Development of the prethalamus is crucial for thalamocortical projection formation and is regulated by Olig2

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ABSTRACT
Thalamocortical axons (TCAs) pass through the prethalamus in the first step of their neural circuit formation. Although it has been supposed that the prethalamus is an intermediate target for thalamocortical projection formation, much less is known about the molecular mechanisms of this targeting. Here, we demonstrated the functional implications of the prethalamus in the formation of this neural circuit. We show that Olig2 transcription factor, which is expressed in the ventricular zone (VZ) of prosomere 3, regulates prethalamus formation, and loss of Olig2 results in reduced prethalamus size in early development, which is accompanied by expansion of the thalamic eminence (TE). Extension of TCAs is disorganized in the Olig2-KO dorsal thalamus, and initial elongation of TCAs is retarded in the Olig2-KO forebrain. Microarray analysis demonstrated upregulation of several axon guidance molecules, including EphA3 and EphA5, in the Olig2-KO basal forebrain. \textit{In situ} hybridization showed that the prethalamus in the wild type excluded the expression of EphA3 and EphA5, whereas loss of Olig2 resulted in reduction of this EphA3-negative area and the corresponding expansion of the EphA3-positive TE. Dissociated cultures of thalamic progenitor cells demonstrated that substrate-bound EphA3 suppresses neurite extension from dorsal thalamic neurons. These results indicate that Olig2 is involved in correct formation of the prethalamus, which leads to exclusion of the EphA3-expressing region and is crucial for proper TCA formation. Our observation is the first report showing the molecular mechanisms underlying how the prethalamus acts on initial thalamocortical projection formation.

KEY WORDS: Dorsal thalamus, Thalamic eminence, EphA3, Microarray, \textit{In situ} hybridization, Mouse

INTRODUCTION
The cerebral cortex and dorsal thalamus have reciprocal connections, which are essential morphological bases for cortical functions. Thalamocortical axons (TCAs) send sensory information and feedback of motor programming from the caudal brain areas, and these connections are organized in a topographical manner (Vanderhaeghen and Polleux, 2004). Formation of the topographic connections is regulated by several axon guidance molecules (Braisted et al., 2000, 2009; Dufour et al., 2003; Vanderhaeghen and Polleux, 2004; Torii and Levitt, 2005; Umura et al., 2007). Developing thalamic neurons send axons towards the ventral telencephalon through the prethalamus (or ventral thalamus). Special guidance cells named corridor cells in the ventral telencephalon guide TCAs to the pallium (López-Bendito et al., 2006). Thus, the ventral telencephalon is regarded as an important intermediate target for the formation of reciprocal connections.

TCAs need to pass through the prethalamus on exiting from the dorsal thalamus to the ventral telencephalon as the prethalamus occupies exiting points of TCAs. The prethalamus has been supposed to be an intermediate target of TCAs (Deng and Elberger, 2003; Molnár et al., 2012); however, evidence is scarce that identifies the molecular mechanisms underlying the axon guidance role of the prethalamus, and functions of the prethalamus in thalamocortical projection are not fully understood (Leyva-Diaz and Lopez-Bendito, 2013). Mouse lines showing defects in prethalamus formation would be a useful model for analyzing the functional role of the prethalamus in thalamocortical projection formation.

Olig2 is a bHLH transcription factor that is essential for oligodendrocyte and somatic motoneuron development (Lu et al., 2002; Takebayashi et al., 2002b; Zhou and Anderson, 2002), and is also involved in dorsoventral patterning of the spinal cord, which is required for pMN domain specification. In the diencephalon, Olig2 is expressed in the VZ of the prethalamus at early fetal stages, such as E9.5 in mice (Ono et al., 2008). These Olig2+ cells differentiate into GABAergic neurons in the thalamic reticular nucleus (TRN) as well as into macroglial cells in the diencephalon, whereas loss of Olig2 does not affect GABAergic neuron differentiation (Takebayashi et al., 2008). The function of Olig2 in this area has not been elucidated. Here, we report that loss of Olig2 results in hypoplasia of the prethalamus, which leads to defects of TCA extension. The prethalamus is devoid of EphA3 and EphA5 expression whereas ventrally adjacent thalamic eminence (TE) expresses EphA3 and EphA5 (referred to here as EphA positive) and, in the E13.5 Olig2-KO diencephalon, EphA-positive TE expanded dorsally. Furthermore, the substrate-bound EphA3 suppresses neurite extension in cultured thalamic neurons. These results together indicate that Olig2 regulates proper formation of the prethalamus, which leads to exclusion of the EphA3-expressing non-permissive region for TCA and is crucial for proper TCA formation.

