Supporting Information

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SI Methods

Conventional and Quantitative RT-PCR. Total RNA was extracted from cultured ES cells or ES-derived cells using the RNeasy Minikit (Qiagen). Following extraction, all samples were treated with TURBO DNase (Ambion) to decrease likelihood of DNA contamination. Single-stranded cDNA (1.5 ng) was synthesized using Omniscript reverse transcriptase (Qiagen) and Oligo-dT primers. Nonquantitative and quantitative RT-PCR analyses were performed as previously reported [Kondo T, Johnson SA, Yoder MC, Romand R, Hashino E (2005) Sonic hedgehog and retinoic acid synergistically promote sensory fate specification from bone marrow-derived pluripotent stem cells. *Proc Natl Acad Sci USA* 102:4789–4794]. Primers sequences used in this study are available upon request.

Immunocytochemistry. Specimens were incubated in blocking solution and then with a monoclonal Tau antibody (Labvision) or TUJ1 antibody (Covance). Immuno-reactivity with a primary antibody was visualized by using an Alexa 568- or 488-conjugated anti-mouse IgG. For visualizing filamentous actin and cellular nuclei, the specimens were counterstained with Alexa 568-conjugated phalloidin and DAPI, respectively.

Western Blotting. Cultured cells were lysed with RIPA buffer (1% Nonidet P-40, 0.1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 1 mM EDTA, 20 μg/ml of leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1 mM NaF, and 100 nM calyculin A, and 10 mM Tris-HCl, pH 7.4) with a protease inhibitor mixture (Pierce). The same amount ($\approx$20 μg) of samples was subjected to SDS/PAGE, and the separated proteins were electrophoretically transferred onto polyvinylidine difluoride (PVDF) membrane (Bio-Rad). The membrane was incubated sequentially in blocking buffer, a primary antibody and alkaline phosphatase-conjugated secondary antibody. Primary antibodies used in this study include: anti-Tlx3 (Santa Cruz Biotechnology), anti-Mash1 (BD PharMingen), anti-Ngn1 (Abnova), anti-NeuroD (Santa Cruz Biotechnology), anti-GluR4 (Chemicon), anti-TUJ1, anti-Tau (Santa Cruz Biotechnology), anti-Oct4 (Chemicon), anti-Sox2 (abcam), anti-Musashi1 (R&D Systems), anti-HuC (Invitrogen), anti-NeuN (Chemicon), anti-Calretinin (Santa Cruz Biotechnology), anti-Synaptophysin (Zymed), anti-NSE (Labvision) and anti-β-actin (Sigma). The immunoreactive bands were visualized with the SuperSignal West Pico or -Femto chemiluminescent detection system (Pierce) and exposed to x-ray film.

Flow Cytometry. ES cells and ES-derived cells were dissociated by a brief exposure to trypsin-EDTA. After being blocked, cells were incubated with one of the following primary antibody: anti-CD24-phycoerythrin (PE), anti-CD56-PE (abcam) and mouse immunoglobulinG (IgG) isotype control. Alternatively, cells were fixed and permeabilized using the Cytofix/Cytoperm Fixation/Permeabilization kit (BD Biosciences), followed by incubation with anti-GluR2 (Chemicon) or anti-Tau. The unconjugated antibody was detected using an Alexa 568-conjugated anti-rabbit (or mouse) IgG. Cell sorting and analysis were performed with a FACSCalibur flow cytometry system (BD Biosciences).

