RESEARCH ARTICLE

Pitx2c ensures habenular asymmetry by restricting parapineal cell number

Laurence Garric1,2, Brice Ronsin1,2, Myriam Roussigné1,2, Sabrina Booton3, Joshua T. Gamse3, Pascale Dufourcq1,2,* and Patrick Blader1,2,*

ABSTRACT

Left-right (L/R) asymmetries in the brain are thought to underlie lateralised cognitive functions. Understanding how neuroanatomical asymmetries are established has been achieved through the study of the zebrafish epithalamus. Morphological symmetry in the epithalamus is broken by leftward migration of the parapineal, which is required for the subsequent elaboration of left habenular identity; the habenular nuclei flank the midline and show L/R asymmetries in marker expression and connectivity. The Nodal target pitx2c is expressed in the left epithalamus, but nothing is known about its role during the establishment of asymmetry in the brain. We show that abrogating Pitx2c function leads to the right habenula adopting aspects of left character, and to an increase in parapineal cell numbers. Parapineal ablation in Pitx2c loss of function results in right habenular isomerism, indicating that the parapineal is required for the left character detected in the right habenula in this context. Partial parapineal ablation in the absence of Pitx2c, however, reduces the number of parapineal cells to wild-type levels and restores habenular asymmetry. We provide evidence suggesting that antagonism between Nodal and Pitx2c activities sets an upper limit on parapineal cell numbers. We conclude that restricting parapineal cell number is crucial for the correct elaboration of epithalamic asymmetry.

KEY WORDS: Nodal, Pitx2, Epithalamus, Left-right, Parapineal, Zebrafish

INTRODUCTION

The epithalamus is composed of the left and right habenulae, and the pineal complex, which is itself composed of the pineal gland and the parapineal nucleus (Concha and Wilson, 2001). Although the habenulae link the anterior forebrain to the ventral midbrain, the pineal complex is a photoreceptive structure involved in regulating circadian rhythms. In zebrafish, the epithalamus is highly asymmetric along the left-right (L/R) axis (for a review, see Roussigné et al., 2012). Indeed, the habenulae display a variety of L/R differences in the expression of various markers and transgenes. The pitx2c target gene encodes a homeobox transcription factor that is a target of Nodal signalling, and that is mutated in individuals with Axenfeld-Rieger syndrome who display ocular and craniofacial abnormalities (Semina et al., 1996; Shiratori et al., 2001). In mouse, Pitx2 mutants display phenotypes that include alterations in heart positioning and right isomerisation of the lungs (Kitamura et al., 1999; Lin et al., 1999; Lu et al., 1999; Liu et al., 2001). In zebrafish embryos, two isoforms of Pitx2 are generated from a single gene through the use of distinct promoters. The expression patterns of the two isoforms are largely similar, but differences exist in structures that display asymmetries along the left-right axis; the parapineal phenocopies some aspects of Axenfeld-Rieger syndrome, nothing is known about a role for pitx2c in the development of L/R asymmetry in the zebrafish epithalamus (Bohsack et al., 2012; Liu and Semina, 2012).

Here, we show that there is an increase in parapineal cell number in the pitx2c loss-of-function context that correlates with the acquisition of aspects of left character in the right habenula. Artificially restricting the number of parapineal cells in this context rescues the habenular phenotype. We propose that restricting parapineal cell number is crucial for the correct elaboration of asymmetry in the epithalamus and that Pitx2c participates in this process.

RESULTS

pitx2c morphant embryos display partial left isomerism of the habenulae

To address a potential role for Pitx2 in the elaboration of L/R asymmetry in the developing zebrafish brain, we employed a...
morpholino knockdown strategy that targets the Pitx2c isoform specifically. Morphant embryos develop in a manner that is globally indistinguishable from wild-type and control morphants (data not shown). Furthermore, the asymmetric expression of the Nodal pathway genes is unchanged, suggesting that early events in the establishment of L/R asymmetry are unaffected (supplementary material Fig. S1) (Thisse and Thisse, 1999; Essner et al., 2000; Long et al., 2003).

