Title
TCF7L2 regulates postmitotic differentiation programs and excitability patterns in the thalamus

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Statement

The study describes a role of TCF7L2 in neuronal differentiation of thalamic glutamatergic neurons at two developmental stages, highlighting its involvement in the postnatal establishment of critical thalamic electrophysiological features.

Abstract

Neuronal phenotypes are controlled by terminal selector transcription factors in invertebrates, but only a few examples of such regulators have been provided in vertebrates. We hypothesised that TCF7L2 regulates different stages of postmitotic differentiation in the thalamus, and functions as a thalamic terminal selector. To investigate this hypothesis, we used complete and conditional knockouts of Tcf7l2 in mice. The connectivity and clustering of neurons were disrupted in the thalamo-habenular region in Tcf7l2-/- embryos. The expression of subregional thalamic and habenular transcription factors was lost and region-specific cell migration and axon guidance genes were downregulated. In mice with a postnatal Tcf7l2 knockout, the induction of genes that confer thalamic terminal electrophysiological features was impaired. Many of these genes proved to be direct targets of TCF7L2. The role of TCF7L2 in terminal selection was functionally confirmed by impaired firing modes in thalamic neurons in the mutant mice. These data corroborate the existence of master regulators in the vertebrate brain that control stage-specific genetic programs and regional subroutines, maintain regional transcriptional network during embryonic development, and induce terminal selection postnatally.
Introduction

Studies of invertebrates have shown that terminal differentiation gene batteries in individual classes of neurons are induced and maintained by specific transcription factors called terminal selectors, that are expressed throughout the life (Hobert and Kratsios, 2019). However, regulatory strategies of postmitotic maturation and terminal selection in vertebrates are unclear. Till date, only a few terminal selectors have been identified in vertebrates (Cho et al., 2014; Flames and Hobert, 2009; Kadkhodaei et al., 2009; Liu et al., 2010; Lodato et al., 2014; Wyler et al., 2016). Neurons of the thalamus and habenula are derived from a single progenitor domain (prosomere 2) and are glutamatergic (Watson et al., 2012), except for GABAergic interneurons in the rostral thalamus (Evangelio et al., 2018). The thalamus is a sensory relay centre and part of cortico-subcortical loops that process sensorimotor information and produce goal-directed behaviours (Sherman, 2017). The habenula controls reward- and aversion-driven behaviours by connecting cortical and subcortical regions with the monoamine system in the brainstem (Benekareddy et al., 2018; Hikosaka, 2010). During postmitotic differentiation, thalamic and habenular neurons segregate into discrete nuclei (Shi et al., 2017; Wong et al., 2018), develop a variety of subregional identities (Guo and Li, 2019; Nakagawa, 2019; Phillips et al., 2019), extend axons toward their targets (Hikosaka et al., 2008; López-Bendito, 2018), and acquire electrophysiological characteristics postnatally (Yuge et al., 2011). The knowledge of the mechanisms that control postmitotic development in this region is important, because its functional dysconnectivity, which possibly originates from the period of postmitotic maturation, is implicated in schizophrenia, autism and other mental disorders (Browne et al., 2018; Steullet, 2019; Whiting et al., 2018; Woodward et al., 2017).

The network of postmitotically induced transcription factors that regulate the maturation of prosomere 2 neurons has only begun to be deciphered. Gbx2 and Pou4f1 are early postmitotic markers of thalamic and habenular neurons, respectively. GBX2 plays a transient regulatory role in the initial acquisition of thalamic molecular identities and thalamocortical axon guidance, and then its expression is downregulated in the majority of the thalamus (Chatterjee et al., 2012; Chen et al., 2009; Li et al., 2012; Mallika et al., 2015; Miyashita-Lin et al., 1999). In contrast, POU4F1 (alias BRN3A) is not essential for the growth of habenular axons, but it maintains the expression of the glutamate transporter gene Vglut1/Slc17a7 and other habenula-abundant genes in adults, though its impact on electrophysiological responses of habenular neurons was not tested (Quina et al., 2009; Serrano-Saiz et al., 2018). Subregional transcription factors RORA and FOXP2 regulate some aspects of postmitotic differentiation in thalamic subregions during embryogenesis.
(Ebisu et al., 2016; Quina et al., 2009; Vitalis et al., 2017), but their role in terminal differentiation in this region was not investigated.

Tcf7l2, a risk gene for schizophrenia and autism (Bem et al., 2019) that encodes a member of the LEF1/TCF transcription factor family (Cadigan and Waterman, 2012), is the only shared marker of prosomere 2 neurons (Nagalski et al., 2013; Nagalski et al., 2016). Its function was tested only during early postmitotic period in zebrafish (Beretta et al., 2013; Husken et al., 2014) and mice (Lee et al., 2017; Tran et al., 2020). In Tcf7l2−/− mouse embryos, some markers were misexpressed in the region of prosomere 2, and the formation of axonal tracts was disrupted. TCF7L2 expression is maintained throughout life and the TCF7L2 motif is overrepresented in putative enhancers of adult thalamus-enriched genes (Nagalski et al., 2016; Wisniewska et al., 2012), suggesting that this factor can play a role of prosomere 2 terminal selector.

The present study used complete and conditional knockout mice to explore the role of Tcf7l2 in postmitotic anatomical maturation, maintenance of molecular diversification, adoption of neurotransmitter identity, and postnatal acquisition of electrophysiological features in the thalamus and habenula. We show that TCF7L2 orchestrates the overall morphological differentiation process in this region by regulating stage-specific gene expression directly or via subregional transcription factors. We also report that TCF7L2 functions as a terminal selector of postnatally-induced thalamic electrophysiological characteristics but not glutamatergic (VGLUT2) identity.
Results

Generation of mice with the complete and conditional knockouts of Tcf7l2

Tcf7l2 expression in prosomere 2 is induced in mice during neurogenesis (Cho and Dressler, 1998) and maintained in the thalamus and medial habenula throughout life (Nagalski et al., 2013). In wild type (WT) embryos on E12.5, we observed high levels of TCF7L2 protein in the superficial portion of the thalamus and habenula, which is populated by postmitotic neurons (Fig. 1A). We also observed several TCF7L2-positive cells in the prethalamus. Possibly, these cells migrate from the GABAergic rostral thalamus to take part in the formation of the intergeniculate leaflet and ventral lateral geniculate nuclei that derive from prethalamic and rostral thalamic progenitors (Jeong et al., 2011). At late gestation, TCF7L2 was observed in the entire caudal thalamus (hereinafter referred to as the thalamus; a glutamatergic domain) and medial habenula (Fig. 1B). Relatively lower levels of TCF7L2 were present in the ventrobasal complex (VB), nucleus reuniens, and recently identified perihabenula (Fernandez et al., 2018). Low levels of TCF7L2 were also present in the lateral habenula and the derivatives of the rostral thalamus.

To investigate the role of TCF7L2 as a selector of morphological and electrophysiological characteristics of prosomere 2, we used two knockout models in mice. The complete knockout of Tcf7l2 was generated by insertion of the tm1a(KOMP)Wtsi allele with lacZ cassette upstream of the critical 6th exon of the Tcf7l2 gene (Fig. 2A). Tcf7l2tm1a allele led to the lack of TCF7L2 protein, confirmed by immunostaining and Western blot, and ectopic expression of β-galactosidase from the lacZ locus (Fig. 2B-C). Tcf7l2tm1a mice die after birth. To create a thalamus-specific postnatal knockout of Tcf7l2, we first crossed Tcf7l2+/tm1a mice with a flippase-expressed strain and then with mice that expressed CRE recombinase from the Cholecystokinin (Cck) gene promoter (Fig. S1). Cck is upregulated postnatally and its expression overlaps with the expression of Tcf7l2 in the thalamus (Allen Brain Atlas, 2011; Nishimura et al., 2015). The expression from the CckCre locus, visualised in CckCre:tdTomatofl/+ reporter line, was high in lateral parts of the thalamus, and lower in thalamic medial and midline parts, including anterodorsal (AD), paraventricular (PV) and parafasicular (PF) nuclei (Fig. S2). Cck-driven knockout of Tcf7l2 was induced postnatally and completed in the thalamus by P14 (Fig. 2G-H). In the resulting CckCre:Tcf7l2fl/fl mice, TCF7L2 was absent in most thalamic nuclei in adults, except for the PV and PF (Fig. S3). In the AD and midline nuclei, Tcf7l2 was partially knocked out.
Normal neurogenesis but disrupted anatomy and connectivity of prosomere 2

To confirm that proliferation and neurogenesis occurred normally within prosomere 2 in Tcf7l2−/− mice, we stained E12.5 brain sections with antibodies specific for the KI-67 antigen and TUJ1, markers of proliferating progenitors and young postmitotic neurons, respectively. Prosomere 2 was identified with Tcf7l2 probe both in WT and knockout (KO) embryos, taking advantage of preserved expression from the targeted Tcf7l2 locus (Fig. 3A). The complete knockout of Tcf7l2 did not cause any apparent defects in proliferation or neurogenesis in prosomere 2 on E12.5 (Fig. 3B), consistent with previous results in another Tcf7l2 knockout strain (Lee et al., 2017).

