

Netrin-4 regulates thalamocortical axon branching in an activity-dependent fashion

Yasufumi Hayano^{a,b}, Kensuke Sasaki^a, Nami Ohmura^c, Makoto Takemoto^a, Yurie Maeda^a, Toshihide Yamashita^b, Yoshio Hata^c, Kazuhiro Kitada^d, and Nobuhiko Yamamoto^{a,1}

^aLaboratory of Cellular and Molecular Neurobiology, Graduate School of Frontier Biosciences, Osaka University, Suita, Osaka 565-0871, Japan; ^cDivision of Integrative Bioscience, Institute of Regenerative Medicine and Biofunction, Tottori University Graduate School of Medical Sciences, 86 Nishicho, Yonago 683-8503, Japan; ^bDepartment of Molecular Neuroscience, Graduate School of Medicine, Osaka University, Suita, Osaka 565-0871, Japan; and ^dDepartment of Biological Sciences, Graduate School of Science, Hokkaido University, Kita-ku, Sapporo 060-0810, Japan

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Axon branching is remodeled by sensory-evoked and spontaneous neuronal activity. However, the underlying molecular mechanism is largely unknown. Here, we demonstrate that the netrin family member netrin-4 (NTN4) contributes to activity-dependent thalamocortical (TC) axon branching. In the postnatal developmental stages of rodents, *ntn4* expression was abundant in and around the TC recipient layers of sensory cortices. Neuronal activity dramatically altered the *ntn4* expression level in the cortex in vitro and in vivo. TC axon branching was promoted by exogenous NTN4 and suppressed by depletion of the endogenous protein. Moreover, *unc-5* homolog B (*Unc5B*), which strongly bound to NTN4, was expressed in the sensory thalamus, and knockdown of *Unc5B* in thalamic cells markedly reduced TC axon branching. These results suggest that NTN4 acts as a positive regulator for TC axon branching through activity-dependent expression.

development | neocortex | axon guidance | neuronal wiring

Axon branching is an essential process to determine the final pattern of neuronal connections. Previous studies have demonstrated that axon branching is controlled not only by axon guidance-related molecules (1–6) but also by neuronal activity, such as firing and synaptic activity (7–10). However, how neuronal activity is converted into the molecular signals that underlie axon branching is still largely unknown.

The thalamocortical (TC) projection is a well-characterized system in which to address this issue. TC axons originating from sensory thalamic nuclei form elaborate arbors, primarily in layer 4 of the neocortex (11). Lamina-specific axon branching occurs from the onset of development and is universal in the mammalian cortex (12–17), indicating that a rigid developmental program is predominant for laminar specificity. TC axon branching is also known to be modified by neuronal activity. In the visual system, geniculocortical axon arbors can be remodeled drastically by manipulating visual experience and cortical-cell activity (18–22). A similar feature has been demonstrated in the somatosensory system. In mutant mice in which synaptic transmission or downstream signaling mechanisms are disrupted, TC axon arbors are affected primarily along the tangential axis whereas their laminar pattern is not obviously influenced (23–26). We have also shown in vitro that the loss of firing and synaptic activities substantially suppresses TC axon branching in the target layer (27). All of these findings imply the existence of a target-derived, branch-promoting molecule whose expression is regulated by neuronal activity. In this study, we attempted to identify this hypothetical molecule and to reveal the molecular mechanism of its action.

Results

Identification of a Target-Derived Molecule Whose Expression Is Regulated by Neural Activity. Because we have shown previously that spontaneous activity promotes TC axon branching in cocultures of the thalamus and cortex (27, 28), we attempted to identify gene(s) whose expression is down-regulated in cortical-

