

RESEARCH REPORT

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

Luca Guglielmi¹, Anja Bühler², Enrico Moro³, Francesco Argenton⁴, Lucia Poggi² and Matthias Carl^{1,2,*}

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 Tcf712 mediates Wnt signaling in developing habenular neurons (Hüsken et al., 2014). Intriguingly, although *tcf712* mRNA is widely expressed in the diencephalon also at earlier stages of development (Young et al., 2002), Tcf712 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 prethalamus 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

¹Heidelberg University, Medical Faculty Mannheim, Department of Cell and Molecular Biology, 68167 Mannheim, Germany. ²University of Trento, Department of Cellular, Computational and Integrative Biology (CIBIO), 38123 Trento, Italy.

³University of Padova, Department of Molecular Medicine, 35121 Padova, Italy.

⁴University of Padova, Department of Biology, 35121 Padova, Italy.

*Author for correspondence (matthias.carl@unitn.it)

DOI: 10.1242/dev.182865; L.P., 0000-0001-6058-1781; M.C., 0000-0002-9936-5813

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:nls-mCherry)* 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 GSK3 β 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 GSK3 β inhibitor (2',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.

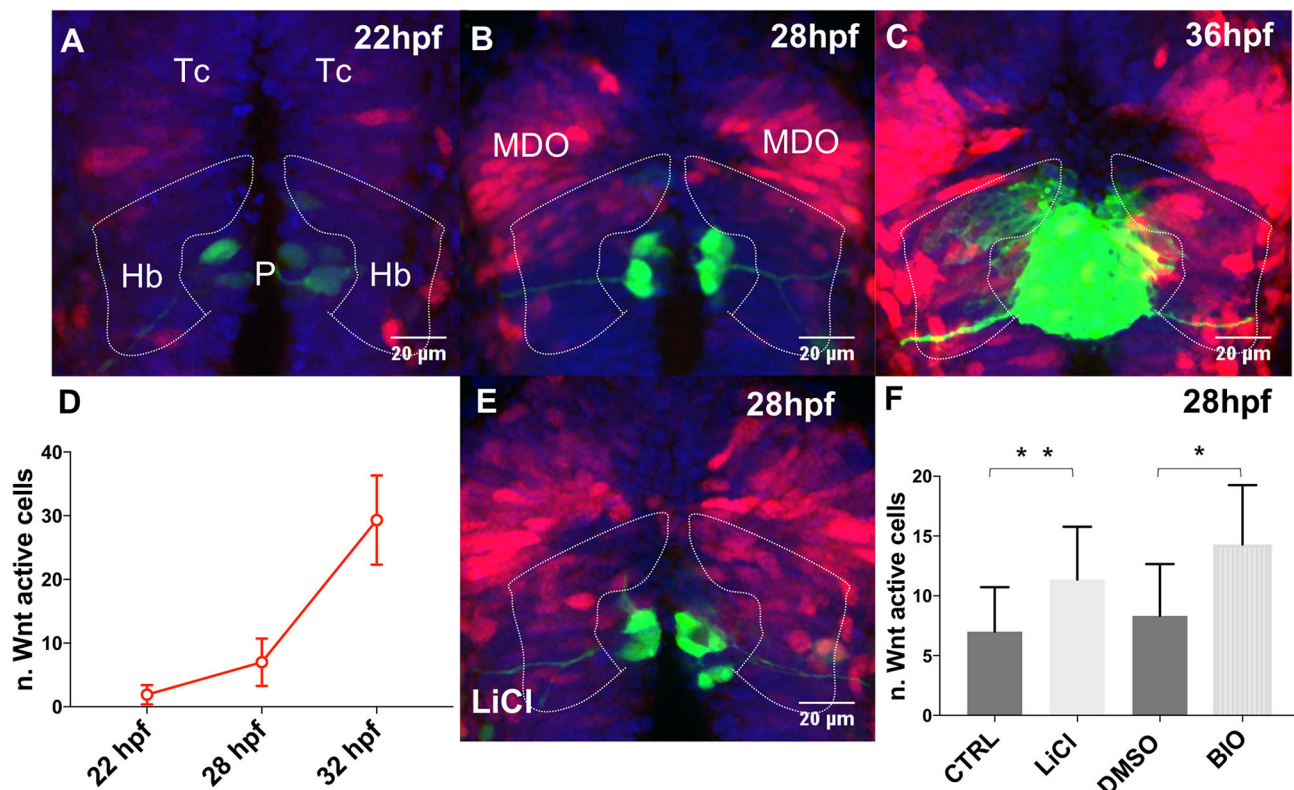


Fig. 1. Wnt signaling activity in and around the developing habenulae. (A–C,E) Projections of confocal z-stacks, dorsal views, anterior is towards the top focused onto the diencephalon of *tg(7xtcf-Xla.Siam:nls-mCherry)*; *tg(flh:GFP)*; *tg(foxD3:GFP)* transgenic embryos at stages indicated. Nuclei are 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.

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, *cxc4b* 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 dHb neurons were severely reduced or absent, while *kctd8* and *brn3a:GFP* in the subsequently generated dHb 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 dHb 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 dHb 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 dHb 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,