To investigate whether TCF7L2 is required for the initial acquisition of postmitotic molecular identities in the thalamus and habenula, we examined the expression of the Gbx2 gene and POU4F1 protein (the earliest markers of postmitotic neurons in the thalamus and habenula, respectively) during neurogenesis. Both Gbx2 mRNA and POU4F1 were highly expressed in the prosomere 2 area in WT and Tcf7l2−/− embryos on E12.5, indicating that their expression is not induced by TCF7L2 (Fig. 3C). The number of POU4F1-positive cells visibly increased, suggesting that prosomere 2 cells more readily adopted habenular fate in Tcf7l2−/− embryos at this stage. Gbx2- and POU4F1-positive areas expanded extensively into each other’s territory, implying a defect in thalamo-habenular boundary formation.

To further investigate if TCF7L2 regulates structural maturation of prosomere 2, we focused on late gestation (E18.5), when nucleogenesis is already concluded and axonal connections are well developed in this region. The anatomy of the area was analysed by Nissl staining (Fig. 3D-E and S4). The boundaries between prosomere 2 and neighbouring structures, i.e., the prethalamus (rostral boundary) and pretectum (caudal boundary), were not morphologically detected in sagittal sections from Tcf7l2−/− embryos. On coronal sections, the habenula was fused with the thalamus, and nuclear groups within prosomere 2 were not well demarcated. The whole region was reduced in the radial dimension and elongated dorsoventrally, resulting in an oval-like shape. Then, we analysed circuit formation in the thalamo-habenular region by tracing thalamocortical axons with DiI and immunostaining brain sections with an antibody specific for L1 cell adhesion molecule (L1CAM) that marks growing axons. Consistent with a previous report (Lee et al., 2017), thalamocortical axons were not detected in KO brains (Fig. 3F). The bundles of stria medullaris, which include afferent fibres from the basal forebrain and lateral hypothalamus to the habenula, and were previously reported to be normal in Tcf7l2−/− embryos on E16.5 (Lee et al., 2017), were disorganised and less compact on E18.5 (Fig. 3G). The major habenular efferent tract, i.e., the fasciculus
retroflexus was split into thinner fascicles. The L1CAM staining also showed major disruption in the general topography of axonal connections in the whole thalamic area. This demonstrated that TCF7L2 is critically involved in the development of habenular and thalamic anatomy and connectivity.

**Impaired cell sorting in the diencephalon**

Because anatomical boundaries of prosomere 2 were blurred in *Tcf7l2*−/− embryos, we hypothesized that cells in this region do not segregate properly. To identify borders of prosomere 2 and its main subdivisions at molecular levels, we analysed the expression pattern of several diencephalic markers by *in situ* hybridisation. We used a *Tcf7l2* probe to stain the pretectum (prosomere 1) and prosomere 2, *Gbx2* probe to stain the glutamatergic thalamus, *Nkx2-2* and *Sox14* probes to stain the rostral thalamus and *Pax6* probe to stain the prethalamus (prosomere 3) and pretectum. These stainings confirmed that the thalamo-habenular area was fused and malformed in *Tcf7l2*−/− embryos (Fig. 4A-B). Also, we noticed that *Gbx2* staining was still present in the periventricular area, where the youngest neurons are located, but absent in the intermediate and superficial portions of the thalamus (Fig. 4B), suggesting premature downregulation of *Gbx2*. The rostral thalamus area was elongated laterally (Fig. 4C). The boundary between prosomere 2 and 3, which was demarcated by the expression of *Pax6*, was disrupted (Fig. 4D).

To examine the boundaries at the cellular level, we stained brain sections with anti-PAX6, anti-SIX3 (marking the prethalamus), anti-NKX2-2 and anti-POU4F1 antibodies. Prosomere 2 cells were identified with anti-TCF7L2 (WT mice) or anti-β-galactosidase (KO mice) antibodies. In control embryos, the thalamic area was delineated rostro-ventrally by a narrow strip of PAX6-positive cells in a prethalamic subdomain (Fig. 4E), which separated the thalamus from SIX3-positive prethalamic area (Fig. 4F), and the NKX2-2-positive area of the intergeniculate leaflet and ventral lateral geniculate nucleus did not overlap with TCF7L2-high area of the caudal thalamus (Fig. 4G). Habenular cells were easily identified by POU4F1 staining, and the differences in cell densities distinguished the lateral from medial part (Fig. 4H). In contrast, in *Tcf7l2* KO embryos, many PAX6-, SIX3-, NKX2-2- and POU4F1-positive cells were intermingled into the neighbouring thalamic territories (Fig. 4E-H). Sparse distribution of these cells, in particular POU4F1-positive cells, pointed to their unusual migration rather than identity switch. Consequently, the border between prosomere 2 and 3 was devoid of its sharpness, and thalamo-habenular border did not exist. This indicated that TCF7L2 plays a critical role in the segregation of cells in subregional clusters in prosomere 2.
**Disrupted prosomere 2-specific regulatory network and altered expression of morphogenesis effector genes**

To investigate the possible role of TCF7L2 in the regulation of a genetic program of region-specific maturation in prosomere 2, we analysed global gene expression by RNA-seq in the thalamo-habenular region in WT and *Tcf7l2* −/− embryos on E18.5. 210 genes were significantly downregulated and 113 were upregulated in KO embryos by ≥ 0.4 or ≤ -0.4 log₂ fold-change (FC) (Fig. 5A, Table S1). Gene ontology (GO) term analysis of the differentially expressed genes (DEGs) revealed an overrepresentation of genes that are involved in transcription factor activity, anatomical structure development, neuron differentiation, axon guidance, cell adhesion, regulation of cell migration, regulation of transcription and synaptic signalling (Fig. 5A, Table S2). To determine whether the E18.5 DEGs from these groups are specific for prosomere 2, we inspected the corresponding in situ hybridisation images of brain sections in the Allen Brain Atlas. 100% of the downregulated genes in the selected groups were enriched in the thalamus, habenula, or both (Fig. 5A). Among them were prosomere 2-specific transcription factor genes, including known regulators of thalamic or habenular development - *Rora*, *Foxp2*, *Etv1* and *Nr4a2* (Ebisu et al., 2016; Quina et al., 2009; Vitalis et al., 2017), cell adhesion molecules *Cdh6*, *Cdh8* and *Ctnn6* (Bibollet-Bahena et al., 2017) and axon guidance genes such as *Epha4*, *Ntng1*, and *Robo3* that encode important regulators of the guidance of thalamic or habenular efferent connections and the segregation of neurons in this region (Belle et al., 2014; Braisted et al., 2000; Dufour et al., 2003; Lehigh et al., 2013). Also excitability genes, such as thalamus-enriched serotonin transporter gene *Slc6a4* that is expressed only during embryogenesis to regulate arborisation of thalamocortical axons (Chen et al., 2015) were downregulated in *Tcf7l2* KO embryos. In contrast, the list of the upregulated genes was dominated by the ones that were specifically expressed along thalamic borders or depleted from the thalamus, such as *Reln* that is involved in neuronal migration and positioning (Hirota and Nakajima, 2017). An increased level of the rostral thalamus markers *Nkx2-2*, *Sox14* and *Lhx5* was also observed, and was likely caused by the expansion of this domain into the caudal thalamic area (Fig. 4C).

To confirm that the knockout of *Tcf7l2* caused misexpression of prosomere 2-enriched or depleted genes, we validated several of the identified E18.5 DEGs by in situ hybridisation. We observed strong ectopic expression of *Reln* and decreased expression of *Cdh6* in the thalamus in the mutant embryos (Fig. 5B). The expression of subregional markers of the caudal thalamus - the transcription factor genes *Lef1*, *Prox1*, *Foxp2* and *Rora*, was virtually absent in the thalamic area in *Tcf7l2* −/− embryos (Fig. 5B). Furthermore, *Tcf7l2* knockout
abolished the expression of habenular markers *Lef1* (Fig. 5B) and *Etv1* (Fig. 5C) in different habenular subregions. These results revealed that TCF7L2 controls a network of prosomere 2 subregional transcription factors and regulates region-specific axon guidance and cell migration related genes.

**Normal acquisition of glutamatergic identity but an impaired expression of postnatally induced synaptic and excitability genes in the thalamus**

We then asked if TCF7L2 acts also as a terminal selector of thalamic phenotype, which is underlined by the expression of region-specific genes that determine neurotransmitter identity and electrophysiological characteristics. Habenular and thalamic neurons in rodents (except for GABAergic interneurons that are derived from the rostral thalamus (Evangelio et al., 2018)) are glutamatergic and express high levels of a vesicular glutamate transporter VGLUT2 (Fremeau et al., 2001; Herzog et al., 2001). To determine whether TCF7L2 is involved in the adoption of glutamatergic fate in the thalamus and habenula, we examined the expression patterns of *Vglut2/Slc17a6* and *Gad67/Gad1* (a marker of GABAergic neurons) in the diencephalon in *Tcf7l2−/−* embryos and *CckCre:Tcf7l2fl/fl* P60 adult mice. Both knockout strains exhibited a pattern of GABAergic and glutamatergic cell distribution that was similar to the wild type condition, with predominant *Vglut2/Slc17a6* expression in prosomere 2 (Fig. 6A and S5A). Thus, TCF7L2 is not involved in the specification and maintenance of VGLUT2-identity in prosomere 2.

