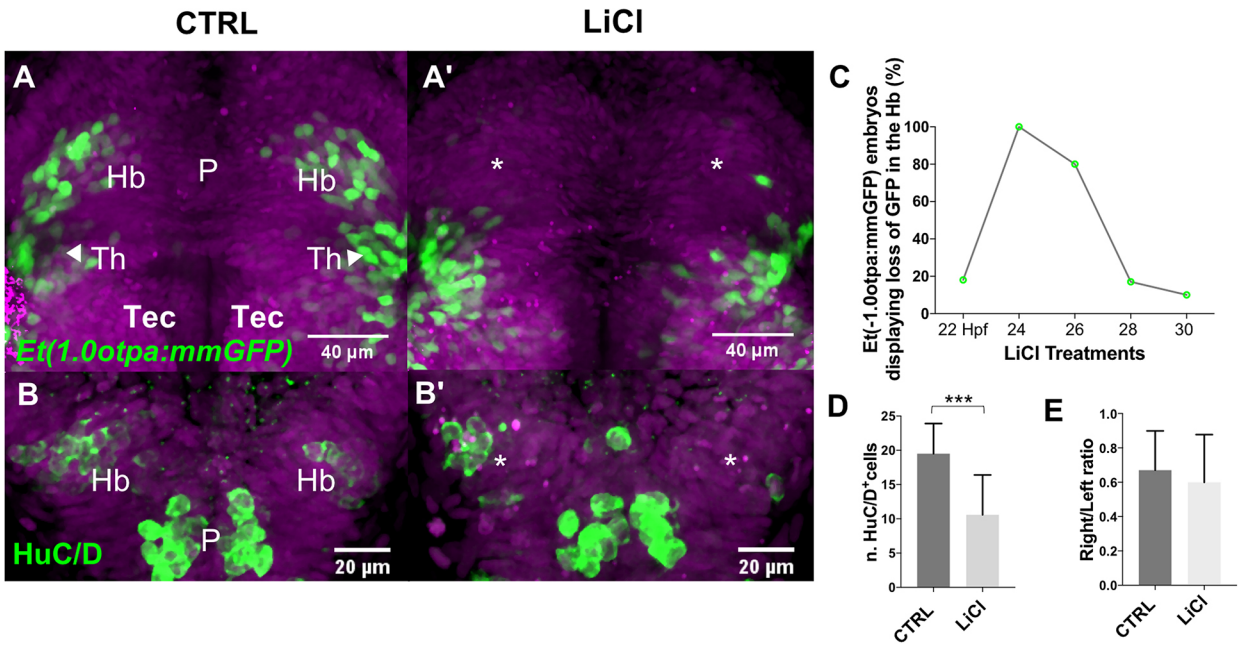

Fig. 2. Premature intrinsic activation of Wnt signaling delays habenular neuron differentiation. (A-B′) Projections of confocal *z*-stacks, dorsal views, anterior is towards the top focused onto the diencephalon of (A,A′) *Et(-1.0otpa:mmGFP)* and (B,B′) *tg(flh:GFP); tg(foxD3:GFP)* transgenic embryos. Nuclei are DAPI labeled (purple). (A,A′,C) Transient Wnt signaling activation causes a specific loss of GFP-expressing habenular neurons at 48 hpf. (B,B′,D,E) The number of HuC/D-positive differentiating habenular neurons is reduced at 36 hpf; their left-right asymmetric development remains unchanged. CTRL, control; Hb, habenulae; P, pineal; Tec, optic tectum; Th, thalamus.

