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Barhl2 determines the early patterning of the diencephalon by regulating *Shh*

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Summary

The diencephalon is the primary relay network transmitting sensory information to the anterior forebrain. During development, distinct progenitor domains in the diencephalon give rise to the pretectum (p1), the thalamus and epithalamus (p2) and the prethalamus (p3), respectively. *Shh* plays a significant role in establishing the progenitor domains. However, the upstream events influencing the expression of *Shh* are largely unknown. Here, we show that *Barhl2* homeobox gene is expressed in the p1 and p2 progenitor domains and the in zona limitans intrathalamica (ZLI), and regulates the acquisition of identity of progenitor cells in the developing diencephalon. Targeted deletion of *Barhl2* results in the ablation of *Shh* expression in the dorsal portion of ZLI and causes thalamic p2 progenitors to take the fate of p1 progenitors and form pretectal neurons. Moreover, loss of *Barhl2* leads to the absence of thalamocortical axon projections, the loss of habenular afferents and efferents, and a gross diminution of the pineal gland. Thus, by acting upstream of *Shh* signaling pathway, *Barhl2* plays a crucial role in patterning the progenitor domains and establishing the positional identities of progenitor cells in the diencephalon.

Introduction

The thalamus is a major relay center in the brain that regulates the transfer of sensory and motor information from peripheral sensory systems to the cortex. The thalamus and epithalamus together serve many functions including but not limited to learning, motor control, regulating sleep-awake cycle, and regulating dopaminergic systems for mood disorders [1]. The thalamus and epithalamus develop from the diencephalon that can be divided into three progenitor domains or ‘prosomeres’ along the anterior-posterior (A-P)

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axis [2–4]. Prosomere (p) 1, p2 and p3 give rise to pretectum, thalamus and epithalamus and prethalamus, respectively.

Wedged in between the p2 and p3 domains is the zona limitans intrathalamica (ZLI), which acts as an ‘organizer’ to regulate diencephalic regionalization and patterning [5]. The ZLI is identified by the expression of the morphogen *sonic hedgehog* (*Shh*). There is significant evidence implicating the early expression of *Shh* both in the ZLI and the diencephalic basal plate as an important cue in establishing thalamic anlage [6–10]. Graded *Shh* expression is also required for specific regionalization within the thalamus with a higher concentration of the morphogen being required for the development of the rostral thalamus (rTh) compared to a much lower concentration for that of the caudal thalamus (cTh) [7]. *Shh* also influences the development of p3 progenitor domain and hence the prethalamus by causing the expression of genes different from those expressed by the thalamus [6, 11]. While we understand the importance of *Shh* expression in the ZLI in establishing diencephalic progenitor domains, the upstream molecular mechanisms regulating *Shh* signaling and hence patterning of the diencephalon is not completely understood.

Previous studies have shown that the BarH-like homeodomain 2 (BARHL2) transcription factor plays critical roles in the specification of retinal cell types [12] and in regulating the divergence of proprioceptive spinal cord dorsal interneurons into its subtypes [13]. The expression of *Barhl2* has also been identified in the developing diencephalon [14, 15]. To address the role of *Barhl2* in patterning the diencephalon, we used $Barhl2^{lacZ}$ and $Barhl2^{Cre}$ knock-in mouse lines that were described in our previous studies [12, 13]. We show here that *Barhl2*-null animals have thalamic and epithalamic insufficiencies reflected by the absence of thalamocortical axon projections. We also show that progenitor cells from the p2 thalamic domain acquire some of the attributes of p1 pretectal neurons in the absence of *Barhl2*. Furthermore, we provide evidence of *Barhl2* being upstream of *Shh* signaling and playing a direct role in patterning the p1 and p2 progenitor domains.

Results

Barhl2-null animals lack thalamocortical axon projections

Barhl2 is expressed in p1 and p2 progenitor domains and the ZLI at early stages of the developing diencephalon (Fig. 1a) Expression of *Barhl2* was seen as early as E10.5 in the developing diencephalon. As development proceeds, its expression is seen in p1 and p2 progenitor domains and the ZLI. To probe the importance of *Barhl2* in the diencephalic progenitor domains, we first examined the thalamus in the *Barhl2*-null animals at E17.5-E18.5, embryonic time points at which thalamic nuclei are well defined, and found that the *Barhl2*-null animals lacked thalamocortical axon projections as revealed by DiI anterograde tracing (Fig. 1b) and Netrin-G1 staining (Fig. 1c). Furthermore, while the segregation of thalamocortical afferents into vibrissal patterned barrels was readily visualized in the somatosensory cortex of the control animals, they were missing in the *Barhl2*-null mice, confirming the lack of cortical projections from the thalamus (Fig. 1d). The anterior thalamic nuclei were not specified at E17.5 as seen by the loss of expression of PROX1 (Fig. S1).

