

RESEARCH ARTICLE

The role of T-cadherin in axonal pathway formation in neocortical circuits

Yuki Hayano¹, Hong Zhao¹, Hiroaki Kobayashi¹, Kosei Takeuchi², Shigemi Norioka³ and Nobuhiko Yamamoto^{1,*}

ABSTRACT

Cortical efferent and afferent fibers are arranged in a stereotyped pattern in the intermediate zone (IZ). Here, we studied the mechanism of axonal pathway formation by identifying a molecule that is expressed in a subset of cortical axons in the rat. We found that T-cadherin (T-cad), a member of the cadherin family, is expressed in deep-layer cell axons projecting to subcortical structures, but not in upper layer callosal axons projecting to the contralateral cortex. Ectopic expression of T-cad in upper layer cells induced axons to project toward subcortical structures via the upper part of the IZ. Moreover, the axons of deep-layer cells in which T-cad expression was suppressed by RNAi projected towards the contralateral cortex via an aberrant route. These results suggest that T-cad is involved in axonal pathway formation in the developing cortex.

KEY WORDS: Neocortex, Pathfinding, Adhesion, Fasciculation, Cadherin

INTRODUCTION

During development, axons with similar destinations form bundles and pathways in the central nervous system (CNS). In the mammalian neocortex, afferent and efferent fibers, which connect the subcortical and cortical regions, form layers in the intermediate zone (IZ). Thalamic afferents run in the most superficial layer of the IZ, whereas efferent fibers occupy the deeper layers (Woodward et al., 1990; Catalano et al., 1991; Henke-Fahle et al., 1996; Molnar et al., 1998b). Efferent fibers are further segregated within the IZ according to their destinations (Woodward et al., 1990; Bicknese et al., 1994; Niquille et al., 2009); callosal axons travel in the lower layers toward the contralateral cortex, while corticofugal axons oriented to the brainstem and spinal cord occupy the upper layers (Bicknese et al., 1994). This pathway segregation in the IZ originates during the onset of cortical development and is thought to be a fundamental mechanism for neural circuit formation.

In general, pioneer axons first navigate toward their targets during cell migration (Schwartz et al., 1991; Noctor et al., 2004; Lickiss et al., 2012), and subsequent axons then grow along preexisting axons to form fiber bundles (Bentley and Keshishian, 1982; Taghert et al., 1982; McConnell et al., 1989; Pike et al., 1992). Preexisting non-neuronal cells are also involved in guiding a particular population of axons (Bentley and Caudy, 1983; Sato et al., 1998). In both cases, cell-cell adhesion is thought to play an important role in the formation

of selective axon pathways (Dodd and Jessell, 1988; Van Vactor, 1998; Wang and Marquardt, 2013). Indeed, several adhesion molecules are known to contribute to axonal fasciculation in the CNS by being expressed in a subset of axons (Bastiani et al., 1987; Patel et al., 1987; Chédotal et al., 1995; Cohen et al., 1998; Weiner et al., 2004; Maness and Schachner, 2007). In cortical circuits, cell surface and extracellular matrix molecules, which might be involved in axonal fasciculation, are expressed in distinct axonal populations in the IZ. For instance, glycosylphosphatidylinositol (GPI)-anchored netrin (netrin-G1) is specifically expressed in thalamocortical axons (Nakashiba et al., 2002). Neurocan, a chondroitin sulfate proteoglycan family member, and L1 are also strongly expressed in thalamocortical axons (Oohira et al., 1994; Fukuda et al., 1997; Wiencken-Barger et al., 2004). On the other hand, corticocortical and corticofugal axons express Tag-1 (Cntn2 – Mouse Genome Informatics), a member of the immunoglobulin superfamily (Fukuda et al., 1997). More recently, neuropilin 1 and nectin have also been shown to be expressed in callosal axons (Hatanaka et al., 2009; Niquille et al., 2009; Piper et al., 2009). However, the molecules that are expressed in a subset of afferent or efferent fibers are not fully characterized. Moreover, the molecular mechanisms underlying pathway guidance in the IZ are largely unknown.

To address this issue, we searched for molecules that are involved in cortical circuit formation using a monoclonal antibody (mAb) technique. We found that a cell adhesion molecule, T-cadherin (T-cad), is expressed in a subpopulation of corticorecipient and corticofugal axons during specific developmental stages. *In vivo* functional analyses, including ectopic expression and knockdown, further revealed that T-cad contributes to axonal pathfinding by cortical projection neurons.

RESULTS**mAb 6C9 recognizes a subset of axons in the developing brain**

To search for molecules that are expressed in subsets of axons in cortical circuits, mAbs were generated by immunizing a mouse with homogenates of newborn rat brains. After immunohistochemical screening of 1000 clones, one of the antibodies, mAb 6C9, showed a unique staining pattern in the developing brain. As shown in Fig. 1A, strong immunoreactivity was found in the IZ of the developing rat cortex. In particular, fiber-like structures were strongly stained in the IZ, but not in the ventricular zone (VZ) (Fig. 1B). This staining pattern was found primarily in embryonic and early postnatal stages (see below). Thus, the antigen for mAb 6C9 was identified as being expressed in afferent and/or efferent fibers in the developing rat cortex, although no fiber was recognized by mAb 6C9 in the mouse brain.

To identify the 6C9 antigen, we purified it by immunoaffinity chromatography (see Materials and Methods). The purified antigen was detected as a single ~90-kDa band by SDS-PAGE (Fig. 1C, left). Western blots confirmed that this band corresponded to the

¹Neuroscience Laboratories, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan. ²Department of Biology, Aichi Medical University, Karimata-Yazako, Nagakute, Aichi 480-1195, Japan. ³Laboratories of Biomolecular Networks, Graduate School of Frontier Biosciences, Osaka University, 1-3 Yamadaoka, Suita, Osaka 565-0871, Japan.

*Author for correspondence (nobuhiko@fbs.osaka-u.ac.jp)

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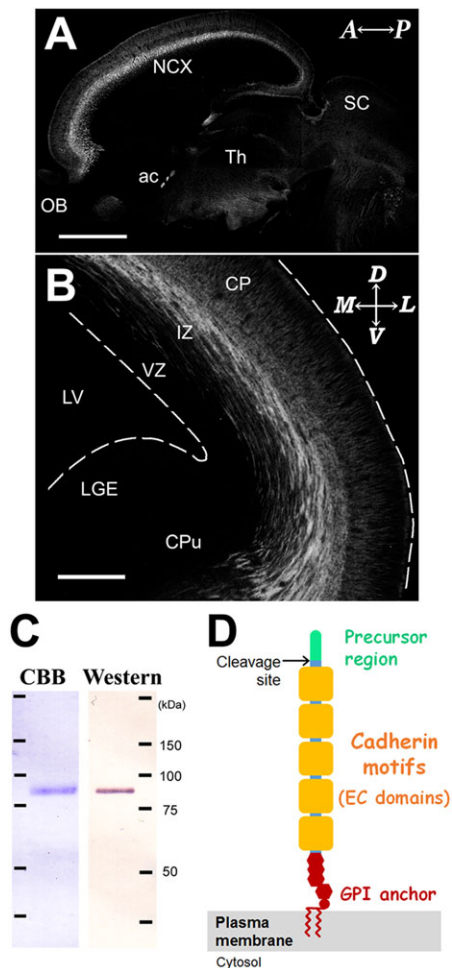