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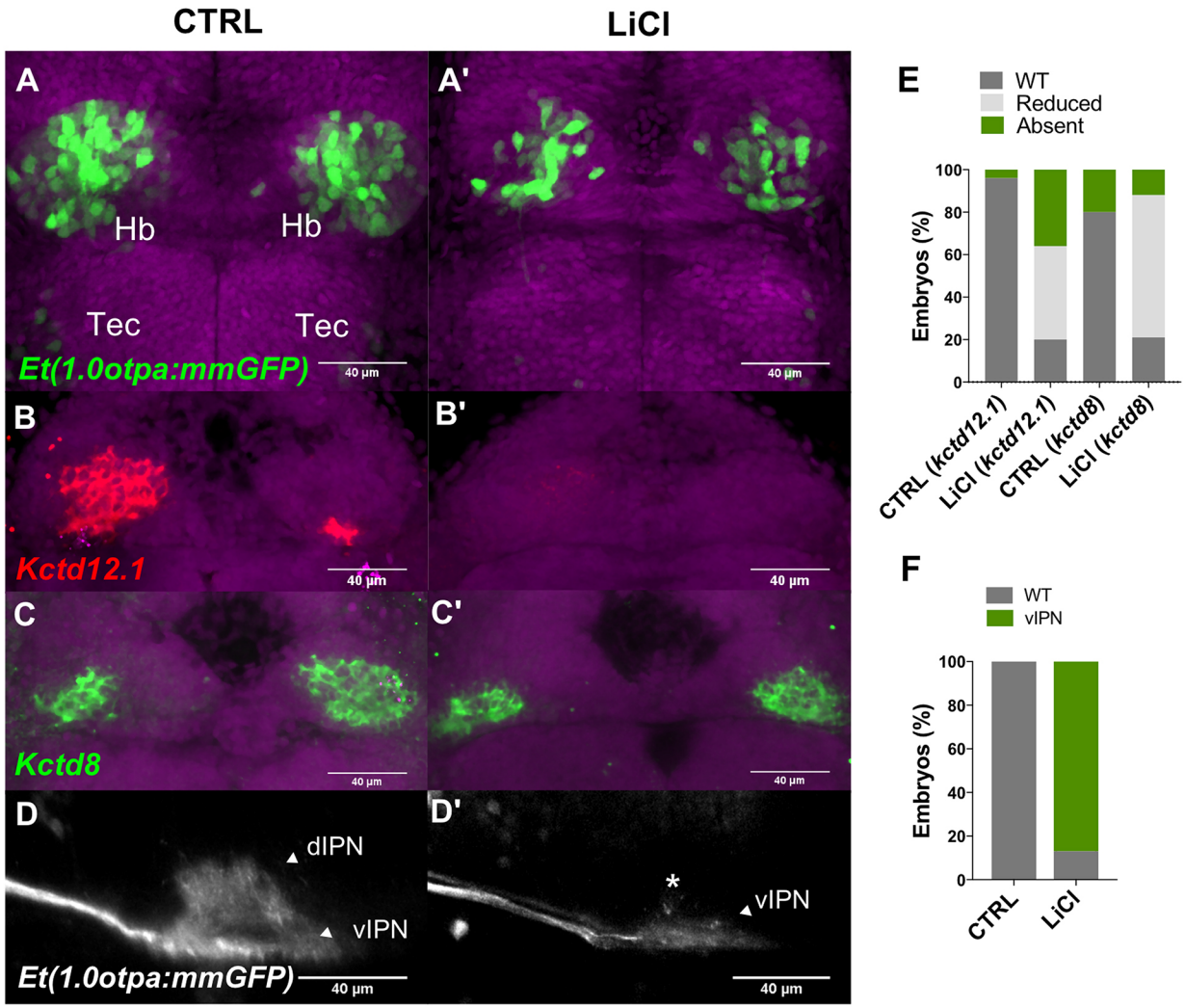

Fig. 3. Premature activation of Wnt signaling causes a reduction of dHbl neurons. (A-D′) Projections of confocal *z*-stacks. (A-C′) Dorsal views, anterior is towards top focused onto the diencephalon of (A-D′) embryos at 3 dpf. Nuclei are DAPI labeled (purple). (A,A′) LiCl treatment causes delayed habenular neuron differentiation. (B-C′,E) Markers for (B,B′) dHbl and (C,C′) dHbm neurons are reduced as represented in E. (D,D′) Lateral views of IPN innervation by habenular efferent axons, anterior is leftwards. Treated embryos show vIPN innervation, as represented in F. d, dorsal; Hb, habenula, IPN, interpeduncular nucleus; Tec, optic tectum; v, ventral.