p1 progenitor domain markers are ectopically expressed in the presumptive p2 progenitor domain

We then investigated the fate of p2 progenitor cells that give rise to the thalamus and epithalamus. DBX1 is expressed in the thalamic progenitor cells in the ventricular zone and is expressed in a graded fashion with strong expression in the dorsal side of the caudal thalamus and a significantly weaker expression in the ventral side of the caudal thalamus [16]. In *Barhl2*-null brains at E12.5, the expression of DBX1 was not restricted to the ventricular zone, but was also present in the mantle zone. In addition, the graded expression pattern of DBX1 was lost (Fig. 2a). We found the same to be true at earlier time points at E10.5 and E11.5 (Fig. S2). PAX7 expression, which marks the post-mitotic cells of the p1 domain [16] in the control at E12.5, was expressed throughout the presumptive p2 domain in the *Barhl2*-null (Fig. 2a). Consistently, LIM1 and *Pax3*, also markers for post-mitotic p1 neurons [17], were ectopically expressed in the presumptive p2 domain in the *Barhl2*-null (Fig. 2b, c). While LHX2 and LHX9 is normally expressed in post-mitotic caudal thalamic neurons [18], its expression in the caudal thalamus was severely attenuated in the null (Fig. 2b, S2). Taken together, these results suggest that the p2 progenitor cells acquire some of the attributes of p1 pretectal neurons in the *Barhl2*-null diencephalon.

Barhl2 acts upstream of *Shh* to control the patterning of p1 and p2 progenitor domains and the development of ZLI

During the establishment of the p1 and p2 domains, *Neurog2* is expressed in the ventricular surface of p2 progenitor cells and in the ZLI. The expression of *Neurog2* inhibits that of *Ascl1* [19]. Hence *Ascl1* is complementarily expressed in the p1 domain, the rostral thalamus region of the p2 domain and the p3 domain [14, 16]. Upon *Barhl2* deletion, the compartmentalized expression patterns of *Neurog2* and *Ascl1* were disrupted (Fig. 3a, b). The attenuation of *Neurog2* expression with a concomitant up-regulation of *Ascl1* in the presumptive p2 domain provides further evidence that the p2 progenitor cells likely acquire pretectal traits. However, there still remained a small region of the diencephalon that expressed both DBX1 and ASCL1 (Fig. S3).

We further examined the expression of *Gbx2*, which is expressed in the thalamic progenitors [20, 21], and found its expression abolished in the *Barhl2*-null (Fig. 3c). Previous studies have shown that *Shh* plays an essential role in regulating *Gbx2* expression and that blocking *Shh* signaling in the dorsal portion of ZLI leads to a reduction in *Gbx2* expression [6]. We thus compared the expression of *Shh* in the control and *Barhl2*-null, and found that *Shh* expression was ablated in the dorsal region and was restricted to the ventral portion of the ZLI (Fig. 3d), suggesting that the progression of ZLI formation is attenuated prematurely owing to the ventral restriction of *Shh*. Taken together, these results indicate that *Barhl2* acts upstream of *Shh* to regulate the progression of ZLI and the patterning of p1 and p2 domains.

Loss of *Barhl2* affects the development of epithalamus structures

To determine whether loss of *Barhl2* affected other future components of the p2 domain namely the habenula and pineal gland (collectively called the epithalamus), we examined the expression of markers of epithalamus structures in *Barhl2*-null mice. *Pou4f1* is a habenular marker expressed in the alar plate of the caudal p2 domain at the border between p1 and p2