slice cultures by blocking firing and synaptic activity. Based on prior molecular screening and gene-expression analyses in the developing cortex, *cadherin-6* (*cdh6*), *ephrin-A5* (*efna5*), *netrin-4* (*ntn4*), *semaphorin7a* (*sema7a*) and *kit ligand* (*kitlg*) were selected as candidate genes that are expressed in and around the TC recipient layer and may directly affect axon behavior (29–33). Their expression in cortical explants was examined using quantitative PCR analysis after 2 wk in culture, when TC axons form extensive branches (27, 34, 35). *ntn4* expression was significantly reduced by chronic application of tetrodotoxin (TTX, 100 nM), a voltage-gated sodium channel blocker (relative mRNA expression compared with the untreated control; 0.69 ± 0.06 , $n = 4$, $P < 0.05$, Dunnett test) (Fig. 1A and Fig. S1). DL-2-amino-5-phosphonovalerate (APV), an antagonist of the NMDA-type glutamate receptor (NMDAR), also decreased *ntn4* expression (relative mRNA expression; 0.62 ± 0.03 , $n = 3$, $P < 0.05$, Dunnett test) (Fig. 1A). These results suggest that firing and synaptic activity are required for *ntn4* expression in the cortex. In contrast, transcripts of the other candidate genes were not reduced by activity blockade (Fig. S1); indeed, the expression levels of three genes increased with one or the other of the pharmacological treatments.

Developmental Expression of *ntn4*. As a netrin family member, NTN4 is thought to be involved in cellular survival in the CNS, but its expression is not well-characterized in the developing brain (33, 36, 37). We investigated the expression pattern at postnatal developmental stages, using transgenic rats in which the β -galactosidase (β -gal) coding region was inserted into the

Significance

The environmental control of neuronal wiring is one of the most intriguing issues in neuroscience. However, the molecular mechanisms are largely unknown. Here, we demonstrate that the expression of the netrin family member netrin-4 (NTN4) is activity-dependent in the developing cortex and promotes terminal branching of thalamocortical axons. Evidence further shows that *unc-5* homolog B (*Unc5B*), a putative receptor of NTN4, is expressed in the developing thalamus and mediates NTN4 signaling. These results suggest that NTN4 is the key molecule that underlies activity-dependent axon branch formation in neocortical circuits.

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¹To whom correspondence should be addressed. Email: nobuhiko@fbs.osaka-u.ac.jp.

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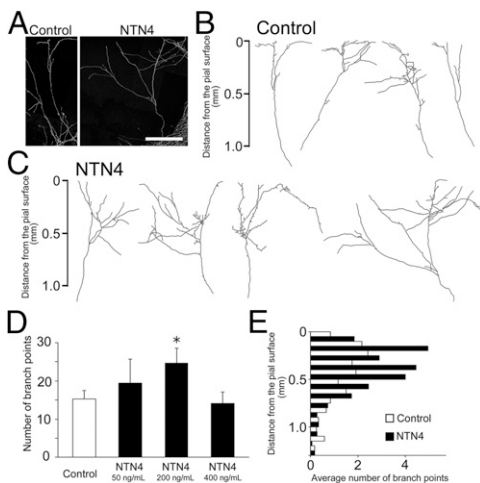


Fig. 3. TC axon branching is promoted by exogenous NTN4. (A) Representative individual TC axons after 2 wk in vitro in the presence or absence of NTN4. Axon branching increased after NTN4 application (200 ng/mL) (C), compared with control (B). (Scale bar: 0.5 mm.) (D) Quantification of branch numbers in control ($n = 22$) and NTN4-treated ($n = 12$ for 50 ng/mL, $n = 18$ for 200 ng/mL, and $n = 14$ for 400 ng/mL) cultures. * $P < 0.05$, Dunnett test. (E) Laminal distribution of branching points in untreated and treated cultures. Histograms show the mean number of branching points in each bin (0.1 mm) as a function of cortical depth (22 axons for control and 18 axons for NTN4 application).

increased the total branch length (3.4 ± 0.3 mm for control; 4.9 ± 0.6 mm for NTN4) and branch width (0.54 ± 0.06 mm for control; for 1.1 ± 0.19 mm for NTN4). The majority of branching points were found in the upper layers, including the presumed layer 4, irrespective of the presence or absence of exogenous NTN4 (Fig. 3E).