Fig. 1. mAb 6C9 antigen distribution in the developing brain. One of the monoclonal antibodies (mAb 6C9) against developing rat brain showed a unique distribution of the antigen, identified as T-cad. Immunostaining of sagittal (A) and coronal sections (B) of E18 rat brain revealed T-cad expression in the IZ throughout the neocortex (NCX). Owing to the size of the sample, the image in A is a composite of several fields of view. (C) A protein purified by affinity chromatography with mAb 6C9 was visualized as a single band by CBB staining after SDS-PAGE and western blotting. T-cad structure is illustrated diagrammatically in D. The mAb recognizes the mature form of the protein, which is generated by cleavage of the precursor form on the N-terminal side of the first cadherin repeat. Scale bars: 100 μ m in A; 250 μ m in B. A, anterior; P, posterior; D, dorsal; V, ventral; M, medial; L, lateral. OB, olfactory bulb; ac, anterior commissure; Th, thalamus; SC, superior colliculus; CP, cortical plate; IZ, intermediate zone; VZ, ventricular zone; LV, lateral ventricle; LGE, lateral ganglionic eminence; CPu, caudate putamen.

6C9 antigen (Fig. 1C, right), and an N-terminal amino acid sequencing of the purified polypeptide yielded 13 residues, SIVVSPILIPENQ. This sequence matches a proportion of T-cad (Cadherin-13 or H-cadherin), which possesses five cadherin motifs and is bound to the plasma membrane via a C-terminal GPI anchor (Fig. 1D). Moreover, as the 13-residue amino acid sequence was detected in an affinity-purified protein from a brain homogenate, we conclude that a full-length T-cad precursor is cleaved on the N-terminal side of the first cadherin domain (Fig. 1D, arrow) to become the mature form *in vivo*.

Expression of T-cad in specific axonal pathways

Because T-cad expression had only been previously described in the spinal cord (Fredette and Ranscht, 1994; Takeuchi et al., 2000) and in adult mRNA (Matsunaga et al., 2013; Rivero et al.,

2013), we needed to thoroughly examine the protein expression pattern in the newborn rat cortex. Immunohistochemistry with mAb 6C9 demonstrated that T-cad was strongly expressed in the IZ (Fig. 1B, Fig. 2A). Strong immunoreactivity was also found in the internal capsule (IC) (Fig. 2C), which contains thalamocortical and corticothalamic fibers, and in the cerebral peduncle (Cp), which is composed of corticospinal axons (Fig. 2D). By contrast, there was no immunoreactivity in the corpus callosum (CC), which contains cortical fibers connecting both hemispheres (Fig. 2B). In addition, T-cad expression was also detected in the anterior commissure (Fig. 1A) and the habenular commissure (data not shown). These observations indicate that T-cad is expressed in a subpopulation of corticorecipient and corticofugal axons but is not expressed in callosal axons.

To examine which cortical neuron types express *T-cad*, *in situ* hybridization was performed in the developing cortex. Strong expression of *T-cad* was found in cortical plate (CP) cells of embryonic day (E) 15.5 rat brain, but not in the IZ or VZ (Fig. 3A,D). Robust expression of T-cad was maintained in the CP, and dispersed signals were observed in the subplate (SP) at E18.5 (Fig. 3B,E). At postnatal day (P) 1, the signal was intense in a subpopulation of layer V cells and weak in layer VI (Fig. 3C,F), but undetectable in the upper layers, which consist of layer II/III and IV cells. Moreover, *T-cad* was expressed in a few thalamic nuclei, such as the lateral geniculate nucleus (DLG and VLG in Fig. 3C) and the reticular thalamic nucleus (Rt in Fig. 3C). Thus, *T-cad* is expressed in a subpopulation of deep layer neurons and in a small fraction of thalamic neurons.

The laminar property of T-cad-positive neurons was further studied by comparing it with the expression of layer-specific markers. Because endogenous T-cad protein is present on the surface of the cell bodies only at earlier stages (Fig. 4), immunohistochemistry for COUP-TF interacting protein 2 (Ctip2; Bcl11b – Mouse Genome Informatics) (Arlotta et al., 2005; Chen et al., 2005; Molyneaux et al., 2005), a marker protein for subcerebral projection neurons in layer V, was performed on E15.5 rat neocortex. Roughly half of T-cad-positive cells in the CP expressed Ctip2 (Fig. 4A–D). Expression of T-brain-1 (Tbr1) (Hevner et al., 2001; Han et al., 2011; McKenna et al., 2011), a marker protein for layer VI corticothalamic projection neurons, was also detected in T-cad-positive cells in the CP and the SP of E18.5 rat neocortex (Fig. 4E–H). Thus, T-cad-expressing cells mostly consist of Ctip2-positive (layer V) and Tbr1-positive (layer VI) subcerebral projection neurons.

Distribution of T-cad-expressing axons in the IZ

The distribution of T-cad-expressing axons in the IZ was subsequently compared with Netrin-G1- and Tag-1-positive axons, which correspond to thalamocortical and corticofugal axons, respectively (Wolfer et al., 1994; Lebrand et al., 1996; Fukuda et al., 1997; Nakashiba et al., 2002). T-cad-expressing axons were found in the upper part of the IZ at E20, when corticofugal and thalamocortical neurons extend axons to the IZ (Fig. 5B). In the same section, Tag-1 expression was localized in the lower part of the IZ, but overlapped partly with T-cad-expressing axons (Fig. 5B–D). T-cad-positive axons also overlapped with Netrin-G1-positive axons in the upper layer of the IZ (Fig. 5H). The two populations were also partly colocalized in the IC (Fig. 5I,J). Therefore, T-cad-expressing fibers, composed of subcerebral projection fibers and a subpopulation of thalamocortical axons, occupy the upper part of IZ.

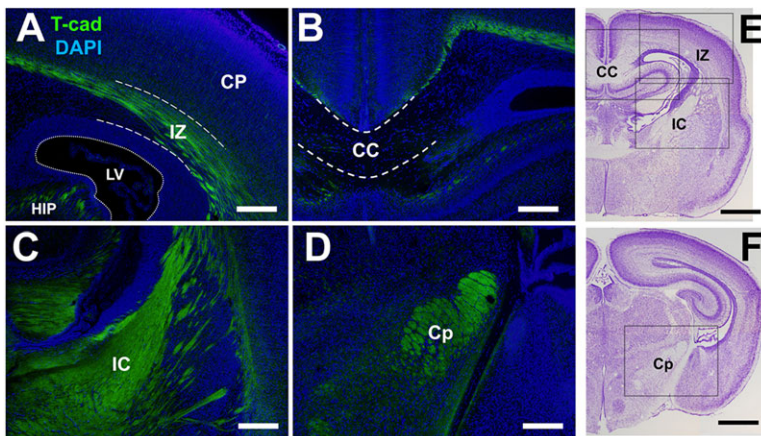


Fig. 2. Distribution of T-cad-expressing axons in various brain regions. (A–D) Immunostaining of T-cad with mAb 6C9 (green) and counterstaining with DAPI (blue) were performed on coronal sections of P0 rat brain. Fiber-like staining is shown in the IZ (A), IC (C) and Cp (D), but not in the CC (B). Boxed areas on Nissl-staining sections (E, F) correspond to A–D. Scale bars: 250 μ m in A–D; 1 mm in E, F. Cp, cerebral peduncle; CC, corpus callosum; HIP, hippocampus; IC internal capsule; IZ, intermediate zone.