[22]. In the *Barhl2*-null mutant, *Pou4f1* is ectopically expressed in the p1 progenitor domain and further into the presumptive p2 domain (Fig. 4a). A similar expansion in the expression of the *deleted in colorectal cancer (Dcc)*, the receptor for netrin ligand expressed in the floor plate [23], was seen in the habenular neurons (Fig. 4b). The expression of *neuropilin-2* (*Nrp2*), which is required along with *semaphorin 3F* (*Sema3F*) for habenular axon guidance [23, 24], was also abolished in the *Barhl2*-null (Fig. 4c). A functional habenula is thus not established in the *Barhl2*-null since the afferent input to the habenula via the stria terminalis (Fig. 4d) and the efferent connections from the habenula via the fasciculus retroflexus (Fig. 4e, f) were severely attenuated. The posterior commissure connecting the pretectal nuclei was also enlarged in the *Barhl2*-null mutant compared to the control (Fig. 4f). Additionally, *Pax6* expression in the developing diencephalon [25] revealed the absence of a pineal gland in the *Barhl2*-null (Fig. 4g). While the p1 and p2 progenitor domains were affected, an analysis of expression of *Dlx1* showed that the p3 progenitor domain remained unaffected in the *Barhl2*-null animals (Fig. 4h). Taken together, loss of *Barhl2* leads to a loss of the developmental components of the p2 domain. A diagrammatic summary of molecular factors affected due to the loss of *Barhl2* is illustrated in Fig. 4i.

Discussion

The thalamus relays sensory information from peripheral nervous system to the cortex to bring about conscious perception and is indispensible for the vitality of an animal. The habenula acts as a relay in connecting the forebrain with the midbrain and hindbrain and is implicated in several disorders such as depression and schizophrenia. The pineal gland mainly regulates the sleep-wake cycle. Furthermore, thalamocortical afferents affect the expression of developmental genes in the neocortex and are crucial for the establishment of neocortical identities [26]. In this study we show that *Barhl2* is essential for the development of these diencephalic structures and that in *Barhl2*-null mice, cells in the p2 progenitor domain acquire attributes of p1 progenitor domain while p3 progenitor domain remains unaffected.

In the absence of *Barhl2*, expression of p1 progenitor domain neuron markers such as *Pax7, Pax3* and *Lim1* expands into the presumptive p2 progenitor domain. Previous studies have shown that a loss of *Gbx2* disrupts the border between the epithalamus and pretectum [21], which is consistent with our results and may explain the cell fate mixing between cells in p1 and p2 prosomeres. Moreover, *Barhl2*-nulls also display a severe attenuation of *Lhx2* expression, a marker for p2 progenitor domain neurons (Fig. 2). This mis-specification can be attributed to the mis-expression of neural bHLH genes *Neurog2* and *Ascl1* in the *Barhl2*-nulls (Fig. 3 a, b). While the expression of *Neurog2* and *Pax6* in the *Barhl2*-nulls is largely missing from the presumptive p2 domain, the appearance of patchy expression of the markers in parts of the p2 prosomere domain cannot be ignored. Previous studies have demonstrated that the ZLI functions as an ‘organizer’ that prevents the mixing of neighboring cell types. It is hence plausible that the dorsal truncation of the ZLI leads to some cell mixing between the p2 and p3 prosomeres, leading to the presence of *Neurog2* and *Pax6* positive p3 cells in the presumptive p2 domain. Hyperfasciculation of the posterior commissure further lends credence to a fate switch of the p2 progenitors into the p1 pretectal neurons (Fig. 4f). Since the pretectal nuclei are interconnected by the posterior commissure

and there is an expansion of the overall p1 domain at the expense of p2 progenitors, it is plausible that the additional pretectal neurons contribute to the hyperfasciculated posterior commissure. However, the p3 progenitor domain remains unaffected in *Barhl2*-nulls, as seen by the expression of *Dlx1*, a marker for p3 domain progenitor neurons. This is a surprising result since a disruption in *Shh* signaling affects the development of both p2 and p3 progenitor domains [6, 11]. One plausible explanation for this might be that the ventral expression of *Shh* in the ZLI contributes in part to the normal development of p3 progenitor domain in *Barhl2*-null animals.

We have showed that in the absence of *Barhl2*, the p2 progenitor domain structures: the thalamus and the epithalamus are severely attenuated. Furthermore, loss of expression of markers for thalamic progenitor cells *Gbx2* (Fig. 1c) and *Pax6* [27] (Fig. 1g) in the presumptive p2 domain in *Barhl2*-null animals supports the claim that *Barhl2*-null animals lack a thalamus. While *Barhl2* seems to affect *Pax6* expression in the p2 domain of the developing diencephalon, whether this ties into the regulation of *Shh* in the ZLI is currently unknown. Furthermore the absence of thalamocortical afferents (Fig. 1b, 1c) and vibrissal staining in the somatosensory cortex proves the absence of a functional thalamus in the *Barhl2*-nulls (Fig. 1d). Similarly we display the loss of a habenular and pineal gland epithalamic structures using markers such as *Pou4f1, Dcc, Nrp-2* and *Pax6* (Fig. 4). Taken together, *Barhl2* is a key regulator of *Shh* and controls the early patterning of the diencephalon. In the absence of *Barhl2*, p2 prosomere domain progenitors acquire attributes of p1 progenitor domain neurons. It is thus crucial for the development of p2 domain structures: the thalamus and epithalamus.