To examine whether NTN4 directly affects TC axon branching, we applied NTN4 to dissociated thalamic cell cultures (Fig. S3A and B). As a result, the number of branches from the longest processes, which were shown to be axons in the previous study (39), was significantly increased by NTN4 application (200 ng/mL) (Fig. S3C). The total branch length was also increased (Fig. S3D). This result strongly suggests that NTN4 acts on TC axons directly as a branch-promoting factor.

An intriguing possibility is that NTN4 acts as a mediator of activity-dependent branching. To test this possibility, the effect of exogenous NTN4 was studied in the presence of TTX (Fig. S4). As shown previously, the activity blockade inhibited axon branching in culture (27), but the addition of NTN4 rescued the phenotype considerably (Fig. S4). Therefore, NTN4 can promote axon branching downstream of neuronal activity.

Endogenous NTN4 in the Cortex Is Required for TC Axon Branching.

To investigate the role of endogenous NTN4 in TC axon branching, geniculocortical axon arbors were studied in 3-wk-old wild-type and *ntn4* mutant rats by injecting an axon tracer, biotinylated dextran amine (BDA), into the dorsal part of the lateral geniculate nucleus (Fig. 4A). Although numerous geniculocortical axons were labeled by this method, we were able to trace individual axonal fragments across layer 4 of the primary visual cortex (Fig. 4B and C). As shown in Fig. 4D, many branches had emerged from individual axon fragments in layer 4 of the wild-type cortex, but branching was less prominent in the KO. Quantitative analysis demonstrated that the number of branches emerging from each axon fragment across layer 4 was significantly smaller in the KO (Fig. 4E) (24.5 ± 8.5 for wild-type, $n = 10$; 14.5 ± 7.6 for *ntn4* KO, $n = 12$, $P < 0.01$, Student *t* test). Likewise, the total branch length of each axon fragment was also reduced in the KO (Fig. 4F) (866 ± 386 μ m for wild-type, $n = 10$;

564 ± 217 μ m for *ntn4* KO, $n = 12$, $P < 0.05$, Student *t* test). Thus, arbor formation in geniculate axons was suppressed in *ntn4* KO.

The TC projection in the somatosensory cortex in wild-type and *ntn4* KO was also studied by immunohistochemistry with an antibody against the serotonin transporter (5-HTT), a specific marker of developing TC axons. Because 5-HTT expression in thalamic axons is negligible in 2-wk-old animals, this analysis was performed at P10. At this stage, 5-HTT staining clearly showed barrel structures in the wild-type (Fig. S5A) (40, 41). Although similar 5-HTT staining was observed in the *ntn4* KO, the intensity was much weaker (Fig. S5B). Quantitative analysis of 5-HTT staining confirmed that the intensity of 5-HTT in layer 4 was significantly lower in the *ntn4* KO than in wild-type (ratio of peak value in KO to wild-type; 0.70 ± 0.12 , $n = 3$, $P < 0.05$, Student *t* test) (Fig. S5C and D). This result also suggested that TC axon branching is less complex in the KO in vivo.

To specify the effect of NTN4 knockdown in the cortex, the action of endogenous NTN4 was further investigated by examining individual TC axon branching in chimeric cocultures of wild-type thalamus and *ntn4*-deficient cortex (Fig. 5A). After 2 wk in culture, EYFP-labeled TC axons were found to have simpler branching in the *ntn4*-deficient cortical explants (Fig. 5A). The number of branch points in the KO cortex was reduced considerably, compared with that in the wild-type cortex (15.3 ± 2.2 , $n = 22$ for wild-type; 4.5 ± 0.8 , $n = 47$ for KO; $P < 0.01$, Dunnett test) (Fig. 5B and Table S1). Likewise, the total branch length (3.4 ± 0.3 mm for wild-type; 1.6 ± 0.1 mm for KO) and branch width (0.54 ± 0.06 mm for wild-type; 0.19 ± 0.03 mm for

