Supporting Information

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SI Materials and Methods

Gene-Expression Analysis. After 2 wk in vitro, the cortical and thalamic explants were separated with scissors. Subsequently, mRNAs were extracted from each explant (PureLin RNA Mini; Invitrogen). After cDNA synthesis (High-capacity cDNA RT Kit; Roche), gene expression was quantified by TaqMan Gene Expression Assay (Applied Biosystems). Rat GAPD was used as an endogenous control to normalize gene expression (Applied Biosystems). To amplify specific sequences of interested genes, the following primer pairs and a universal probe were used: for *ntn4*, 5′-CCCATGTACTGGCGGAGA-3′ and 5′-GCGGAGGTTGGTGATCTTC-3′; for *sema7a,* 5′-GCTCAATGTATCCCGAGTGG-3′ and 5′-GCTTTCAGGAAGGTGTTCCA-3′; for *cdh6,* 5′-CCGTGAACATCACACTGACA-3′ and 5′- ACTCGGGAGTCTTAAACTGATAGG-3′; and for *kitlg*, 5′-TTAGGAATGACAGCAGTAGCAGTAATA-3′ and 5′-AAATGAGAGCCGGCAGTG-3′. For *efna5*, a custom-designed primer pair and probe were used (assay ID Rn00588118_m1; Applied Biosystems).

Axon Labeling by Electroporation and Image Analysis. To label thalamic axon, a plasmid containing the coding region of enhanced yellow fluorescent protein (EYFP) (pCAGGS:EYFP, 1.5 μg/μL) was applied to the thalamic explants, and electrical pulses (five to seven trains of 200 square pulses of 1 ms duration at 200 Hz, 400–500 μA) were then delivered through a glass micropipette (tip diameter of 150–200 μm). EYFP-labeled axons were observed by confocal microscopy (MRC-600; Bio-Rad and C2; Nikon) with a filter set for fluorescein isothiocyanate (excitation, 488 nm; emission long-pass filter, 515 nm). Images were collected with 10× and 40× objective lenses at 1- to 5-μm steps to obtain the entire axon arbors (2–20 optical sections).

Individually distinguishable axons were drawn using ImageJ software. All measurements of axonal morphology, such as branch length, were performed using ImageJ. Short processes (<5 μm) from the axon shaft were excluded from the analysis.

Dissociated Cell Culture of Thalamic Neurons. Dissociated cell culture of thalamic neurons was prepared as described previously (1). In brief, blocks of the dorsal thalamus were dissected from embryonic day 15 (E15) rat embryos and minced thoroughly. The tissues were washed extensively with Dulbecco's PBS and incubated in 0.1% trypsin-containing Dulbecco's PBS. After trituration, dissociated thalamic cells were plated at 5,000 cells/cm^2 on poly-L-ornithine–coated dishes (2 cm^2 per well) in DMEM/F12 with B27 supplement (Invitrogen). The recombinant protein, mouse netrin-4 (NTN4) (200 ng/mL; R&D Systems) was applied to the culture medium 1 d after cell plating. After 4 d culture, cells were fixed with 4% paraformaldehyde in 0.1 M PBS. Fixed cultures were washed three times with 0.05 M PBS and incubated with 0.05 M PBS containing 5% (vol/vol) normal goat serum and 0.1% Triton X-100 for 30 min. Then, they were incubated with 0.05 M PBS containing 5% normal goat serum, 0.1% Triton X-100 and mouse anti-acetylated-tubulin (Ac-Tub) antibody (1:7,500; Sigma) overnight at 4 °C. After three washes, the cultures were incubated with FITC-conjugated anti-mouse IgG (FI-2000, 1:300; Vector Laboratories Inc.) for 3 h.

Pharmacological Treatments and Protein Applications. To suppress firing and synaptic activity, either DL-2-amino-5-phosphonovalerate (APV) (100 μM; Tocris) or tetrodotoxin (TTX) (100 nM; Seikagaku-Kogyo) was applied during the second week in culture. To increase neuronal activity, high potassium solution (50 mM) was applied to the culture for 3 h. The recombinant NTN4 (200 ng/mL) was applied to the culture medium during the second week in culture. Drug- or recombinant protein-containing media was exchanged every other day.