We show that the loss of *Barhl2* leads to the down-regulation of *Shh*, suggesting that *Barhl2* could regulate the early patterning of diencephalon by acting upstream of *Shh*. Interestingly, the expression of *Shh* is not abolished in the absence of *Barhl2* (Fig. 3d, S3). Rather, it is restricted to the ventral portion of the ZLI. *Shh* is expressed initially in the basal plate of p1-p3 and later in the ZLI as patterning of the diencephalon proceeds [10, 28]. Once initiated in the developing ZLI, it expands dorsally thus forming a barrier between p2 and p3 domains [29]. One possibility is that *Barhl2* could have a role in regulating the dorsal expansion of *Shh* expression but not the initiation of *Shh* expression in the ZLI. Since *Barhl2* expression begins at E10.5 in the developing diencephalon and expression of *Shh* begins at E9 in the ZLI [29], it is likely that loss of *Barhl2* restricts the dorsal expansion of ZLI. Recently published studies have identified a *Shh* ZLI enhancer of evolutionary origin and have demonstrated that BARHL2 directly binds to this enhancer in E10.5 mouse embryo brains [30]. Moreover, studies in *Xenopus* have shown that *Barhl2* restricts the dorsal expansion of the ZLI by affecting the competence of neuroepithelial cells to respond to the secreted form of SHH from the alar plate [31]. Previous studies have also shown that *Gbx2* expression is regulated by *Shh* and that blocking *Shh* signaling in the dorsal portion of ZLI attenuates *Gbx2* expression [6]. Additionally, studies have shown that *Shh* promotes thalamic specification by activating *Gbx2* [32] and that the dorsal extension of *Shh* expression in the ZLI is crucial for normal gene expression in the thalamus [6]. Here we have shown that the loss of *Barhl2* abolishes *Shh* expression in the dorsal portion of ZLI and that loss of *Barhl2* also leads to an attenuation of *Gbx2* expression in p2 domain. Our results taken in conjunction with previously published data argue for the role of *Barhl2* in regulating the

patterning of the developing diencephalon by directly controlling the expression of *Shh* in the *Barhl2* to *Shh* to *Gbx2* regulatory pathway.

Experimental procedures

Animals

All animal procedures in this study were approved by the University Committee of Animal Resources at the University of Rochester. *Barhl2*$^{lacz/+}$ and *Barhl2*$^{Cre/+}$ mice were generated previously (10). Embryos were designated as E0.5 at noon on the day vaginal plug was first observed in the breeding mother. Pups were designated P0 on the day of birth.

Immunohistochemistry and In situ hybridization

Time-mated embryos were dissected in cold PBS and fixed in 4% (w/v) paraformaldehyde at 4°C overnight, following which they were equilibrated in 30% (w/v) sucrose at 4°C overnight and embedded in optimal cutting temperature (OCT) compound and sectioned at 18–30 μm on a cryostat. Sections were collected on slides and were processed for immunohistochemistry (IHC) or *in situ* hybridization (ISH).

The following antibodies were used for IHC: Anti-DBX1 (1:1,000, gift from Y. Nakagawa), Anti-DCC (1:500, Santa Cruz), Anti-GFP (1:1000, Abcam), Anti-L1 (1:200, Chemicon), Anti-LHX2 (1:200, Santa Cruz), Anti-LHX9 (1:200, Santa Cruz), Anti-LIM1 (1:500, DSHB), Anti-ASCL1 (1:200, R&D Systems), Anti-NKX2.2 (1:200, DSHB), Anti-PAX7 (1:200, DSHB), Anti-PROX1 (1:500, Covance), Anti-SERT (1:500, Immunostar), Anti-SHH (1:500, R&D Systems). Alexa conjugated antibodies (1:1,000, Molecular probes) were used for visualization.

