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Tcf7l2 plays crucial roles in forebrain development through regulation of thalamic and habenular neuron identity and connectivity

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ABSTRACT

The thalamus acts as a central integrator for processing and relaying sensory and motor information to and from the cerebral cortex, and the habenula plays pivotal roles in emotive decision making by modulating dopaminergic and serotonergic circuits. These neural compartments are derived from a common developmental progenitor domain, called prosomere 2, in the caudal forebrain. Thalamic and habenular neurons exhibit distinct molecular profile, neurochemical identity, and axonal circuitry. However, the mechanisms of how their progenitors in prosomere 2 give rise to these two populations of neurons and contribute to the forebrain circuitry remains unclear. In this study, we discovered a previously unrecognized role for *Tcf7l2*, a transcription factor known as the canonical Wnt nuclear effector and diabetes risk-conferring gene, in establishing neuronal identity and circuits of the caudal forebrain. Using genetic and chemical axon tracers, we showed that efferent axons of the thalamus, known as the thalamocortical axons (TCAs), failed to elongate normally and strayed from their normal course to inappropriate locations in the absence of *Tcf7l2*. Further experiments with thalamic explants revealed that the pathfinding defects of *Tcf7l2*-deficient TCAs were associated at least in part with downregulation of guidance receptors *Robo1* and *Robo2* expression. Moreover, the fasciculus retroflexus, the main habenular output tract, was missing in embryos lacking *Tcf7l2*. These axonal defects may result from dysregulation of *Nrp2* guidance receptor. Strikingly, loss of *Tcf7l2* caused a post-mitotic identity switch between thalamic and habenular neurons. Despite normal acquisition of progenitor identity in prosomere 2, *Tcf7l2*-deficient thalamic neurons adopted a molecular profile of a neighboring forebrain derivative, the habenula. Conversely, habenular neurons failed to maintain their normal post-mitotic neuronal identity and acquired a subset of thalamic neuronal features in the absence of *Tcf7l2*. Our findings suggest a unique role for *Tcf7l2* in generating distinct neuronal phenotypes from homogeneous progenitor population, and provide a better understanding of the mechanism underlying neuronal specification, differentiation, and connectivity of the developing caudal forebrain.

1. Introduction

The precise assembly of neural circuits during development is initiated by the specification and differentiation of distinct classes of neurons from progenitor cells (Jessell and Sanes, 2000). Neurons then exit from progenitor domains, migrate to more superficial regions of the neural tube, and send their axons through the developing nervous system to reach specific synaptic targets. The thalamus and epithalamus are essential diencephalic derivatives that play unique roles in connecting the rostral forebrain with the caudal CNS regions. The thalamocortical axons (TCAs) and fasciculus retroflexus (FR) (also called the habenulopeduncular tract) belong to principal output tracts emerging from the thalamus and an epithalamic nucleus habenula, respectively. The thalamus functions as a major relay station for various incoming sensory information, conveying it from the periphery to primary sensory regions of the cerebral cortex *via* the TCAs, and regulates consciousness, sleep, and awareness (Jones, 2007). By contrast, the habenula acts as a crucial link between the limbic system/basal ganglia to midbrain/hindbrain, and regulates dopaminergic and serotonergic activities by receiving input signals *via* the stria

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medullaris and sending output *via* the FR (Nishikawa and Scatton, 1985; Nishikawa et al., 1986; Lecourtier and Kelly, 2007; Hikosaka et al., 2008).