RESULTS
Reduced size of the prethalamus in Olig2-KO mice
We first explored early development of the prethalamus in the Olig2-KO mouse to examine whether Olig2-KO mice can be used to
analyze functions in the prethalamus for thalamocortical projection formation. The prethalamus is demarcated by Dlx2, as well as by Islet1/2, expression (Bulfone et al., 1993).

As Olig2 expression in the diencephalon is observed as early as E9.5 (Ono et al., 2008; supplementary material Fig. S1), whole-mount Dlx2 in situ hybridization was performed in the E10.5 forebrain. Dlx2+ prethalamus was much smaller in Olig2-KO mice (n=3) than in wild-type animals (n=4) (Fig. 1A,B, arrows). To observe prethalamus formation more precisely, coronal sections of the E11.5 forebrain were double-stained with Dlx2 in situ hybridization and Islet1/2 immunohistochemistry. In wild-type or heterozygous mice, Dlx2+ cells were observed in the middle part of the diencephalon (Fig. 1C,E,G,J). Islet1/2+ cells were located laterally to Dlx2+ cells (Fig. 1I). Because no significant difference was observed between wild-type and Olig2 heterozygous animals, they are both referred to as normal control animals. Dlx2+ cells in the Olig2-KO diencephalon were also observed at a similar level; however, as shown in the whole-mount in situ hybridization, the Dlx2+ area was much smaller than that in normal control animals (Fig. 1D,F,H,J). The mean area of the Dlx2+ region in each section of Olig2-KO was ~60% smaller than that of the wild type (Fig. 1K).

In addition, the Islet1/2+ region was also greatly decreased in the Olig2-KO prethalamus (Fig. 1LJ). The prethalamus in E13.5 Olig2-KO mice was also smaller (not shown); thus, loss of Olig2 results in hypoplasia of the prethalamus in early development, as early as E10.5.

We then examined whether reduced proliferation or elevated apoptosis contributes to the hypoplasia of the Olig2-KO prethalamus. Sections of control and Olig2-KO mice at E10.5 and E11.5 were double labeled with Dlx2 in situ hybridization and cleaved caspase 3 immunohistochemistry (a marker of apoptotic cells) or pH3 immunohistochemistry (a marker of mitotic cells). At E10.5, cleaved caspase 3+ spots were more abundantly observed in the prethalamus of Olig2-KO than in control mice (supplementary material Fig. S2A-E), whereas pH3+ cell density was similar between the control and Olig2-KO (supplementary material Fig. S2G). At E11.5, the density of cleaved caspase 3+ cells was similar between the control and Olig2-KO prethalamus, whereas that of pH3+ cells was slightly higher in the Olig2-KO prethalamus than in the normal control (supplementary material Fig. S2F,H). These results indicate that transiently elevated apoptosis at E10.5 may be, at least in part, involved in reduction of the size of the prethalamus in Olig2-KO mice.

**Dorsal shift of the border between the prethalamus and thalamic eminence in the Olig2-KO diencephalon**

To better understand the defects of prethalamus formation in Olig2-KO mice, areas adjacent to the prethalamus were examined by the expression of region marker molecules. The thalamic eminence (TE) is a dorsal part of prosomere 3, although, in coronal sections of the fetal diencephalon, TE is observed ventral to the prethalamus (Fig. 2; López-Bendito and Molnár, 2003; Puellas, 2001). The TE is demarcated by the expression of calretinin (Abbott and Jacobowitz, 1999), Tbr1 and Tbr2 (Eomes – Mouse Genome Informatics) (Bulfone et al., 1995; Puellas, 2001). Tbr2 is expressed in basal progenitors of the TE, and calretinin and Tbr1 are expressed in the mantle layer. In normal control animals, Tbr2 expression was observed ventrally to the Olig2+ domain (Fig. 2A).

In the Olig2-KO diencephalon, Tbr2 was also expressed ventrally to the CreER expression that recapitulates intrinsic Olig2 expression (Fig. 2B), and the Tbr2+ area was much wider than that in normal control animals (Fig. 2A, B, flanked by arrows), whereas the CreER+ prethalamus was narrower (Fig. 2B, flanked by arrowheads). Although Lhx5 expression was reported to demarcate the prethalamus, in our observation, Lhx5 was expressed in the dorsal border of the prethalamus and the main body of the TE whereas the main part of the prethalamus was devoid of Lhx5 expression (supplementary material Fig. S3A,F). In the E12.5 Olig2-KO diencephalon, the Lhx5-negative area was much reduced in size and was compatible with the reduction of the Dlx2+ area (supplementary material Fig. S3B,G). We then measured the relative positions of Tbr2- and Olig2- or Cre-expressing domains within the dorsoventral axis of the E13.5