The following probes were used for ISH: *Barhl2* (Gift from M. Xiang), *Pax3* (Gift from L. Puelles), *Neurog2* (657-1497 of NM009718.2), *Ascl1* (Gift from A. Joyner), *Shh* (Gift from A. McMahon), *Gbx2* (Gift from A. Joyner), *Pou4f1* (267-1497 of NM011143.4), *Dcc* (Gift from Z.F. Chen), *Nrp2* (984-1911 of AF022857), *Pax6* (Gift from X. Zhang), *Lhx2* (1149-1806 of AF124734), and *Dlx1* (1501-2277 of NM010053).

DiI tracing

To visualize thalamocortical axon projections, DiI crystals (Molecular Probes) were applied to the thalamus of E17.5 brain and placed in 4% (w/v) paraformaldehyde for 14 days. 50 μm sections were obtained on a vibratome and were visualized under the 543 nm filter in a ZEISS stereoscope.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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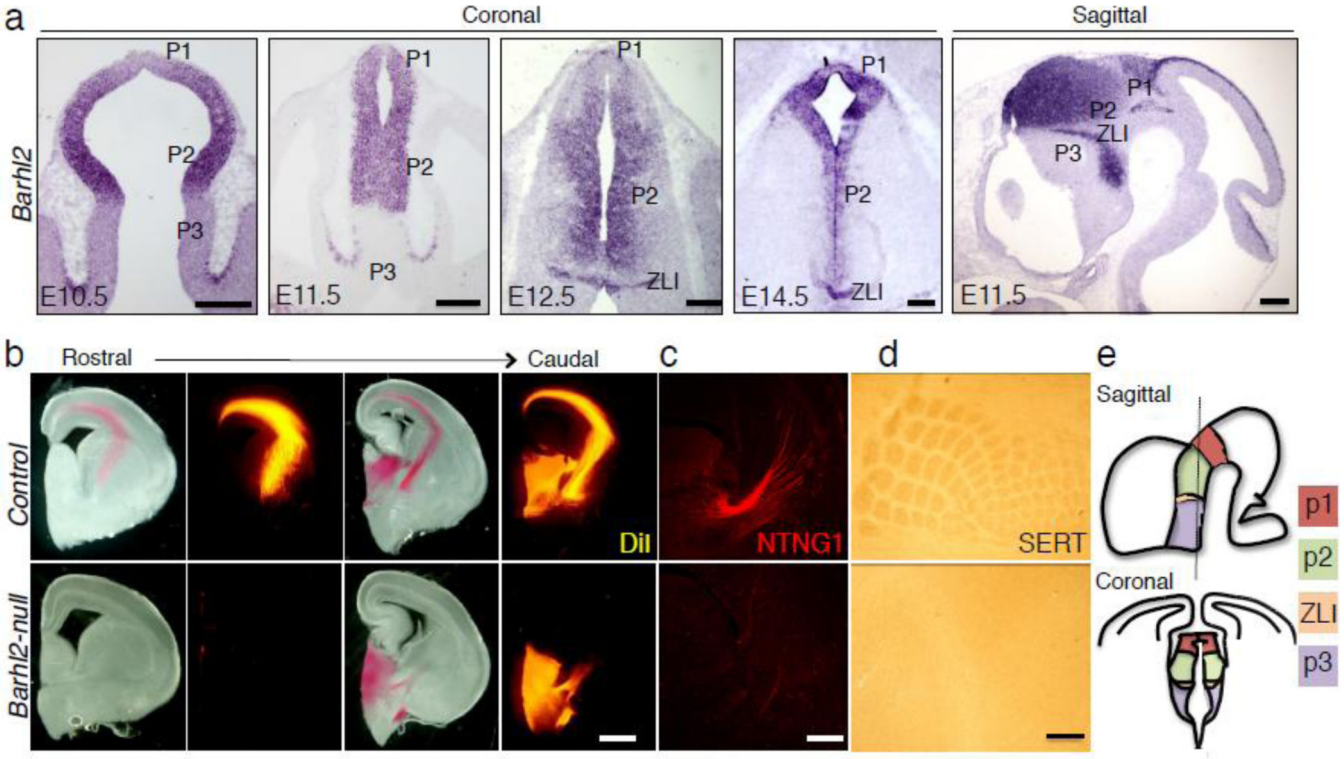

Figure 1.