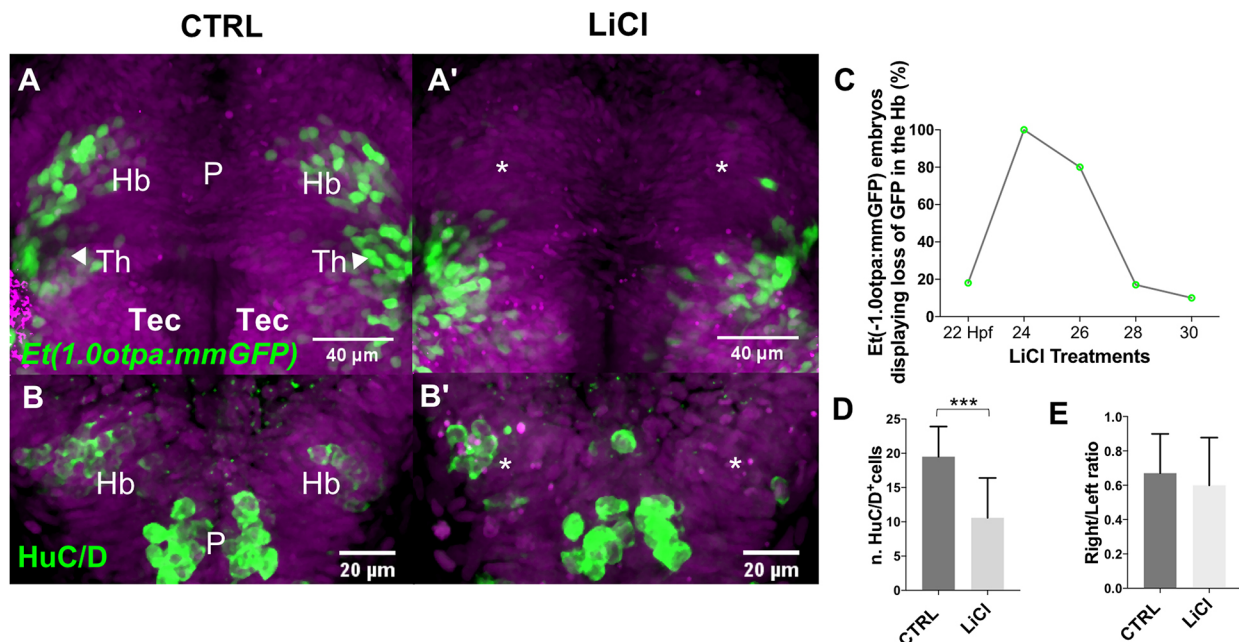


Fig. 2. Premature intrinsic activation of Wnt signaling delays habenular neuron differentiation. (A-B') Projections of confocal z-stacks, dorsal views, anterior is towards the top focused onto the diencephalon of (A,A') *Et(-1.0otpa:mmGFP)* and (B,B') *tg(flh:GFP); tg(foxD3:GFP)* transgenic embryos. 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.

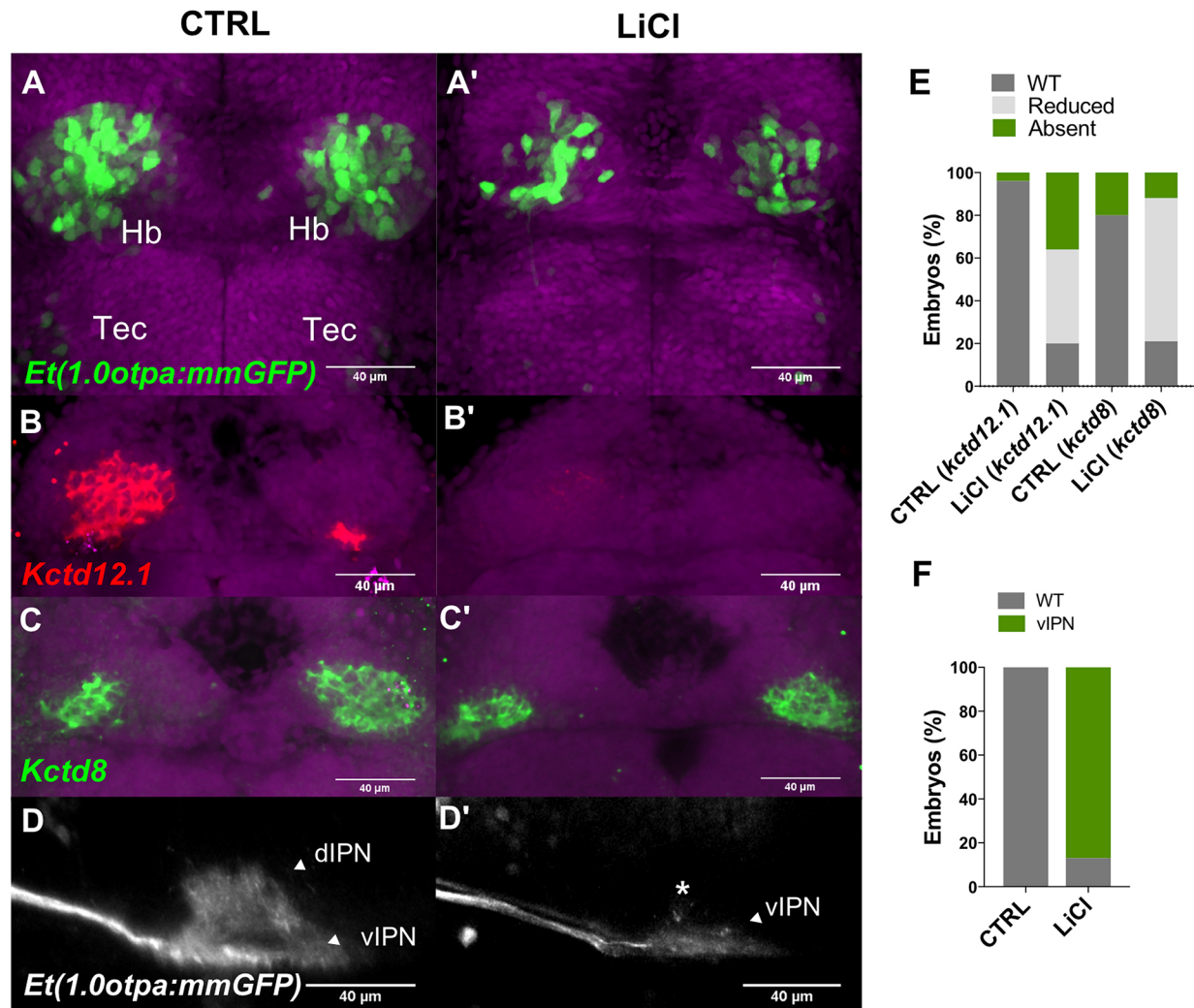


Fig. 3. Premature activation of Wnt signaling causes a reduction of dHbl neurons. (A-D') Projections of confocal z-stacks. (A-C') Dorsal views, anterior is towards top focused onto the diencephalon of (A-D') embryos at 3 dpf. Nuclei are DAPI labeled (purple). (A,A') LiCl treatment causes delayed habenular 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 Tcf712 protein

expression (Hüsken et al., 2014) and hence an excellent candidate antagonist 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, *cxc4b* 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