Next, we analysed the expression of genes displaying L/R asymmetric patterns in the habenulae in pitx2c morphants. As previously described, the expression of the potassium channel tetramerization domain containing 8 (kctd8/dexter) gene is broader in the right than the left habenula of wild-type embryos at 72 hours post-fertilisation (hpf) and this pattern remains unchanged in embryos injected with either a control morpholino or morpholinos targeting pitx2c (Fig. 1A-C, Fig. 1A-C′) (Gamse et al., 2005); the expression of a second right asymmetric potassium channel tetramerization domain containing gene, kctd12.2, is similarly unaffected (supplementary material Fig. S2A-C). Conversely, although in wild-type and control morphant embryos the habenular expression volumes of a third Kctd gene, kctd12.1/leftover, are asymmetric with broader expression in the left habenula at 72 hpf, in pitx2c morphant embryos the asymmetry index is significantly reduced (Fig. 1D, Fig. 1D′, Fig. 1F, Fig. 1F′) (Gamse et al., 2003). However, although pitx2c is expressed exclusively in the left dorsal diencephalon, the shift toward symmetry in pitx2c morphant embryos is the result of an expansion of kctd12.1 expression in the right rather than a decrease of expression in the left habenula (Fig. 1E versus Fig. 1D,F). Similarly, a habenular neuropil of left character appears in the right habenula in pitx2c morphants (supplementary material Fig. S2D,D′). Not all markers expressed preferentially in the left epithalamic behave in a more symmetric manner in the pitx2c knockdown context; nrp1a expression remains robustly asymmetric (supplementary material Fig. S2E-G). Finally, expression of the pan-habenular marker brn3a is unaffected in pitx2c morphants at 72 hpf, suggesting that overall habenular size is unchanged in the absence of Pitx2c function (supplementary material Fig. S2H-J) (Aizawa et al., 2005; Roussigné et al., 2009). Taken together, these results indicate that Pitx2c is required for the establishment of a subset of L/R habenular asymmetries.

**Parapineal cell numbers are increased in pitx2c morphant embryos**

The elaboration of left habenular character requires input from the parapineal (Concha et al., 2003; Gamse et al., 2003). Moreover, in

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**Fig. 1. Partial left isomerisation in pitx2c morphants.** (A-C) Single confocal sections of the epithalamus showing in situ hybridisation against kctd8 in wild-type, pitx2c morphant and control morphant embryos at 72 hpf. No differences are detected between the different contexts. (A′-C′) Three-dimensional renderings of volumes of kctd8 expression in the habenulae of the respective confocal acquisition sets. (D-F) Single confocal sections of the epithalamus showing in situ hybridisation against kctd12.1 in wild-type, pitx2c morphant and control morphant embryos at 72 hpf. pitx2c morphants display broader expression of kctd12.1 in the right habenula than either of the control contexts. (D′-F′) Three-dimensional renderings of volumes of kctd12.1 expression in the habenulae of the respective confocal acquisition sets. (G) Asymmetry index (AI) with regard to the volumes of kctd8 or kctd12.1 expression for individual wild-type (wt), control or pitx2c morphant embryos. Horizontal black line represents the median AI for each context and the dotted line indicates symmetry. Only the AI for the expression of kctd12.1 in pitx2c morphants is significantly more symmetric than control conditions; ***P<0.001 using a t-test. Embryos are viewed dorsally with the anterior to the top.
some mutant contexts, cases of left isomerism of kctd12.1 expression are associated with a duplication of the parapineal on the right side of the dorsal diencephalon (Gamse et al., 2003). We, thus, asked whether the partial left habenular isomerism we detect in pitx2c morphant embryos correlates with changes in the development or migratory behaviour of the parapineal.

The expression of parapineal markers, such as gfi1.2 and Et(krt4:EGFP)\textsuperscript{jowell}, is detected in morphants at 72 hpf, indicating that the parapineal is formed in the pitx2c knock-down context (Fig. 2A,B; supplementary material Fig. S3A,B) (Dufourcq et al., 2004; Choo et al., 2006). Furthermore, reduction of pitx2c function does not affect the orientation of parapineal migration. We did find, however, that expression of the Tg(fox3-GFP)\textsuperscript{f15} transgene appears in parapineal cells with an approximately 12-hour delay in pitx2c morphants relative to wild type (Fig. 2D\textsuperscript{″} versus Fig. 2E\textsuperscript{″}); robust expression of GFP from the Tg(fox3-GFP)\textsuperscript{f15} is detected at later stages in pitx2c morphants (data not shown). These data suggest that Pitx2c is required for the correct schedule of neural specification in the parapineal.