Loss of thalamocortical axons in *Barhl2*-null mice. (**a**) In situ hybridization of caudal sections at various time points and of representative sagittal section at E11.5 shows *Barhl2* expression in the developing diencephalon. **(b)** DiI tracing of thalamocotrical axons at E18.5 shows a loss of cortical projections in *Barhl2*-null mice. **(c)** Anti-NTNG1 immunohistochemistry displays the loss of thalomo-cortical afferents in *Barhl2*-nulls at E17.5. **(d)** Anti-SERT immunohistochemistry of tangential cortical sections reveals the loss of distinct vibrissal patterned barrels in *Barhl2*-null mice at P8. **(e)** Schematic representation of sagittal and coronal sections of the diencephalon outlines the positions of different progenitor domains and ZLI. Scale bars in a, c and d equals 200 μm and in b equals 500 μm.

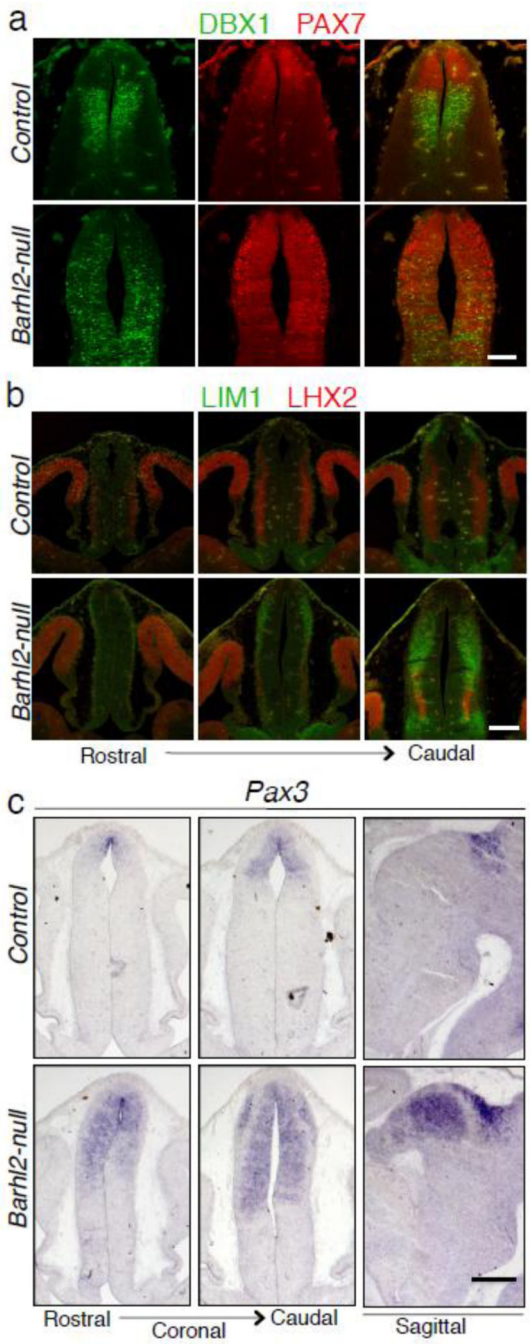

Figure 2.

Mis-expression of pretectal neuron markers in the absence of *Barhl2*. **(a)** Immunolabeling reveals that the confined expression of DBX1 (green) in thalamic progenitor cells is disrupted and that PAX7 (red) expression expands from the p1 domain to the presumptive p2 domain in the *Barhl2*-null mice at E12.5. **(b** and **c)** A similar fate switch is seen by expression of LIM1 (green in **b**) and *Pax3* **(c)**, markers for prectectal neurons and LHX2 (red in **b**) expression is down-regulated in the dorsal thalamic neurons of the *Barhl2*-null mice at E12.5. Scale bars equal 200 μm.