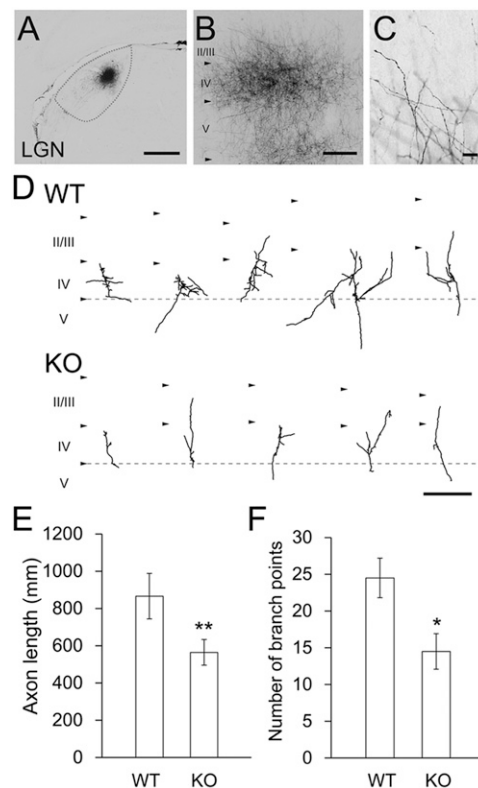


Fig. 4. Endogenous NTN4 regulates TC axon branching in vivo. In vivo labeling of single TC axons. (A) BDA injection site in the LGN at P19. (B) Labeled TC axons in visual cortex. (C) Higher-magnification image of TC axon terminals. (Scale bars: A and B, 0.5 mm; C, 0.1 mm.) (D) Typical examples of reconstructed TC axon arbors of wild-type (Upper, WT) and *ntn4* KO animals (Lower, KO). All examples represent single arbors in layer 4 of the V1 cortex in 50- μ m-thick coronal sections. Quantitative analysis of TC axon length (E) and the number of axon branching point (F). * $P < 0.05$, ** $P < 0.01$, Student *t* test.

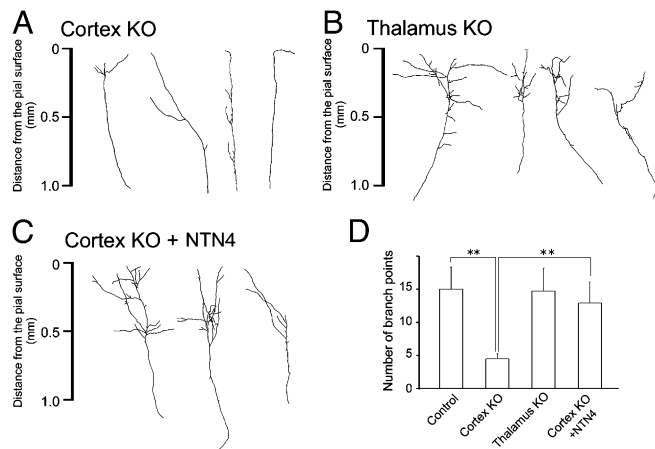


Fig. 5. NTN4 is required in cortical explants for TC axon branching. (A–C) Typical examples of individual TC axons are shown in chimeric cocultures of wild-type and *ntn4* KO. (A) Wild-type thalamus was cocultured with *ntn4* KO cortex. (B) *Ntn4* KO thalamus was cocultured with wild-type cortex. (C) NTN4 recombinant protein (200 ng/mL) was added to the culture medium in cocultures of wild-type thalamus and the KO cortex. (D) Quantification of branching points in each case. ** $P < 0.01$, Dunnett test.

KO) were significantly reduced in the *ntn4*-deficient cortex (Fig. 5A and B and Table S2). This decrease of branch complexity is very similar to that observed in *ntn4* KO rats in vivo (Fig. 4F). In contrast, branch complexity was unaffected in cocultures of *ntn4*-deficient thalamus and wild-type cortex (number of branch points, 14.7 ± 3.4 , $n = 22$) (Fig. 5B and D and Table S2). Moreover, addition of NTN4 recombinant protein to the culture medium rescued the phenotype of *ntn4* KO (number of branch points, 11.7 ± 3.1 ; $n = 11$) (Fig. 5C and D and Table S2). These in vivo and in vitro results clearly indicated that endogenous NTN4 in the cortex contributes to normal TC axon branching.