T-cad expression during development

The developmental time course of T-cad expression was analyzed by western blotting of E15–P34 rat neocortex (Fig. 6A). T-cad expression was first noticeable at E15, peaked at E18 and P0, sharply decreased, and then increased until P14. The first peak in T-cad expression coincided with the time of afferent and efferent growth, and the protein was mainly localized in axons (Figs 1, 2 and 6). Based on these characteristics, we focused on T-cad function in cortical circuit formation. The time course of T-cad expression in rat neocortex was examined by immunohistochemistry with mAb 6C9 (Fig. 6B). T-cad expression was first observed in the CP and IZ at E15, around the time that deep layer neurons are migrating radially. Expression in cell bodies was observed only at the early stages (E15–18). Consistent with the western blot results, T-cad expression in the IZ was prominent between E18 and P2, although the expression increased at the late developmental stages.

Ectopic expression of T-cad in upper layer cells alters the projection pattern

In order to investigate the function of T-cad in axonal projections, the protein was ectopically expressed in upper layer cells, which otherwise do not express T-cad. A vector encoding rat T-cad was co-transfected with an *eyfp* expression vector by *in utero* electroporation into E14.5 mouse VZ cells, which are destined to become layer II/III cells. In this experiment, the mouse was used

to detect ectopically expressing T-cad using immunohistochemistry with mAb 6C9, as this antibody recognizes rat, but not mouse T-cad. As a control, an *eyfp*- or a *dsred*-encoding vector was transfected into the VZ cells of the rest of the littermates. First, we confirmed that cortical laminar organization was not influenced by ectopic expression. T-cad-overexpressing cells settled correctly in the upper layers, although migration was delayed and occasionally accumulated in the SVZ and IZ (supplementary material Fig. S1A, Fig. S2A,E and Fig. S3A,B). These electroporated cells expressed a marker protein for upper layer cells and did not express a deep-layer cell marker, indicating that cell fate is not changed by ectopically expressed T-cad (supplementary material Fig. S2).

Shortly after birth (P3–P6), axonal projections from T-cad-overexpressing cells were compared with those from control cells. In the controls, most axons were found to extend medially in the IZ (Fig. 7A, arrowhead) and crossed the midline along the CC. Although some labeled axons were occasionally found in the striatum (McGeorge and Faull, 1989), such projection was negligible compared with the contralateral projection (Fig. 7D). By contrast, in the *T-cad*-transfected cortex, a substantial number of labeled fibers oriented laterally and projected into the IC (Fig. 7E). The abnormal projections originated from cells which migrated into the CP (supplementary material Fig. S3C,D) and accumulated in the SVZ and IZ (supplementary material Fig. S3A,B, asterisk). The axons from the CP turned abnormally to the lateral direction,

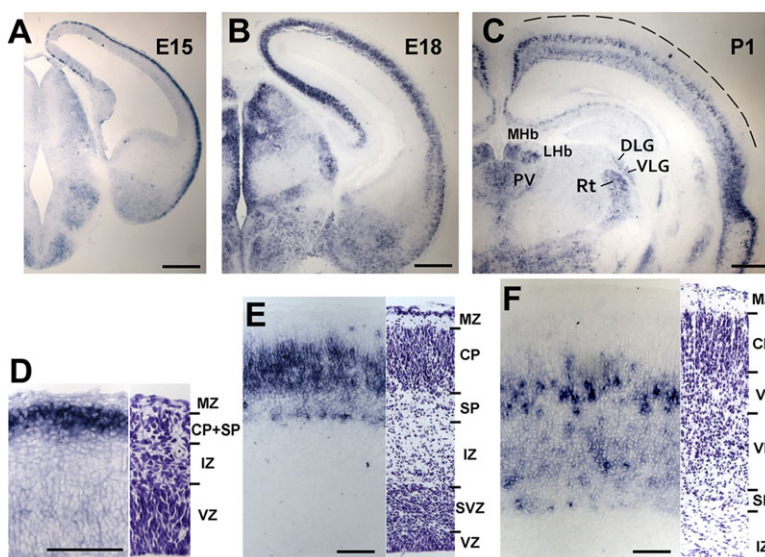


Fig. 3. T-cad expression in a subset of deep-layer cells.

(A–C) *In situ* hybridization revealed strong expression of T-cad mRNA in the cortex and thalamic nuclei at late embryonic stages (E15.5 and E18.5) and a neonatal stage (P1). Dashed line in C indicates the pial surface of the neocortex. (D) At E15.5, *T-cad* expression is found in the CP and SP, but not in the IZ, SVZ and VZ. (E) Robust expression of *T-cad* was maintained in the deep layers, and dispersed signals were observed in the SP at E18.5. (F) *T-cad* was expressed exclusively in a subpopulation of layer V and VI neurons at P1. The adjacent Nissl-stained section shows the laminar structure (right). Scale bars: 500 μ m in A–C; 100 μ m in D–F. DLG, dorsal nucleus of lateral geniculate body; VLG, ventral nucleus of lateral geniculate body; Rt, Reticular thalamus; MHb, medial habenular nucleus; Lhb, lateral habenular nucleus; PV, paraventricular thalamic nucleus. MZ, marginal zone; SVZ, subventricular zone; V, layer V; VI, layer VI; SP, subplate.

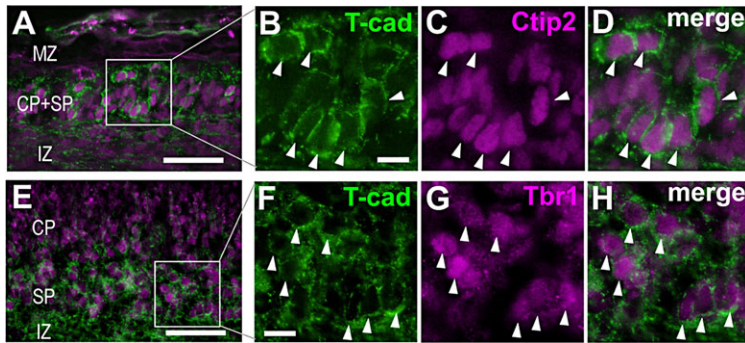


Fig. 4. Expression of deep layer markers in T-cad-positive cells. Double staining of T-cad and marker proteins was performed on coronal sections (10 μm thick) prepared from E15.5 (A–D) and E18.5 (E–H) rat embryos. T-cad expression was found on the surface of cell bodies in the SP and the CP only in the early stages. (A–D) Ctip2, a marker for subcerebral projection neurons in layer V, was expressed in the T-cad-positive cells (arrowheads). (E–F) Tbr1, a marker protein for layer VI corticothalamic projection neurons, was also detected in T-cad-positive cells (arrowheads). Scale bars: 50 μm in A,E; 20 μm in B,F.

extended in the upper part of the IZ, which is the pathway of T-cad-positive subcortical projection neurons (supplementary material Fig. S3C,D, arrowheads), and entered into the IC. Ectopic T-cad expression in these axons was confirmed by immunohistochemistry with mAb 6C9 (Fig. 7C). These abnormally projecting fibers seem to gather tightly in the IC (Fig. 7C,E). Aberrant projections were further observed in the Cp of the *T-cad*-transfected brain (Fig. 7H,I), whereas there were almost none in the Cp of the control brain (Fig. 7G). The frequencies of these abnormal projections are quantified in Fig. 8. Medially projecting axons in the CC and IZ were observed in both T-cad-overexpressing and control brains (Fig. 8A,B). By contrast, there were more axons in the IC of the

T-cad-overexpressing brain than in the control (Fig. 8C). Furthermore, a moderate number of axons were found in the Cp, whereas there were almost none in the controls (Fig. 8D).