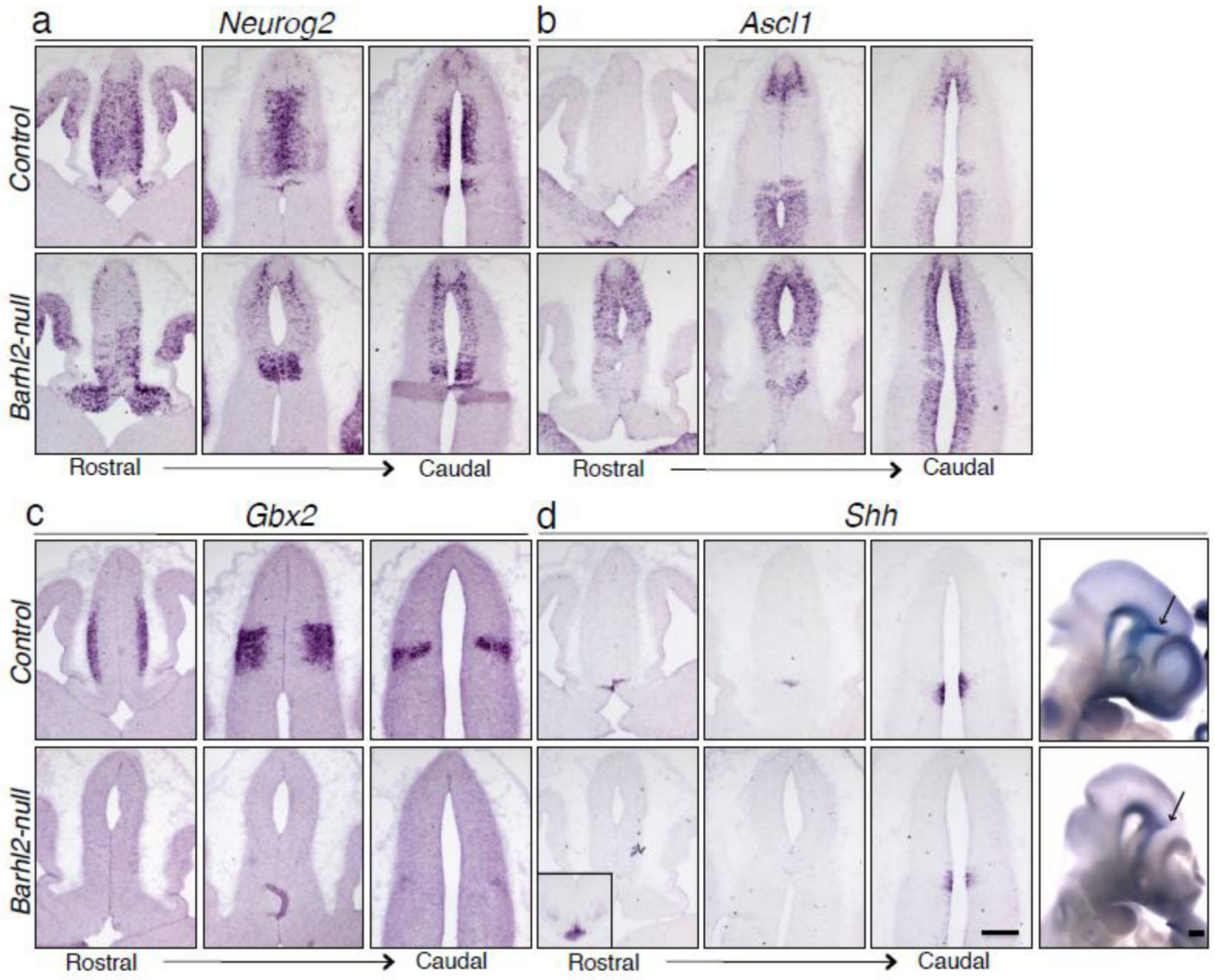

Figure 3.

Barhl2 acts upstream of *Shh* and regulates pro-neural gene expression. In the *Barhl2*-null mice at E12.5, the compartmentalized expression of *Neurog2* in the ZLI and the caudal thalamic p2 neurons is disrupted **(a)**. The distinctive expression of *Ascl1* in the pretectal p1 neurons, rostral thalamic p2 neurons and prethalamic p3 neuorns is disrupted **(b)**. *Gbx2* expression in p2 thalamic neurons is nearly abolished **(c)**. *Shh* expression in the ZLI is restricted to the ventral portion of ZLI while its dorsal expression is lost **(d)** both in coronal sections and whole-mount images, inset showing the normal expression of *Shh* in the notochord. Scale bars equal 200 μm.