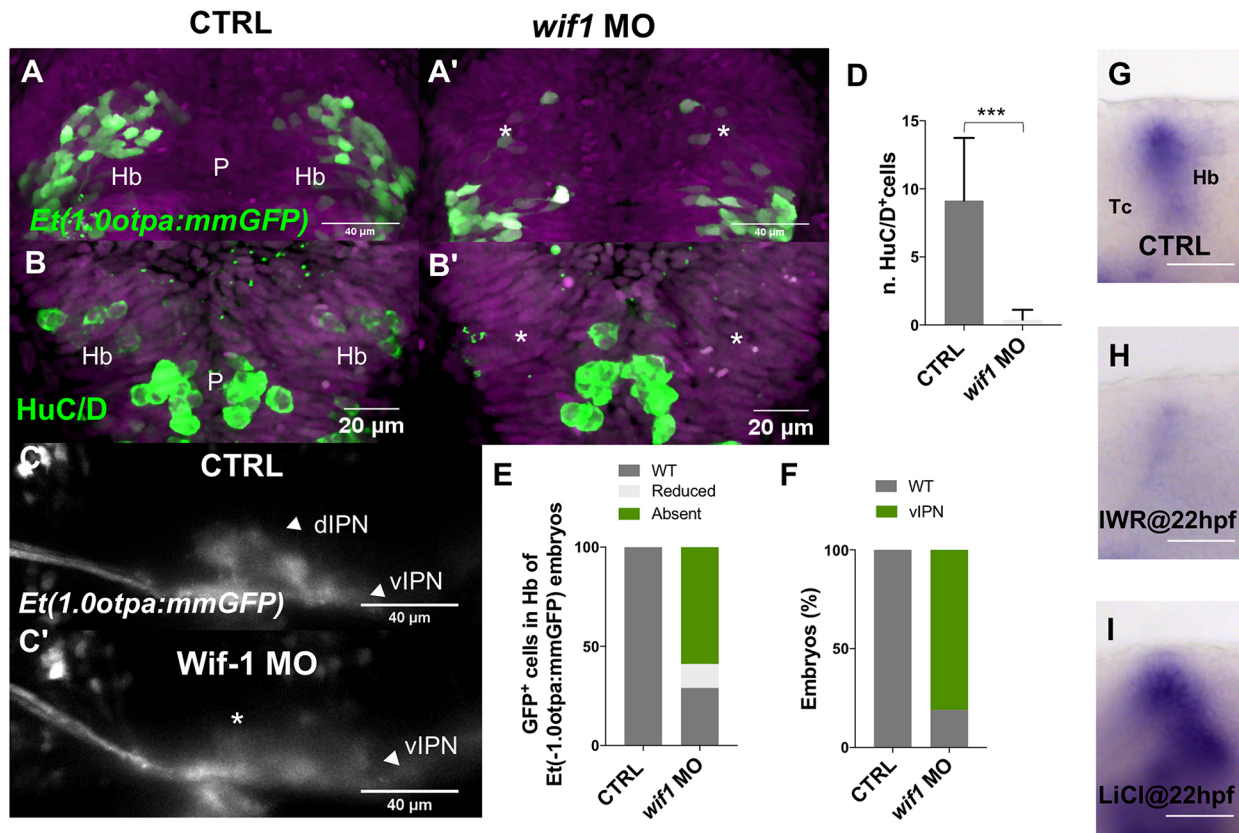


Fig. 4. Wif1 controls dHb neuron differentiation and is regulated by Wnt signaling. (A-C') Projections of confocal z-stacks. (A-B') Dorsal views, anterior is towards the top focused onto the diencephalon of embryos at 3 dpf. Nuclei are DAPI labeled (purple). (A-B',D,E) *Wif1* hypomorphic embryos exhibit 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 μ m. 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. *Tcf712* 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 *Tcf712-Wif1* expression in habenular precursors, *Wif1* expression was largely unchanged in *tcf712* 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

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)^{w711}*; *tg(foxD3:GFP)^{z104}* (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 *cxc4b* (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-HuCD 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',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 μ M 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-HuCD immunostaining in combination with nuclear DAPI staining. Confocal z-stacks (50 μ m) were acquired by using the pineal and epithalamic morphology as a landmark. Left and right HuCD-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 μ m confocal z-stacks were acquired in

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 \pm 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 \times and 40 \times 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 \times 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.

Acknowledgements

We thank our colleagues for transgenic fish, support and discussion, in particular A. Faro, A. Paolini, E. F. Taveras, M. Tsalavouta, G. Weidinger and S. W. Wilson. We are grateful to the Department of Cellular, Computational and Integrative Biology, CIBIO, University of Trento and to the Medical Faculty Mannheim, Heidelberg University, for financial support.

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.

Funding

This work was supported by a European Society for Fish Models in Biology and Medicine travel fellowship to L.G., by a Deutsche Forschungsgemeinschaft long-term fellowship to A.B. (BU3753/1-1) and by a Deutsche Forschungsgemeinschaft investigator grant to M.C. (CA298/6-1).

Supplementary information

Supplementary information available online at <http://dev.biologists.org/lookup/doi/10.1242/dev.182865.supplemental>