Wnt signaling is required for dHbm differentiation (Hüsken et al., 2014).

The Wnt inhibitory factor 1 promotes dHbl neuron generation

The habenulae are positioned in close proximity to the mid-diencephalic organizer (MDO; see also Fig. 1), a source of secreted Wnt molecules (Peukert et al., 2011; Hagemann and Scholpp, 2012). In the light of our findings, we hypothesized that nascent habenular neurons need to be protected at segmentation stages from incoming Wnt ligands. This would require a secreted Wnt inhibitor molecule, such as Wnt inhibitory factor 1 (Wif1), which can directly bind to canonical and non-canonical Wnt ligands (i.e. Wnt3a, Wnt4, Wnt5a, Wnt7a, Wnt8, Wnt9a and Wnt11) and prevent their binding to receptors (Hsieh et al., 1999; Kawano and Kypta, 2003). Wif1 is a crucial tumor suppressor linked to multiple types of cancer, but it also functions in developmental processes, including neurogenesis (Poggi et al., 2018).

We find that *Wif1* is expressed specifically in cells of the developing habenulae between 20 hpf and 36 hpf (Fig. S2). Thus, *Wif1* mRNA is temporally complementing Tcf7l2 protein expression (Hüsken et al., 2014) and hence an excellent candidate antagonizer of Wnt signaling during the early stages of habenular neuron development. We analyzed embryos hypomorphic for Wif1 using a previously established and well controlled for Wif1 morpholino (Yin et al., 2012). Consistent with a role for Wif1 in habenular neuron generation, Wif1 interference mimicked transient LiCl- or BIO-mediated upregulation of Wnt/β-catenin signaling. At 48 hpf, GFP expressing habenular neurons in Et(-1.0otpa:mmGFP) transgenic embryos were severely reduced or missing and HuC/D-expressing differentiating habenular neurons were strongly reduced (control, 9.14±4.6, n=7; MoWif-1: 0.38±0.74, n=9, P<0.0001) (Fig. 4A-B′,D,E, Fig. S3A,A′ and Table S4). The normal number of pineal cells adjacent to the habenulae confirmed that Wif1 downregulation specifically affected habenular neuron development (control, 12.63±1.51, n=8; MoWif-1, 11.88±1.46, n=11, P=0.328) (Fig. 4 and Fig. S3A,A′). In addition, *cxcr4b* expression in habenular precursor cells and the morphology of the habenular region appeared largely normal, as judged from nuclei staining (Fig. S3B,B′). Subsequently, at 3 dpf, markers for differentiated habenular neurons were reduced and habenular