Unc5B Acts as a Putative Receptor of NTN4. We studied the expression in the thalamus of putative receptors that reportedly bind to NTN4: *deleted in colorectal carcinoma* (*dcc*), *neogenin-1* (*neo1*), and *unc5* family (36, 42). At P7, *dcc*, *neo1*, and *unc5b* were strongly expressed in the ventrobasal (VB) thalamus and the dorsal part of the lateral geniculate nucleus (dLGN) (Fig. 6A and Fig. S64, Upper). *unc5b* expression was sustained at P14 although the expression of *dcc* and *neo1* became weaker and more diffuse (Fig. 6A and Fig. S64, Lower). Thus, it is likely that developing sensory thalamic cells express receptor molecules that can respond to NTN4.

Receptor binding to NTN4 was examined by a cell surface binding assay, in which HEK293T cells expressed one of the above three candidate receptors. His-tag NTN4 was found to bind strongly to Unc5B-expressing cells, but to neither DCC- nor Neo1-expressing cells (Fig. S6C).

The role of endogenous Unc5B in TC axon branching was further studied using organotypic coculture preparations. Thalamic cells were cotransfected with an *unc5b* shRNA-expressing plasmid and the *eyfp* plasmid, and TC axon branching was observed after 14 d in culture. Knockdown of endogenous Unc5B significantly suppressed branch number, compared with scramble shRNA (Fig. 6B, C, and E) (3.8 ± 0.6 , $n = 24$ for shRNA; 15.8 ± 4.8 , $n = 16$ for the scrambled shRNA, $P < 0.05$, Dunnett test). Moreover, cotransfection with an shRNA-resistant *unc5b* rescued the phenotype (Fig. 6D and E) (12.2 ± 3.1 , $n = 18$ for resistant *unc5b* with shRNA, $P < 0.05$, Dunnett test). In contrast, transfection of *dcc* shRNA did not decrease TC axon branching (13.4 ± 3.7 , $n = 8$) (Fig. 6E). Thus, it is likely that Unc5B is

expressed in sensory thalamic cells and contributes to axon branching as a NTN4 receptor.

Discussion

The present study indicates that NTN4 is a branch-promoting molecule that is expressed in the sensory cortex with activity dependence. Previous observations led us to hypothesize that branch-promoting activity for TC axons is distributed in the TC recipient layer and is up-regulated by neuronal activity (27, 43). Although it had previously shown that guidance molecules and neuronal activity work together to pattern precise connections in the retino-tectal projection (44–47), the molecular basis of this cooperation was unresolved. The present findings clearly demonstrate the existence of a molecule, NTN4, that combines both elements.

NTN4 has been shown to play an important role in angiogenesis (42, 48), lung branching (49), and salivary gland formation (50). In these systems, NTN4 contributes to morphogenesis via mechanisms that also pertain to axon guidance (36, 37, 42) although its action can be both stimulatory and inhibitory. The present study has added a previously unidentified aspect, neuronal activity dependence, to the conventional function of NTN4. What neuronal activity is required for *ntn4* expression? As shown by mRNA expression in the pharmacological blockade, both firing and synaptic activity are likely to be necessary for *ntn4* expression. Consistent with this notion, our previous work has shown that the same pharmacological treatments suppress TC axon branching in coculture preparations (27).

To date, several molecular mechanisms in pre- and post-synaptic cells have been demonstrated to regulate axon branching (1–6, 51–54). Our results show that NTN4 synthesized by cortical cells has branch-promoting activity for TC axons in the mammalian brain. The fact that exogenous NTN4 slightly but significantly promoted axon branching in the upper layers (Fig. 3) implies that the effect of NTN4 may be more prominent for the formation of terminal axon branches.