References

- Aizawa, H., Bianco, I. H., Hamaoka, T., Miyashita, T., Uemura, O., Concha, M. L., Russell, C., Wilson, S. W. and Okamoto, H. (2005). Laterotopic representation of left-right information onto the dorso-ventral axis of a zebrafish midbrain target nucleus. *Curr. Biol.* **15**, 238-243. doi:10.1016/j.cub.2005.01.014
- Aizawa, H., Goto, M., Sato, T. and Okamoto, H. (2007). Temporally regulated asymmetric neurogenesis causes left-right difference in the zebrafish habenular structures. *Dev. Cell* **12**, 87-98. doi:10.1016/j.devcel.2006.10.004
- Aizawa, H., Amo, R. and Okamoto, H. (2011). Phylogeny and ontogeny of the habenular structure. *Front. Neurosci.* **5**, 138. doi:10.3389/fnins.2011.00138
- Amo, R., Aizawa, H., Takahoko, M., Kobayashi, M., Takahashi, R., Aoki, T. and Okamoto, H. (2010). Identification of the zebrafish ventral habenula as a homolog of the mammalian lateral habenula. *J. Neurosci.* **30**, 1566-1574. doi:10.1523/JNEUROSCI.3690-09.2010
- Benekareddy, M., Stachniak, T. J., Bruns, A., Knoflach, F., von Kienlin, M., Künnecke, B. and Ghosh, A. (2018). Identification of a corticohabenular circuit regulating socially directed behavior. *Biol. Psychiatry* **83**, 607-617. doi:10.1016/j.biopsych.2017.10.032
- Beretta, C. A., Brinkmann, I. and Carl, M. (2011). All four zebrafish Wnt7 genes are expressed during early brain development. *Gene Expr. Patterns* **11**, 277-284. doi:10.1016/j.gexp.2011.01.004
- Beretta, C. A., Dross, N., Gutierrez-Triana, J. A., Ryu, S. and Carl, M. (2012). Habenula circuit development: past, present, and future. *Front. Neurosci.* **6**, 51. doi:10.3389/fnins.2012.00051
- Beretta, C. A., Dross, N., Bankhead, P. and Carl, M. (2013). The ventral habenulae of zebrafish develop in prosomere 2 dependent on Tcf712 function. *Neural Dev.* **8**, 19. doi:10.1186/1749-8104-8-19
- Beretta, C. A., Dross, N., Guglielmi, L., Bankhead, P., Soulika, M., Gutierrez-Triana, J. A., Paolini, A., Poggi, L., Falk, J., Ryu, S. et al. (2017). Early commissural diencephalic neurons control habenular axon extension and targeting. *Curr. Biol.* **27**, 270-278. doi:10.1016/j.cub.2016.11.038
- Bianco, I. H., Carl, M., Russell, C., Clark, J. D. W. and Wilson, S. W. (2008). Brain asymmetry is encoded at the level of axon terminal morphology. *Neural Dev.* **3**, 9. doi:10.1186/1749-8104-3-9
- Boerboom, D., White, L. D., Dalle, S., Courty, J. and Richards, J. A. S. (2006). Dominant-stable β -catenin expression causes cell fate alterations and Wnt signaling antagonist expression in a murine granulosa cell tumor model. *Cancer Res.* **66**, 1964-1973. doi:10.1158/0008-5472.CAN-05-3493
- Bonkowski, J. L., Wang, X., Fujimoto, E., Lee, J. E., Chien, C.-B. and Dorsky, R. I. (2008). Domain-specific regulation of foxP2 CNS expression by left1. *BMC Dev. Biol.* **8**, 103. doi:10.1186/1471-213X-8-103
- Carl, M., Bianco, I. H., Bajoghli, B., Aghaallaei, N., Czerny, T. and Wilson, S. W. (2007). Wnt/Axin1/ β -catenin signaling regulates asymmetric nodal activation, elaboration, and concordance of CNS asymmetries. *Neuron* **55**, 393-405. doi:10.1016/j.neuron.2007.07.007
- Cavodeassi, F. and Houart, C. (2012). Brain regionalization: of signaling centers and boundaries. *Dev. Neurobiol.* **72**, 218-233. doi:10.1002/dneu.20938
- Chatterjee, M., Guo, Q., Weber, S., Scholpp, S. and Li, J. Y. H. (2014). Pax6 regulates the formation of the habenular nuclei by controlling the temporospatial expression of Shh in the diencephalon in vertebrates. *BMC Biol.* **12**, 13. doi:10.1186/1741-7007-12-13
- Concha, M. L. and Wilson, S. W. (2001). Asymmetry in the epithalamus of vertebrates. *J. Anat.* **199**, 63-84. doi:10.1046/j.1469-7580.2001.19910063.x
- Concha, M. L., Russell, C., Regan, J. C., Tawk, M., Sidi, S., Gilmour, D. T., Kapsimali, M., Sumoy, L., Goldstone, K., Amaya, E. et al. (2003). Local tissue interactions across the dorsal midline of the forebrain establish CNS laterality. *Neuron* **39**, 423-438. doi:10.1016/S0896-6273(03)00437-9
- Cowling, V. H., D'Cruz, C. M., Chodosh, L. A. and Cole, M. D. (2007). c-Myc transforms human mammary epithelial cells through repression of the Wnt inhibitors DKK1 and SFRP1. *Mol. Cell Biol.* **27**, 5135-5146. doi:10.1128/MCB.02282-06
- Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-68. doi:10.1038/380066a0
- Cui, Y., Yang, Y., Ni, Z., Dong, Y., Cai, G., Foncelle, A., Ma, S., Sang, K., Tang, S., Li, Y. et al. (2018). Astroglial Kir4.1 in the lateral habenula drives neuronal bursts in depression. *Nature* **554**, 323-327. doi:10.1038/nature25752
- Diep, D. B., Hoen, N., Backman, M., Machon, O. and Krauss, S. (2004). Characterisation of the Wnt antagonists and their response to conditionally activated Wnt signalling in the developing mouse forebrain. *Brain Res. Dev. Brain Res.* **153**, 261-270. doi:10.1016/j.devbrainres.2004.09.008
- Dreosti, E., Vendrell Llopis, N., Carl, M., Yaksi, E. and Wilson, S. W. (2014). Left-right asymmetry is required for the habenulae to respond to both visual and olfactory stimuli. *Curr. Biol.* **24**, 440-445. doi:10.1016/j.cub.2014.01.016
- Gamse, J. T., Thisse, C., Thisse, B. and Halpern, M. E. (2003). The paraneural mediates left-right asymmetry in the zebrafish diencephalon. *Development* **130**, 1059-1068. doi:10.1242/dev.00270
- Gamse, J. T., Kuan, Y.-S., Macurak, M., Brösamle, C., Thisse, B., Thisse, C. and Halpern, M. E. (2005). Directional asymmetry of the zebrafish epithalamus guides dorsoventral innervation of the midbrain target. *Development* **132**, 4869-4881. doi:10.1242/dev.02046
- Gilmour, D. T., Maischein, H.-M. and Nüsslein-Volhard, C. (2002). Migration and function of a glial subtype in the vertebrate peripheral nervous system. *Neuron* **34**, 577-588. doi:10.1016/S0896-6273(02)00683-9
- Hagemann, A. I. H. and Scholpp, S. (2012). The tale of the three brothers – Shh, Wnt, and Fgf during development of the thalamus. *Front. Neurosci.* **6**, 76. doi:10.3389/fnins.2012.00076
- Heisenberg, C.-P., Houart, C., Takeuchi, M., Rauch, G. J., Young, N., Coutinho, P., Masai, I., Caneparo, L., Concha, M. L., Geisler, R. et al. (2001). A mutation in the Gsk3-binding domain of zebrafish Masterblind/Axin1 leads to a fate transformation of telencephalon and eyes to diencephalon. *Genes Dev.* **15**, 1427-1434. doi:10.1101/gad.194301
- Hsieh, J.-C., Kodjabachian, L., Rebbert, M. L., Rattner, A., Smallwood, P. M., Samos, C. H., Nusse, R., Dawid, I. B. and Nathans, J. (1999). A new secreted protein that binds to Wnt proteins and inhibits their activities. *Nature* **398**, 431-436. doi:10.1038/18899
- Huang, S.-M. A., Mishina, Y. M., Liu, S., Cheung, A., Stegmeier, F., Michaud, G. A., Charlat, O., Willellette, E., Zhang, Y., Wiessner, S. et al. (2009). Tankyrase inhibition stabilizes axin and antagonizes Wnt signaling. *Nature* **461**, 614-620. doi:10.1038/nature08356
- Hüsken, U. and Carl, M. (2013). The Wnt/ β -catenin signaling pathway establishes neuroanatomical asymmetries and their laterality. *Mech. Dev.* **130**, 330-335. doi:10.1016/j.mod.2012.09.002
- Hüsken, U., Stickney, H. L., Gestri, G., Bianco, I. H., Faro, A., Young, R. M., Roussigne, M., Hawkins, T. A., Beretta, C. A., Brinkmann, I. et al. (2014).