To investigate the hypothesis that TCF7L2 regulates terminal gene batteries, we compared global gene expression profiles in the thalamus between *CckCre:Tcf7l20/−* and WT mice on P60 by RNA-seq. 310 genes were significantly downregulated and 227 were upregulated in KO mice by ≥ 0.4 or ≤ -0.4 log2 FC (Fig. 6B, Table S3). GO term enrichment analysis of the P60 DEGs revealed significant enrichment with terms that clustered into groups of synaptic proteins and regulators of membrane conductance: regulation of ion transport, voltage-gated channel activity, regulation of membrane potential, G-protein coupled receptor signalling pathway, regulation of trans-synaptic signalling and regulation of synapse organisation (Fig. 6B, Table S4). Transcription factor genes were not overrepresented in the P60 DEGs. However, 6 thalamus-enriched subregional transcription factor genes were significantly downregulated or upregulated in *CckCre:Tcf7l20/−* mice on P60 (Fig. 6C), including *Rreb1* that is expressed in the thalamus postnatally, *Lef1* and *Rora*, the latter confirmed by in situ hybridisation (Fig. 6D and S5B).
To investigate if TCF7L2 regulates thalamus-specific or generic neuronal features, we examined spatial expression profiles of the identified excitability/synaptic genes in this cluster in the Allen Brain Atlas. The vast majority of the downregulated genes are expressed specifically in the thalamus (Fig. 6B), such as *Kcnc2* and *Cacna1g*, which encode subunits of Kv3.2 voltage-gated potassium channels and Ca\(_{v}\)3.1 voltage-gated calcium channels, respectively (Kasten et al., 2007; Kim et al., 2001). The downregulation of Ca\(_{v}\)3.1 was further confirmed by immunohistochemistry (Fig. 6E and S5C). Also, the habenular and thalamic glutamate transporter gene *Vglut1/Slc17a7* was downregulated, but not *Vglut2/Slc17a6* that encodes the main thalamic glutamate transporter, consistently with the *in situ* hybridisation results (Fig. 6A and S5A). Conversely, only a few of the upregulated excitability/synaptic genes were thalamus-enriched.

To investigate the hypothesis that TCF7L2 regulates a genetic program of terminal selection that is activated postnatally, we crossed the selected group of genes with a list of genes that were differentially expressed between E18.5 and P60 in WT mice. Almost 90% of the synaptic/excitability P60 DEGs that were thalamus-enriched were induced after embryogenesis, confirming that TCF7L2 functions as a terminal selector during postnatal development. Thus, postnatally, TCF7L2 only partly regulates the expression of thalamic transcription factors but controls a battery of genes that are induced postnatally and shape terminal electrophysiological identities of thalamic neurons.

**Direct regulation of thalamic terminal effector genes by TCF7L2**

To understand how TCF7L2 regulates terminal effector genes in the thalamus, we performed a ChIP-seq analysis on the thalami isolated from adult WT mice on P60. We used the same antibody that we used for Western blot and immunofluorescence/immunohistochemistry in this study, and which was validated with samples from the mutant animals (Fig. 2B-G). This antibody was previously used by other authors on different cell types (Frietze et al., 2012; Geoghegan et al., 2019; Norton et al., 2011). Analysis resulted with 4625 peaks in the anti-TCF7L2 precipitated samples with fold enrichment (FE) ≥ 10 over input sample, which annotated to 3496 unique genes (Table S5). Analysis of motif enrichment (the AME algorithm from the MEME suite) showed significant overrepresentation of the consensus motif for TCF7L2 in the sequences bound by the anti-TCF7L2 antibody. This motif was detected in almost 85% of the TCF7L2 ChIP-seq peaks (Fig. 7A), validating the experiment. In addition, we used a thalamic sample from *Cck\(^{Cre}\):Tcf7l2\(^{-/-}\)* mice. 94.3% of the peaks identified in the wild type condition were not detected in this sample, proving specific target recognition in our assay.
To find the most frequent motifs in the TCF7L2 ChIP-seq peaks, we performed de novo motif discovery with MEME-ChIP. 17 motifs were identified. The most significantly overrepresented motif was identical to the TCF7L2/TCF7L1 consensus binding site ($E$ value = 4.3e$^{-425}$; Fig. 7B). The motifs of GCR (NR3C1), RREB1 and RORA were also overrepresented ($E$ value = 1.3$^{-32}$, 2.3$^{-30}$, 2.2$^{-23}$, respectively; Fig. S6A). These transcription factors are enriched in the thalamus, their expression was altered in $Cck^{Cre}:Tcf7l2^{fl/fl}$ mice, and their genes were identified by the ChIP-seq, suggesting that not only are they downstream targets of TCF7L2 but also cooperate with TCF7L2 in gene expression regulation. The peaks annotated to genes that were expressed in the thalamus on P60 were most frequently localised in intronic regions that may act as intragenic enhancers (Fig. S6B and Table S5). The remaining peaks (i.e., annotated to non-expressed genes) were mainly annotated to predicted genes and pseudogenes, and were located in distal intergenic regions, suggesting that they represent distal enhancers of unidentified genes.

GO term enrichment analysis performed on genes significantly bound by TCF7L2 AND expressed at P60 revealed an overrepresentation of genes related to the regulation of membrane potential and synaptic signalling, calcium and potassium ions transmembrane transport, also the regulation of neuron projection development and cell adhesion (Table S6). Moreover, genes bound by TCF7L2 were highly overrepresented in the P60 DEGs (Fig. 7C). The ChIP-seq peaks were detected in 31% genes that were upregulated, and in 45% genes that were downregulated, included many thalamus-enriched genes that are involved in synaptic signalling or membrane excitability (Fig. 7D-E and Table S5). These genes were either broadly expressed in the thalamus (such as $Cacna1g$, $Gabrd$, $Knce2$, $Syt7$, $Gabra4$, $Grm1$, $Grid2ip$ or $Synpo2$) or restricted to thalamic subregions (such as $Grm1$ to the anterodorsal and mediodorsal nuclei, $Cacng3$ to the PV and midline nuclei, $Kcnab2$ to the PF, AD and ventral nuclei, and $Kcnd2$ to the AD, PV and habenula). This confirmed that TCF7L2 is directly involved in the activation of genes that define pan-thalamic terminal identity and subregional identities in the thalamus. The DEGs with no annotated ChIP-seq peaks can be indirect targets of TCF7L2 or are regulated by TCF7L2-dependent distal enhancers.

Severe impairments in excitability of thalamic neurons in adult mice

To functionally test the role of TCF7l2 in the regulation of thalamic neurons electrophysiological properties, we used whole-cell patch-clamp recordings in $Cck^{Cre}:Tcf7l2^{fl/fl}$ mice from targeted thalamocortical neurons in the VB. We first tested the basic properties of VB neurons. Resting potential and capacitance were similar in WT and KO mice, but input
resistance significantly decreased in KO mice (Fig. 8A). We then examined whether the targeted neurons were able to evoke action potentials (APs; e.g., tonic, burst, and rebound burst modes) that are typical for thalamocortical cells. We used depolarising current steps to evoke both sustained trains of APs (tonic) and burst firing at the beginning of a train (Fig. 8B-C). Thalamic cells produced fewer APs in Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} mice in both tonic and burst firing modes (Fig. 8D). Rebound bursts, which are crucial for the response of thalamocortical neurons to inhibitory input, were evoked by steps of hyperpolarising current (Fig. 8E). Most neurons from Tcf7l2 KO mice did not show any rebound bursts at the hyperpolarising membrane potential (~ -65 mV; Fig. 8F). Hyperpolarising steps that were applied at the resting membrane potential (~ -57 mV) evoked rebound burst spiking in VB neurons in Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} mice, but the number of spikes was approximately twice as low as those of WT mice (Fig. 8G). These dramatic impairments in electrophysiological responses demonstrated that TCF7L2 is essential for the establishment of unique excitability and firing patterns in thalamocortical neurons.

Discussion

Little is understood about how the lengthy process of postmitotic differentiation is regulated in the vertebrate brain. The present study identifies TCF7L2 as a master regulator of regional transcription factors in the thalamus and habenula, and a selector of stage-specific developmental programs that switch postnatally from morphological to electrophysiological maturation.

TCF7L2 and a network of transcription factors regulate morphological maturation of the thalamus and habenula

TCF7L2 is the only developmentally regulated transcription factor that is expressed postmitotically throughout prosomere 2 (Nagalski et al., 2016). TCF7L2 is not necessary for the induction of caudal thalamic and pan-habenular identities that are defined by Gbx2 and Pou4f1 expression, respectively, because the expression of these markers was not abolished in Tcf7l2 KO embryos during neurogenesis (E12.5). The maintenance of Gbx2 expression in the intermediate and superficial thalamic portions may depend on TCF7L2 at later stages, because Gbx2 staining in the mutant thalami persisted only in the periventricular area, where the youngest neurons are located. An apparent increase in the number of POU4F1 cells in Tcf7l2\textsuperscript{-/-} embryos on E12.5 suggests that TCF7L2 could play a regulatory role in cross-
repressing thalamic and habenular identities, by promoting thalamic fate, in agreement with a previous conclusion (Lee et al., 2017). However, decreased expression of sub-habenular markers, e.g., *Etv1* and *Nr4a2*, as well as a thalamo-habenular marker *Lef1*, and sub-thalamic markers, e.g., *Foxp2*, *Prox1* and *Rora* in *Tcf7l2−/−* embryos on E18.5 indicates that TC7L2 plays a positive role in the development and diversification of both thalamic and habenular identities. *Tcf7l2* knockout did not inhibit the expression of rostral thalamic markers, *Nkx2-2*, *Sox14* and *Lhx5*, indicating a different role of TCF7L2 in this particular subdomain of prosomere 2.