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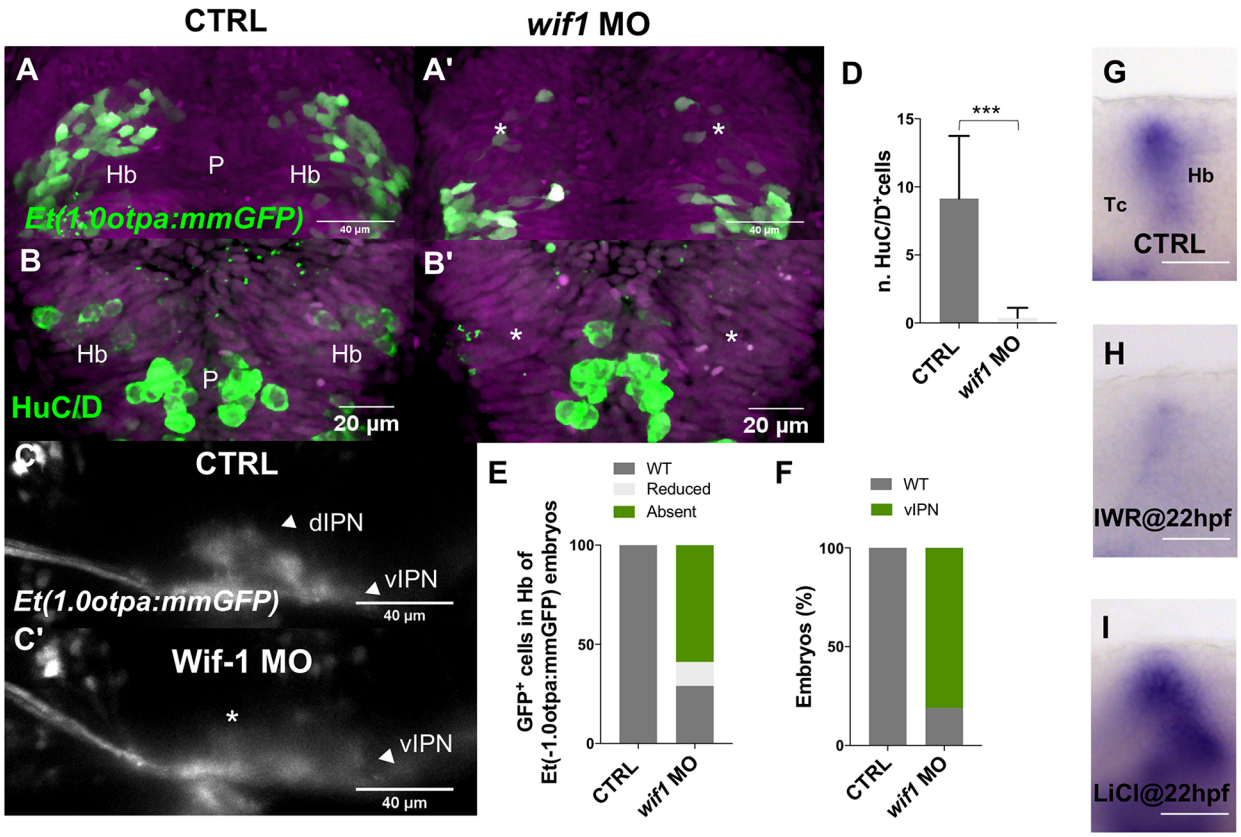

Fig. 4. Wif1 controls dHb neuron differentiation and is regulated by Wnt signaling. (A-C′) Projections of confocal *z*-stacks. (A-B′) Dorsal views, anterior is towards the top focused onto the diencephalon of embryos at 3 dpf. Nuclei are DAPI labeled (purple). (A-B′,D,E) Wif1 hypomorphic embryos exhibit delayed habenular neuron differentiation (asterisks in A′,B′). (C,C′) Lateral views focused on the IPN of *Et(-1.0otpa:mmGFP)* embryos with anterior towards the left. (C,C′,F) Wif1 knockdown embryos show predominant vIPN innervation. Asterisk highlights the missing dIPN innervation. (G-I) Lateral views focused on the diencephalon of 25 hpf (G) untreated and (H,I) treated embryos, labeled for *Wif1* expression. Scale bar: 60 µm. Labeling procedures were conducted in parallel for comparison. d, dorsal; Hb, habenula, IPN, interpeduncular nucleus; P, pineal; v, ventral.

efferent axons innervated the target of dHbm axons: the ventral IPN (Fig. 4C,C′,F, Fig. S3C-F′ and Tables S3 and S4). These data suggest that Wif1 suppresses Wnt/β-catenin signaling in habenular precursor cells. Interference with Wif1 function results in premature activation of the pathway, a delay in habenular neuron specification and their subsequent development into predominantly dHbm neurons on both sides of the brain. Tcf7l2 is not expressed at this stage of brain development, but Wif1 has previously been shown to inhibit the expression of the Wnt signaling downstream transcriptional cofactor *lef1* (Yin et al., 2012), which is expressed in the early developing dorsal diencephalon (Bonkowsky et al., 2008). It is tempting to speculate that Lef1 may mediate the premature Wnt activity, causing the observed effect on neurogenesis.