- Tcf7l2 is required for left-right asymmetric differentiation of habenular neurons. *Curr. Biol.* **24**, 2217–2227. doi:10.1016/j.cub.2014.08.006
- Jones, E. G. and Rubenstein, J. L. R. (2004). Expression of regulatory genes during differentiation of thalamic nuclei in mouse and monkey. *J. Comp. Neurol.* **477**, 55–80. doi:10.1002/cne.20234
- Kawano, Y. and Kypta, R. (2003). Secreted antagonists of the Wnt signalling pathway. *J. Cell Sci.* **116**, 2627–2634. doi:10.1242/jcs.00623
- Kiecker, C. and Lumsden, A. (2012). The role of organizers in patterning the nervous system. *Annu. Rev. Neurosci.* **35**, 347–367. doi:10.1146/annurev-neuro-062111-150543
- Kuan, Y.-S., Roberson, S., Akitake, C. M., Fortuno, L., Gamse, J., Moens, C. and Halpern, M. E. (2015). Distinct requirements for Wntless in habenular development. *Dev. Biol.* **406**, 117–128. doi:10.1016/j.ydbio.2015.06.006
- Licchesi, J. D. F., Van Neste, L., Tiwari, V. K., Cope, L., Lin, X., Baylin, S. B. and Herman, J. G. (2010). Transcriptional regulation of Wnt inhibitory factor-1 by Miz-1/c-Myc. *Oncogene* **29**, 5923–5934. doi:10.1038/ncr.2010.322
- Mattes, B., Weber, S., Peres, J., Chen, Q., Davidson, G., Houart, C. and Scholpp, S. (2012). Wnt3 and Wnt3a are required for induction of the mid-diencephalic organizer in the caudal forebrain. *Neural Dev.* **7**, 12. doi:10.1186/1749-8104-7-12
- McNabb, A., Scott, K., von Ochsenstein, E., Seufert, K. and Carl, M. (2012). Don't be afraid to set up your fish facility. *Zebrafish* **9**, 120–125. doi:10.1089/zeb.2012.0768
- Meijer, L., Skaltsounis, A.-L., Magiatis, P., Polychronopoulos, P., Knockaert, M., Leost, M., Ryan, X. P., Vonica, C. A., Brivanlou, A., Dajani, R. et al. (2003). GSK-3-selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* **10**, 1255–1266. doi:10.1016/j.chembiol.2003.11.010
- Moro, E., Ozhan-Kizil, G., Mongera, A., Beis, D., Wierzbicki, C., Young, R. M., Bournele, D., Domenichini, A., Valdivia, L. E., Lum, L. et al. (2012). In vivo Wnt signaling tracing through a transgenic biosensor fish reveals novel activity domains. *Dev. Biol.* **366**, 327–340. doi:10.1016/j.ydbio.2012.03.023
- Muncan, V., Faro, A., Haramis, A.-P. G., Hurlstone, A. F. L., Wienholds, E., van Es, J., Korving, J., Begthel, H., Zivkovic, D. and Clevers, H. (2007). T-cell factor 4 (Tcf7l2) maintains proliferative compartments in zebrafish intestine. *EMBO Rep.* **8**, 966–973. doi:10.1038/sj.embor.7401071
- Okamoto, H., Agetsuma, M. and Aizawa, H. (2012). Genetic dissection of the zebrafish habenula, a possible switching board for selection of behavioral strategy to cope with fear and anxiety. *Dev. Neurobiol.* **72**, 386–394. doi:10.1002/dneu.20913
- Peukert, D., Weber, S., Lumsden, A. and Scholpp, S. (2011). Lhx2 and Lhx9 determine neuronal differentiation and compartment in the caudal forebrain by regulating Wnt signaling. *PLoS Biol.* **9**, e1001218. doi:10.1371/journal.pbio.1001218
- Picker, A., Brennan, C., Reifers, F., Böhl, H., Holder, N. and Brand, M. (1999). Requirement for zebrafish *acerebellar/FGF8* in midbrain polarization, mapping and confinement of the retinotectal projection. *Development* **126**, 2967–2978.
- Poggi, L., Casarosa, S. and Carl, M. (2018). An eye on the Wnt inhibitory factor Wif1. *Front. Cell Dev. Biol.* **6**, 167. doi:10.3389/fcell.2018.00167
- Quinlan, R., Graf, M., Mason, I., Lumsden, A. and Kiecker, C. (2009). Complex and dynamic patterns of Wnt pathway gene expression in the developing chick forebrain. *Neural Dev.* **4**, 35. doi:10.1186/1749-8104-4-35
- Roussigne, M., Bianco, I. H., Wilson, S. W. and Blader, P. (2009). Nodal signalling imposes left-right asymmetry upon neurogenesis in the habenular nuclei. *Development* **136**, 1549–1557. doi:10.1242/dev.034793
- Shimogori, T., Vansant, J., Paik, E. and Grove, E. A. (2004). Members of the Wnt, Fz, and Frp gene families expressed in postnatal mouse cerebral cortex. *J. Comp. Neurol.* **473**, 496–510. doi:10.1002/cne.20135
- Stambolic, V., Ruel, L. and Woodgett, J. R. (1996). Lithium inhibits glycogen synthase kinase-3 activity and mimics wingless signalling in intact cells. *Curr. Biol.* **6**, 1664–1669. doi:10.1016/S0960-9822(02)70790-2
- Stoick-Cooper, C. L., Weidinger, G., Riehle, K. J., Hubbert, C., Major, M. B., Fausto, N. and Moon, R. T. (2007). Distinct Wnt signaling pathways have opposing roles in appendage regeneration. *Development* **134**, 467–478. doi:10.1242/dev.001123
- Sutherland, R. J. (1982). The dorsal diencephalic conduction system: a review of the anatomy and functions of the habenular complex. *Neurosci. Biobehav. Rev.* **6**, 1–13. doi:10.1016/0149-7634(82)90003-3
- Tan, M., Wu, J. and Cai, Y. (2013). Suppression of Wnt signaling by the miR-29 family is mediated by demethylation of WIF-1 in non-small-cell lung cancer. *Biochem. Biophys. Res. Commun.* **438**, 673–679. doi:10.1016/j.bbrc.2013.07.123
- Thisse, B. and Thisse, C. (2004). Fast Release Clones: A High Throughput Expression Analysis. *ZFIN Direct Data Submission* (<http://zfin.org/>). <https://zfin.org/ZDB-PUB-040907-1>
- Thisse, C. and Thisse, B. (2005). High Throughput Expression Analysis of ZF-Models Consortium Clones. *ZFIN Direct Data Submission* (<http://zfin.org/>).
- Thisse, C. and Thisse, B. (2008). High-resolution in situ hybridization to whole-mount zebrafish embryos. *Nat. Protoc.* **3**, 59–69. doi:10.1038/nprot.2007.514
- Thisse, B., Pflumio, S., Fürthauer, M., Loppin, B., Heyer, V., Degraeve, A., Woehl, R., Lux, A., Steffan, T., Charbonnier, X. Q. and Thisse, C. (2001). Expression of the zebrafish genome during embryogenesis. *ZFIN Direct Data Submission* (<http://zfin.org/>). <https://zfin.org/ZDB-PUB-010810-1>
- Turner, K. J., Bracewell, T. G. and Hawkins, T. A. (2014). Anatomical dissection of zebrafish brain development. *Methods Mol. Biol.* **1082**, 197–214. doi:10.1007/978-1-62703-655-9_14
- Vaes, B. L., Dechering, K. J., van Someren, E. P., Hendriks, J. M., van de Ven, C. J., Feijen, A., Mummery, C., Reinders, M., Olijve, W. and Vanzoelen, E. (2005). Microarray analysis reveals expression regulation of Wnt antagonists in differentiating osteoblasts. *Bone* **36**, 803–811. doi:10.1016/j.bone.2005.02.001
- Weidinger, G., Thorpe, C. J., Wuennenberg-Stapleton, K., Ngai, J. and Moon, R. T. (2005). The Sp1-related transcription factors sp5 and sp5-like act downstream of Wnt/β-catenin signaling in mesoderm and neuroectoderm patterning. *Curr. Biol.* **15**, 489–500. doi:10.1016/j.cub.2005.01.041
- Yang, Y., Cui, Y., Sang, K., Dong, Y., Ni, Z., Ma, S. and Hu, H. (2018). Ketamine blocks bursting in the lateral habenula to rapidly relieve depression. *Nature* **554**, 317–322. doi:10.1038/nature25509
- Yin, A., Korzh, V. and Gong, Z. (2012). Perturbation of zebrafish swimbladder development by enhancing Wnt signaling in Wif1 morphants. *Biochim. Biophys. Acta Mol. Cell Res.* **1823**, 236–244. doi:10.1016/j.bbamcr.2011.09.018
- Young, R. M., Reyes, A. E. and Allende, M. L. (2002). Expression and splice variant analysis of the zebrafish tcf4 transcription factor. *Mech. Dev.* **117**, 269–273. doi:10.1016/S0925-4773(02)00180-6
- Zirn, B., Samans, B., Wittmann, S., Pietsch, T., Leuschner, I., Graf, N. and Gessler, M. (2006). Target genes of the WNT/β-catenin pathway in Wilms tumors. *Genes Chromosomes Cancer* **45**, 565–574. doi:10.1002/gcc.20319