A direct regulation of the above subregional markers by TCF7L2 is supported by our previous *in vitro* results showing that TCF7L2 and the closely related factor LEF1 can regulate promoters of thalamic factors *Gbx2*, *Foxp2* and *Rora* (the latter one confirmed in the present study by our ChIP-seq on P60), and habenular factors *Pou4f1*, *Nr4a2* and *Etv1* (Nagalski et al., 2016). The mechanism of differential regulation of genes by TCF7L2 in subregions of prosomere 2 is not known. A differentiating factor might be the level of TCF7L2, which varies between thalamic nuclei during embryogenesis. Another possibility is cooperation with independently-induced transcription factors that are restricted to smaller areas in prosomere 2. GBX2 is a good candidates, considering that *Foxp2* and *Rora* were also downregulated in *Gbx2−/−* embryos (Mallika et al., 2015). We speculate that different levels of GBX2, whose expression decreases in the developing thalamus in the latero-medial gradient (Li et al., 2012) might contribute to subregional diversification of thalamic identities by TCF7L2. Striking similarities between phenotypes in *Tcf7l2−/−* and *Gbx2−/−* embryos, such as the elongated shape of the thalamo-habenular region, impaired cell segregation and the absence of thalamocortical projections (Chatterjee et al., 2012; Chen et al., 2009), corroborates the conclusion that TCF7L2 and GBX2 cooperate in the thalamus during embryogenesis. Less severe impairments in the establishment of thalamocortical connections were reported in *Foxp2* (Ebisu et al., 2016) and *Rora* (Vitalis et al., 2017) KO mice, and mice with either knockout exhibited molecular identity impairments that were restricted to subregions of the thalamus. This implies that postmitotic differentiation in the thalamus is regulated by a hierarchical network of regional and subregional transcription factors.

Similarities also exists between *Tcf7l2−/−* and *Pou4f1−/−* embryos. Subregional habenular markers *Etv1* and *Nr4a2* were also downregulated in *Pou4f1−/−* embryos (Quina et al., 2009), and the expression of habenular axon guidance genes *Rgma* and *Epha8* decreased *Pou4f1−/−* as well as *Tcf7l2−/−* embryos (Quina et al., 2009). However, a comparison between the effects of *Tcf7l2* and *Pou4f1* knockouts in the habenula is not straightforward.
Anatomical impairments were much more severe in Tcf7l2−/− embryos, but much of this phenotype may be attributed to secondary effects that result from the spread of POU4F1-positive cells throughout lateral part of prosomere 2 in Tcf7l2−/− embryos.

Presumably, cell non-autonomous and secondary mechanisms contribute to morphological malformation of the thalamo-habenular region. Considering that Pax6-positive prethalamic cells do not express Tcf7l2 in wild type embryos, abnormal intermingling of these cells into thalamic territory must be cell non-autonomous. The same may apply to the impaired segregation of rostral thalamic and habenular cells. Mechanisms that regulate cell migration and nucleogenesis in the diencephalon are poorly understood. We speculate that misexpression of cell adhesion genes in the thalamus, such as ectopic expression of Reln and decreased expression of thalamus-specific genes Cdhl6, Cdhl8, and Cntn6, could turn the thalamus into a permissive environment for cells migrating from the neighboring Reln-positive structures, i.e., prethalamus, rostral thalamus, habenula and, possibly, pretectum. Considering that topographic axonal connections can create physical boundaries in the developing brain, disorganised stria medullaris or afferent connections from the retina, pretectum and midbrain, where Tcf7l2 is expressed at high levels (Nagalski et al., 2013; Vacik et al., 2011), may also play a role.

A previous research showed that the aberrant growth of thalamocortical axons toward the hypothalamus instead of the ventral telencephalon in Tcf7l2−/− embryos resulted from unresponsiveness of thalamic cells to Slit repulsive ligands, due to decreased expression of genes that encode Slit receptors Robo1 and Robo2 (Lee et al., 2017). We did not observe any changes in the levels of Robo1 and Robo2 mRNA. The expression of these genes is specific for prosomere 2 only at earlier stages (Allen Brain Atlas: Developing Mouse Brain, 2008); hence it may not depend on TCF7L2 at late gestation. Instead, we observed decreased expression of genes that encode habenular axon-navigating molecules Robo3 and Rgma and thalamic axon-navigating molecules that are later induced and subregion-specific, e.g., Ntng1, EPHA1, 3, 4, 8. Eph receptor A4 (EPHA4) regulates topographical sorting of VB axons in the ventral telencephalon at late gestation (Dufour et al., 2003). This implicates TCF7L2 in controlling the sequential steps of thalamocortical axon navigation and subregional sorting.
**TCF7L2 controls the acquisition of characteristic excitability patterns in the thalamus**

Many genes that were downregulated in mice with the postnatal knockout of Tcf7l2 and/or identified as direct TCF7L2 targets in ChIP-seq assay encode postnatally-induced and thalamus-enriched proteins involved in neural signal transmission. The examples are voltage-gated T-type Ca_{v3.1} calcium channels and K_{v3.2} potassium channels (encoded by thalamus-enriched Cacna1g and Kcnc2, respectively). This is consistent with our previous *in silico* predictions and ChIP-qPCR which showed that β-catenin, which is a cofactor of LEF1/TCF transcription factors, interacts with promoters of several excitability/synaptic genes, including in particular Cacna1g (Wisniewska et al., 2010; Wisniewska et al., 2012).

Excitability and synaptic transmission parameters are specific to different classes of neurons and together ensure the proper functioning of neural circuits. Functional experiments in the present study demonstrate that thalamocortical relay neurons lose their proper tonic and burst firing patterns in the absence of TCF7L2. The downregulation of Cacna1g and Kcnc2 most likely contributed to this phenotype, given that Cacna1g knockout resulted with the absence of burst firing (Kim et al., 2001), whereas K_{v3.2} channel inhibition suppressed the firing rate in tonic mode (Kasten et al., 2007) in thalamocortical neurons, similar to Tcf7l2 knockout. These results implicate TCF7L2 in the regulation of postnatal genes that control thalamic terminal excitability patterns.

The thalamus is molecularly distinguishable from other brain structures, but many thalamus-enriched genes are differentially expressed between thalamic nuclei or groups of nuclei (Nagalski et al., 2016; Phillips et al., 2019). TCF7L2 was proved to regulate genes that are broadly expressed in the thalamus and those that are specifically expressed in groups of thalamic nuclei. Although Tcf7l2 was not knocked out in PV and PF, and was less efficiently knocked out in the AD or midline nuclei, ChIP-seq analysis identified TCF7L2 peaks in excitability/synaptic genes whose expression is enriched specifically in these regions, implicating TCF7L2 in the direct control of subregional as well as pan-regional terminal selection in the thalamus. Cooperation with subregional thalamic transcription factors, such as RORA, NR3C1 and RREB1, as suggested by the overrepresentation of the corresponding binding motifs in the TCF7L2 ChIP-seq peaks, could contribute to TCF7L2-dependent regulation of differentially expression thalamic genes, but this question needs further investigation.
TCF7L2 is a terminal selector in the thalamus

TCF7L2 meets the criteria of a thalamic terminal selector: (i) its expression is induced during neurogenesis and maintained in thalamic neurons throughout life (Nagalski et al., 2013), (ii) it directly binds to thalamic terminal differentiation genes, (iii) and its activity is required for electrophysiological maturation of the thalamus. Besides, TCF7L2 orchestrates morphological maturation of the thalamo-habenular region during embryogenesis. This double function differentiates TCF7L2 from the majority of other known terminal selectors, which play minor roles in cell migration or axon guidance (Hobert, 2016), for example habenular selector POU4F2 (Serrano-Saiz et al., 2018). Other examples of terminal selectors that regulate axon guidance are the glutamatergic selector of corticospinal neurons FEZF2 and serotoninergic selector PET1 (Donovan et al., 2019; Lodato et al., 2014).

TCF7L2 is essential for the postnatal induction of terminal functional properties of thalamic neurons. However, unlike classic terminal selectors, TCF7L2 does not control neurotransmitter identity, which in the thalamus depends on VGLUT2. A recent research reported that cells in a thin superficial portion of the thalamus switched to GABAergic identity in Tcf7l2−/− embryos, shown by the colocalisation of Gad1 and Gbx2-driven tdTomato signal (Tran et al., 2020). However, the staining resolution does not allow concluding that the signals colocalised in the same cells; and according to the most recent research the origin of thalamic GABAergic cells may be assigned to prethalamic, rostral thalamic and even pretectal domains (Jager et al., 2016; Puelles et al., 2020). More importantly, normal pattern of Vglut2 and Gad1 expression in mice with the postnatally induced knockout of Tcf7l2 demonstrates that TCF7L2 does not play a role in maintaining glutamatergic identity in thalamic neurons. This implies that in the case of glutamatergic neurons in the thalamus, the selection of different terminal features is uncoupled. It is known form the studies of invertebrate and vertebrate neurons that the adoption of neurotransmitter identity and other terminal features are often linked (Serrano-Saiz et al., 2013). For example, in contrast to thalamic cells and TCF7L2, PET1 controls the early postmitotic adoption of neurotransmitter phenotype and the postnatal acquisition of excitability features by serotonergic cells in the raphe nucleus (Hendricks et al., 2003; Liu et al., 2010; Wyler et al., 2016), and neurotransmitter metabolism genes together with ion channel genes constitute a co-varying module in midbrain dopaminergic neurons (Tapia et al., 2018). Furthermore, studies on glutamatergic neurons in mice concluded that POU4F1 and FEZF2 are selectors of VGLUT1 identity in the medial habenula and corticospinal neurons, respectively, as well as regulators of many other neuron subtype–specific genes (Chen et al., 2008; Lodato et al., 2014; Serrano-Saiz et al., 2018). There are only a few examples of
separate regulation of neurotransmitter identity and other terminal features. In *C. elegans*, unc-3 regulates cholinergic identity in command interneurons, but not other terminal features (Pereira et al., 2015), and ttx-3 controls the terminal differentiation of neurosecretory-motor neurons, but not their serotonergic identity (Zhang et al., 2014). More research is needed to build models of the postmitotic regulation of neurons in vertebrates and compare regulatory strategies between vertebrates and invertebrates.