We also found that the number of cells in the parapineal in pitx2c morphants differs from wild-type embryos. Indeed, although 17±0.94 and 9±0.75 cells are labelled respectively, with gfi1.2 and Et(krt4:EGFP)\textsuperscript{jowell}, in controls a significantly larger number of cells are labelled in pitx2c morphants with these markers (25±1.59 and 12±1.57 cells; Fig. 2C; supplementary material Fig. S3). To address the origin of the increased number of parapineal cells, we performed time-lapse confocal analysis on wild-type and pitx2c morphant embryos (Fig. 2D-E\textsuperscript{″}). At 24 hpf, a stage just prior to parapineal migration, no difference in the number of parapineal cells was detected between wild-type and pitx2c morphant embryos, indicating that reducing pitx2c function does not influence the initial number of parapineal cells (Fig. 2D,E,F). As the parapineal migrates, however, supernumerary cell divisions lead to a significant increase in the number of cells per parapineal in morphant versus control embryos (Fig. 2F; supplementary material Movie 1 versus Movies 2 and 3). The extra cell divisions tend to occur during the first 6 hours of migration but can be detected up to 14 hours after migration is initiated. No signs of apoptosis of parapineal neurons was noted in our time-lapse data sets, suggesting that cell death does not eliminate parapineal neurons in wild-type embryos. Furthermore, all parapineal cells identified at the end of the time-lapse datasets are derived from cell divisions of the initially delaminated cell population, indicating that supernumerary cells do not join the parapineal during its migration from elsewhere in the epithalamus. In conclusion, our data indicate that Pitx2c limits the mitotic capacity of parapineal cells once they have been determined.

Previous studies have shown that habenular asymmetry is established in embryos lacking Nodal signalling in the epithalamus, despite them failing to express pitx2c (Concha et al., 2000; Liang et al., 2000). Furthermore, treatment of embryos from 16 hpf with SB431542, a small molecule inhibitor of Nodal signalling, blocks pitx2c expression in the epithalamus but does not result in an enlarged parapineal (Fig. 3A-C). One explanation for these
paradoxical results could be that Nodal signalling promotes an increase in cell number in the parapineal and that Pitx2c acts to impede this activity; in the absence of epithalamic Nodal activity this level of control would no longer be required. If the threshold of Nodal signalling needed to promote increased parapineal cell number was exceeded, this might allow the expansion of aspects of left character in the right habenula, as has been previously proposed (Concha et al., 2003; Gamse et al., 2003). Indeed, the results from our partial ablation studies suggest that Pitx2c restricts the number of cell of the parapineal and that this appears essential for the correct elaboration of habenular asymmetry.

**Restricting parapineal cell numbers in pitx2c morphants rescues habenular asymmetry**

To begin to address whether the partial left habenular isomerism detected in pitx2c morphants is due to the concomitant changes detected in the parapineal, we performed parapineal ablation experiments. In wild-type embryos, ablation of parapineal precursors prior to their migration leads to a ‘double right’ pattern of kctd12.1 expression (Fig. 5A,B) (Concha et al., 2003; Gamse et al., 2003); volumetric analysis shows that such ablations result in symmetric kctd12.1 expression pattern (Fig. 5A′,B′,E). Ablation of either the left or right half of the parapineal anlage prior to migration reduces parapineal cell numbers but had no effect on the resulting asymmetric development of the habenulae (Fig. 5C′-D′,E,J) (Concha et al., 2003). Whereas a ‘double left’ pattern of kctd12.1 expression is detected in pitx2c morphants, complete ablation of parapineal precursors in this context results in a ‘double right’ kctd12.1 phenotype (Fig. 5E-G′). Thus, in pitx2c morphants the expansion of left character in the right habenula seems to require the parapineal.