In conjunction with morphological features, the profile of molecular markers expressed by the developing forebrain has enabled precise defining of progenitor cell populations in the caudal diencephalon (Bulfone et al., 1993; Kitamura et al., 1997; Nakagawa and O'Leary, 2001; Vue et al., 2007; Chen et al., 2009; Jeong et al., 2011; Suzuki-Hirano et al., 2011). Our previous study and those of others have elucidated the roles of extracellular signals such as Shh, Wnt/β-catenin, and Fgf8 in the rostro-caudal regionalization of the caudal diencephalon (Kiecker and Lumsden, 2004; Vieira et al., 2005; Kataoka and Shimogori, 2008; Vue et al., 2009; Szabo et al., 2009; Jeong et al., 2011; Bluske et al., 2012; Chatterjee et al., 2014). We have recently reported that *Ascl1* and *Helt* mediate *Shh* function in a cooperative manner in regulating thalamic progenitor identity by suppressing the fate of a more rostral diencephalic derivative, the prethalamus (Jeong et al., 2011; Song et al., 2015). The thalamus and epithalamus originate from the alar plate of prosomere 2 (p2), and are further subdivided into the rostral (rTH)/caudal (cTH) thalamus and the habenula/pineal gland, respectively. A previous study demonstrated that *Pax6* acts upstream of *Shh* to control differentiation of the p2 domain into the thalamus and habenula (Chatterjee et al., 2014). A homeobox gene *Gbx2* plays essential roles in partitioning of the caudal diencephalon by promoting thalamic differentiation and suppressing habenular fate (Chen et al., 2009; Mallika et al., 2015). However, how the thalamus and habenula segregate from the same p2 domain remains poorly understood.

Tcf7l2 (transcription factor 7-like 2) is a member of the *Tcf/Lef* family of transcription factors, which activate Wnt target genes upon binding to β-catenin (Korinek et al., 1998a; Huelsken and Birchmeier, 2001). Mice lacing *Tcf7l2* are neonatal lethal likely due to abnormal intestine development (Korinek et al., 1998b), and exhibit pituitary hyperplasia (Brinkmeier et al., 2007). Since its identification as a diabetes susceptibility gene (Grant et al., 2006), numerous epidemiological studies have confirmed that *TCF7L2* is one of the most significant genetic diabetes risk factors found to date. Aside from population studies, previous studies have highlighted the function of *TCF7L2* in peripheral organs such as the pancreas, liver, adipocytes, and intestine (Korinek et al., 1998b; Johansson et al., 2006; da Silva Xavier et al., 2009; Boj et al., 2012). However, relatively little is explored about its role within the developing forebrain.

In this study, we genetically analyzed the influence of *Tcf7l2* on axonal development in the developing thalamus and habenula. In the absence of *Tcf7l2*, thalamocortical axons failed to elongate normally and strayed from their normal course to inappropriate locations. Moreover, the main habenular efferent axons were missing in embryos lacking *Tcf7l2*. These axonal defects may result in part from dysregulation of guidance receptors *Robo1/2* and *Nrp2*. Furthermore, thalamic and habenular neurons failed to maintain their post-mitotic fates and acquired molecular characters of each other in the absence of *Tcf7l2*. Collectively, our study unveils pivotal roles of *Tcf7l2* in the proper establishment of neuronal identity and circuitry in the caudal forebrain.

2. Results

2.1. Tcf7l2 *is expressed regionally in the developing diencephalon*

As the distribution of Tcf7l2 protein in the developing forebrain has yet to be described, we sought to determine the expression of the Tcf7l2 protein when distinct anatomical subdivisions of the diencephalon begin to be established. *In situ* hybridization and immunohistochemistry showed that *Tcf7l2* mRNA was expressed in the same pattern as Tcf7l2 protein (Fig. 1). Notably, *Tcf7l2* was expressed broadly in the caudal diencephalon, but stronger expression was detected laterally in the mantle layer of the thalamus (Fig. 1E). To determine the rostral and caudal limits of Tcf7l2 expression in more detail, we compared it with other proteins expressed specifically in the pretectum, epithalamus, thalamus and prethalamus (Fig. 1; Nakagawa and O'Leary, 2001; Vue et al., 2007; Chatterjee et al., 2014). Double immunofluorescence showed that expression of Tcf7l2 protein overlaps heavily with that of Gbx2, which is a useful marker for the caudal thalamic mantle zone (Fig. 1F-K). Tcf7l2 expression further extended rostrally into rTH and caudally into the pretectum and epithalamus, as demonstrated by double staining with Ascl1 or Nrp2 (Fig. 1L-Q), but the level of Tcf7l2 expression was lower in these regions than within cTH. Tcf7l2 was weakly detected in the prethalamus, but not in the zona limitans intrathalamica (zli) (Fig. 1D,N). In summary, in the caudal diencephalon, Tcf7l2 was expressed at high levels in thalamic neurons projecting to cerebral cortex, as well as at lower levels in the pretectum and epithalamus.