Supplementary tables

Table S1 related to figure 2: LiCl treatments of Et(-1.0otpa:mmGFP) embryos

Hpf	WT	Reduced or absent GFP expression in the dHb (48hpf)	n
22	16 (82%)	3 (18%)	19
24	0	80 (100%)	80
26	3 (20%)	12 (80%)	15
28	18 (77%)	6 (17%)	24
30	20 (90%)	2 (10%)	22

Table S2 related to figure 3: Effect of LiCl treatments on dHb neuron markers

Marker	WT	Reduced	Absent	n
HuC/D (48hpf)	1 (11%)	8 (89%)	0	9
<i>Cxcr4b</i> (48hpf)	28 (93%)	2 (7%)	0	30
<i>kctd12.1</i> (72hpf)	11 (20%)	24 (43%)	20 (37%)	55
Et(gata2a:EGFP) (72hpf)	8 (20%)	18 (45%)	14 (35%)	40
<i>kctd8</i> (72hpf)	10 (20%)	32 (67%)	6 (13%)	48
Tg(hsp70-brn3a:GFP) (72hpf)	23 (85%)	4 (15%)	0	27
<i>Kctd12.2</i> (72hpf)	39 (63%)	15 (24%)	8 (13%)	62

Table S3 related to figure 3, 4: Premature Wnt activation results in vIPN innervation

Treatment	WT	Only ventral	n
LiCl	4 (13%)	27 (87%)	31
Wif1 MO	5 (19%)	21 (81%)	26
BIO	1 (25%)	3 (75%)	4
tg(hsp70l:wnt8a-GFP)@22hpf	9 (100%)	0	9

Table S4 related to figure 4: Wif1 knock down phenocopies LiCl treatments

Marker	WT	Reduced	Absent	n
Et(-1.0otpa:mmGFP) (48hpf)	13 (29%)	5 (12%)	25 (59%)	43
HuC/D (48hpf)	0	5 (100%)	0	5
<i>Cxcr4b</i> (48hpf)	17 (94%)	1 (5%)	0	18
<i>kctd12.1</i> (72hpf)	26 (37%)	30 (42%)	13 (19%)	71
Et(gata2a:EGFP) (72hpf)	7 (23%)	7 (23%)	17 (54%)	31
<i>kctd8</i> (72hpf)	21 (46%)	16 (38%)	9 (18%)	46
Tg(hsp70-brn3a:GFP) (72hpf)	6 (60%)	4 (40%)	0	10
<i>Kctd12.2</i> (72hpf)	22 (58%)	14 (37%)	2 (5%)	38

Table S5 related to figure 4: *Wif1* expression is regulated by Wnt signaling

Treatment/ mutant	WT	Reduced	Increased	n
tg(hsp701:wnt8a-GFP) @22hpf	0	0	46 (100%)	46
LiCl@22hpf	10 (30%)	0	23 (70%)	33
BIO@22hpf	4 (44%)	0	5 (56%)	9
<i>Axin1</i> ^{-/-}	8 (32%)	0	17 (68%)	25
IWR@22hpf	8 (24%)	36 (76%)	0	44
tg(hsDkk1:GFP) @22hpf	2 (6%)	31 (94%)	0	33
<i>Tcf7l2</i> (whole incross clutches)	135 (100%)	0	0	135

Table S6 related to figure 4: *Wif1* suppresses Wnt signaling during dHb development

Treatment/ <i>Kctd12.1</i>	WT	Reduced	Absent	n
hs - control	79 (95%)	4 (5%)	0	83
tg(hsp701:wnt8a-GFP) @22hpf	63 (71%)	25 (28%)	1 (1%)	89
<i>wif1</i> MO	42 (88%)	6 (12%)	0	48
<i>wif1</i> MO + tg(hsp701:wnt8a-GFP) @22hpf	14 (39%)	21 (58%)	1 (3%)	36
Treatment/ <i>Kctd8</i>	WT	Reduced	absent	n
hs - control	52 (70%)	22 (30%)	0	74
tg(hsp701:wnt8a-GFP) @22hpf	47 (57%)	36 (43%)	0	83
<i>wif1</i> MO	40 (67%)	17 (28%)	3 (5%)	60
<i>wif1</i> MO + tg(hsp701:wnt8a-GFP) @22hpf	20 (46%)	18 (42%)	5 (12%)	43