**Conclusion**

The present study sheds new light on vertebrate regulatory strategies in the postmitotic differentiation of molecularly diverse neurons that share a glutamatergic identity. We found that temporarily separated developmental events and molecular diversification of neurons within a region can be controlled by a single regional transcription factor, as exemplified by TCF7L2 and prosomere 2. Finally, we showed that electrophysiological maturation can be uncoupled from the selection of neurotransmitter identity. Considering that Tcf7l2 is associated with mental disorders, our findings also provide a new insight into the aetiology of thalamic and habenular dysfunction that are observed in these disorders.

**Materials and Methods**

**Animals.** We used C57BL/6NTac-Tcf7l2tm1a(EUCOMM)Wtsi/WtsiIeg (Tcf7l2tm1a) mouse strain (Skarnes et al., 2011), with a trap cassette upstream of the critical exon 6 of the Tcf7l2 gene. To generate CckCre:Tcf7l2fl/fl strain, in which the knockout of Tcf7l2 is induced in the thalamic area perinatally (Allen Brain Atlas, 2011), Tcf7l2tm1a/+ animals were first crossed with flippase-expressing mice (ROSA26::FLPe knock in strain; JAX stock #009086; (Farley et al., 2000)), and then with Ccktm1.1(cre)Zjh/J mice (CckCre:Tcf7l2+/+, JAX stock #012706; (Taniguchi et al., 2011)), which express Cre recombinase from the Cck promoter. CckCre:Tcf7l2+/+ animals were used as wild type. To generate CckCre:tdTomatofl/+ reporter strain, CckCre:Tcf7l2+/+ mice were crossed with homozygous Ai9(RCL-tdT) strain. Mice were maintained on a 12 h/12 h light/dark cycle with *ad libitum* access to food and water. For the experimental procedures all mice were selected by PCR-based genotyping: Tcf7l2tm1a & Tcf7l2fl alleles: tcf_F – GGAGAGAGACGGGGTTTGTG; tcf_R – CCCACCTTTGAATGGGAGAC; floxed_PNF – ATCCGGGGGTACCGCGTCGAG; Tm1c_R – CCGCCTACTGCGACTATAGAGA; CckCre allele: 11214 – GAGGGGTCTATATGTGGGT; 11215 – GGGAGGCCAGATTGCTGTT; 9989 – TGGTTTGTCCAAACTCATCAA. All of the experimental procedures were conducted in compliance with the current normative standards of the European Community (86/609/EEC) and the Polish Government (Dz.U. 2015 poz. 266).
Brain fixation and brain slice preparation. Embryos were collected on embryonic day 12.5 (E12.5) or E18.5. Noon on the day of appearance of the vaginal plug was considered E0.5. Timed-pregnant dams were sacrificed by cervical dislocation, the embryos were removed and decapitated. E18.5 brains were dissected out and fixed overnight in 4% paraformaldehyde (PFA; Cat. no. P6148, Sigma-Aldrich) in 0.1 M phosphate-buffered saline (PBS; pH 7.4; Cat. no. PBS404, BioShop) at 4°C. E12.5 heads were fixed whole. Adult mice were sacrificed on P60-P75 (further referred to as P60) by pentobarbital sedation and perfusion with PBS and 4% PFA. Their brains were dissected out and fixed overnight in 4% PFA. For cryostat sections, the brains were sequentially transferred into 15% and 30% sucrose in PBS at 4°C until they sank. Next, E12.5 and E18.5 tissues were embedded in 10% gelatine/10% sucrose solution in PBS (Ferran et al., 2015a; Ferran et al., 2015b). Postnatal brains were transferred to O.C.T (Cat. no. 4583, Sakura Tissue-Tek). Tissues were frozen in -60°C isopentane. Sections (20 μm – embryos, 40 μm - adult) were obtained using a Leica CM1860 cryostat. Embryonic sections were mounted directly on Superfrost-plus slides (Cat. no. J1800AMNZ, Menzel-Gläser). Adult tissue was collected as free-floating sections into an anti-freeze solution (30% sucrose/30% glycerol in PBS). For DiI axon tracing, E18.5 immersion-fixed brains were kept in 4% PFA at 4°C. 3-5 embryos/mice per genotype from at least two litters were used in each analysis by Nissl staining, DiI axon tracing, immunohistochemistry or in situ hybridisation. Stained sections were visualized under a Nikon Eclipse Ni-U microscope.

Nissl staining. Brain sections were dehydrated in a series of ethanol solutions (50%, 70%, 95%, and 99.8%), cleared in xylene and rehydrated. The sections were rinsed in tap water and stained with 0.13% (w/v) Cresyl violet solution (Cat. no. CS202101, Millipore) for 4 min, rinsed and dehydrated again as described above. The slices were washed in xylene and mounted using EuKitt.

Chromogenic in situ hybridisation. In situ hybridisation was performed in cryosections as previously described (Ferran et al., 2015b; Puelles et al., 2016), using digoxigenin-UTP-labelled antisense riboprobes (sense & antisense), synthesized with the DIG RNA labelling Kit (Cat. no. 11175025910, Roche). Plasmids for the synthesis of Cdhl, Foxp2, Gad1/Gad67, Gbx2, Lef1, Prox1, Rora, Tcf7l2 and Vglut2/Slc17a6 probes come from the collection of José Luis Ferran; the other plasmids were kind gifts from: James Li from the Department of Genetics and Developmental Biology, University of Connecticut Health Center, Farmington, CT, USA (Etv1) (Chatterjee et al., 2014); Seth Blackshaw from the Johns Hopkins University
School of Medicine, Baltimore, MD, USA (*Nkx2-2* and *Sox14*) (Shimogori et al., 2010); Tomomi Shimogori from the RIKEN Center for Brain Science, RIKEN, Saitama, Japan (*Reln*) (Chiara et al., 2012); David Price from the Centre for Integrative Physiology, University of Edinburgh, UK (*Pax6*) (Walther and Gruss, 1991). The *Tcf7l2* probe spans the first 8 exons of *Tcf7l2* gene, therefore it detects also *Tcf7l2* transcripts which are truncated after exon 5 in the mutant mice. The stained sections were washed in xylene and mounted using EuKitt (Cat. no. 03989, Sigma-Aldrich).

**Fluorescent immunohistochemistry.** Frozen sections were washed in PBS with 0.2% Triton X-100 (PBST) and blocked with 5% normal donkey serum (NDS) for 1 h. The slides were then incubated with primary antibodies mixed in 1% NDS overnight at 4°C. Antibodies against TCF7L2 (1:500; Cat. no. 2569, Cell Signaling), Ca<sub>a,3.1</sub> (1:500; Cat. no. MABN464, Sigma-Aldrich, NeuroMab clone N178A/9), β-galactosidase (1:100; Cat. no. AB986, Merck Millipore), L1CAM (1:500; Cat. no. MAB5272, Merck Millipore), PAX6 (1:100; Cat. no. PRB-278P, Biolegend), KI-67 (1:100; Cat. no. AB9260, Merck Millipore), TUJ1 (1:65; Cat. no. MAB1637, Merck Millipore), NKX2-2 (1:50; Cat. no. 74.5A5, DSHB), SIX3 (1:100; Cat. no. 200-201-A26S, Rockland), POU4F1 (1:300; (Fedtsova and Turner, 1995)) were used. Sections were then incubated for 1 h with appropriate secondary antibody conjugated with Alexa Fluor 488 or 594 (1:500; Cat. no. A-21202, A-21207, and A-11076, ThermoFisher Scientific). The slides were additionally stained with Hoechst 33342 (1:10000; Cat. no. 62249, ThermoFisher Scientific), washed and mounted with Vectashield Antifade Mounting Medium (Cat. no. H1000, Vector Laboratories).