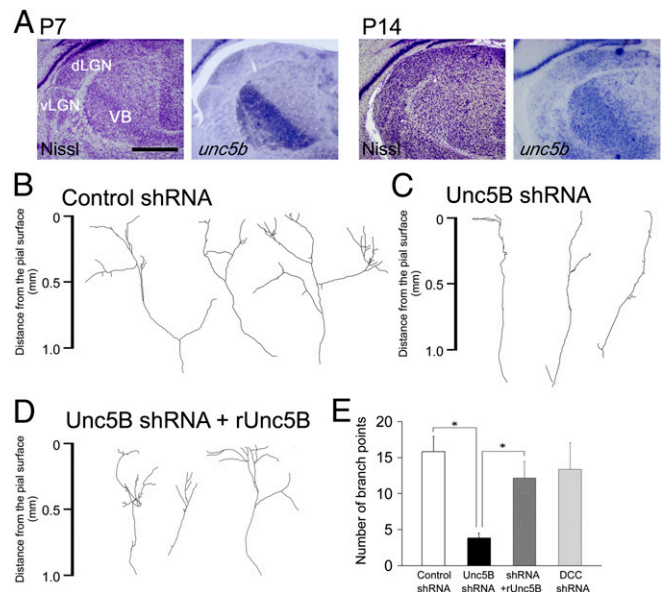


Fig. 6. Unc5B is involved in TC axon branching by being expressed in the thalamus. (A) Expression pattern of *unc5b* was examined at P7 and P14 by in situ hybridization and compared with the distribution of thalamic nuclei as shown by Nissl staining. (Scale bar: 1 mm.) (B and C) Typical examples of TC axons transfected with control shRNA (B) and with *unc5b* shRNA (C). (D) TC axons cotransfected with *unc5b* shRNA and shRNA-resistant *unc5b* (*runc5b*). (E) Quantification of branching points in B–D and TC axons transfected with *dcc* shRNA. vLGN, the ventral part of the LGN. * $P < 0.01$, Dunnett test.

Our present result further shows that Unc5B is expressed in sensory thalamic cells and is involved in TC axon branching. In retinotectal axon branching, DCC mediates the netrin-1-induced branch-promoting signal (55). Similarly, growth of TC and callosal axons at the embryonic stages is promoted by netrin-DCC signaling (56–59). However, the present data show that DCC is not strongly expressed in the thalamus at P14, and its binding to NTN4 is substantially weaker than that of Unc5B (Fig. 6). Thus, it is likely that Unc5B acts as a mediator for NTN4-induced branch-promoting activity, perhaps by activating of RhoA (60, 61). The expression of *unc5b* is also found in upper layer cells of the developing cortex (Fig. S6), suggesting the possibility that neurite growth of these neurons can be promoted by NTN4. Such growth might strengthen synaptic connections and increase neuronal activity in local circuits, which can in turn further enhance TC axon branching (10). However, we emphasize that NTN4 promotes branch formation via direct interaction with TC axons, due to the increase in branch formation of dissociated thalamic cells after NTN4 application (Fig. S3). This view is further supported by the inhibition of axon branching in *unc5b* knockdown cells (Fig. 6).

The developmental characteristics of *ntn4* expression imply a specific role in TC axon branching. The fact that its expression in rodents is stronger during postnatal 2–4 wk suggests that NTN4 does not contribute to initial branch formation of TC axons because fundamental branching is established by the end of the first postnatal week (12, 16, 17, 23, 40). This temporal expression is considered to depend on not only neuronal activity but also a developmental program, considering that spontaneous firing and sensory-evoked activity are present in the cortex even during the first postnatal week (62–65). Similarly, the expression of brain-derived neurotrophic factor (BDNF), which can affect axon branching (7, 66), is also known to depend on neuronal activity (67) but is regulated developmentally (68, 69). Indeed, *bdnf* expression begins to increase in 2- to 3-wk-old rat cortex (68). Thus, it is likely that NTN4 is involved in activity-dependent TC axon branching in the juvenile stages of cortical development.

A series of evidence has demonstrated that activity-independent mechanisms are also responsible for TC axon branching (70–72). It has been reported that TC axons begin to form branches in *snap-25* KO in which synaptic transmission is disrupted (73). In accordance with this finding, SEMA7A acts as an early branch-promoting factor through activity-independent expression (Fig. S1) (32). EFNA may also work together for initial TC axon branching regardless of neuronal activity (74). Taken together, multiple branch-promoting molecules including NTN4 are expressed during different developmental phases and are likely to contribute to axon branching in distinct contexts (39).