Supplementary Figures

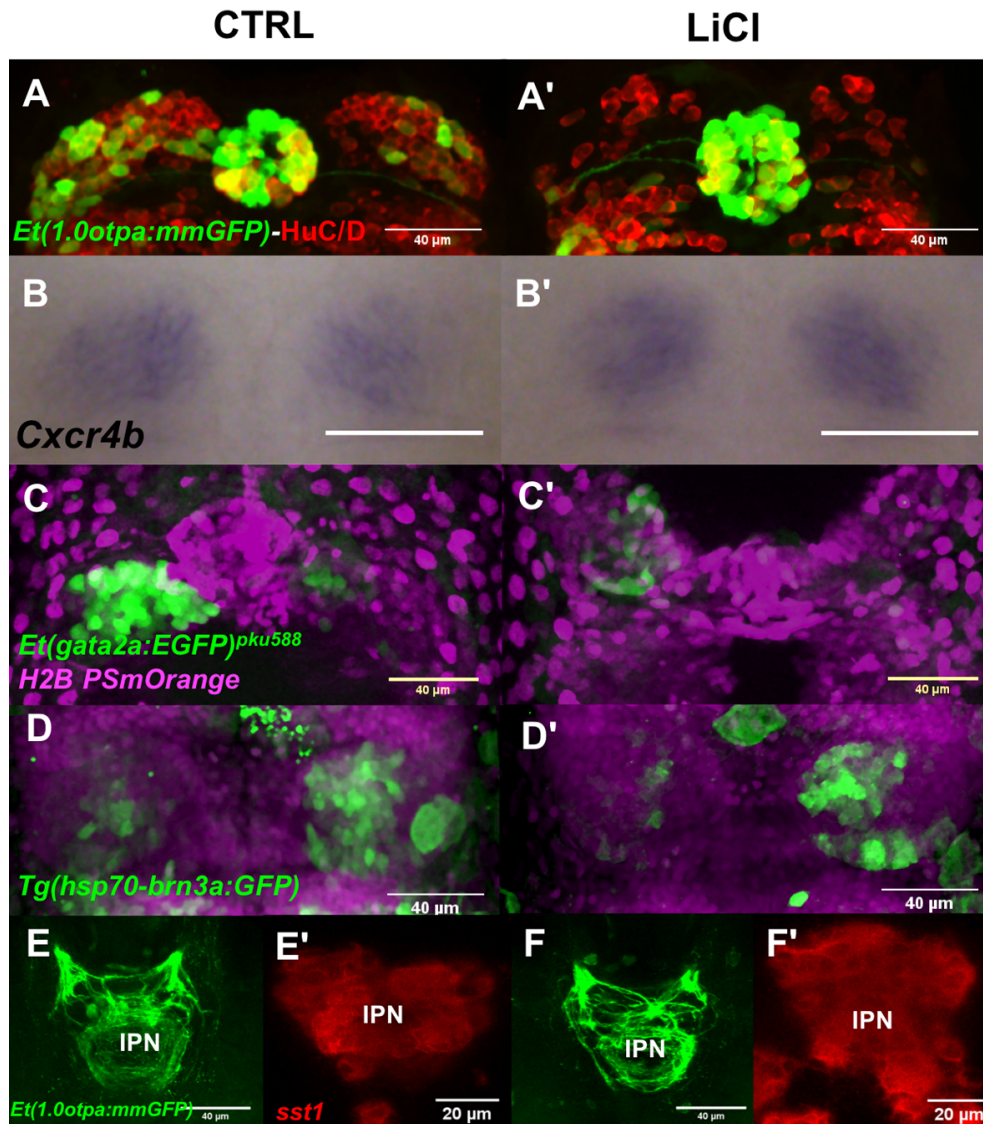


Figure S1, related to Figure 2 and 3: Premature activation of Wnt signaling affects dHb neuron differentiation

(A-F') Dorsal views, anterior to the top focused on the (A-D') developing habenulae and (E-F') IPN. (A, A', C-F') Projections of confocal z-stacks. (A-B') At 48 hpf, LiCl treated embryos exhibit the specific lack of GFP expressing habenular cells and a reduction in HuC/D positive differentiating neurons in the presence of unaffected *cxcr4b* expression in habenular precursor cells (Roussigne et al., 2009). (C, C') At 72 hpf, GFP expressing dHbl neurons and (D, D') less evident also dHbm neurons are reduced upon LiCl induced Wnt signaling activation. (E-F') Innervation of the normally formed IPN by habenular efferent axons as evidenced by normal expression of *somatostatin1* (*sst1*) in the IPN.

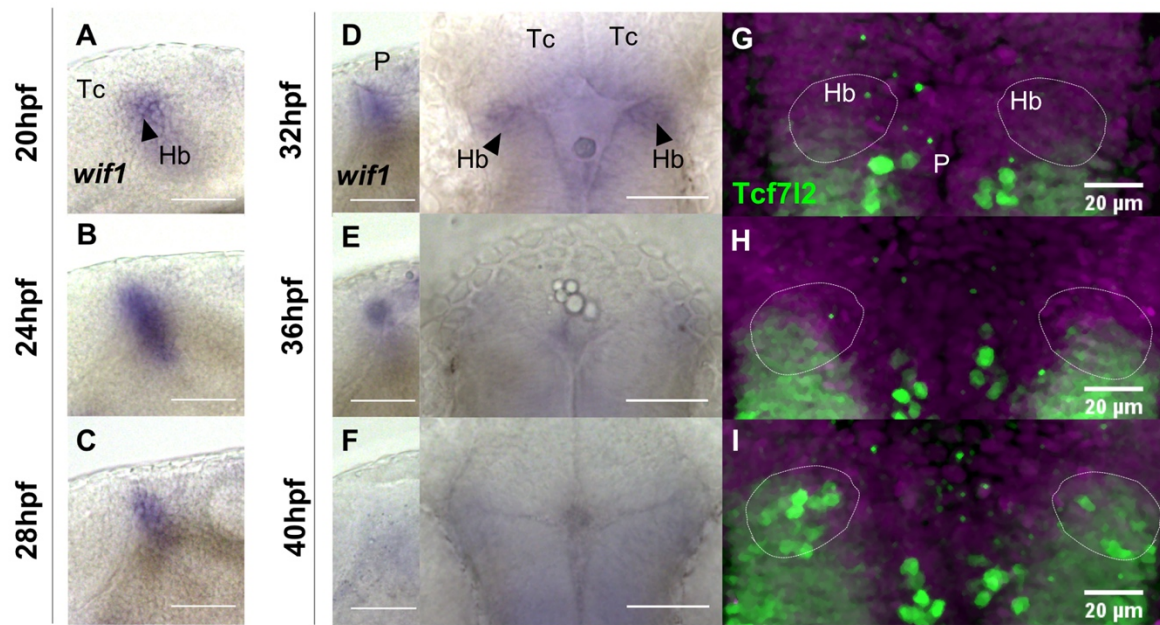


Figure S2: *Wif1* expression temporally complements *Tcf7l2* expression
 (A-C) and (D-F left) lateral views, anterior to the left and (D-F right) and (G-I) projections of confocal z-stacks (habenular region is encircled), dorsal views with anterior to the top focused on the diencephalon of wild type embryos at stages indicated on the left. *Wif1* is discretely expressed in the developing habenular region (arrowheads) until *Tcf7l2* expression is initiated at 36 hpf. Hb, habenula; P, pineal; Tc, telencephalon.