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**Fig. 1. Defective prethalamus development in the Olig2-KO mouse.**

(A,B) Whole-mount in situ hybridization of E10.5 forebrain with Dlx2. Arrows indicate prethalamus. The prethalamus of the Olig2-KO mouse shows hypoplasia. (C-J) Double staining with Dlx2 in situ hybridization (purple) and Islet1/2 immunohistochemistry (brown). (C,E,G,I) E11.5 wild-type mouse. (D,F,H,J) E11.5 Olig2-KO mouse. The Dlx2+ and Islet1/2+ area is much smaller in Olig2-KO mouse than in normal control animals. Cb, cerebellum; DTh, dorsal thalamus; GE, ganglionic eminence; Pth, prethalamus; TE, thalamic eminence. (K) Quantitative analysis of E11.5 Dlx2+ prethalamus region, showing 60% reduction of the area in the Olig2-KO mouse. Data are mean±s.e.m. (wild type, n=3; Olig2-KO, n=3; Student’s t-test). Scale bars: 1 mm in B; 500 µm in H; 100 µm in J.
Fig. 2. Altered formation of the prethalamus and TE in Olig2-KO mice.
(A,B) Comparison of formation of the prethalamus and thalamic eminence in normal control and Olig2-KO mouse at E13.5. Both are composite pictures, in which left and right halves are adjacent sections immunostained with Islet1/2 (green in left half), Olig2 or Cre (red in left half), and Tbr2 (red in right half). The prethalamus is shown by Islet1/2 and the TE by Tbr2. The VZ of the prethalamus is indicated by arrowheads and that of the TE by arrows. The mutant embryo brain was taken at a slightly caudal level compared with the wild-type embryo brain. Scale bar: 500 µm in B. (C) Relative position of borders of the dorsal thalamus (yellow), prethalamus (red) and TE (blue) in coronal sections of the diencephalon. Total height of the diencephalon in the coronal section is regarded as 100% height, and relative position is expressed as percentage from the bottom (mean±s.d.; wild type, n=3; Olig2-KO, n=3; Student’s t-test).

Fig. 3. Fate change of Olig2 lineage cells in the absence of Olig2.
(A,B) E13.5 Olig2-hetero;Rosa26GFP and Olig2-KO;Rosa26GFP mouse diencephalon, stained with calretinin (red) and GFP (green). Boxed areas in A and B are magnified in C and D, respectively. (C,D) Higher magnification pictures. In the heterozygous mouse, GFP+ and calretinin+ cells are mutually exclusive (C), whereas overlapping distribution is apparent in Olig2-KO mouse (D). Inset shows representative double-labeled cells (arrows). Scale bars: 500 µm in B; 200 µm in D; 20 µm in inset.

GFP+ (supplementary material Fig. S4C,D) and therefore Olig2 lineage cells in the Olig2-KO diencephalon showed dual phenotypes, both prethalamus and TE.

Although the border between the prethalamus and the TE was dorsally shifted in Olig2-KO mice, other areas of the forebrain seemed to be properly formed. For example, the Olig3+ dorsal thalamus was positioned dorsal to the prethalamus (Vue et al., 2009; supplementary material Fig. S3C,H). Whereas the ventral and dorsal borders of the Olig3+ area were unchanged in the Olig2-KO mouse (supplementary material Fig. S3C,H), the relative width of the Olig3 domain within the dorsoventral axis was slightly reduced in the Olig2-KO diencephalon (supplementary material Fig. S3K). Shh and Fgf8 expression in ZLI was unchanged in the absence of Olig2 (supplementary material Fig. S3D,E,I,J). In addition, Islet1/2+ cells, including corridor cells (López-Bendito et al., 2006), were positioned normally in the ventral telencephalon at E13.5 in both the normal control and Olig2-KO mice (supplementary material Fig. S5). Overall, the prethalamus and TE are specifically malformed in the early forebrain development of the Olig2-KO mouse, although a slight defect was noticed in the dorsal thalamus.