Immunohistochemistry. Coronal cryosections (50 μm thickness) were prepared from *nnt4*-homozygous animals and their wild-type littermates. After two washes with 0.01 M PBS, the sections were treated with a blocking solution (0.01 M PBS containing 2% BSA and 0.25% Triton X-100) for 2 h and then incubated with a rabbit polyclonal antibody against 5-HTT (1:2,000; Calbiochem) at 4 °C for 2 d. After three washes, the sections were incubated with Cy3-conjugated secondary antibody (1:400; Chemicon) at 4 °C for 1day. The sections were washed extensively and mounted with a glycerol-containing buffer.

In Situ Hybridization. In situ hybridization was performed with digoxigenin (DIG)-labeled cRNA probes of *deleted in colorectal carcinoma* (*dcc*), *neogenin-1* (*neo1*), *unc5b*, and *unc5c*, as described previously (2). The following sequences were used for these RNA probes: 3,515–4,420 bp of *dcc* (×85788), 3,495–4,544 bp of *neo1* (Y09535), 1,614–2,030 bp for *unc5b* (NM_029770), and 1,297–1,911 bp of *unc5c* (NM_009472). After fixation, the brains were cut into 20-μm coronal sections with a cryostat. The sections were subjected to refixation, acetylation, and prehybridization followed by hybridization with a solution containing the DIG-labeled probe (1 μg/mL) at 60 °C overnight. After extensive washes with high stringency buffers, the sections were incubated overnight at 4 °C with alkaline phosphatase-conjugated anti-DIG antibody (1:2,000; Roche). After washing, the color reaction was carried out at room temperature with BM Purple (Roche). The reaction was terminated by immersing the fixative. Finally, the sections were immersed in 70%, 80%, 90%, and 100% (vol/vol) ethanol, and xylene, and then embedded. Adjacent sections were subjected to Nissl staining with cresyl violet.

Cell Surface Binding Assay. The cell surface binding experiment was performed as described previously (3). Before transfection, human embryonic kidney 293T (HEK293T) cells were plated on a poly-L-ornithine–coated dish. Transfection of the following expression constructs was performed using Lipofectamine 2000 (Invitrogen): *dcc* (a generous gift from Tim Kennedy, McGill University, Quebec), *neo1* (a generous gift from Eric R. Fearon, University of Michigan, Ann Arbor), pcDNA3-*unc5b-venus* (encoding rat UNC5B fused with Venus, a generous gift from Kyonsoo Hong, New York University, New York), and pEGFP-N1 (TaKaRa Bio). Forty-eight hours after transfection, the cells were washed with PBS and then incubated with 2 μg/mL recombinant polyhistidine-tagged mouse NTN4 protein (R&D Systems) in PBS supplemented with 1% normal goat serum and 2 μg/mL heparin sodium at 37 °C for 1 h. After washing with PBS, the cells were fixed with 4% paraformaldehyde in PBS at room temperature for 15 min. After blocking with PBS containing 5% normal goat serum and 0.1% Triton X-100, the overexpressed receptors and His-tagged NTN4 protein were stained with anti-GFP monoclonal antibody (1:1,000; Nacalai) and anti-His-tag polyclonal antibody (1:500; MBL), respectively. The receptors and NTN4 were then visualized with the secondary antibodies Alexa Fluor 488-conjugated goat anti-rat IgG (1:500; Invitrogen) and Cy3-conjugated donkey anti-rabbit IgG (1:600; Chemicon), respectively. The cells were finally counterstained with 4′,6-diamidino-2-phenylindole (DAPI) to visualize their nuclei.

Recording of Spontaneous Activity by Multielectrode Dish. Extracellular recording of cultured slices was performed by multielectrode dish (MED) as described previously (4, 5). In brief, extracellular voltages were recorded at several locations for more than 5 min during the second week. Negative potentials with amplitudes above a set threshold (1.5× the maximal amplitude of the baseline noise) were counted as spikes with Axograph software (Axon Instruments).

Genotyping of *ntn4* Mutant Rats. Genotyping for *ntn4* mutant rats was carried out by PCR amplification with the following primers: 5′-TCCCGGTTCTTGCTAAGCAGAGG-3′ and 5′-TGGTGCACGAGCACGAACTAGG-3′ for the wild-type allele; and 5′-CTTGTGTCATGCACAAAGTAGATGTCC-3′ and 5′-TGGTGCACGAGCACGAACTAGG-3′ for the mutant allele. After denaturation at 95 °C for 5 min, the mixture was incubated for 35 cycles of 1 min each at 95 °C, 55 °C, and 72 °C, followed by a 7-min extension at 72 °C.