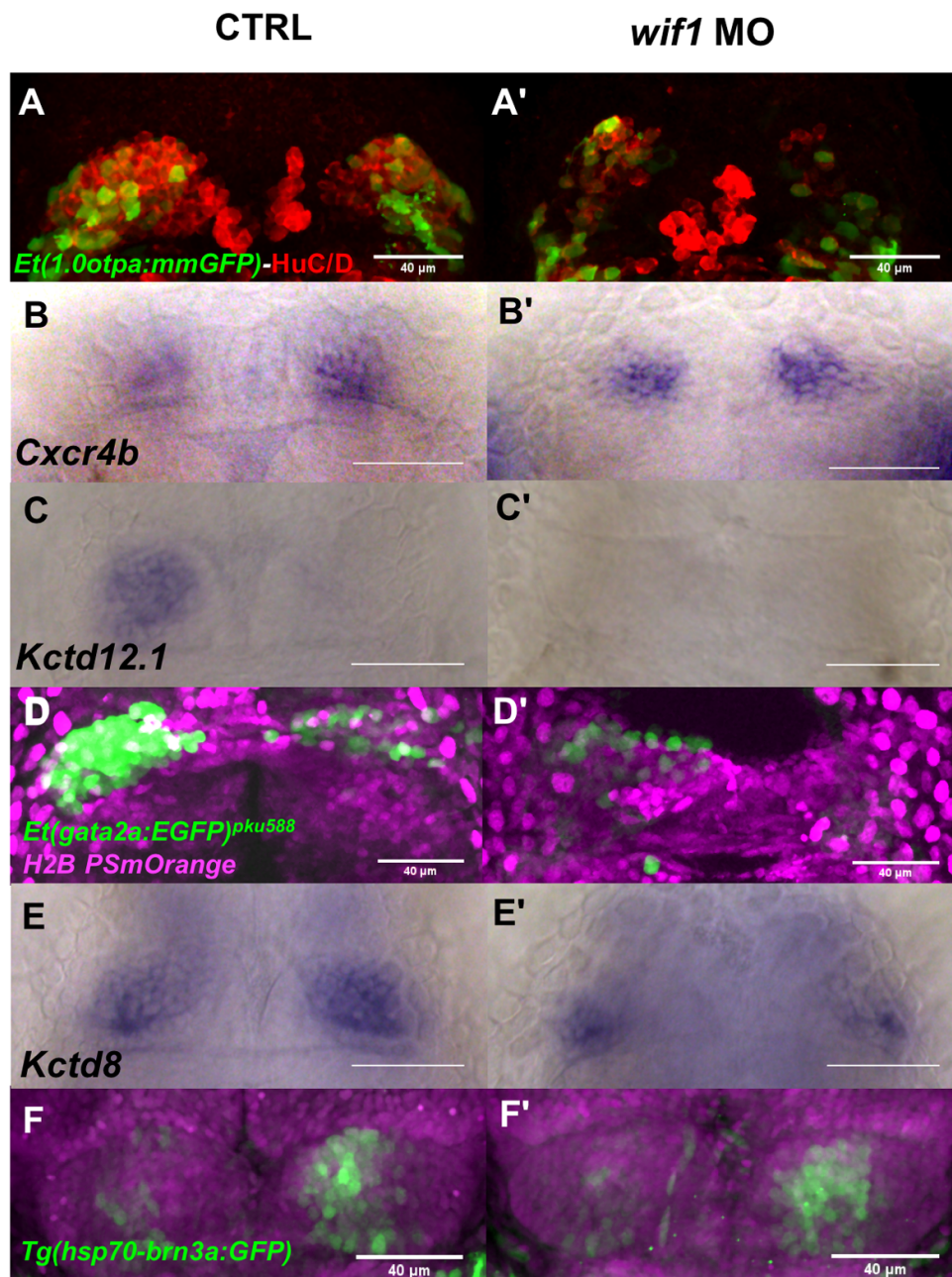


Figure S3, related to Figure 4: Wif1 interference alters habenular neuron differentiation

(A-F') Dorsal views, anterior to the top focused on the developing habenulae. (A, A', D, D' F, F') Projections of confocal z-stacks. (A-B') At 48 hpf, *Wif1* hypomorphic embryos exhibit the reduction of GFP expressing habenular cells and a reduction in HuC/D positive differentiating neurons in the presence of largely unaffected *cxcr4b* expression in habenular precursor cells (Roussigne et al., 2009). (C-F') At 72 hpf, the dHbl markers *kctd12.1* and *pkus88* are reduced or absent upon *Wif1* knock down, while *kctd8* and *brn3a:GFP* in dHbm neurons are less strongly affected.

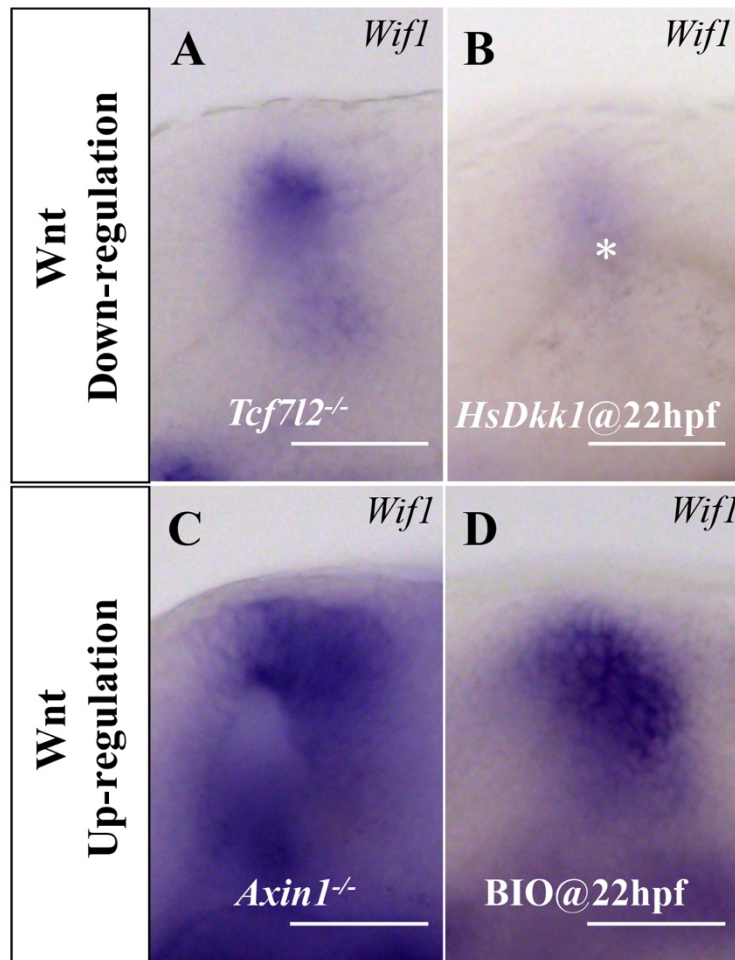


Figure S4, related to Figure 4: *Wif1* expression is regulated by Wnt signaling

(A-D) Lateral views with anterior to the left focused on the diencephalon at 26 hpf labeled for *Wif1* expression. (A) Wnt downregulation in *tcf7l2* mutants does not affect *Wif1* expression (compare also with the control embryo in Fig. 4G), which is consistent with the temporally complementing expression of *Tcf7l2* and *Wif1*. (B) Transient transgenic Wnt suppression results in a decrease of *Wif1* expression in the habenulae (asterisk). (C, D) Wnt upregulation causes an increase of the *Wif1* expression domain. Labelling procedures were conducted in parallel for comparability.