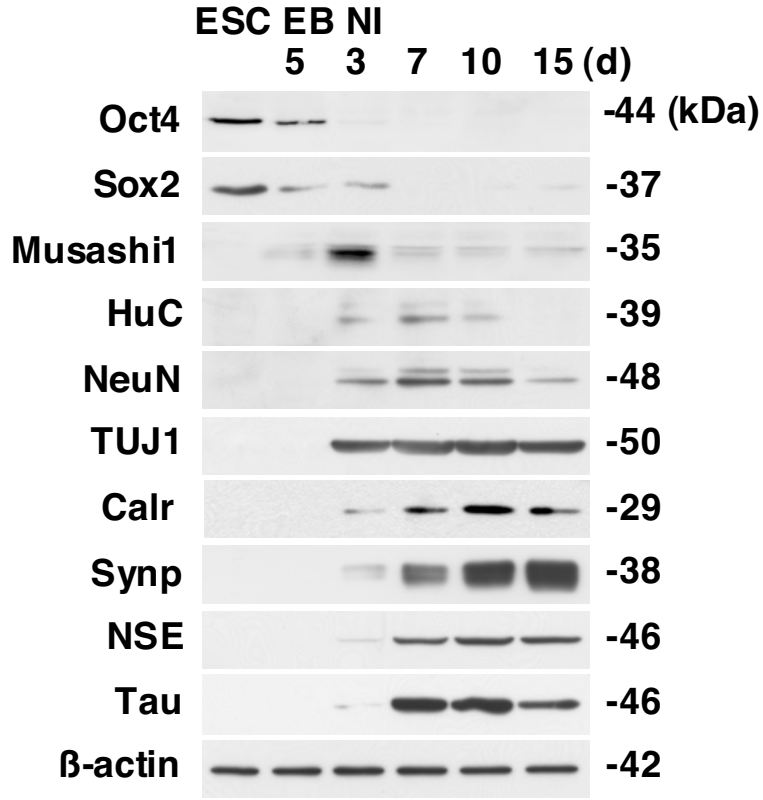

Fig. S1. Temporal expression patterns of ES cell- and neural-marker proteins in ES-derived cells at various time points before and during neural differentiation. Western blot analysis for ES cell markers (Oct4, Sox2), early neural markers (Musashi1, HuC, NeuN) and mature neural markers (TUJ1, Calretinin (Calr), Synaptophysin (Synp), NSE and Tau) in undifferentiated ES cells (ESC), ES-derived cells grown in differentiation medium (EB) and neural induction medium (NI) for 3,7,10 and 15 days.