Defect of TCA extension in the Olig2-KO diencephalon
We next examined whether the prethalamus has an impact on the formation of the thalamocortical projection. Axons in the fetal forebrain were stained with anti-neurofilament M (NF-M) antibody. At E13.5, when TCAs start to extend into the prethalamus and the ventral telencephalon (López-Bendito et al., 2006), NF-M+ axons extended dorsosventrally and in parallel in the mantle layer, and they gradually converged in the ventral part (Fig. 4.A,B, arrowheads). In age-matched Olig2-KO mice, NF-M+ axons were disorganized and followed a tortuous course in the dorsal thalamus; they sometimes formed small aggregates (Fig. 4.C,D, arrowheads). To elucidate whether these NF-M+ axons are TCAs, they were co-immunostained with anti-netrin G1 antibody (Niimi et al., 2007) because netrin G1 is...
of projection formation would be disorganized in Olig2-KO mice. We thus examined TCA at E13.5 using Dil axonal tracing. DiI-labeled TCAs were observed, orienting dorsolaterally in the ventral telencephalon of wild-type animals (Fig. 5A). By contrast, in Olig2-KO mice, DiI-labeled TCAs had just crossed the telencephalon-diencephalon boundary (TDB), and had not yet reached the lateral ganglionic eminence (Fig. 5B). Measuring the length of the TCAs in the ventral telencephalon between the growing tip and the TDB, Olig2-KO TCAs were much shorter (around 60% reduction) than those of wild-type animals (Fig. 5C). The result clearly shows a reduction of TCA extension at early stages in the absence of Olig2 (Fig. 5A,B, insets).

We further examined whether TCAs reach the cerebral cortex and whether topographic thalamocortical projection is formed in the Olig2-KO mouse. Dil was injected into the middle part of the E18.5 cortex, and DiA was injected into the frontal and occipital poles of the same brain (Fig. 6A,B, insets). The frontal cortex had a connection with the medial part of the thalamus, the middle part with the central part, and occipital cortex with the lateral part in both normal control and Olig2-KO mice (Fig. 6C,D). Therefore, the topography of the reciprocal projection was roughly preserved in the absence of Olig2. However, axon arrangement was markedly disorganized in the Olig2-KO thalamus: whereas axons were arranged in parallel in the wild-type thalamus, those in Olig2-KO formed a thick fasciculus and crossed each other with abnormal overlapping course of axons (Fig. 6C-F). On closer examination, retrogradely labeled cell bodies were observed in the Olig2-KO dorsal thalamus (Fig. 6F, arrows), indicating that TCAs in Olig2-KO reached the cerebral cortex in spite of the initial delay of their extension.

**Disorganized TCAs extend from non-Olig2 lineage cells**

Olig2 is strongly expressed in the VZ of the prethalamus and weakly in the caudoventral part of the dorsal thalamus, whereas TCAs have defects of axon extension; therefore, we examined whether the axonal extension defect in the Olig2-KO dorsal thalamus is induced in a cell-autonomous or a non-cell-autonomous manner. If TCA defects in Olig2-KO mice are induced in a cell-autonomous manner, they should be shown by Olig2 lineage neurons. To analyze this, we again used Olig2-KO;Rosa26GFP mice, and tamoxifen was administered to pregnant mice at E11.5 and/or E12.5 to label Olig2+ cells. At E13.5, when NF-M+ axons were disorganized in the lateral part of the dorsal thalamus of Olig2-KO;Rosa26GFP mouse, axon aggregates were found in the nucleus-free zones (supplementary material Fig. S6A,B, arrows). Nevertheless, processes of GFP+ Olig2-lineage cells were present in the prethalamus showing radial extension, but were not present in cell-free zones (supplementary material Fig. S6C,D, asterisks). At E17.5, when NF-M+ axons were disorganized in the lateral part of the dorsal thalamus of Olig2-KO;Rosa26GFP mouse, axon aggregates were found in the nucleus-free zones (supplementary material Fig. S6A,B). Abnormally oriented axons extending randomly were GFP negative (supplementary material Fig. S6F, arrowheads). These results indicated that axons showing disorganized elongation do not belong to Olig2 lineage neurons, suggesting non-cell autonomous defect of axonal extension in the Olig2-KO dorsal thalamus.

**Altered expression of axon guidance molecules in the Olig2-KO prethalamus**

As axon arrangement was impaired in the Olig2-KO mouse dorsal thalamus, and axonal disorganization occurred in a non-cell-autonomous manner, it is highly probable that the expressions of axon guidance molecules are altered in the terrain through which expressed by developing dorsal thalamic neurons (Nakashiba et al., 2002). Indeed, NF-M+ axons co-expressed netrin G1 in both the wild-type and Olig2-KO dorsal thalamus (Fig. 4B,D, arrowheads), indicating that the disorganized axons in Olig2-KO mice were TCAs (Fig. 4D). By E17.5, wild-type TCAs had formed a thin fasciculus in the dorsal thalamus and prethalamus including TRN (E), whereas those in the Olig2-KO mouse formed abnormal thick axon bundles orienting randomly (arrowheads in F).