1. Maruyama T, Matsuura M, Suzuki K, Yamamoto N (2008) Cooperative activity of multiple upper layer proteins for thalamocortical axon growth. *Dev Neurobiol* 68(3): 317–331.
2. Zhong Y, et al. (2004) Identification of the genes that are expressed in the upper layers of the neocortex. *Cereb Cortex* 14(10):1144–1152.
3. Takemoto M, et al. (2011) Laminar and areal expression of unc5d and its role in cortical cell survival. *Cereb Cortex* 21(8):1925–1934.
4. Uesaka N, Hayano Y, Yamada A, Yamamoto N (2007) Interplay between laminar specificity and activity-dependent mechanisms of thalamocortical axon branching. *J Neurosci* 27(19):5215–5223.
5. Yamada A, et al. (2010) Role of pre- and postsynaptic activity in thalamocortical axon branching. *Proc Natl Acad Sci USA* 107(16):7562–7567.

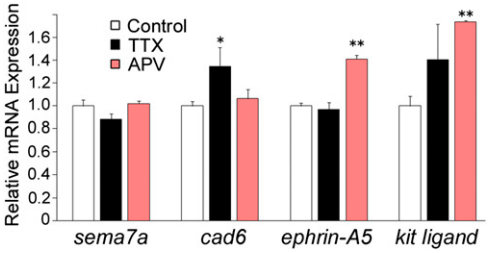

Fig. S1. Activity-dependent expression of other candidate genes. Transcripts of candidate genes [*semaphorin7a* (*sema7a*), *cadherin-6* (*cdh6*), *ephrin-A5* (*efna5*), and *kit ligand* (*kitlg*)] other than *ntn4* in cortical explants were also studied in normal culture medium (white, $n = 6$), or in the presence of TTX (black, $n = 4$) or APV (red, $n = 3$). Histograms represent relative mRNA expression levels of these genes. For *sema7a*, 0.88 ± 0.05 (TTX), 1.02 ± 0.02 (APV); for *cdh6*, 1.34 ± 0.16 (TTX), 1.06 ± 0.08 (APV); for *efna5*, 0.97 ± 0.06 (TTX), 1.41 ± 0.03 (APV); for *kitlg*, 1.41 ± 0.31 (TTX), 1.73 ± 0.01 (APV). Note that, in contrast to that of *ntn4* (Fig. 1*A*), the levels of none of them were significantly reduced by either TTX or APV application. $*P < 0.05$, $**P < 0.01$, Dunnett test.

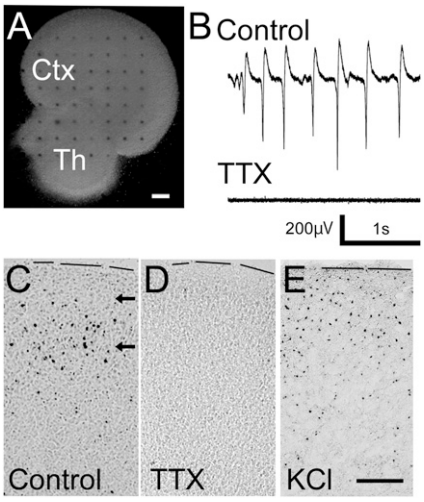

Fig. S2. Activity-dependent *ntn4* expression in vitro. (*A*) Electrical activity was recorded through the implanted multielectrodes underneath the explants of thalamus (Th) and cortex (Ctx). In the normal culture medium, spontaneous activity occurred frequently (*B*, *Upper*) but was completely suppressed in the presence of TTX (*B*, *Lower*). The distribution of β-gal$^+$ cells was investigated in cultured cortical explants of ntn4-heterozygous rats after 2 wk in culture. β-gal$^+$ cells were distributed in the upper layers of the cortical explant in normal culture medium (*C*) but completely disappeared in TTX-treated cultures (*D*). In contrast, high potassium depolarization increased the number of β-gal$^+$ cells (*E*). Dashed lines indicate the pial surface of each cortical explant. Arrows indicate the presumed layer 4 boundaries. (Scale bars: 0.3 mm.)