The possibility that the T-cad-overexpression phenotype is due to its homophilic binding was studied using cocultures of cortical cells and L-cells (fibroblast cell line). We found that neurites from T-cad-overexpressing cortical cells tended to grow on adjacent T-cad-overexpressing L-cells (supplementary material Fig. S4A,B). By contrast, when cortical neurons were simply transfected with the *eyfp* expression vector, neurites did not grow preferentially on L-cells regardless of whether or not they express T-cad (supplementary material Fig. S4C–E). Thus, it is likely that homophilic binding is responsible for preferential axonal extension.

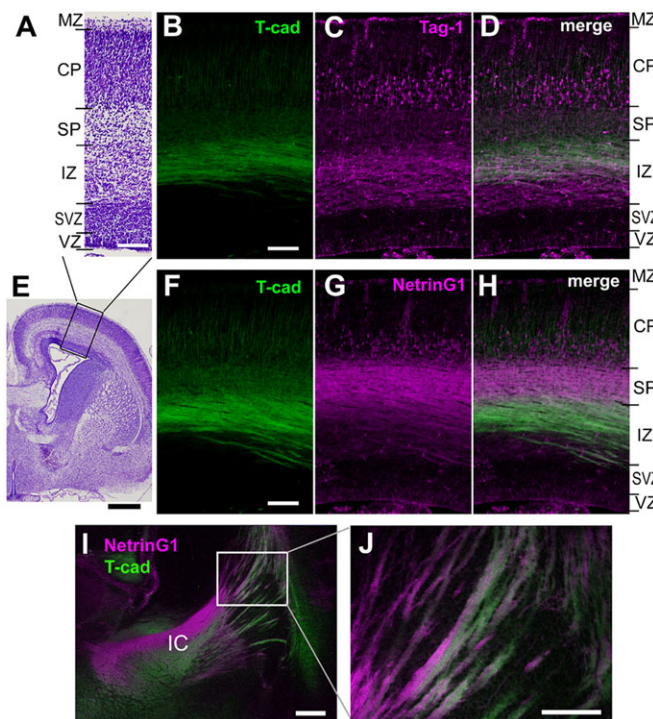


Fig. 5. Distribution of T-cad-expressing axons in the IZ. Immunostaining for T-cad and marker proteins were performed on serial sections of E20 rat brain. Nissl staining shows the layer structure of the cortex (A,E). (B,F) T-cad protein was localized in the axons at E20, and cell bodies were hardly immunostained with 6C9 mAb. T-cad-expressing fibers were distributed in the upper part of the IZ. (C) Cortical efferent fibers, visualized by immunostaining for Tag-1, occupy the lower part of the IZ. (D) T-cad and Tag-1 expression are merged. T-cad positive fibers overlap partly with Tag-1-positive fibers in the middle part of the IZ (white). (G) Thalamocortical fibers, visualized by immunostaining for Netrin-G1, extend the upper part of the IZ and the SP. (H) The two populations overlap partly in the uppermost layer of the IZ. (I,J) Partial overlap is also observed in the IC. Scale bars: 100 μm in A,B,F,J; 500 μm in E; 200 μm in I.

Knockdown of T-cad in deep-layer cells causes an aberrant projection

The role of T-cad was further studied by knocking down endogenous T-cad in deep-layer cells. Rat tissue was used because mAb 6C9 efficiently shows expression of endogenous T-cad. One of the effective constructs, shRNA689 (Fig. 9A), was electroporated into the cells destined to be deep-layer cells. Scrambled shRNA or *dsred* vectors were transfected into cortical cells of the rest of the littermates. Brain sections were immunostained with 6C9 antibody in order to detect endogenous T-cad (Fig. 9C,F,I).

Deep-layer cells are fundamentally composed of callosal neurons, which project to the contralateral hemisphere, and ipsilaterally subcortical projection neurons. When the *dsred* plasmid was electroporated in E15.0 rat cortex, 63% of DsRed-positive cells expressed T-cad (supplementary material Fig. S5A,C). This indicates that 63% of the transfected cells are subcortical projection neurons expressing T-cad, whereas the remaining 37%, originally T-cad-negative cells, are callosal neurons. On the other hand, only 20% of the shRNA-transfected cells were obviously T-cad-positive (supplementary material Fig. S5C), indicating that T-cad expression is completely diminished *in vivo* in ~70% of T-cad-positive cells by the present knockdown method.

At P0, labeled deep-layer cell axons projected both medially and laterally in the IZ of both the control (Fig. 9B) and the shRNA-transfected brains. In the control and scrambled shRNA-transfected brains (boxed area in Fig. 9B), medially oriented fibers that project to the contralateral cortex were observed in the deep IZ (Fig. 9C–H). In the shRNA-transfected brain, surprisingly, part of labeled deep-layer cell axons traveled in the SP toward the contralateral cortex (arrows in Fig. 9J,K), although most labeled axons traveled in the deep IZ (Fig. 9I–K). This abnormal projection corresponds to the T-cad-negative thalamocortical axon pathway (Fig. 5F–H). As callosal axons never travel in this pathway, the abnormal projection must be due to T-cad-downregulated subcerebral fibers. The frequency distributions of

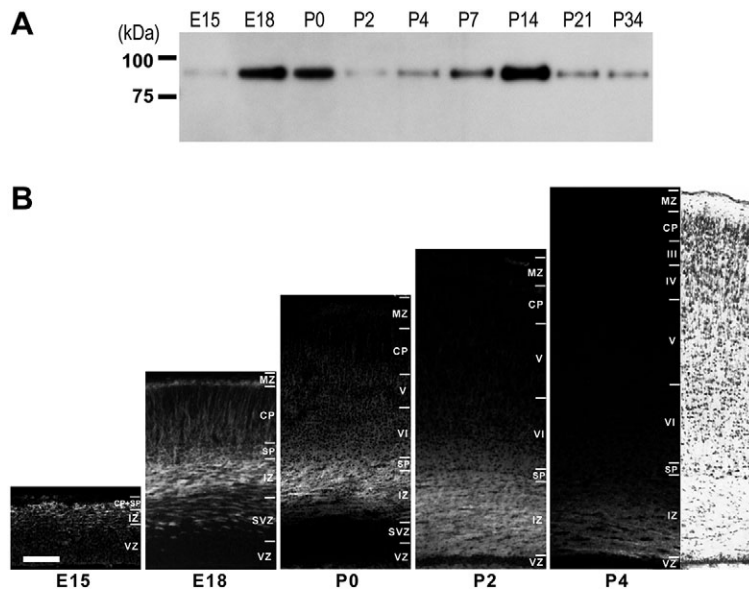


Fig. 6. Developmental changes of T-cad expression in the cortex. (A) A developmental time course of T-cad expression was confirmed by western blotting analysis of rat brain lysate. Expression of T-cad was first observed at E15 and became prominent from E18 to neonatal stages. Although it is decreased after P2, the intense signal appeared again at P14. Each sample (20 μ g) was applied to SDS-PAGE except for lane 1 (100 μ g, E15). (B) T-cad expression in the rat neocortex was visualized with mAb 6C9 during the first peak of the expression. The protein was localized mainly in the axons during late embryonic stages and postnatal stages. T-cad signals are clearly defined until P0 on the axons in the IZ. The signal becomes weaker after P2. Layer structure was defined by Nissl staining of the adjacent sections. Scale bar: 100 μ m. III, layer III; IV, layer IV.

such abnormal projections were compared with control and shRNA-subjected brains (Fig. 9L). As a result, the abnormality was found in all of the shRNA-transfected brains, although the extent was varied among the samples. In the control and the scrambled shRNA brains, abnormal projection was not observed. Thus, the knockdown of T-cad altered both the direction and the pathway of originally T-cad-positive subcerebral axons.