**DAB immunohistochemistry.** Free-floating sections were washed in PBST, incubated in 0.3% H<sub>2</sub>O<sub>2</sub> for 10 min, blocked with 3% normal goat serum (NGS) for 1 h and incubated with primary antibodies against TCF7L2 (1:1000) or Ca<sub>a,3.1</sub> (1:500) in 1% NGS overnight at 4°C. Next, sections were incubated for 1 h with biotinylated goat anti-rabbit antibody (Cat. no. BA-1000, Vector Laboratories) in 1% NGS, and then for 1 h in Vectastain ABC reagent (Cat. no. PK-6100, Vector Laboratories). Staining was developed using 0.05% DAB (Cat. no. D12384, Sigma-Aldrich) and 0.01% H<sub>2</sub>O<sub>2</sub>. Next, sections were mounted onto Superfrost Plus slides, dehydrated in a series of ethanol solutions (50%, 70%, 95%, and 99.8%), washed in xylene and mounted using EuKitt.
**DiI axon tracing.** PFA-fixed brains were separated into hemispheres and small DiI crystals (Cat. no. D-3911, ThermoFisher Scientific) were placed in the exposed thalamic surface. Tissue was then incubated in 4% PFA at 37°C for 18-21 days. The hemispheres were then embedded in 5% low-melting-point agarose and cut into 100 μm thick coronal sections in a vibratome. The sections were counterstained with Hoechst, mounted onto glass slides, and secured under a coverslip with Vectashield Antifade Mounting Medium.

**Western blot analysis.** Protein extracts were obtained from 6 animals per genotype from at least two litters. The thalamo-habenular regions were dissected from the brains (Fig. S7A-B) and homogenised in ice-cold RIPA buffer. Protein concentrations were determined using Bio-Rad protein assay (Cat. no. 5000006, Bio-Rad Laboratories). Clarified protein homogenate (50 μg) was loaded on 10% SDS-polyacrylamide gels. Separated proteins were then transferred to Immun-Blot PVDF membranes (Cat. no. 1620177, Bio-Rad Laboratories), which were then blotted with anti-TCF7L2 (1:1000; Cat. no. 2569, Cell Signaling), anti-β-actin (1:500; Cat. no. A3854, Sigma-Aldrich) and anti-GAPDH (1:1000; Cat. no. SC-25778, SantaCruz) antibodies. The staining was visualized with peroxidase substrate for enhanced chemiluminescence (ECL) and 200 μM coumaric acid. Images were captured using Amersham Imager 600 RGB (General Electric).

**Quantification of Ki-67.** Ki-67-positive cells were counted manually in the prosomere 2 region in E12.5 brain sections from control and Tcf7l2-/- animals from three different litters (3 mice, 4 sections each per genotype). Areas of each prosomere 2 section were measured in ImageJ and the number of Ki-67-positive cells by 1 mm² was calculated. Two-tailed Student’s t-test was used to test for the difference between two groups.

**RNA isolation and RNA-seq analysis.** Mice were collected on E18.5 and P60. The thalamo-habenular regions were dissected-out immediately (Fig. S7A-B), and the RNA was extracted using QIAzol (Cat. no. 79306, Qiagen) and the RNeasyMini Kit (Cat. no. 74106, Qiagen). The quality of RNA was verified with Bioanalyzer (Agilent). RNA samples from three animals (two litters) for each genotype were sequenced on the same run of Illumina HiSeq2500. The reads were aligned to the mouse genome mm10 assembly from UCSC, using HISAT (Kim et al., 2015) and their counts were generated using HTSeq (Anders et al., 2015). Differential gene expression analysis was performed with DeSeq2 (Love et al., 2014). Genes with log₂(FC) ≥ 0.4 and log₂(FC) ≤ -0.4 and FDR adjusted p value (q value) ≤ 0.05 were considered to be the differentially expressed up- and downregulated genes.
**Functional enrichment analysis.** Gene ontology enrichment was performed using an open access online tool GOrilla (http://cbl-gorilla.cs.technion.ac.il/). Two unranked lists of genes, one target and one universal (all detected transcripts) were used, assuming a hypergeometric distribution of the unranked genes. GO-Term enrichments were tested with Fisher’s exact test, and FDR adjusted $p$ value ($q$ value) $\leq 0.01$ were considered significant.

**ChIP-seq.** Mice were collected on P60 and sacrificed by cervical dislocation. The thalami were dissected-out (Fig. S7B), chopped and fixed for 10 minutes in 1% formaldehyde (Cat. no. 114321734, Chempur), followed by 10 minutes of quenching with stop solution (Cat. no. 53040, Activ Motif). Chromatin was isolated from cellular nuclei (Cotney and Noonan, 2015), and then sonicated into 200-500 bp fragments with Covaris S220. Chromatin from 6 mice (two litters) was pooled for each replicate. Samples from two independent replicates, each containing 10 µg of DNA, were immunoprecipitated with 375 ng (10 µl) of an antibody specific for TCF7L2 (C48H11; Cat. no. 2569, Cell Signaling) or 5ug of normal rabbit IgG (Cat. no. 12-370, Sigma). Incubation with protein G agarose beads and elution of precipitated chromatin was conducted according to ChIP-IT High Sensitivity kit protocol (Cat. no. 53040, Activ Motif). Cross-links were reversed and both eluates and input controls were treated with RNase and Proteinase K, according to (Cotney and Noonan, 2015). DNA from 2 biological replicates was purified with Monarch PCR & DNA Cleanup Kit (Cat. no. T1030, NEB). Libraries were prepared with KAPA HyperPrep Kit and KAPA Dual-Indexed adapters (Cat. no. 7962363001, KK8722, Kapa Biosciences). Enrichment by 15 cycles of amplification was applied and the final library was size-selected using Kapa Pure Beads to obtain the average size of 350 bp. Libraries were tested for quality with High Sensitivity DNA kit (Cat. no. 5067-4626, Agilent) and then were sequenced on Illumina NovaSeq 6000 instrument in pair-end mode: 2x100 cycles. After trimming, raw reads were mapped to the reference genome, mm10 (UCSC) with the use of BWA (Li and Durbin, 2009). Duplicated reads were identified and removed with the use of Picard tool (Pentland et al., 1994). Peak calling was performed with the use of MACS2 (Zhang et al., 2008). Peaks were assigned to genes with annotatePeak function from the ChIPseeker package using the UCSC (University of California, Santa Cruz) Genome Browser). Because TCF7L2 was enriched in intronic regions, we adjusted the annotation algorithm to prioritise the association of peaks with introns. The EdgeR package (Robinson et al., 2010) was used for statistic differential binding analysis between input and TCF7L2 samples. To identify the most significant peaks we filtered the data for $q$ value $\leq$ 0.01, what resulted with 14 801 annotated to 6 728 unique genes. At last, we filtered the data
FE over the input control ≥ 10, which resulted with 4625 peaks annotated to 3496 unique genes. Motif enrichment and motif discovery analyses were performed using the MEME suite (http://meme.nbcr.net) on filtered peaks, $q$ value ≤ 0.01, FE ≥ 10. TF7L2 motif enrichment in ChIP-seq peak sequences was tested with AME (McLeay and Bailey, 2010) using default options. For de novo motif discovery, MEME-ChIP (comprising MEME (Bailey and Elkan, 1994), DREME (Bailey, 2011) and CentriMO (Bailey and Machanick, 2012)) was used with DNA HOCOMOCO Mouse (v11 CORE) database (Kulakovskiy et al., 2013; Ma et al., 2014). Statistical significance was tested with Fisher's exact test, and $p$ values were corrected for multiple testing ($E$ value).

In vitro slice electrophysiology. Brain slices (300 μm thick) from control and Cck$^{Cre::Tcf7l2^{0/0}}$ mice (4 animals per genotype) of both sexes on P21-23 were prepared by an “along-row” protocol in which the anterior end of the brain was cut along a 45° plane toward the midline (Ying and Goldstein, 2005). Slices were cut, recovered and recorded at 24°C in regular artificial cerebrospinal fluid (ACSF) composed of: 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 2.5 mM CaCl2, 1 mM NaH2PO4, 26.2 mM NaHCO3, 11 mM glucose equilibrated with 95/5% O2/CO2. Somata of thalamocortical neurons (WT n=13-16; Cck$^{Cre::Tcf7l2^{0/0}}$ n=17-20) in the VB were targeted for whole-cell patch-clamp recording with borosilicate glass electrodes (resistance 4-8 MΩ). The internal solution was composed of: 125 mM potassium gluconate, 2 mM KCl, 10 mM HEPES, 0.5 mM EGTA, 4 mM MgATP, and 0.3 mM NaGTP, at pH 7.25-7.35, 290 mOsm. Patch-clamp recordings were collected with a Multiclamp 700B (Molecular Devices) amplifier and Digidata 1550A digitizer and pClamp10.6 (Molecular Devices). Recordings were sampled and filtered at 10 kHz. Analysis of action potentials was performed in Clampfit 10.6. Intensity to Voltage (I-V) plots were constructed from a series of current steps in 40 pA increments from -200 to 600 pA from a holding potential of -65 mV or at the resting membrane potential (around -57 mV). Two-tailed Mann-Whitney test was used to test for the difference in resting membrane voltage, membrane capacitance, numbers of action potentials and spiking frequency. Two-tailed Student’s t-test was used to test for the difference in series resistance (after confirming the normal distribution of the data).
Data availability

The RNA-seq raw FASTQ files are available at the EMBL-EBI data repository – ArrayExpress, under E-MTAB-8755 number. The ChIP-seq files are in the process of uploading at the NCBI Gene Expression Omnibus data repository. The number will be provided as soon as the datasets are accepted.

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Conflict of interest statement:

No competing interests declared.