If the increase in parapineal cell number underlies the appearance of left character in the right habenula in pitx2c morphants, we hypothesised that reducing it in this context should rescue habenular asymmetry. For this, we performed partial ablations of parapineal precursors in the pitx2c morphant context. Such ablations result in parapineals whose cell number falls within the range sufficient for imposing correct habenular asymmetry in wild-type embryos (Fig. 5J); unlike partial ablation in wild-type embryos, however, more parapineal cells remain after ablation of the right than the left parapineal anlage in pitx2c morphants (Fig. 5I). Examination of the expression of kctd12.1 indicates that L/R asymmetry is efficiently restored in morphant embryos where the parapineal has been partially ablated (Fig. 5E,H-I′). Taken together, our results suggest that Pitx2c restricts the number of cell of the parapineal and that this appears essential for the correct elaboration of habenular asymmetry.

**DISCUSSION**

Elaboration of epithalamic asymmetry requires reciprocal interactions between the anlage of the left habenula and the parapineal (for a review, see Roussigné et al., 2012). Previously, laser ablation studies suggest that there is a minimum parapineal cell number required for the acquisition of left habenular character (Concha et al., 2003; Gamse et al., 2003). Here, we provide the first evidence that setting an upper limit of parapineal cell number is equally important. Our results reveal one mechanism for restricting the number of parapineal cells involves Pitx2c.

Abrogation of Pitx2c function leads to an increase in parapineal cell number and a concomitant expansion of aspects of left character in the right habenula, but where and when Pitx2c acts is unknown. Although formal proof is lacking, there are several reasons to believe that the role of Pitx2c on parapineal cell numbers is achieved via an autonomous function in the left epithalamus. First, the expression of the pitx2a isoform overlaps extensively both spatially and temporally with that of pitx2c in most tissues, including the ventral diencephalon, and previous studies suggest that the two proteins are largely functionally redundant (Essner et al., 2000). Such redundancy does not exist in the epithalamus, however, as only the pitx2c isoform is expressed in this brain region. Second, with the exception of the epithalamus, the expression of pitx2c is symmetric in the brain whereas the effects we describe appear to be unilateral. Indeed, the results from our partial ablation studies suggest that...
abrogation of Pitx2c function affects left parapineal precursors preferentially; although ablation of the left or right half of the parapineal anlage in wild-type embryos results in a similar number of residual parapineal cells being present, in the pitx2c morphant context statistically more residual parapineal cells are found if the right half of the parapineal anlage is ablated rather than the left half. An autonomous role for Pitx2c in left parapineal progenitors is also supported by the pattern of expression of pitx2c, which overlaps with these cells prior to parapineal migration (Liang et al., 2000). Further experiments, using conditional loss-of-function techniques such as photo-morpholinos, will be needed to address this issue in more detail.

Our results suggest that Pitx2c function restricts the mitotic capacity of parapineal cells during migration. How this restriction is achieved in molecular terms, however, remains unclear. Indeed, the best-known Pitx2 targets would appear to increase cell division and, as such, loss of Pitx2c would be expected to reduce the number of mitoses (Kioussi et al., 2002; Baek et al., 2003); Pitx2 is overexpressed in many cancers and is generally thought to promote cell proliferation. Recent proteomic and transcriptomic approaches have produced datasets of potential interactors and novel targets of Pitx2 from a variety of different cell types, including those affected in individuals with Axenfeld-Rieger syndrome, but the pertinence of these with respect to asymmetric development in the epithalamus remains to be determined (Huang et al., 2010; Paylakhi et al., 2011). The apparent antagonistic activities of Nodal and Pitx2c on parapineal cell numbers could perhaps provide clues. For example, Nodal and Pitx2c might regulate genes involved in cell-cycle progression, but in opposite fashions. Unfortunately, pertinent targets of Nodal signalling in the epithalamus other than pitx2c itself and lefty1 are lacking. A final possibility would be that Pitx2c antagonises transduction of Nodal signals. Although Lefty1 is itself a feedback inhibitor of Nodal signalling, it is unlikely to act downstream of Pitx2c as we have shown that morpholino knockdown of pitx2c does not affect its expression.