DISCUSSION

In the present study, we identified the cell-surface molecule T-cad in specific pathways of the developing neocortex. In cortical efferent projections, T-cad was expressed specifically in axons originating from subcerebral projection neurons in deep layers. Functional

analysis demonstrated that ectopic expression of T-cad in upper layer cells resulted in abnormal projections toward subcortical structures. Moreover, the deep-layer cell axons in which T-cad expression was suppressed altered their trajectory. These results suggest that T-cad plays a role in pathway formation of corticofugal axons.

T-cad expression pattern in the developing cortex

To date, some classical cadherins, such as cadherin-6 and cadherin-8, are known to be expressed in the CNS (Suzuki et al., 1997; Inoue et al., 1998; Nakagawa et al., 1999), although their functions are still unclear. On the other hand, expression of T-cad in the spinal cord repels axons of motor neurons (Fredette and Ranscht, 1994; Fredette et al., 1996). The complete expression pattern and function of T-cad

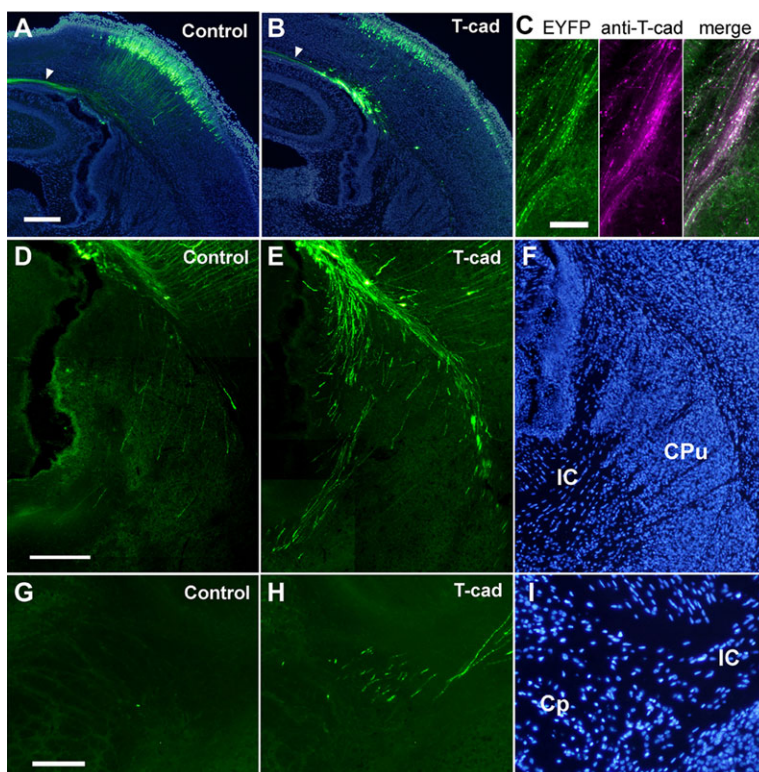


Fig. 7. Abnormal projection from T-cad-overexpressing upper layer cell axons. (A) In control P3 mice, almost all EYFP-labeled upper layer cell axons projected medially toward the contralateral cortex (arrowhead), but few projected into the IC (D). (G) No labeled axons were detected in the Cp. By contrast, T-cad-overexpressing upper layer cell axons projected both medially (arrowhead in B) and laterally (E); a few were also found in the Cp (H). (C) A higher magnification of the IC in T-cad-overexpressing brain at P6 confirms that EYFP-positive fibers express T-cad. F and I show DAPI staining of E and H, respectively. "Fig (D,E) Panels are montages of several images at different focal planes to allow axonal processes to be seen in focus throughout the sample. Scale bars: 300 μ m in A; 100 μ m in C, G; 200 μ m in D.

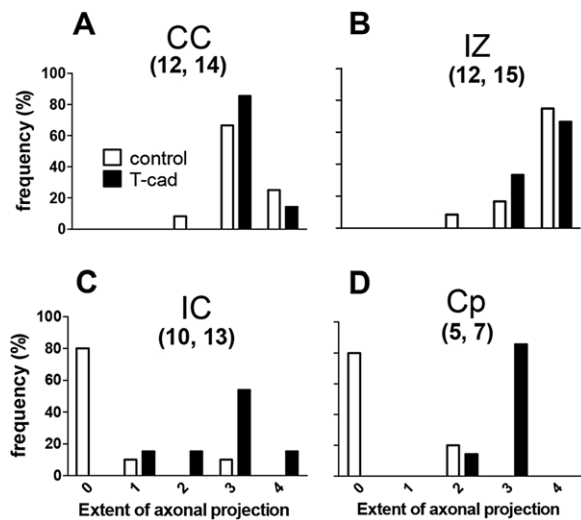


Fig. 8. Quantification of abnormal projections of T-cad-overexpressing axons. The number of labeled axonal segments in each brain region was classified into five categories based on the amount of axons labeled, with 0 indicating virtually no visible segments, 1–3 indicating up to 10–30 segments, and 4 indicating fasciculation of numerous axons (see Materials and Methods). The frequency distributions were studied in CC (A), IZ (B), IC (C) and Cp (D). White and black histograms represent the distributions in control and T-cad-overexpressing cortex, respectively. The numbers of samples (animals) used for control and T-cad-overexpressing cortex are indicated in parentheses.

in the CNS are not well characterized. In the present study, we first showed that T-cad is expressed in subsets of cortical and subcortical neurons in the developing brain. T-cad is primarily expressed in axons, but it is also expressed in cell bodies or dendrites at early developmental stages. Other adhesion molecules have also been shown to be expressed specifically in axons. In the developing cortex, L1 and TAG-1, immunoglobulin superfamily members, are expressed strongly in thalamic and corticofugal fibers, respectively (Wolfer et al., 1994; Fukuda et al., 1997; Fujimori et al., 2000; Ohya et al., 2004). Several studies have demonstrated more specific relationships between axon subtypes and molecular expression. Npn1, a receptor for semaphorin 3A (Sema3A), is expressed in callosal axons in the upper layers, which project to the contralateral hemisphere (Hatanaka et al., 2009; Niquille et al., 2009; Piper et al., 2009). Moreover, the adhesion molecule nectin is expressed exclusively in callosal axons that originate from deep-layer cells (Prandovszky et al., 2008; Molyneaux et al., 2009). Our present results reveal a new feature: T-cad, a member of the cadherin superfamily, is expressed in a subset of subcortically projecting axons, but not in callosal axons (Figs 2 and 4). Thus, multiple cell surface molecules, including T-cad, are expressed differentially in callosal and non-callosal axons.