Wif1 functions in a feedback loop

Starting from around 22 hpf, we find increasing numbers of Wnt active cells at the MDO over time (Fig. 1A-C). This implies that the early MDO adjacent habenular precursors are not only protected from Wnt ligands. The system has to have the ability to react to varying amounts of Wnt ligands until dHb neuron differentiation at 36 hpf (Hüsken et al., 2014). Wnt signaling has been shown to regulate its own activity during processes in development and disease (Cowling et al., 2007; Licchesi et al., 2010). *In vitro*, the self-regulation normally involves multiple molecules, also including, at times, Wif1 (Diep et al., 2004; Vaes et al., 2005; Boerboom et al., 2006; Zirn et al., 2006). In forms of cancer, Wif1 is regulated by feedback loops between microRNAs and DNA methyltransferases (Dnmts), which hypermethylate the Wif1 promoter and thus shut down *Wif1* expression (Tan et al., 2013).

To assess whether a feedback mechanism is applied *in vivo* during habenular development, we examined *Wif1* expression in embryos with altered Wnt signaling. Transient stabilization of Axin 1 using IWR drug treatments or heat shock induction of *dkk1* expression (Stoick-Cooper et al., 2007; Huang et al., 2009), both of which result in the inhibition of Wnt/β-catenin signaling, leads to decreased *Wif1* expression (Fig. 4G,H and Fig. S4B and Table S5). In contrast, and in line with the temporally complementing Tcf7l2-*Wif1* expression in habenular precursors, *Wif1* expression was largely unchanged in *tcf7l2* mutant embryos (Fig. S4A). Conversely, activation of Wnt signaling in BIO- or LiCl-treated embryos causes an expansion of *Wif1* expression (Fig. 4I and Fig. S4D and Table S5). Similarly, embryos mutant for *Axin1* (Heisenberg et al., 2001; Carl et al., 2007) show expanded *Wif1* expression (Fig. S4C and Table S5). These findings indicate that Wif1 is part of a Wnt regulatory loop, which may buffer habenular precursor cells from extracellular Wnt ligands. To further support this hypothesis, we activated Wnt/β-catenin signaling by heatshock-activating Wnt8a in *tg(hsp70l:Wnt8a-GFP)* transgenic embryos (Weidinger et al., 2005). Transient Wnt8a induction resulted in enlarged *Wif1* expression domains throughout the embryo, including the habenulae (Fig. S5A,A′). Intriguingly, these embryos exhibited no gross abnormalities in the timing of

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habenular neuron differentiation or axonal connectivity. About a quarter of treated embryos showed a mild reduction of habenular marker gene expression (Fig. S5B-D′ and Tables S3 and S6). Our findings are consistent with the idea that, unlike the intracellular activation of the pathway, excess extracellular Wnt ligands can be rapidly compensated for by increased extracellular Wif1 protein levels. To further support this hypothesis, we injected Wif1 morpholinos into *tg(hsp70l:Wnt8a-GFP)* transgenic embryos to cause mildly hypomorphic Wif1 conditions, leaving habenular marker gene expression largely unaffected (Fig. S5C,E). Heat-shock activation of *Wnt8a* in these Wif1 hypomorphic embryos led to a marked increase in the number of embryos showing reduced habenular marker gene expression (Fig. S5C,E′). Although further experiments are needed to show direct evidence for Wnt8a/Wif1 binding in this process, it appears that, upon the initial activation of *Wif1* transcription by Wnt8a, the two proteins subsequently interact extracellularly to prevent further activation of Wnt signaling within nascent habenular neurons. Given the widespread Wnt8a-induced *Wif1* expression, this dynamic buffering system might also be at work in other tissues in which both *Wif1* and Wnt ligands are expressed, e.g. the midbrain, notochord or hypothalamus. We did not find any evidence for an involvement of Wnt signaling in the early initiation or later decrease of *Wif1* expression. The timing of *Wif1* expression was not affected in *mbl/Axin1* mutants with upregulated Wnt signaling. Consistently, the onset of *Wif1* expression at 20 hpf was not affected when embryos were treated with IWR at 18 hpf to block Wnt signaling. Conversely, ectopic *Wnt8a* activation did not result in temporally extended *Wif1* expression in the dorsal diencephalon or premature Tcf7l2 protein activation. One possible explanation for *Wif1* onset and downregulation in this process of neurogenesis may be the involvement of (Wnt signaling independent) Wif1 promoter (de)methylation reported in cancer cells (Tan et al., 2013). At least two of the numerous Dnmt genes in the zebrafish genome, *dnmt1* and *dnmt3bb.1*, are expressed in the developing dorsal diencephalon at the time of habenular neuron generation (Thisse et al., 2001) and could be involved.