Last, it is not obvious whether the increase in parapineal cell number in the absence of Pitx2c is the sole explanation for the effect on habenular asymmetry. Another explanation would be that the parapineal remains at the midline longer in pitx2c morphants, leaving it time to impose left character on the right habenula. For two reasons, however, we believe that this is unlikely. First, although in some pitx2c morphant embryos the parapineal appears to spend more time at the midline prior to migrating, this is not always the case. Second, genetic contexts have been reported in which there is a significant delay in parapineal migration without a concomitant effect on the acquisition of left and right habenular identity. For example, homozygous one-eyed pinhead mutant embryos in which the requirement for Oep during early developmental stages has been rescued – known as Roep embryos – display a significant delay in parapineal migration without the expansion of left character in the right habenula (Gamse et al., 2002; Gamse et al., 2003); the parapineal of 17% of Roep embryos remains at the midline until at least 48 hpf with no undue effect on habenular asymmetry. Another explanation would be that the increase in parapineal cell number in the absence of Pitx2c is the sole explanation for the effect on habenular asymmetry. We, therefore, favour the idea that the increase in parapineal cell number increases the capacity of the parapineal to emit signals that impose left character on the right habenula. This is counter-intuitive, however, as pitx2c expression is limited to the left epithalamus. Furthermore, restoring wild-type parapineal cell numbers by laser ablation efficiently restores habenular asymmetry. We, therefore, favour the idea that the increase in parapineal cell number increases the capacity of the parapineal to emit signals that impose left habenular identity even across the dorsal midline. However, it remains unresolved why the right habenula adopts only a specific aspect of left character when Pitx2c activity is abrogated.

Conclusions

Pitx2 is a canonical Nodal target that has been implicated in the establishment of various aspects of L/R asymmetry in a variety of model systems. Here, we provide the evidence that Pitx2c is involved in limiting parapineal cell number during the establishment
of L/R asymmetry in the zebrafish epithalamus. Intriguingly, in this context Pitx2c appears to act via negative feedback against Nodal activity. It waits to be seen if a similar role for Pitx2 exists in other models of L/R asymmetry.

**MATERIALS AND METHODS**

**Fish lines and maintenance**

Embryos were raised and staged according to standard protocols (Kimmel et al., 1995). The transgenic lines Tg(foxd3:GFP)zf15, Et(krt4:EGFP)sqet11 and TgBAC(flh:flh-Kaede)vu376 have been described previously (Gilmour et al., 2002; Choo et al., 2006; Clanton et al., 2013). For later-staged embryos, PTU was used to block the formation of pigment.

**In situ hybridisation and antibody labelling**

Antisense probes for kctd8, kctd12.1, kctd12.2 (Gamse et al., 2003; Gamse et al., 2005), nrp1a (Kuan et al., 2007), brn3a (Aizawa et al., 2005), gfi1.2 (Dufourcq et al., 2004), lefty1 (Thirse and Thirse, 1999), southpaw (Long et al., 2003) and pitx2 (Essner et al., 2000) were generated using standard
procedures. Immunohistochemical stainings were performed as previously described (Masai et al., 1997), using either anti-GFP (Torrey Pines Biolabs) or anti-acetylated tubulin (Sigma) as primary antibodies with the appropriate Alexa Fluor 488-conjugated secondary antibody (Molecular Probes). For nuclear staining, embryos were incubated in ToPro (Molecular Probes).

**Volumetric analysis**
To quantify volumes, in situ hybridisation in control and morphant embryos was performed with Fast-Red (Sigma). Confocal stacks were generated from single sections every 0.18 μm, and from each stack were rendered using Velocity 3D Image Analysis Software. To determine the asymmetry index (A) for individual embryos, the volume of the left (L) minus the right (R) expression domain was divided by the sum of both left and right volumes, i.e. (L–R)/(L+R). Student’s t-test was performed using Prism 5 software to compare A1 values between datasets (GraphPad Software).

**Morpholinos**
Morpholino oligonucleotides were designed to target sequences comprising the Pitx2c ATG or a second sequence in the 5′ UTR (Gene Tools); a control morpholino, where mis-matches were introduced in the ATG morpholino sequence, was also designed.

**Parapineal ablation and SB431542 treatment**
Ablation of parapineal precursors was performed in TgBAC(flh:flh-Kaede)vu376 transgenic embryos using a Leica SP5 multiphoton confocal microscope. Embryos were subsequently grown to the appropriate stage, fixed and labelled by in situ hybridisation to assess parapineal cell numbers and hubellar asymmetry. Nodal signalling was modulated by treating embryos with the small molecule inhibitor SB431542 from 16 hpf as previously described (Inman et al., 2002; Roussigne et al., 2009).

**References**
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