T-cad expression is regulated not only spatially, but also temporally; it ceases at early postnatal stages in rat (Fig. 6), when major cortical circuits are established. In particular, T-cad is strongly expressed in late embryonic stages, when efferent and afferent fibers are accumulating in the IZ. Together with its spatial distribution, the temporal expression pattern implies that T-cad might be involved in pathway guidance in the IZ (see below). We also found that T-cad expression in the cortex increases again at late developmental stages (Fig. 6A), which is consistent with previous reports (Matsunaga et al., 2013). Previous studies also suggest that T-cad might play a distinct role in neural circuit formation, such as synaptic rearrangement (Zhong et al., 2004; Rivero et al., 2013).

Axonal adhesion by T-cad

To date, axon guidance molecules have been shown to aid in axonal fasciculation and pathway finding via attractive and repulsive properties (Satoda et al., 1995; Kawasaki et al., 2002; Cloutier et al., 2004; Imai et al., 2009; Jaworski and Tessier-Lavigne, 2012). Cell adhesion molecules play a more direct role in axon bundling through axon-axon interactions (Buttiglione et al., 1998; Marthiens et al., 2005; Schmitz et al., 2008). In chick CNS, several cadherins have been shown to affect pathway guidance (Treibert-Zimmermann et al., 2002). Chick tectofugal pathways contain several axonal populations, each of which projects to a distinct target and expresses particular cadherin family members. Moreover, ectopic cadherin expression in a given fiber type can cause an abnormal projection, indicating that cadherin family members contribute to the formation of axon bundles. Together with these previous findings, the present study strongly suggests that T-cad is involved in pathway formation of mammalian cortical circuits. This action of T-cad can be explained by its adhesion property (see below).

T-cad lacks a cytoplasmic domain that is present in other cadherin family members for connecting with the cytoskeleton; however, a calcium-dependent homophilic interaction has been suggested for T-cad in previous studies (Vestal and Ranscht, 1992; Sacristán et al., 1993; Ciatto et al., 2010). T-cad might, therefore, contribute to axonal fasciculation via this homophilic binding property. The result in cocultures of cortical neurons with L-cells supports this view (supplementary material Fig. S4). Moreover, such a homophilic binding property might also contribute to afferent and efferent pathway formation *in vivo* (Molnar et al., 1998a, 2012). However, we cannot exclude the possibility that ligand-receptor interactions between T-cad-expressing axons and adjacent cells could be involved in axonal pathway formation, as T-cad is known to act as a receptor molecule in the metabolic system (Hug et al., 2004; Denzel et al., 2010).

In the overexpression study described here, ectopically expressed T-cad caused a migration delay of the transfected cells (supplementary material Fig. S1). The exogenous expression was detected not only in the axons (Fig. 7C), but also in the cell bodies of delayed and aggregated cells (data not shown). The migration delay of T-cad-overexpressing cells might also be due to cell adhesion caused by ectopically expressing T-cad on cell bodies, as the expression on the cell bodies is more intense in the delayed cells than in the normally migrating upper layer cells.

Role of T-cad in axonal sorting during development

A prominent aspect of cortical axonal formation is the selection of mediolateral orientation. Callosal axons originating in the upper layers project medially in the IZ, whereas subcortically projecting axons, which express T-cad, are directed laterally towards the IC and the Cp. The present findings indicate that T-cad contributes to pathway formation of deep-layer cell axons. In addition, T-cad might also be involved in directed axon growth of these cells. Indeed, a proportion of T-cad-overexpressing upper layer cell axons turned laterally, ran in the upper part of the IZ (supplementary material Fig. S3C), then traveled in the IC and Cp (Fig. 7), which is the pathway for the corticospinal tract. Furthermore, originally subcerebral cortical axons were oriented toward the contralateral cortex by the knockdown (Fig. 9). The trajectory was also distinct from the original pathway of subcerebral cortical axons (Fig. 9I–K). Thus, it is likely that T-cad plays a role in both direction and pathway of subcortically projecting axons.

However, it is unlikely that T-cad is the only determinant of this laterally oriented axon growth because most T-cad-overexpressing

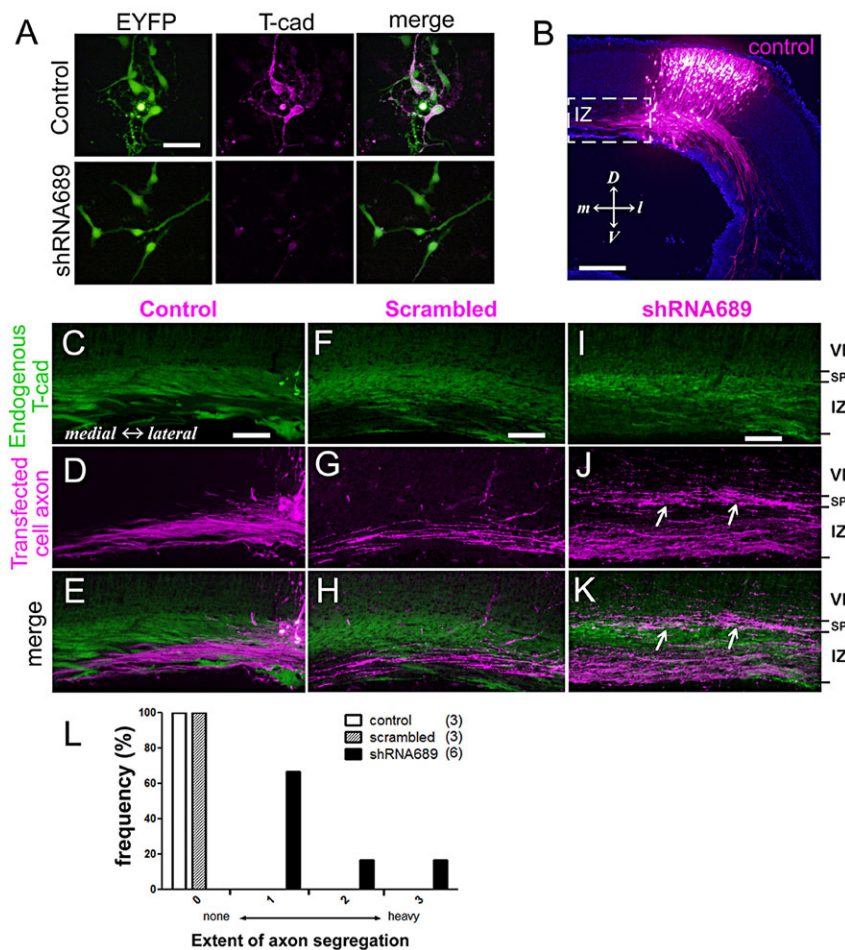


Fig. 9. Axonal projections of T-cad-downregulated deep layer neurons. (A) Suppression of T-cad by shRNA was confirmed in a primary cell culture of rat embryonic neocortical neurons. Cells were cotransfected with one of the shRNA vectors (pCAG-EYFP/mU6-Tcad689) and T-cad expression vector (pCAGGS/T-cad). (B) Deep-layer cell axons (magenta) projected both medially and laterally in normal brain. The section was counterstained with DAPI. (C–K) Higher magnification views of medially oriented deep layer axons in IZ (same positions as boxed area in B). Endogenous T-cad-expressing axons, immunostained by 6C9 mAb (green in C–K), occupy the upper part of the IZ, whereas medially oriented deep-layer cell axons (magenta) run in the lower part in control brain (C–E) and scrambled shRNA brain (F–H). (J) Part of axons originating from deep-layer cells transfected with the shRNA vector (piGENEmU6/Tcad689) abnormally traveled in the SP (arrows). As callosal axons never travel in this pathway, the abnormal projection must be due to T-cad-downregulated subcerebral fibers. Sections shown were prepared from P0 rat brains. (L) The amount of abnormal projections into the SP was classified into four categories (0–3). The numbers of animals are indicated in parentheses. Scale bars: 50 μ m in A; 250 μ m in B; 100 μ m in C,F,I.