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Figure 1. TCF7L2 protein levels are high in postmitotic neurons in the thalamo-habenular region. (A) Immunofluorescent staining of TCF7L2 in coronal brain sections from E12.5 embryos. Arrowheads show TCF7L2 in the mantle zone. (B) Immunofluorescent staining of TCF7L2 in coronal brain sections from E18.5 embryos. Asterisks mark nuclei of the caudal thalamus with relatively lower levels of TCF7L2 protein: *, perihabenula; **, nucleus reuniens; ***, ventrobasal complex. Cx, cortex; f, fornix; Hb, habenula; Hp, hippocampus; HTh, hypothalamus; ic, internal capsule; lHb, lateral habenula; mHb, medial habenula; Pt, pretectum; PTh, prethalamus; rTh, rostral thalamus; Th, thalamus. Scale bars represent 0.25 mm (A) and 0.5 mm (B).
Figure 2. Generation of Tcf7l2−/− and CckCre:Tcf7l2fl/fl mouse strains. (A) Schematic representation of Tcf7l2tm1a allele generated by EUCOMM, in which a trap cassette with the lacZ and neoR elements was inserted upstream of the critical exon 6 of the Tcf7l2 gene. Exons and introns are represented by vertical black and horizontal grey lines, respectively. Blue arrows indicate transcription start sites. Regions that encode the β-catenin binding domain and HMG-box are marked by red lines above the exons. (B) Immunofluorescent staining of
TCF7L2 and β-galactosidase in coronal brain sections from E18.5 WT and Tcf7l2⁻/⁻ embryos. (C) Western blot analysis of TCF7L2 in the thalamo-habenular region from E18.5 WT and Tcf7l2⁻/⁻ embryos. Higher molecular weight bands represents the full-length protein (FL-TCF7L2), lower bands represents dominant negative isoform of TCF7L2 (dnTCF7L2). Asterisk shows signals from an incompletely stripped staining. (D) Western blot analysis of TCF7L2 in the thalamo-habenular region from P60 WT (CckCre: Tcf7l2+/+) and CckCre: Tcf7l2⁰/⁻ mice. (E) DAB immunohistochemical staining of TCF7L2 in coronal brain sections from P60 WT and CckCre: Tcf7l2⁰/⁻ mice – magnified view of the thalamus (F) and habenula. (G) Immunofluorescent staining of TCF7L2 in coronal sections from WT and CckCre: Tcf7l2⁰/⁻ mice on E18.5, P4 and P14, showing the progression of the CckCre-driven knockout. White arrows show the ventrolateral region of the thalamus where TCF7L2 is depleted by P4. (H) Simplified time course of the development of the thalamus with representative mouse brain schemes. Cx, cortex; Hb, habenula; Hp, hippocampus; ic, internal capsule; lHb, lateral habenula; mHb, medial habenula; PTh, prethalamus; sm, stria medullaris; Th, thalamus. Scale bars represent 0.5 mm.
Figure 3. TCF7L2 controls the establishment of proper anatomy and axonal connections of the thalamo-habenular region at late gestation but does not affect proliferation and neurogenesis. (A) In situ hybridisation with a Tcf7l2 probe in E12.5 coronal brain sections (truncated Tcf7l2 mRNA is expressed in Tcf7l2^/- embryos). (B) Immunofluorescent staining of the proliferation marker KI-67 and neural marker TUJ1 in E12.5 coronal brain sections. Right panel - quantification of proliferating cells in prosomere 2 (WT n=3 (4 sections each); Tcf7l2^/- n=3 (4sections each); t-test; p value = 0.0615). (C) In situ hybridisation with a Gbx2 probe and immunofluorescent staining of POU4F1 in consecutive coronal brain sections from the same embryo on E12.5. Spreading of the Gbx2-positive and POU4F1-positive regions into each other’s territory in Tcf7l2^/- embryos is emphasised by bars. (D) Nissl staining in E18.5 sagittal and (E) coronal brain sections. (F) DiI tracing of thalamocortical tracts in the E18.5 brains. (G) Immunofluorescent staining of the axonal marker L1CAM in the thalamo-habenular region on E18.5. ac, anterior commissure; Cx, cortex; f, fornix; fr, fasciculus retroflexus; Hb,
habenula; Hp, hippocampus; HTh, hypothalamus; ic, internal capsule; lHb, lateral habenula; mHb, medial habenula; P, pons; Pt, pretectum; PTh, prethalamus; rTh, rostral thalamus; sm, stria medullaris; TCA, thalamocortical axons; Th, thalamus; Th-Hb, thalamo-habenular region. Scale bars represent 0.25 mm (A-C) and 0.5 mm (D-G).
Figure 4. TCF7L2 controls the establishment of anatomical borders in the thalamus and habenula. (A) In situ hybridisation with a Tcf7l2 probe in consecutive E18.5 coronal brain sections. (B) In situ hybridisation with Gbx2 and (C) Nkx2-2 and Sox14 and (D) Pax6 probes in E18.5 coronal brain sections. (E) Immunofluorescent costaining of PAX6 and TCF7L2 (WT embryos) or β-galactosidase (Tcf7l2−/− embryos) in E18.5 coronal brain sections. White arrowheads show PAX6-positive cells invading the thalamus in Tcf7l2−/− embryos. (F) Immunofluorescent staining of PAX6 and SIX3 in E18.5 coronal brain sections. White arrows show SIX3-positive cells and white arrowheads show PAX6-positive cells which intermingle into the thalamic region. (G) Immunofluorescent costaining of NKX2-2 and TCF7L2 (WT mice) or β-galactosidase (Tcf7l2−/− mice) in E18.5 coronal brain sections. White arrowheads
show NKX2-2-positive cells from rostral thalamus invading the thalamus in $Tcf7l2^{-/-}$ embryos. 