**Retarded and disorganized thalamocortical projection in the Olig2-KO forebrain**

The Olig2-KO mouse prethalamus shows defects as early as E10.5, before the onset of TCA formation, suggesting that the initial stage of projection formation would be disorganized in Olig2-KO mice.
TCAs pass, including the prethalamus and the TE. We therefore conducted a microarray analysis for changes in the gene expression of axon guidance molecules in the diencephalon or basal forebrain comparing the transcriptomes of wild-type and Olig2-KO mice at E13.5 (Table 1). Efna, Epha and Sema genes, and Unc5c were shown to change their expression in the absence of Olig2. Using Allen Institute for Brain Science Web in situ hybridization data (http://developingmouse.brain-map.org/), we confirmed that Epha3 and Epha5 were expressed in the E13.5 diencephalon. We then examined the expression pattern of these molecules in the Olig2-KO diencephalon by in situ hybridization. In the E13.5 wild-type forebrain, the prethalamus is devoid of mRNA for Epha3 and Epha5, and the Ephas-negative region continues to the TDB (Fig. 7A,C, asterisks), and both Epha3 and Epha5 are expressed in the TE (Fig. 7A,C; supplementary material Fig. S7A,C,E,G,I,K,M,O; Kudo et al., 2005). In the age-matched Olig2-KO forebrain, the Epha3- and Epha5-expressing TE was dorsally expanded and, conversely, the Ephas-negative prethalamus were reduced in size (Fig. 7B,D; supplementary material Fig. S7B,D,F,H,J,L,N,P). Thus, malformed formation of the prethalamus and TE was accompanied with the altered expression of axon guidance molecules, likely leading to the altered extension of TCAs. EphrinA5 (Efna5), which encodes one of the ligands for EphA3, was expressed in the dorsal thalamus, which seemed to be unchanged in the absence of Olig2 (Fig. 7E,F).

Sema5a and Unc5c also showed changed expression in microarray analysis (Table 1); however, in situ hybridization analysis demonstrated that the expression patterns of Sema5a and Unc5c were not altered in the E13.5 diencephalon of Olig2-KO mice (not shown).

**EphA3 suppresses axonal extension from cultured thalamic neurons**

The above results strongly suggest that EphA3 and EphA5 are candidate molecules affecting the initial extension of TCAs. We next examined the roles of EphA3 in the neurite extension of dorsal thalamic neurons in vitro. Dissociated dorsal thalamic progenitor cells were cultured on the substrate double coated with PLL and either EphA3-Fc chimeric protein or human Fc for 40 h. Neurons were identified with class-III β tubulin immunohistochemistry (Fig. 8A,B). Neurite length on Fc-coated substrate was slightly but definitely different from that on EphA3 (33.3±2.38 µm and 24.4±2.90 µm, respectively, mean±s.e.m.) (Fig. 8C); thus, substrate-bound EphA3 apparently inhibited neurite extension from dorsal thalamic neurons.

**Ectopic Olig2 does not affect EphA3 expression**

We then examined whether Olig2 affects or suppresses EphA3 expression. In this experiment, we used a chick embryo neural tube, because gene expression hierarchy is well known in this region of the chick embryo (Liu et al., 2007; Mizuguchi et al., 2001) when compared with the fetal mouse diencephalon. EphA3 expression in the...
normal E6 chick spinal cord was observed in the ventral VZ and ventral horn (VH). In the VZ, Epha3 expression was partially overlapped with Olig2, whereas motoneurons in the ventral horn, which are of Olig2 lineage, do not express Olig2. Olig2 was transfected to the E3 chick neural tube and E6 spinal cord was examined with GFP, Olig2, Islet1/2 and HB9 immunohistochemistry, and Epha3 in situ hybridization. Olig2 was overexpressed on the electroporated side (supplementary material Fig. S8A,B), which induced ectopic Islet1/2 but did not induce HB9 (not shown). Signal intensity of Epha3 expressed in the VZ and VH was measured with ImageJ software, and the ratio of electroporated side/non-electroporated side was calculated and compared between Olig2-transfected and GFP-transfected samples. Signal intensity was not changed after Olig2 or EGFP transfection in both VZ and the ventral horn (supplementary material Fig. S8F). The ratio was 0.94-1.01 and was not statistically significant. The results indicate that ectopic overexpression of Olig2 does not affect Epha3 expression.

DISCUSSION

In the present study, the Olig2-KO mouse diencephalon was examined morphologically, and we found malformation of the prethalamus and TE, followed by disorganized extension of TCAs. These results indicate that Olig2 controls proper formation of the prethalamus and also that proper formation of the prethalamus controls the initial extension of TCAs. Together, our results provide possible molecular mechanisms underlying initial TCA extension in the prethalamus.