upper layer cell axons extended medially in the IZ (arrowhead in Fig. 7B; supplementary material Fig. S3A). Taken together, our observations suggest that endogenous T-cad is involved in laterally oriented axon growth of subcerebral projection neurons. It has been shown that netrin-1 released by IC cells attracts axons of subcerebral projection neurons (Richards et al., 1997), and one of our previous studies has also demonstrated that graded expression of Sema3A contributes to medially directed axon growth of upper layer cells (Zhao et al., 2011). Such diffusible molecules might therefore cooperate with cell surface molecules such as adhesion proteins.

Does ectopic expression of T-cad alter the identity of upper layer cells? Our data indicate that this is unlikely, as upper layer cells overexpressing T-cad still exhibited expression of an upper-layer-specific gene, but not a deep-layer-specific marker (supplementary material Fig. S2). Moreover, T-cad knockdown did not change the laminar property (supplementary material Fig. S5D). This contrasts with the occurrence of cell fate conversion in response to forced expression of transcription factors (Arlotta et al., 2005; Lai et al., 2008). Moreover, the final laminar locations of cells that are destined for the upper layers were also unaffected by overexpression of T-cad (supplementary material Fig. S1B). T-cad might therefore be more important for fasciculation of subcortically projecting axons or axonal pathway guidance, but not differentiation of neuronal subtypes.

In summary, T-cad is expressed specifically in subcerebral projection neurons and is most likely to be involved in pathway formation during development. Differential expression of cell-surface

molecules such as T-cad might thus be an integral process in the construction of axonal pathways in the mammalian brain.

MATERIALS AND METHODS

Generation of monoclonal antibodies

mAbs were produced from rat brain homogenate according to a standard procedure (Fujita et al., 1982). Brain homogenate prepared from P0 Sprague-Dawley (SD) rats (Nippon Animal Company) was administered to an adult mouse (BALB/c, Nippon Animal Company). Two months later, the spleen was dissociated and fused with myeloma cells. The hybridomas were plated in 96-well dishes and selected on RPMI medium (Invitrogen) containing HAT (hypoxanthine, aminopterin and thymidine) and 10% fetal bovine serum (FBS). Each supernatant was subjected to immunohistochemical screening (see below).

Immunohistochemistry

Newborn rat brains were used for immunohistochemical screening. To study developmental changes in staining patterns for the selected mAb (6C9), brain sections were prepared from E15 to P4 rats. Brain sections of rats subjected to shRNA and of T-cad-overexpressing mice were prepared at the indicated stages. All animals were anesthetized and sacrificed in accordance with the guidelines laid down by animal welfare committees of Osaka University and Japan Neuroscience Society.

Whole brains were fixed in 4% paraformaldehyde/0.1 M phosphate buffer (PB). The tissue was further immersed sequentially in 10, 20 and 30% sucrose solution, and 10 or 20 μ m-thick sections were cut by cryostat. The sections were incubated with primary antibodies at 4°C overnight, then incubated with secondary antibodies for 2 h at room temperature, and finally mounted in an aqueous buffer (50% glycerol, 0.05 M PBS) containing diamidino-2-phenylindole (DAPI). The sections were observed with a

microscope (Axiophot, Zeiss), and the images were captured with a CCD camera (DP70, Olympus).

The following antibodies were used against the indicated proteins: T-cadherin (mouse, culture supernatant of hybridoma 6C9); Netrin-G1 (rabbit, 1:200; a generous gift from Dr Nakashiba, RIKEN-MIT Center for Neural Circuit Genetics); Tag-1 (rabbit, 1:2000; generated against Ig-fusion protein); Ctip2 (Abcam, ab18465; 1:100); Tbr1 (Abcam, ab31940; 1:100); EYFP [for rat sections (Invitrogen, A6455; 1:500); for mouse sections (Nacalai Tesque, 04404-84; 1:1000)]. The secondary antibodies used were Alexa Fluor 488-conjugated goat anti-mouse IgG (Invitrogen, A11029), Cy3-conjugated donkey anti-rabbit IgG (Millipore, AP182C) and Cy3-conjugated goat anti-rat IgG (Millipore, AP183C).

Purification and identification of antigen recognized by monoclonal antibody

To identify the antigen recognized by mAb 6C9, an immunoaffinity column was generated. First, the supernatant of cultured hybridoma was collected and the antibody was purified using a protein A column (Affi-Gel Protein A MAPS II kit, Affi-Gel protein A agarose, BioRad). The purified antibody (2 mg) was then immobilized on 1 ml of Affi-Gel Hz hydrazide gel (Affi-Gel Hz immunoaffinity kit, Bio-Rad) according to the manufacturer's instructions.

To obtain the antigen, brain homogenate was applied to the affinity column. P0 rat brains were homogenized in phosphate buffer (0.05 M PB, 0.5 M NaCl, pH 7.4) and centrifuged at 20,000 g, 4°C for 20 min. One gram of the pellet was ground in chilled acetone and then dried into powder. Two milliliters of an extraction buffer (0.05 M PB, 0.5 M NaCl, 1 mM EDTA, 10 mM CHAPS, protease inhibitor mix, pH 7.4) was added to the acetone powder, and it was then incubated at 4°C for 1.5 h. The extract was centrifuged at 100,000 g, 4°C for 30 min, and the collected supernatant was then applied to the affinity column. The column was washed five times with 1 ml of loading buffer (0.05 M PB, 0.5 M NaCl, 1 mM EDTA, 5 mM CHAPS, pH 7.4). The antigen was eluted with 0.15 M citrate buffer (pH 3.0) containing 5 mM CHAPS. Fractions containing the antigen were determined by Coomassie Brilliant Blue (CBB) staining of an SDS-polyacrylamide gel.

After SDS-PAGE, the antigen was blotted onto a nitrocellulose membrane with semi-dry blotting equipment. After blocking in 5% skim milk/TBS (20 mM Tris-HCl, 0.5 M NaCl, pH 7.5), the membrane was incubated with the primary antibody at 4°C overnight. Alkaline phosphatase-conjugated anti-mouse IgG secondary antibody (Nacalai Tesque, 01800-61) was used at 1:4000 dilution in 1% skim milk/TBST. The color reaction was developed in BCIP-NBT solution (Nacalai Tesque).