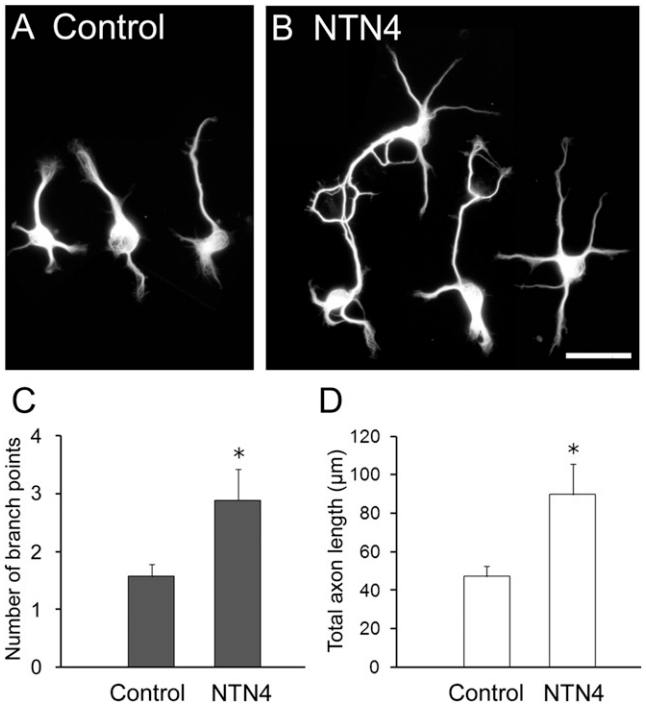

Fig. S3. NTN4 promotes axon branching of dissociated thalamic cells. Dissociated thalamic cells from E15 rat brains were cultured for 4 d in the absence (*A*) or presence (*B*) of recombinant NTN4 (200 ng/mL). Quantification of the number of branch points (*C*) and total branch length (*D*) in control each case. *$P < 0.05$, Student *t* test.

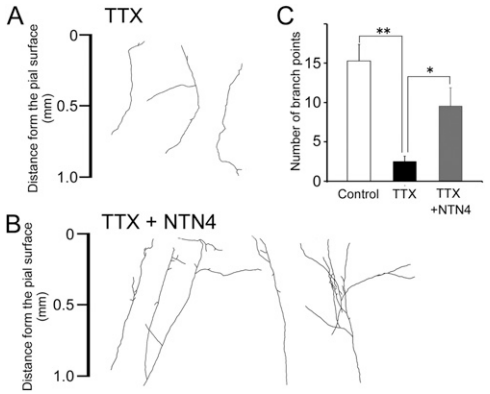

Fig. S4. NTN4 is involved in activity-dependent thalamocortical (TC) axon branching. NTN4 action was examined in the absence of neuronal activity. TC axons formed little branching in the TTX-containing medium (*A*). NTN4 application considerably rescued the phenotype (*B*). (*C*) The quantitative analysis of branch points (number of branch points per individual axon: 2.5 ± 0.7, $n = 21$ for TTX; 9.6 ± 2.3, $n = 10$ for TTX + NTN4; *$P < 0.05$, **$P < 0.01$, Dunnett test).

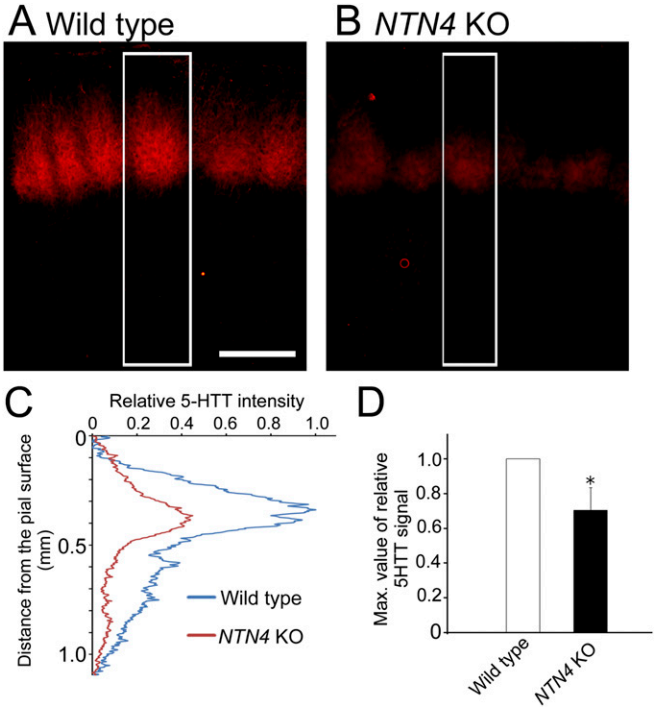

Fig. S5. Genetic deletion of NTN4 reduces 5-HTT immunoreactivity in the somatosensory cortex. 5-HTT immunostaining in the somatosensory cortex at P10 of wild-type (*A*) and *ntn4* mutant rats (*B*). (*C*) Laminar distribution of 5-HTT signals in wild-type (blue) and *ntn4* mutant (red). The *x* axis represents relative intensity of 5-HTT immunopositivity. The *y* axis represents distance from the pial surface. (*D*) The peak values of the relative 5-HTT staining were averaged in wild type ($n = 3$) and *ntn4* mutants ($n = 3$). (Scale bar: 0.2 mm.) $*P < 0.05$, Student *t* test.