Olig2 regulates proper formation of the prethalamus

Olig2 is expressed in VZ cells of the prethalamus, which develops into TRN and the zona incerta (Inamura et al., 2011), which are mostly composed of GABAergic neurons (Ottersen and Storm-Mathisen, 1984). We previously demonstrated that distribution of GABAergic neurons is nearly normal in the Olig2-KO forebrain compared with normal control animals (Ono et al., 2008), and a fate-mapping study demonstrated that Olig2 lineage cells differentiate into GABAergic neurons in the absence of Olig2 (Takebayashi et al., 2008). In the present study, we examined the formation of the prethalamus at much younger stages: E10.5-E13.5. The Dlx2-positive, as well as Lhx5-negative, prethalamus in Olig2-KO mice was markedly reduced in size (Figs 1, 2). Accompanying the size reduction of the prethalamus, the TE expanded dorsally. The reduced size of the prethalamus is probably caused both by elevated apoptosis at E10.5 (supplementary material Fig. S2) and by the fate change of Olig2 lineage cells from prethalamus cells to TE cells (Fig. 3; supplementary material Fig. S4). It is noteworthy that this is the first demonstration of Olig2 function in early forebrain patterning beyond its known role in glial development (Cai et al., 2007; Lu et al., 2002; Ono et al., 2008, 2009). We previously

### Table 1. Microarray expression analysis of control versus Olig2-KO brains at E13.5

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Significant changes were observed in genes encoding these axon guidance molecules.
reported that loss of Olig2 does not affect apoptosis in the pMN domain (Takebayashi et al., 2008). Other parts of the E10.5 forebrain regions, such as the Dlx2-positive ganglionic eminence or (Dlx2-negative) cerebral cortex, revealed a similar level of apoptosis (not shown). Therefore, involvement of Olig2 in the apoptotic pathway might be dependent on the stage or region of the central nervous system. In addition, it is probable that Olig2 interacts with other transcription factors, such as Tbr2, for proper boundary formation in the TE, as has been reported in the developing neural tube (Helms and Johnson, 2003). Further studies are necessary to clarify the mechanisms of Olig2 function in forebrain patterning.

Proper formation of the prethalamus and TE is required for early thalamocortical projection

Another important new finding is that loss of Olig2 results in retardation of initial TCA extension, which leads to abnormal fasciculation and random orientation of TCA at the late fetal stage (Figs 4–6). It is highly probable that the delay of TCA extension is caused by reduction of prethalamus formation and expansion of the TE, both of which are induced by loss of Olig2: (1) the prethalamus occupies an exit region for TCAs; (2) a defect in the thalamocortical projection was probably induced in a non-cell-autonomous manner (see supplementary material Fig. S6); (3) EphA3 and EphA5 expression is observed in the TE but not in the prethalamus; (4) EphA3 and EphA5 expression occupies the route of TCA extension in the Olig2-KO diencephalon (Fig. 7; supplementary material Fig. S7); (5) ephrinA5, a possible counterpart molecule of EphA3, is expressed in the dorsal thalamus; (6) substrate-bound EphA3 suppresses neurite extension from thalamic neurons in vitro (Fig. 8). Eph receptors and ephrin ligands act as a repellant molecule system in neural circuit formation. In addition, ephrin A proteins also recognize and transduce the reverse signaling of EphA proteins, which also includes axon repulsion (Rashid et al., 2005; Xu and Henkemeyer, 2012). Together, these observations suggest that the EphA3- and EphA5-negative prethalamus may provide a permissive or less inhibitory substrate for TCAs, and that occupation of EphA3 and EphA5 in the exit route impairs initial extension of TCAs in the Olig2-KO diencephalon. It is probable that EphA3 negatively regulates TCA elongation, and, therefore, expansion of EphA-positive TE may impair initial TCA extension in the Olig2-KO forebrain (Figs 4, 5).

Another possible functional role of the prethalamus in TCA extension is contact guidance of prethalamic axons to TCAs. Mitrofanis and Baker (Mitrofanis and Baker, 1993) proposed that pioneer fibers from the prethalamus extending into the thalamus guide TCAs to the prethalamus. Our observation might support the possible association of thalamic and prethalamic axons as these axons do not express a combination of repellant molecules; while thalamic axons may express ephrinA5, its counterpart molecules EphA3 and EphA5 are not expressed in prethalamic axons (Fig. 7). It remains unknown whether these axons express molecules that mediate contact attraction.