For amino acid sequence analysis, antigen eluted from the affinity column was concentrated tenfold with an Ultrafree-MC filter (5000 NMWL, UFC3LCC00, Millipore) and applied to SDS-PAGE. The protein was transferred onto a PVDF membrane (Immobilon-PSQ, Millipore) immersed in blotting buffer (25 mM Tris, 20% methanol, 0.05% SDS, 40 mM gamma-amino-n-caproic acid) using semi-dry blotting equipment. The antigen on the membrane was detected by CBB staining, and the visualized band was excised and subjected to N-terminal Edman sequencing (Protein Institute, Osaka University).

In situ hybridization

A fragment containing the full-length rat T-cad open reading frame (ORF) was obtained by RT-PCR from P0 rat brain RNA, and ligated into a pGEM-T Easy vector (Promega) (pGEM/T-cad). A fragment (nucleotides 543–1611 of rat T-cad, GenBank accession number: NM_138889) was subcloned to produce a template for *in vitro* transcription. The template was amplified by PCR using SP6 and T7 primers. A digoxigenin (DIG)-labeled RNA probe was synthesized using SP6 polymerase to produce the antisense probe (DIG-RNA Synthesis kit, Roche). The probe was purified with gel filtration columns (Quick Spin, Roche) and stored at –80°C.

In situ hybridization was performed according to previous work (Zhong et al., 2004). In brief, 10- μ m-thick cryosections were prepared from newborn rat brains. After refixation and acetylation, the sections were hybridized in a buffer containing 1 μ g/ml DIG-labeled RNA probe, at 60°C overnight. After high-stringency washes and blocking, the sections were incubated with alkaline phosphatase-conjugated anti-DIG antibody. Signals

were visualized with BM Purple (Roche). Nissl staining was performed on adjacent sections to determine neocortical layer structure.

Western blot analysis

Cortical tissue was collected from E15, E18, P0, P2, P4, P7, P14 and P35 rat brains and homogenized with ice-cold PBS, which contained protease inhibitors (cOmplete protease inhibitor cocktail, Roche). After centrifugation (~20,000 g) for 30 min at 4°C, the supernatant was discarded. The pellet was resuspended with PBS that contained the protein inhibitor. Protein concentrations were measured in a part of each sample (BCA protein Assay kit, Pierce); then, each sample was added to SDS-PAGE after 2 \times sample buffer had been added. The electrophoresis proteins were transferred to a PVDF membrane. The membrane was incubated with mAb 6C9 followed by incubation with HRP-conjugated secondary antibody (Nacalai Tesque, 01803-44; 1:10,000). Finally, T-cad bands were detected by a chemiluminescent method (ECL Prime Western blotting detection system, GE Healthcare).

In utero electroporation

To overexpress T-cad in cortical cells *in vivo*, a mammalian expression vector encoding rat T-cad was constructed. The full-length T-cad ORF was excised from pGEM/T-cad and ligated into the *EcoRI* site of pCAGGS vector to yield pCAGGS/T-cad. For generating T-cad-overexpressing animals, pCAGGS/T-cad was mixed with an expression vector encoding the fluorescent reporter EYFP (pCAGGS/EYFP) in order to trace transfected cells. The reporter construct alone was electroporated into control animals.

In utero electroporation was performed on E14.5 mouse embryos, according to previous studies (Fukuchi-Shimogori and Grove, 2001; Saito and Nakatsuji, 2001; Tabata and Nakajima, 2001). A pregnant ICR mouse (CLEA Japan) was deeply anesthetized, and plasmid solutions were injected into the VZ. Electroporation was performed at 30 V with five 50 ms pulses at intervals of 95 ms, using an electroporator (BEX, CUY-21).

To knockdown endogenous T-cad in rats, RNAi expression plasmids were constructed. Four target sequences were designed for generating short hairpin-type RNAs (shRNAs) and were then inserted into piGEMemU6 vector (Clontech) in order to be transcribed under the mouse U6 promoter. Three of the plasmids (piGEMemU6/Tcad1631, piGEMemU6/Tcad689 and pCAG-EYFP/mU6-Tcad689), which contained the shRNA sequence corresponding to nucleotides 1631–1651 (shRNA1631) and 689–707 (shRNA689) of rat T-cad mRNA (GenBank accession number NM_138889), were detected by immunocytochemistry (Fig. 9A) and western blotting in order to suppress T-cad expression in cultured cortical cells. The target sequence for shRNA689 is exactly the same as the shRNA, which was found to be free of an off-target effect in a previous study (Paradis et al., 2007). Scrambled shRNA sequence was designed by randomly shuffling the nucleotides of T-cad 689–707.

The shRNA constructs were introduced into cells destined to become deep layer neurons (E15.0 SD rat embryos) by *in utero* electroporation, as described above. The expression of fluorescent reporter was achieved by co-electroporation with pCAGGS/EYFP or by using dual promoter vector (pCAG-EYFP/mU6-Tcad689) to trace shRNA-transfected cells. Another fluorescent reporter construct (pCAGGS/DsRedII) was used for a control.

Coculture of cortical neurons with L-cells

To examine the role of T-cad in cortical axon growth, dissociated cortical cells expressing T-cad were cocultured with T-cad-expressing L-cells. L-cells were plated on poly-ornithine-coated wells (~10,000 cells per 10 mm-diameter well). Twelve to 24 h after plating, L-cells were transfected with the pCAGGS/T-cad and pCAGGS/DsRedII expression plasmids by lipofection (Lipofectamine 2000, Invitrogen). For dissociated cortical cells, E15 rat cortex was dissected and dissociated with trypsinization. These cortical cells were incubated for 1 h in a solution containing pCAGGS/T-cad and pCAGGS/EYFP expression plasmids with lipofectamine. Then, these transfected cortical cells were added to the transfected L-cell cultures (~50,000 cells per well). After 2 days the cultures were fixed and subjected to immunohistochemistry with anti-GFP and anti-DsRed (rabbit anti-RFP, 1:500; PM005, Medical & Biological Laboratories). For the control experiment, only pCAGGS/EYFP and pCAGGS/DsRedII were used to transfect cortical cells and L cells, respectively.

Quantitative analysis of axon growth

To analyze cortical cells that overexpress T-cad, 20 μm -thick cryosections were prepared from animals that had been subjected to *in vivo* electroporation. Ectopic T-cad protein was detected by immunohistochemistry using the anti-T-cad antibody (6C9 mAb), which does not react with endogenous mouse T-cad. In the knockdown study, 6C9 mAb was used to detect endogenous rat T-cad.

To quantify axonal projection of T-cad-overexpressing cells or shRNA-transfected cells, the axonal projection in a given brain region was categorized into five groups for P0–P6 brain sections, according to the following criteria. Mark 0, virtually no axonal segments; Mark 1, up to 10 segments; Mark 2, up to 20 segments; Mark 3, up to 30 segments; Mark 4, fasciculation of numerous axons. The mark value given for each animal was determined from 1–4 sections.

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Competing interests

The authors declare no competing financial interests.

Author contributions

Y.H. and N.Y. planned this study and prepared the manuscript. Y.H. performed most experiments. H.Z. contributed to the quantitative analysis. S.N. performed amino acid sequencing of antigen for mAb 6C9. H.K. and K.T. contributed to the generation of antibodies and the study of T-cad expression profiles.

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Supplementary material

Supplementary material available online at <http://dev.biologists.org/lookup/suppl/doi:10.1242/dev.108290/-/DC1>

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