In conclusion, our data show that tight temporal control of Wnt/β-catenin signaling in nascent habenular neurons is crucial for habenular circuit development. Premature Wnt signaling in habenular precursors at 24-26 hpf causes their delayed differentiation into predominantly dHbm neurons and the innervation of only the vIPN by dHb efferent axons. Both the temporal expression analysis and our functional data are consistent with the hypothesis that, once *Wif1* is expressed, the secreted Wif1 protein protects dHb precursors from incoming Wnt ligands outside the cell. The uncovered Wnt/Wif1 feedback regulation allows the system to adapt quickly to the dynamic changes occurring in the habenular precursor environment at this stage of embryonic development. When habenular precursors become post-mitotic, *Wif1* expression decreases, which is paralleled by the initiation of Tcf7l2 expression. *Wif1* downregulation may be a consequence of or be necessary for the maturation of developing habenular neurons because, at this time, Tcf7l2-mediated Wnt signaling is required for dHbm neuronal fate. Thus, both the early inhibition of Wnt signaling and its subsequent temporally controlled activation are equally important for establishing the neuronal diversity and left-right asymmetry of the habenulae.

MATERIALS AND METHODS

Fish lines and maintenance

Zebrafish were maintained according to standard procedures (McNabb et al., 2012). For inhibition of pigmentation, embryos were incubated in 0.2 mM 1-phenyl-2 thiourea (PTU). AB wild-type zebrafish and the following transgenic and mutant lines were used: *Et(-1.0otpa:mmGFP)^{hd1}* (Beretta et al., 2012, 2017), *tg(7xtcf-Xla.Siam:nlsmCherry)^{ia5}* (Moro et al., 2012), *tg(flh:GFP)^{u711}*; *tg(foxD3:GFP)^{zf104}* (Gilmour et al., 2002; Concha et al., 2003), *tcf7l2^{exl}* (Muncan et al., 2007), *masterblind (mbl)^{tm213}* (Heisenberg et al., 2001), *tg(hsp70l:wnt8a-GFP)^{w34}* (Weidinger et al., 2005), *tg(hsDkk1:GFP)* (Stoick-Cooper et al., 2007), *tg(hsp70-brn3a:GFP)^{rw0110b}* (Aizawa et al., 2007) and *Et(gata2a:EGFP)^{pku588}* (Hüsken et al., 2014). Genotyping of *tcf7l2^{exl}* mutants was performed as previously described (Hüsken et al., 2014). All animal procedures were approved by local review boards (Regierungspräsidium Karlsruhe – permit AZ 35-9185.81/G-60/12 and the Italian Ministry of Health - permit 151/2019-PR).

Whole-mount *in situ* hybridization and antibody labeling

Antisense probes were generated using digoxigenin/fluorescein RNA labeling kits (Roche). Colorimetric reactions were performed using BM Purple AP Substrate (Roche) or Fast Red (Sigma) according to standard procedures (Thisse and Thisse, 2008). For *in situ* hybridization, the following antisense probes were used: *Wif1* (Hsieh et al., 1999), *kctd8*, *kctd12.1* and *kctd12.2* (Gamse et al., 2003, 2005), *sst1* (Thisse and Thisse, 2004), and *cxcr4b* (Roussigne et al., 2009). Antibody labeling was performed using standard procedures (Turner et al., 2014). The following primary antibodies were used: mouse anti-GFP antibody (1:1000, Santa Cruz Biotechnology, B2316), chicken anti-GFP antibody (1:250, Invitrogen, A10262), mouse anti-human TCF3,4 antibody (1:200, Sigma, T5692) and mouse anti-HuC/D antibody (1:100, Invitrogen, A21271) (Hüsken et al., 2014). Antibody binding was detected using the following secondary antibodies: goat anti-mouse and anti-chicken Alexa Fluor 488-conjugated (1:250, Invitrogen, mouse A11001, chick A11039) and donkey anti-mouse Alexa Fluor 568-conjugated (1:250, Invitrogen, A10037). For nuclear staining, embryos were incubated in PBS, 0.8% Triton X-100 and 1% BSA containing DAPI (1:1000, ThermoFisher Scientific).