2.2. Tcf7l2 *is required for outgrowth and pathfinding of TCAs*

Robust expression of Tcf7l2 in caudal thalamic neurons would imply a role in the formation of thalamic neuronal connectivity. We therefore addressed whether loss of *Tcf7l2* has an impact on the development of thalamocortical projections. In mouse embryos, TCAs that emerge from the developing thalamus at embryonic day (E) 12.5 extend rostrally through the prethalamus to the boundary between the diencephalon and ventral telencephalon, and then make a sharp turn to avoid the hypothalamus and enter the telencephalon (Figs. 2A and 3A; Braisted et al., 1999; Tuttle et al., 1999; Auladell et al., 2000; López-Bendito and Molnár, 2003; Garel and Rubenstein, 2004). By E14.5, TCAs navigate through the internal capsule, and then cross the junction between the ventral and dorsal telencephalon (Figs. 2B,C and 3B). After a waiting period, TCAs continue to ascend to invade the cortical plate (Figs. 2D,E and 3D-F), and soon after birth reach their final destinations in cortical layers IV and V. We first examined the gross morphology of the major TCAs by visualizing axons in the embryonic forebrain with anti-α-internexin (αIN) and anti-neurofilament (NF) antibodies (Lee et al., 1987; Kaplan et al., 1990). Immunohistochemistry at E12.5 showed that initial thalamic axons were detected in the prethalamus in both wild-type and *Tcf7l2*-deficient embryos (Fig. 2A,F). At E14.5, wild-type embryos exhibited a normal fasciculation pattern of TCAs extending to the primitive internal capsule in the ventral telencephalon (Fig. 2B,C). By contrast, *Tcf7l2*-deficient embryos exhibited a marked decrease in the number of axons projecting to the ventral telencephalon and failed to form the primitive internal capsule (Fig. 2G,H). At E16.5, thalamic axons innervated the subplate layer of cortex in controls (Fig. 2D,E), whereas in the *Tcf7l2* mutant embryos the axons stopped their growth in the midportion of the ventral telencephalon and did not reach the cortical areas (Fig. 2I,J). In addition to a failure to grow into the dorsal telencephalon, we also observed significant guidance errors in *Tcf7l2* mutant embryos. Thalamocortical axons that entered the ventral telencephalon strayed from their normal courses to inappropriate locations (Fig. 2G-J, arrows).

To determine whether these abnormally projecting axons indeed represent TCAs, we took the advantage of the $Gbx2^{CreER\text{-}Ires\text{-}eGFP}$ allele (referred to herein as $Gbx2^{CG}$), which expresses both CreER and eGFP from the *Gbx2* locus (Chen et al., 2009). Accordingly, we bred $Tcf7l2^{+/-}$ mice with $Gbx2^{CG}$ mice, in which GFP fluorescence is detected almost exclusively in caudal thalamic neurons and their axons during forebrain development (Fig. 3A-F). Immunohistochemistry for GFP revealed prominent defects in TCA outgrowth and guidance in *Tcf7l2* mutants (Fig. 3G-L). Despite similar patterns of GFP expression in the cTH between controls and *Tcf7l2* mutants, fewer GFP^+ axons extended rostrally into the prethalamus and reached the boundary between the diencephalon and telencephalon at E12.5 in *Tcf7l2*-deficient embryos (Fig. 3G). In E14.5–16.5 mutants, many thalamic axons were misrouted