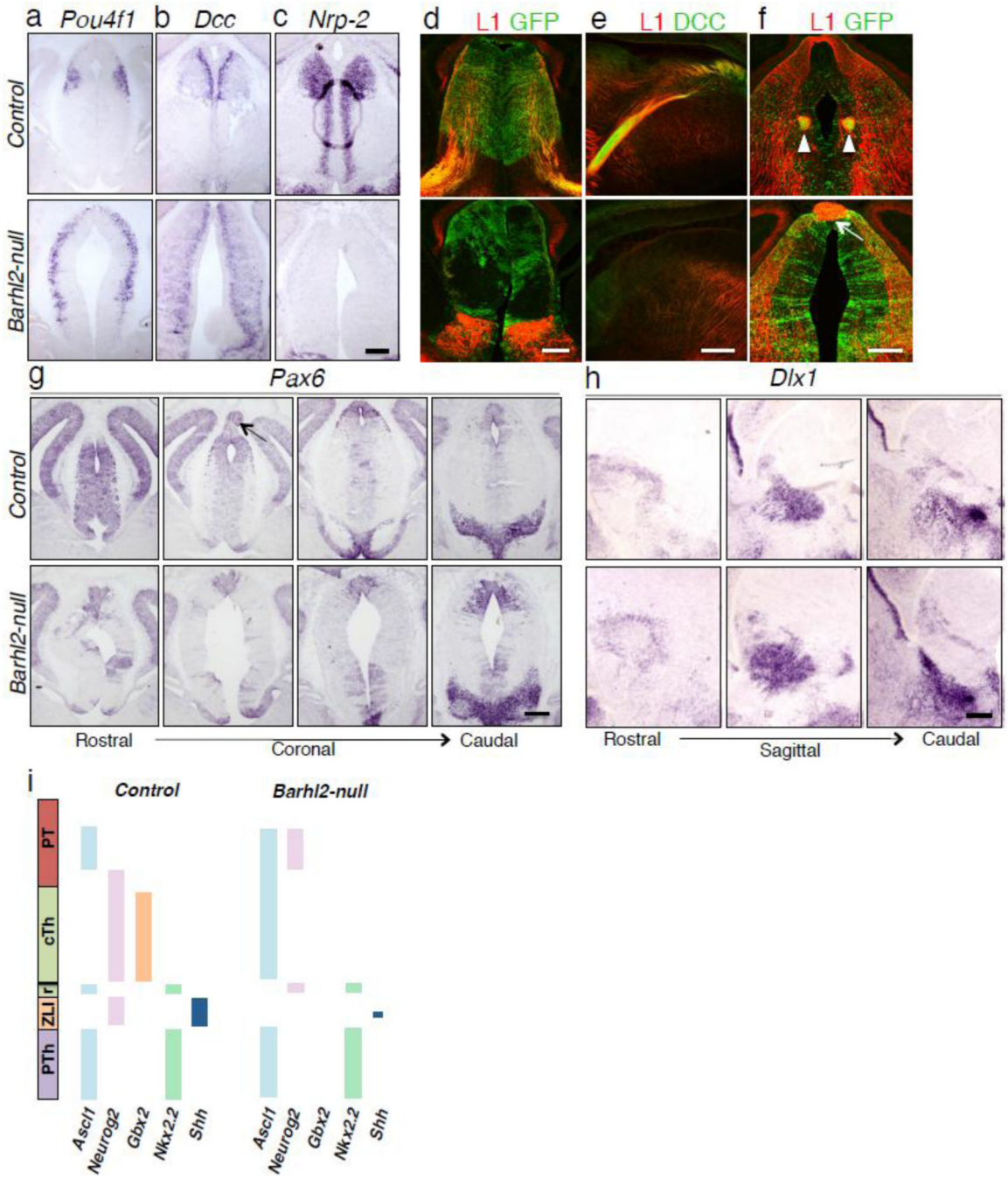

Figure 4.

Absence of *Barhl2* leads to a loss of other p2 progenitor domain structures. (**a-c)** At E14.5, *Pou4f1*, a habenular neuron marker is ectopically expressed in p1 and p2 domain neurons in the *Barhl2*-null mice **(a)**. *Dcc* expression in habebular axons is similarly affected in the *Barhl2*-null mice **(b)**. The expression of *Nrp2*, a habenular axon guidance molecule is abolished in the *Barhl2*-null mice **(c)**. (**d-f**) At E14.5, the expression of L1 in stria terminalis, the habenular afferent is abolished in the *Barhl2*-null mice **(d)**. Efferent output from the habenula provided by the fasciculus retroflexus as seen by the expression of L1 is abolished

in the *Barhl2*-null mice **(e)**. Expression of L1 displays a hyperfasciculation of the posterior commissure (arrow) in the null mutant as compared to the control **(f)**. Arrowheads indicate the presence of fasciculus retroflexus in the control as compared to the mutant **(f)**. Expression of *Pax6* at E12.5 across all progenitor domains is affected in the null mutant and displays a loss of pineal gland (arrow) **(g)**. Expression of *Dlx1*, a marker for the p3 progenitor domain is unaltered in the *Barhl2*-null mutant **(h)**. A summary of affected factors encompassing various progenitor domains in the *Barhl2* mutant as compared to the control is schematically represented **(i)** (Figure schematic adapted and modified from [33]). Scale bars equal 200 μm.