Morpholino and mRNA injections

To knock down Wif1 function, previously established morpholino oligonucleotides for *Wif1* (Yin et al., 2012) were dissolved in water to a final working concentration of 1 mM. A 1 nl MO solution was injected into one-cell stage embryos. For nuclei labeling, 200 pg of *H2B-PSmOrange* mRNA was injected into one-cell stage embryos.

Heat shock and drug treatments

To manipulate Wnt signaling, we used IWR (Sigma) as pharmacological antagonist and BIO [(2′Z,3′E)-6-Bromo-indirubin-3′oxime, Sigma] or LiCl (AppliChem) as pharmacological agonists. Experiments were performed by incubating dechorionated embryos either in IWR-containing solutions (0.1 mM in E3 embryo medium/1% DMSO), starting from 22 hpf for 3 h or in BIO-containing solution (10 µM in E3 embryo medium/0.15% DMSO), starting from 22 hpf for 3 h or 6 h. Control groups were treated with 1% and 0.15% DMSO, respectively. For LiCl treatments, embryos at various stages were exposed to 0.3 M LiCl in E3 embryo medium for 30 min at 28°C, as described previously (Carl et al., 2007). Afterwards, embryos were washed repeatedly with E3 embryo medium and allowed to develop at 28°C. Heat-shock experiments were performed as follows: *tg(hsp70l:Wnt8a-GFP)* and *tg(hsDkk1:GFP)* embryos were incubated for 45 min at 37°C starting from 22 hpf. Transgenic embryos were identified by fluorescence and heatshocked non-transgenic siblings were used as controls.

Cell counting and statistical analyses

To count dHb neurons we used anti-HuC/D immunostaining in combination with nuclear DAPI staining. Confocal *z*-stacks (50 µm) were acquired by using the pineal and epithalamic morphology as a landmark. Left and right HuC/D-positive cells were counted using the software Fiji (NIH). To count Wnt-active cells in *tg(7xtcf-Xla.Siam:nlsmCherry); tg(flh:GFP); tg(foxD3:GFP)* transgenic embryos, 50 µm confocal *z*-stacks were acquired in

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the developing dHb region. *Z*-stacks (20 µm; upper-limit defined by the uppermost pineal cell) were extracted from the raw data and mCherry-positive cells were counted using the software Fiji (NIH). Comparable orientation of the different embryos was ensured by comparing the morphology of the ventricles. Data are mean±s.d. The significance of differences between groups was investigated using Student's *t*-test (two tail, GraphPad software), and the following levels of significance used: **P*<0.05; ***P*<0.02; ****P*<0.001.

Microscopy and image manipulation

For transmitted light pictures, larvae were mounted in glycerol and imaged using differential interference contrast optics (Leica CTR6000; 20× and 40× objectives). For fluorescence confocal microscopy, embryos were mounted in 1% low-melt agarose in glass-bottom dishes (MatTek or LabTek). Embryos were imaged using a TCS SP5 MP (Leica) inverse laser scanning microscope, equipped with a pulsed IR laser for multi-photon excitation (Mai Tai HP, Spectra Physics) and two external filter-based detectors for multi-photon detection. Images were acquired with a 40× water objective (NA 1.1, Leica) and each *z*-stack was acquired with a 1 µM interval. Stack analysis, maximum intensity projections (MIPs), cropping and 2D-views were performed using the Fiji software.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.C.; Methodology: L.G., A.B.; Software: L.G.; Validation: L.G., A.B., M.C.; Formal analysis: L.G.; Investigation: L.G., A.B., L.P.; Resources: E.M., F.A.; Data curation: L.G.; Writing - original draft: M.C.; Writing - review & editing: L.P., M.C.; Visualization: L.G.; Supervision: M.C.; Project administration: M.C.; Funding acquisition: M.C., A.B., L.G.

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Supplementary information

Supplementary information available online at
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