The Olig2-KO forebrain includes other minor defects. For example, the prethalamus region at E11.5 and E12.5 was constricted in Olig2-KO mice (Fig. 1, Fig. 7E,F). This constriction was, however, partially recovered by E13.5 (Figs 2, 3, Fig. 7A–D) [probably owing to elevated proliferation in the Olig2-KO prethalamus (supplementary material Fig. S2H)], and cytoarchitecture, such as radial arrangement of the prethalamic cells and radial fiber extension, was preserved normally in the absence of Olig2 (Fig. 3D; supplementary material Fig. S6). Therefore, the constriction of the prethalamus at early stages may not be the cause of the retardation of TCAs in the Olig2-KO forebrain, although this possibility cannot be ruled out completely. In addition, the dorsal thalamus of the Olig2-KO mouse shows slight hypoplasia (supplementary material Fig. S3K), and the EphA3-positive region is also slightly reduced in the Olig2-KO thalamus (Fig. 7). We could not clarify whether the slight reduction of EphA3 expression in the dorsal thalamus affects the initial delay of TCA extension. However, as EphA3 negatively regulates TCA extension, reduction of EphA3 expression might not have a strong effect on the initial delay of axon elongation. As hypoplasia of the prethalamus is the severest defect within the Olig2-KO diencephalon, it is the most likely cause of the initial retardation and impairment of TCA guidance.

The prethalamus and TE have been supposed to be intermediate targets for formation of the thalamocortical projection, as loss of transcription factors expressed in these regions induces defects of thalamocortical projection, which includes Pax6, Mash1 (Ascl1 - Mouse Genome Informatics), Tbr1 (López-Bendito and Molnár, 2003), Dlx1/2 and Ebf1 (Garel et al., 2002) and Olig2 (present study). Transcription factors regulate region identity in the rostral-caudal axis (Kiecker and Lumsden, 2004; Puelles and Rubenstein, 2003; Rubenstein et al., 1998) and cell-fate determination in the dorsoventral axis (Helms and Johnson, 2003; Tanabe and Jessell, 1996). Recently, evidence has supported that transcription factors regulating cell-type specification also regulate cell migration and neural circuit formation (Chédotal and Rijli, 2009; Shirasaki et al., 2006). Cell-fate determination may include the cell-type-specific behavior of committed cells through regulation of the expression of cytoskeletal molecules and receptors for axon guidance molecules.

In our observations, prethalamus formation is controlled by Olig2, which leads to EphA3- and EphA5-negative terrain for the initial extension of TCAs; therefore, transcription factors also control the milieu or environment for axon elongation, regulating neural circuit formation indirectly. As Olig2 may not suppress or regulate EphA3 expression directly (supplementary material Fig. S8) and as the expression of morphogen molecules that may affect EphA3 expression was not altered in the Olig2-KO diencephalon (supplementary material Fig. S3D,E,LI), it is probable that putative programs for TE formation regulate EphA3 expression. Olig2 is involved in multistep processes of diencephalon formation and may indirectly repress EphA3 expression through interacting with transcription factors involved in TE development.

In conclusion, the present study elucidates that Olig2 is crucial for proper formation of the prethalamus, especially the boundary between the prethalamus and TE, and that proper formation of the prethalamus is crucial for correct extension of TCAs. Therefore,
appropriate regionalization of the prethalamus and TE provides a pathway from the dorsal thalamus to the ventral telencephalon.

MATERIALS AND METHODS

Animals and tissue preparation

The animals used in this study were Olig2\textsuperscript{KICreER} (Takebayashi et al., 2002b), Olig2\textsuperscript{−/−} (Lu et al., 2002), ROSA26-GAP43-EGFP (Nakahira et al., 2006) and wild-type ICR mice (Slc, Shizuoka, Japan). Genotyping was performed as described previously (Takebayashi et al., 2002b; Tsutumi et al., 2008). Fertilized chick eggs were purchased from Yamagishi Corporation (Mie, Japan) and were incubated at 37°C. Embryonic stages of chicks were determined according to Hamburger and Hamilton (Hamburger and Hamilton, 1951). At least three independent experiments were carried out in all histological and culture analyses, including Dil tracing. All animal procedures were approved by the Animal Research Committee of Kyoto Prefectural University of Medicine and of the National Institute for Physiological Sciences.