(H) Immunofluorescent staining of POU4F1 in coronal brain sections. White arrowheads show POU4F1-positive cells spreading into the thalamic area. Cx, cortex; Hb, habenula; lHb, lateral habenula; mHb, medial habenula; Pt, pretectum; PTh, prethalamus; rTh, rostral thalamus; Th, thalamus; Th-Hb, thalamo-habenular region. Scale bars represent 0.5 mm.
Figure 5. TCF7L2 orchestrates genetic program of morphological maturation in the thalamo-habenular region. (A) Differentially expressed genes in the thalamo-habenular region in Tcf7l2-/- embryos on E18.5 compared to wild type embryos. Clustering heatmaps represent expression patterns of the genes from the overrepresented GO terms: (i) transcription factors; (ii) axon guidance and cell adhesion molecules; (iii) genes related to neuron excitability and synaptic transmission. The log2 values of expression related to the median of the row are shown as a red-blue colour scale. Each line represents an independent biological replicate. Localisation of each gene’s expression in the brain, assessed in the Allen Brain Atlas, is marked with dots to the right of the matrices. Th-enriched, expression enriched in the thalamus; Hb-enriched, expression enriched in the habenula; Th boundary, expressed ubiquitously except for the thalamus (black dots) or expressed at the boundary of prosomere 2.
(white dots); Ubiquitous, expressed throughout the brain; Low, overall low expression throughout the brain; N/A, not available in the Allen Brain Atlas. (B) In situ hybridisation with Reln, Cdhl, Lefl, Proxl, Foxp2 and Rora probes in E18.5 coronal brain sections. (C) In situ hybridisation with Etvl probe in E18.5 coronal brain sections. Cx, cortex; Hb, habenula; Hp, hippocampus; Th, thalamus; Th-Hb – thalamo-habenular region. Scale bars represent 0.5 mm.
Figure 6. TCF7L2 controls the expression of terminal excitability genes, but not VGLUT2 identity in the thalamus. (A) In situ hybridisation with Vglut2/Slc17a6 and Gad1/Gad67 probes in E18.5 and P60 coronal brain sections. (B) Differentially expressed genes in the thalamo-habenular region in Cck$^{Cre}:Tcf7l2^{fl/fl}$ mice on P60 compared to control mice. A clustering heatmap represents expression patterns of the genes from the overrepresented GO terms: (i) ion-channels; neurotransmitter receptors and transmitters; G-proteins and synaptic vesicle proteins. The log$_2$ values of expression related to the median of the row are shown as a red-blue colour scale. Each line represents an independent biological replicate. Localisation of each gene’s expression in the brain, assessed in the Allen Brain Atlas, is marked with black dots to the right of the matrices. Th-enriched, expression enriched in the thalamus; Th-depleted, expressed ubiquitously in the brain except for the thalamus; P3-
enriched, enriched in prosomere 3; Ubiquitous, expressed throughout the brain; Low, overall low expression throughout the brain; N/A, not available in the Allen Brain Atlas. Asterisk next to Kcne2 indicates that this gene is expressed specifically in the choroid plexus, so its high expression in one sample is most likely an artefact. (C) Differential expression of selected transcription factors in P60 Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} compared to control based on RNA-seq (Wald statistics - q value ≤ 0.05 *; ≤ 0.0001 ****; error bars indicate standard error for log2 fold change). (D) In situ hybridisation with a Rora probe in P60 coronal brain sections. (E) DAB immunohistochemical staining of Ca\textsubscript{v,3.1} ion channel (encoded by Cacna1g gene) in P60 coronal brain sections. Hb, habenula; Hp, hippocampus; Th, thalamus. Scale bars represent 0.5 mm.
Figure 7. TCF7L2 directly regulates terminal effector genes in the thalamus. (A) Enrichment analysis of the TCF7L2 consensus motif in the TCF7L2 ChIP peaks by the AME algorithm. TP, true positive peaks, where the TCF7L2 motif was identified, FP, false positive peaks, where the TCF7L2 motif was identified in reshuffled peaks data. (B) De novo motif discovery analysis. The most significant motif identified by MEME-ChIP is TCF7L2. (C) Venn
diagram - the overlap between genes identified by RNA-seq (red) and ChIP-seq (blue). The
P60 DEGs are in white, DEGs overlapping with ChIP-seq are in black. (D) Venn diagrams -
the overlap between the upregulated (red) or downregulated (green) P60 DEGs and genes
identified by ChIP-seq (grey). (E) TCF7L2 binding profiles in the Cacna1g, Gabra4, Slc17a7,
Grin2b, Rora and Lef1 genes. Two independent biological replicates are represented as
separate lines: pink/blue - the TCF7L2 ChIP-seq signal; orange/olive – input; green - negative
control (IgG).
Figure 8. TCF7L2 is essential postnatally for the development of burst and tonic firing patterns in thalamocortical relay neurons. (A) Basic electrical properties of cell membrane measured in VB neurons in brain slices: input resistance (WT n=16, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=20; t-test; \(p\) value=0.0443); resting potential (WT n=16, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=20; Mann-Whitney test; \(p\) value= 0.1112); membrane capacitance (WT n=13, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=17; Mann-Whitney test; \(p\) value=0.6725). Representative traces from whole-cell patch-clamp recordings from VB neurons in brain slices at -65 mV membrane potential and increasing depolarising current inputs (burst and tonic spikes) (B), and at -50 mV membrane potential and depolarising current inputs (tonic spikes) (C). (D) Frequency of spikes evoked by increasing depolarising currents at -65 mV, as represented in (B) (WT n=16, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=20, Mann-Whitney test). (E) Representative traces from whole-cell patch-clamp recordings from VB neurons in brain slices at -65 mV or the resting membrane potential (\(V_{\text{rest}}\), ~ -57 mV) and hyperpolarising current inputs (rebound bursts of spikes). The number of spikes per a rebound burst at -65 mV (F) (WT n=16, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=20, Mann-Whitney test) or at the resting membrane potential (\(V_{\text{rest}}\)) (G) (WT n=15, Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} n=20, Mann-Whitney test), as represented in (E). \(p\) value \(\leq\) 0.05 *; \(\leq\) 0.01 **; \(\leq\) 0.001 ***; \(\leq\) 0.0001 ****; error bars in D, F, and G indicate SEM.
Figure S1. Schematic representation of Tcf7l2^tm1a allele and its conversion into Tcf7l2^tm1d allele. Tcf7l2^tm1a allele has a trap cassette with the lacZ and neoR elements inserted upstream of the critical exon 6 of the Tcf7l2 gene. Expression from the Tcf7l2^tm1a leads to the production of a truncated Tcf7l2 mRNA and lack of functional TCF7L2 protein. Mice homozygous for the Tcf7l2^tm1a allele were used as Tcf7l2^-/- knockout mice. After crossing this strain with mice expressing Flippase, the lacZ and neoR elements were removed from the Tcf7l2^tm1a allele, thus creating Tcf7l2^tm1c allele with its critical exon 6 flanked with loxP sites. Mice homozygous for the Tcf7l2^tm1c allele (Tcf7l2^fl/fl) were then crossed with a strain expressing Cre recombinase driven by the Cholecystokinin gene promoter (Cck^Cre). The expression of Cck and Tcf7l2 overlaps in the thalamus. In the resulting Cck^Cre:Tcf7l2^fl/fl strain, Tcf7l2 is knocked out in Cck-positive thalamic neurons. Exons and introns are represented by vertical black and horizontal grey lines, respectively. Blue arrows indicate transcription start sites. Regions that encode the β-catenin binding domain and HMG-box are marked by red lines above the exons.
Figure S2. \textit{Cck}^{\text{Cre}} \textit{expression in the mouse brain on P14.} Expression of tdTomato in \textit{Cck}^{\text{Cre}}:tdTomato\textsuperscript{0/+} reporter line in (A) a single thalamic section, and in consecutive (B) coronal and (C) sagittal brain sections. Cx, cortex; Hb, habenula; Hp, hippocampus; HTh, hypothalamus; lHb, lateral habenula; mHb, medial habenula; P, pons; Pt, pretectum; Th, thalamus. Scale bars represent 0.5 mm.
Figure S3. Depletion of the TCF7L2 protein in the thalamus of Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} on P60. DAB immunohistochemical staining of TCF7L2 in consecutive (A) coronal and (B) sagittal brain sections from WT and Cck\textsuperscript{Cre}:Tcf7l2\textsuperscript{fl/fl} mice. TCF7L2 is successfully depleted in majority of the majority of thalamic nuclei. The magnified views show TCF7L2-positive cells in the anterodorsal nucleus (AD), paraventricular nucleus (PVT) and parafascicular nucleus (PF). Cx, cortex; Hb, habenula; Hp, hippocampus; HTh, hypothalamus; lHb, lateral habenula; mHb, medial habenula; Pt, pretectum; Th, thalamus. Scale bars represent 0.5 mm.
Figure S4. Anatomy of the E18.5 Tcf7l2−/− brain. Nissl staining in consecutive coronal brain sections of E18.5 WT and Tcf7l2−/− embryos on E18.5. Hb, habenula; Th, thalamus; Th-Hb, thalmo-habenular region. Scale bars represent 0.5 mm.
Figure S5. Expression of the Cav3.1 protein and Vglut2, Gad67, and Rora mRNA in P60 CckCre: Tcf7l2flo/flo. (A) In situ hybridisation with Vglut2/Slc17a6 and Gad1/Gad67 probes in sagittal brain sections. (B) In situ hybridisation with Rora probe in consecutive coronal brain sections. (C) DAB immunohistochemical staining of Cav3.1 in consecutive coronal brain sections. Cx, cortex; Hb, habenula; Hp, hippocampus; HTh, hypothalamus; Pt, pretectum; Th, thalamus. Scale bars represent 0.5 mm.
Figure S6. Motif discovery and genomic distribution analysis of TCF7L2 ChIP peaks. (A) 
De novo motif discovery in 4624 TCF7L2 ChIP-seq peaks with MEME-ChIP. Logos of 
significant de novo motifs, \( E \) values, and similarities to motifs from HOCOMOCO database 
(mouse) are shown. (B) Genomic distribution of the TCF7L2 binding sites. Genes that were 
and were not expressed on P60 were analysed separately.
**Figure S7. Sectioning of the thalamo-habenular region.** (A) Sectioning of the thalamo-habenular region from the embryonic brain (E18.5). (a-e) A brain slice containing the thalamus and habenula is dissected by cutting the brain from the bottom view through the preoptic area close to the optic chiasm and through the mammillary bodies; (f-g) The pallium is pulled apart with a spatula and cut away; (h-i) The hypothalamus is removed with a straight cut below the thalamus. (B) Sectioning of the thalamo-habenular region from the adult brain (P60). (a-c) The cortical hemispheres are cut open and pulled apart with a spatula to expose the subcortical structures including thalamus and habenula; (d-e) The pallium and subpallium are removed with cuts at the front and on the sides of the thalamus; (f) The midbrain and hindbrain are removed with a V-shaped cut at the back of the thalamus, made at the level of the pretectum; (g-h) The hypothalamus is removed with a straight cut below the thalamus. Cx, cortex; Hb, habenula; HTh, hypothalamus; Mb, midbrain; OB, olfactory bulb; Th, thalamus.
Table S1. RNA-seq data for E18.5 wild type and Tcf7l2^{-/-} mice (DeSeq2).
all_expressed_WT_KO_E18.5 - lists of all genes with non 0 normalised values or non NA p values; E18.5_sign._log2FC<-0.4_or_>0.4 - differentially expressed genes (q value ≤ 0.05) with arbitrary log_2 fold change cutoffs ≥ 0.4 or ≤ -0.4. n=3 (independent biological replicates).

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Table S2. Gene ontology enrichment analysis of the E18.5 DEGs. Reported GO terms (q value ≤ 0.01) are divided into 3 ontologies: biological process, molecular function and cellular component.

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Table S3. RNA-seq data for P60 wild type and Cck^{Cre}:Tcf7l2^{fl/fl} mice (DeSeq2).
all_expressed_WT_KO_E18.5_P60 - lists of all genes with non 0 normalised values or non NA p values ion E18.5 or P60; all_expressed_WT_KO_P60 - list of all differentially expressed genes on P60; P60_sign._log2FC<-0.4_or_>0.4 - differentially expressed genes (q value ≤ 0.05) with arbitrary log_2 fold change cutoffs ≥ 0.4 or ≤ -0.4. n=3 (independent biological replicates).

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Table S4. Gene ontology enrichment analysis of the P60 DEGs. Reported GO terms (q value ≤ 0.01) are divided into 3 ontologies: biological process, molecular function and cellular component.

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Table S5. TCF7L2 ChIP-Seq data for P60 wild type mice. 14801 significant (q value ≤ 0.01), annotated to 6728 unique genes; 4624 peaks with arbitrary fold enrichment ≥ 10, intersected with the P60 DEGs from the RNA-seq analysis. Two independent biological replicates, for each replicates; chromatin from 6 mice was pooled for each replicate.

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Table S6. Gene ontology enrichment analysis of the genes identified by the TCFL2 ChIP-seq and expressed in the thalamo-habenular region in wild type mice on P60. Reported GO terms (q value ≤ 0.01) are divided into 3 ontologies: biological process, molecular function and cellular component.

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