Supplementary Materials for

**BTBD3 Controls Dendrite Orientation Toward Active Axons in Mammalian Neocortex**

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Supplementary material

Fig. S1
Mouse somatosensory barrel development in early postnatal stage. Thalamocortical axons and cortical neurons were stained by anti-vGlut2 and anti-RORc antibodies, respectively. Coronal section (A-D) and tangential section (E-H) of the cortex is shown at different developmental time points. Scale bar in A is 100 µm.

Fig. S2
(A) Btbd3 knockdown in mice by shRNA construct. Btbd3 shRNA is electroporated at E13.5 and Btbd3 expression was assessed by in situ hybridization at P6, resulting in an approximately 70% reduction in gene expression compare to control brain. Scale bar is 200 µm.
(B) Control YFP plasmid (mock), human BTBD3 or human BTBD3 and shRNA construct was electroporated at E13.5 and brains were harvested at E16.5. Expression of BTBD3 mRNA was tested with using a human BTBD3-specific primer. (C) Morphological changes of primary dendrites during barrel formation. Electroporation was performed at E13.5 to transfect the YFP construct. Brains were harvested at P0, P2, P4 and P6, tangentially sectioned and stained with anti-GFP antibody to reveal dendritic morphology. (D) Number of primary dendrites was counted using the Sholl method. * p < 0.05, ** p < 0.01, t test.

Materials and Methods.

Animals
Outbred ICR (CD-1) timed pregnant mice were obtained from Japan SLC. Midday of the day of vaginal plug discovery is considered embryonic (E) day 0.5. NR1flox/flox mice were crossed with Emx1 cre mice to obtain conditional KO (10). Early postnatal mice were anesthetized with a lethal dose of pentobarbitone (100 mg/kg), and after three failed attempts to elicit a foot withdrawal reflex, the animals were transcardially perfused with 4% paraformaldehyde in phosphate-buffered saline. Normally pigmented, sable ferrets, Mustela putorius
furo, were purchased from Marshall Farms (North Rose, NY). All procedures were performed in accordance with a protocol approved by RIKEN Institutional Animal Care.

**In utero electroporation**

Mouse *in utero* electroporation and ferret electroporation were performed as described previously (18, 28) with combination of sparse labeling method provided high resolution of dendritic morphology (29). For targeting mouse layer IV neurons, electroporation was performed at embryonic day (E) 13.5. For targeting ferret layer IV neurons, electroporation was performed at E34.

**In situ hybridization**

Brains were removed, fixed overnight in 30% sucrose/4% paraformaldehyde, and sectioned in the coronal plane on a Leica sledge microtome at 28µm. *In situ* hybridization was performed as described previously (30).

**Immunohistochemistry**

Anti-RORc (H3925, Perseus Proteomics, Tokyo, Japan) and anti-vGlut2 (135 404, Synaptic Systems, 1/400) primary antibodies were detected with the following secondary antibodies: horse anti-mouse IgG (Vector, FI-2000) and Cy3-conjugated goat anti-rabbit IgG (Chemicon, AP132C).

**Western blotting and immunocytochemistry**

Whole brain was collected after quick decapitation and washed in cold PBS to remove any contaminating blood. Somatosensory cortex was immediately dissected and kept in cold RIPA buffer [20 mM Tris, pH 7.4, 150mM NaCl, 2mM EDTA, 1% NP-40, 1% Na deoxycholate, 0.1% SDS and protease inhibitor cocktail (04080, Nacalai tesque, Japan)]. To collect the cytoplasmic fraction, somatosensory cortex was kept in cold extract buffer and centrifuged [20mM HEPES, pH7.6, 20% Glycerol, 10mM NaCl, 1.5mM MgCl2, 0.2mM EDTA, pH 8.0, 1mM DTT, 0.1% NP-40 and protease inhibitor cocktail (Nacalai tesque)]. After collecting supernatant of cytoplasmic extraction, the pellet is homogenized with cold extract buffer to extract nuclear fraction [20mM HEPES, 20% Glycerol,
500 mM NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.1% Glycerol and protease inhibitor cocktail]. Primary antibodies and concentration for western blotting were, rabbit anti-BTBD3 antibody (HPA042048; ATLAS, 1:500), mouse anti-α-tubulin antibody (T6199; SIGMA-ALDRICH, 1:20,000), mouse anti-c-Raf antibody (610151; BD Transduction Laboratories, 1:2,000), rabbit anti-MEF2D antibody (#AB2263; Millipore, 1:2,000). For secondary antibodies, we used peroxidase-conjugated goat anti-rabbit (# 7074S, Cell Signaling), anti-mouse IgG antibody (#7076S, Cell Signaling, 1:10,000). The signals were visualized using chemiluminescent detection reagents (02230; Chemi-Lumi One Super, Nacalai tesque). Images of the signals were captured with LAS-3000UV mini (Fujifilm).

**DNA Constructs and shRNA**

Full length *Btbd3* (FANTOM clone, D430043L03) was subcloned into Tol2 plasmid vector and electroporated with T2TP vector for permanent transfection (31). Small hairpin (sh) RNA plasmid was purchased from Santa Cruz (BTBD3 shRNA Plasmid (m): sc-141776-SH). *Btbd3-HA* construct is generated using PCR to add HA tag on C-terminus of mouse Btbd3.

**QT-PCR**

Total RNA extractions were carried out with Trizol reagent (Invitrogen, 15596-026) according to the manufacturer’s instructions. RNA obtained from brains was reverse transcribed by using SuperScript III First-Strand Synthesis System (18080-051, Invitrogen). Synthesized cDNA was used for PCR reaction with using following primers.

- Ferret *Btbd3*-F: 5’-TGAAATTGACTTGCTGCTG-3’
- Ferret *Btbd3*-R: 5’-GCTGCCTCAAAAACCACAAT-3’
- Ferret b-actin-F: 5’-GCTGCCTCAAAAACCACAAT-3’
- Ferret b-actin-R: 5’-CTTGATGTCACGCACGATTT-3’
- Human *Btbd3*-F: 5’-TGTGGTTCCATGCGATTT-3’
- Human *Btbd3*-R: 5’-AGGAGCACACAGGCATTCTTT-3’
- mouse b-actin-F: 5’-GACTTTGTACATTTGTTT-3’
- mouse b-actin-R: 5’-TGCACTTTTATTTGGTCTCA-3’
Cell culture and pharmacological treatment
Neuro2a cells were cultured in DMEM medium (# 08458, Nacalai tesque) supplemented with 10% fetal bovine serum, high-glucose and pyrubic acid). The cells were seeded in a 2 well chamber slide (177380, Thermo Scientific, NY, USA) for 24hr and transfected with plasmid vectors by X-tremeGENE HP DNA transfection Reagent (06 366 126 001, Roche). After transfection, cells were cultured for 24hr and cell differentiation was initiated by serum starvation in DMEM. After 24hr, to depolarize the cultured cells, 60mM KCL solution was added to the culture medium. After a 1hr stimulation, cells were fixed in 4% PFA. Cells were treated with 0.1% triton-X in PBS and blocked with 5% goat serum solution in PBS. The cultures were incubated with rabbit anti-HA polyclonal antibody (RBP-101P, CONVANCE, 1: 200). Primary antibody was detected by incubation with Alexa 488-conjugated anti-rabbit IgG (A-21244, Invitrogen) at 1: 200.

Confocal microscopy
40 µm tangential mouse brain sections or coronal ferret brain sections were immunostained with anti-GFP and imaged using confocal microscopy.

Illustrations
Contrast, brightness, and sharpness of entire images were adjusted by using Adobe Photoshop CS5.
References