To obtain Olig2-\textsuperscript{KO} mice, Olig2\textsuperscript{KICreER} heterozygous mice were mated and the day when the vaginal plug was found was regarded as embryonic day 0.5 (E0.5). To label Olig2 lineage cells, Olig2\textsuperscript{KICreER}, ROSA26-GAP43-EGFP double heterozygous mice were mated, and tamoxifen (TM; 3 mg/animal) was injected intraperitoneally into pregnant mothers with fetuses at E10.5, E11.5 or E11.5 and E12.5, as described previously (Masahira et al., 2006). Fetal mouse brains were removed from the uterus. Fetal mouse brains were fixed with 4% paraformaldehyde (PFA) in PBS overnight, and subsequently with 20% sucrose in PBS. Fixed brains were cut coronally with a cryostat at 20 µm, and sections were thaw-mounted onto MAS-coated glass slides (Matsunami Glass, Tokyo, Japan).

\textit{In situ} hybridization

\textit{In situ} hybridization was performed using digoxigenin-labeled antisense riboprobes, as described previously (Ding et al., 2005; Wilkinson, 1998). cDNAs used in this study were as follows: \textit{mDlx2} (NM_010054, nt_746-1355), \textit{meprinA3} (NM_207654, nt204-831), \textit{mEpha3} (Watanabe et al., 2006), \textit{mEpha5} (NM_007937, nt_1135-2100), \textit{mGf8} (Ohuchi et al., 1994), \textit{mOlig3} (Takebayashi et al., 2002a), \textit{mLhx3} (Hirata et al., 2006), \textit{mShh} (Iseki et al., 1996), \textit{mSema5a} (NM_009154, nt_634-1162) and chick \textit{Epha4} (Iwamasa et al., 1999). Sense probes were used as a negative control, which did not show specific signals.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (Ono et al., 2004, 2008). The GFP signal in the Rosa26-GAP43-EGFP reporter mouse brain was enhanced by incubation with fluorescein-conjugated tyramide (DP-71; Olympus). Animals were anesthetized with pentobarbital (100 mg/kg body weight), and fetal mice were removed from the uterus. Fetal mouse brains were fixed with 4% paraformaldehyde (PFA) in PBS overnight, and subsequently with 20% sucrose in PBS. Fixed brains were cut coronally with a cryostat at 20 µm, and sections were thaw-mounted onto MAS-coated glass slides (Matsunami Glass, Tokyo, Japan).

Dissociation culture of dorsal thalamus neurons

The dorsal thalamus at E12.5 was dissected free from the fetal brain, minced into small pieces, treated with 0.1% trypsin for 15 min at 37°C, and then triturated with a fire-polished pasteur pipette. Dissociated cell suspension (1×10^5 cells/ml) was prepared in culture medium (MEM containing 5% fetal bovine serum and 5% horse serum). For analyses of the substrate-bound form of EphA3, plastic dishes (35 mm in diameter) were sequentially coated with poly-L-lysine (PLL, 80 µg/ml; Sigma, St Louis, MO, USA) overnight at 4°C and then with mouse EphA3-human Fc or human Fc (100 nM; R&D Systems, Minneapolis, MN, USA) for 5 h at 37°C. After rinsing the culture substrates with PBS, 2 ml thalamic cell suspension was plated onto the culture dishes. Cultures were maintained for 40 h. They were fixed with 4% PFA and stained with anti-class III β tubulin antibody (1:1000, G7121; Promega) to examine neurite extension. Three independent culture experiments were performed. In each experiment, epifluorescent photomicrographs were taken of 9-15 randomly chosen fields and the length of neurites with more than 50 labeled cells was measured with ImageJ software (NIH).

Electroporation

\textit{In vivo} electroporation was performed as previously described (Ono et al., 2004). E3 chick embryos (HH stage 17-20; Hamburger and Hamilton, 1951) were used. DNA of pCAGGS-EGFP (Gotoh et al., 2011) solution with or
without pCAGGS-mOlig2 (Mizuguchi et al., 2001) was injected into the central canal, and square pulses (30 V, 50 ms; Nihon Koden, SEN-3401) were delivered to the embryos. The embryos were fixed at E6 (HH stage 27-29) and analyzed histologically. Gene expression was analyzed as the ratio of signal intensity between the electroporated side and non-electroporated side, and the ratio of Olig2-transfected samples was compared with that of EGFP-transfected samples.

Image analysis
The area of the Dhh+2 region and neurite length both in vivo and in vitro was measured with ImageJ software after images were captured with a CCD camera as above. In all cases, quantitative analysis was carried out using at least three Olig2-KO or wild-type/heterozygous mice.

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Competing interests
The authors declare no competing financial interests.

Author contributions
The project was designed by K.O., H.T., K.S. and K.I. Histological and culture experiments were performed by K.O., A.C., T.N., H.G., A.U., O.A., Q.Z. and S.I., and microarray analysis was carried out by A.C., C.M.P. and O.A. The manuscript was written by K.O. and H.T. with the remaining authors commenting on the manuscript.

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Supplementary material
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References