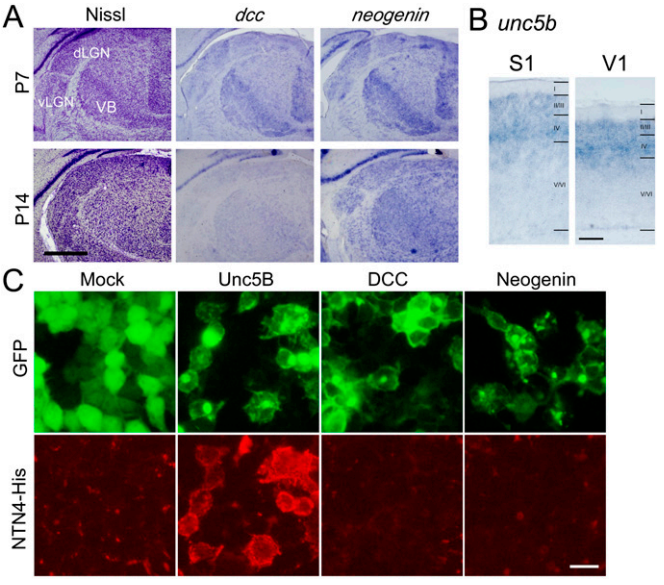

Fig. S6. Expression of the candidate receptors in the thalamus and their binding with NTN4. (*A*) Expression patterns of *dcc* and *neo1* were examined at P7 (*Upper*) and P14 (*Lower*) by in situ hybridization and compared with the distribution of thalamic nuclei as shown by Nissl staining. (Scale bar: 1 mm.) (*B*) *unc5b* expression in the visual and somatosensory cortex at P7. (Scale bar: 0.2 mm.) (*C*) Cell surface binding assay of NTN4 with putative receptors. NTN4-His (red) was incubated with HEK293T cells expressing GFP-tagged Mock, DCC, Neo1, or Unc5B (green). (Scale bar: 50 μm.)

Table S1. The density of β-gal$^+$ cells in the developing sensory cortices

Postnatal days	P0	P4	P7	P14	P28
V1	0	0	0.9 ± 0.6	61.0 ± 13.0	54.1 ± 12.0
S1	0	8.8 ± 2.8*	11.8 ± 0.7*	73.5 ± 10.4	48.0 ± 17.0

Each value represents the mean and SEM of the number of β-gal$^+$ cells per unit area (mm^2). The number of β-gal$^+$ cells was counted in several sections and divided by the area. The average values in the two cortical areas were compared statistically. S1, primary somatosensory cortex; V1, primary visual cortex. *$P < 0.05$ (Student *t* test). P, postnatal day.

Table S2. Branch aspects of TC axons in different types of cocultures

Coculture combination	WT Th, WT CTX	WT Th, KO CTX	KO Th, WT CTX	WT Th, KO CTX + NTN4
n_a	20	47	22	11
n_c	6	7	10	7
Branch points	15.0 ± 3.3	4.5 ± 0.8**	14.7 ± 3.4	11.7 ± 3.1††
Total length, mm	2.7 ± 0.4	1.6 ± 0.1**	2.4 ± 0.3	2.5 ± 0.4††
Density, mm^{-1}	12.5 ± 2.5	4.3 ± 1.6*	13.6 ± 3.2	6.7 ± 1.1
Tip length, μm	62.7 ± 5.6	72.2 ± 5.6	53.2 ± 3.8	69.3 ± 6.5
Width, μm	0.49 ± 0.10	0.19 ± 0.03**	0.40 ± 0.07	0.33 ± 0.05†

Each value represents the mean and SEM. CTX, cortex; Th, thalamus; KO, *ntn4* KO; n_a, number of axons sampled; n_c, number of cultures. **$P < 0.01$, *$P < 0.05$ (compared with WT Th plus WT CTX, Dunnett test). $^{\dagger\dagger}P < 0.01$, $^{\dagger}P < 0.05$ (compared with WT Th plus KO CTX, Dunnett test).