In summary, NTN4 is expressed in a target- and activity-dependent fashion to contribute to TC axon branching in the developing sensory cortex. We propose that NTN4 is a key molecule that underlies activity-dependent neural circuit formation.

Materials and Methods

Animals. Sprague–Dawley (SD) rats were used for organotypic cocultures of the thalamus and cortex. *ntn4* mutant rats generated by transposon-tagged mutagenesis were used for gene expression and loss-of-function analyses (38). All experiments were performed according to the guidelines laid down by the animal welfare committees of Osaka University, Tottori University, and the Japan Neuroscience Society.

Organotypic Coculture of Thalamus and Cortex. Cocultures of the cortex with thalamic explants were prepared as described previously (34). In brief, the dorsal thalamic region was dissected from embryonic day (E) 15 rat embryos, and cortical slices were dissected from the visual and somatosensory cortex of P1 to -2 rats. A thalamic block and a cortical slice were placed on a membrane filter and cultured for 2 wk.

Axon Labeling by Electroporation and Image Analysis. To label individual thalamic axons in the cortical explants, a small number of thalamic cells were transfected with an *eyfp* plasmid as described previously (27). See *SI Materials and Methods* for details.

β-Galactosidase Staining. Whole brains or cultured slices were fixed with 4% (wt/vol) paraformaldehyde in 0.1 M phosphate buffer for 3–4 h, followed by cryoprotection with 15% and 30% (wt/vol) sucrose in 0.01 M phosphate buffer with saline. The frozen tissues were then cut into 50-μm coronal sections with a cryostat. After two washes with 0.01 M phosphate buffer containing 2 mM MgCl₂, the sections were incubated with a staining solution (1 mg/mL X-gal, 5 mM potassium ferricyanide crystalline, 5 mM potassium ferricyanide trihydrate, 2 mM MgCl₂ in 0.01 M phosphate buffer with saline) at 37 °C for a few days. The sections were then treated in 50%, 70%, 95%, and 100% (vol/vol) ethanol, and xylene, and finally embedded. Adjacent sections were subjected to Nissl staining with cresyl violet.

Dark-Rearing Conditioning. NTN4 heterozygotes (Tp/+) were reared in darkness from birth. Exchange of food or cages was performed using night scope. At P21, animals were either deeply anesthetized in darkness and killed as dark-reared animals or exposed to the light for the next 24 h, and then killed as the light-reared group. As a control, animals were reared under 12 h:12 h light-dark cycles.

Tracer Injection and Morphological Analysis of Labeled Axons. Tracer injection and morphological analysis of labeled axons were as described previously (18). In brief, a glass microelectrode filled with a solution containing BDA (Molecular Probe) was positioned stereotaxically to locate a clear visual response from the dorsal part of the LGN, and BDA was injected iontophoretically. After several days, the animals were euthanized with an overdose of Nembutal and perfused transcardially with cold saline and 4% paraformaldehyde in 0.1 M phosphate buffer. The brain was removed and postfixed overnight. The brains were cut into 50-μm-thick sections and subjected to the standard ABC method. All sections were mounted on gelatinized slides. All of the injection sites were confirmed to be localized in the LGN. As many BDA-filled axonal fragments as possible that could be followed individually across layer 4 of the primary visual cortex were selected and traced with the aid of a computer graphic system (NeuroLucida; Microbrightfield). The number of branch points and total branch lengths were measured for each axon fragment.

Knockdown of Endogenous Unc5B Expression. To knock down endogenous Unc5B expression, short hairpin-type RNA (shRNA)-expressing plasmids were constructed. Three target sequences were designed for generating shRNAs and were inserted into the piGEMemU6 vector (Clontech). A plasmid that contains the target sequence corresponding to nucleotides 486–506 of rat *unc5b* mRNA was used in this study. shRNA-resistant *unc5b* (*rUnc5B*) sequence was generated by using PCR with KOD-plus DNA polymerase (Toyobo) and was inserted into the pcDNA3.1 vector.

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