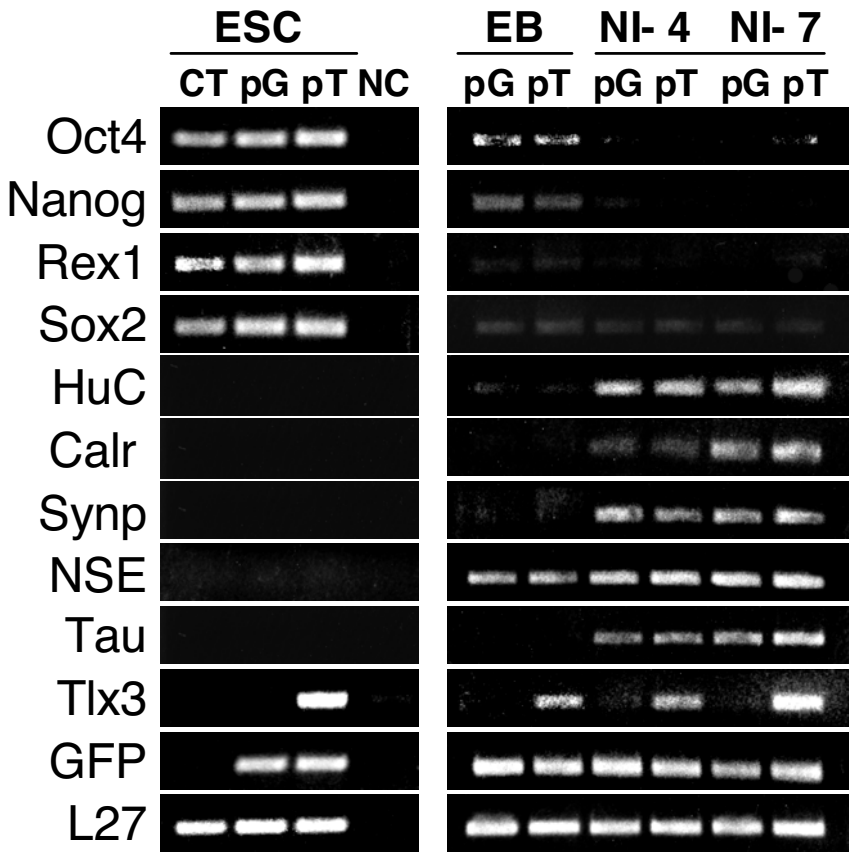

Fig. S2. Comparison of the expression of ES cell- and neural-marker genes between ES-derived cells expressing the Tlx3 construct and control construct. Conventional RT-PCR analysis for ES cell markers (*Oct4, Nanog, Rex1* and *Sox2*), neural markers (*HuC, Calr, Synp*, *NSE,* and *Tau*), expression vector controls (*Tlx3* and *GFP*) and house-keeping gene (*L27*) in undifferentiated ES cells (ESC), ES-derived cells in the EB stage (EB), and ES cell-derived neurons at day 4 (NI-4) and day 7 (NI-7) after the start of neural induction. CT, untranfected ES cells; pG, ES cells or ES-derived cells expressing the control vector; pT, ES cells or ES-derived cells expressing the *Tlx3* expression vector; NC, negative control.

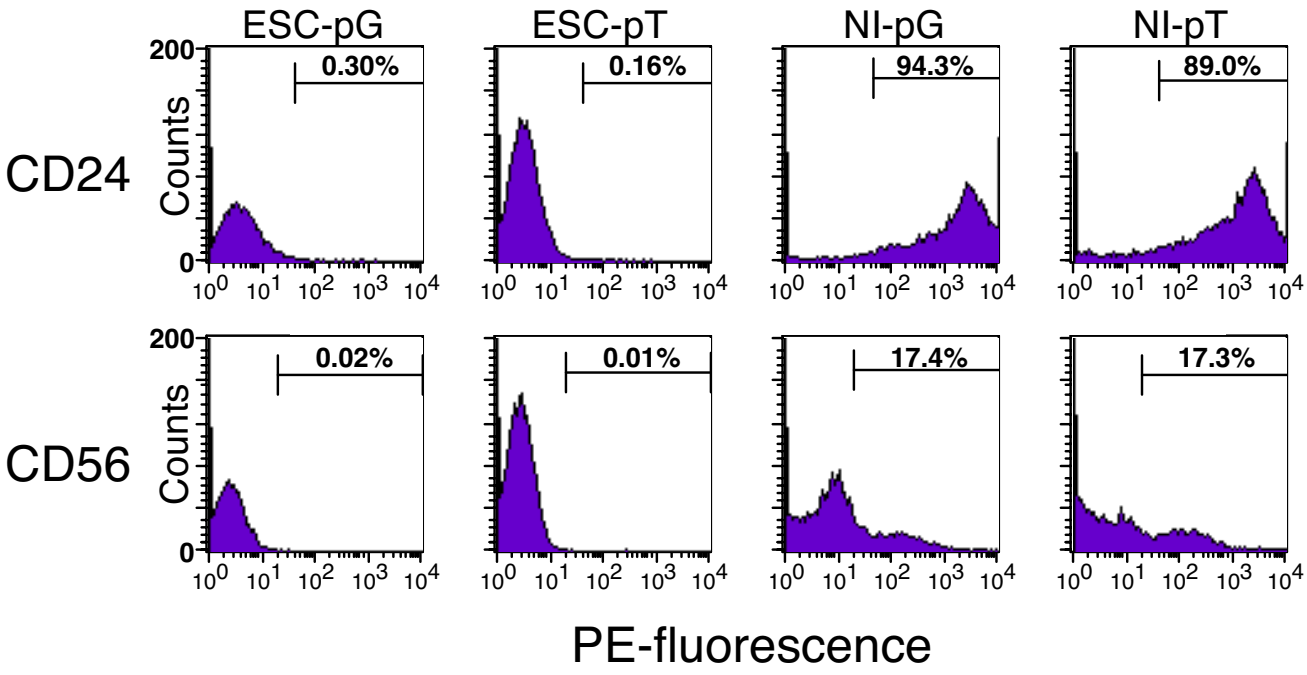

Fig. S3. Flow cytometry profiles of the neural surface markers, CD24 and CD56, in undifferentiated ES cells (ESC-pG and ESC-pT) or ES cell-derived neurons at neural induction day 7 (NI-pG and NI-pT). The percentage of both CD24- and CD56-positive cells is increased after neural induction regardless of the presence or absence of Tlx3.

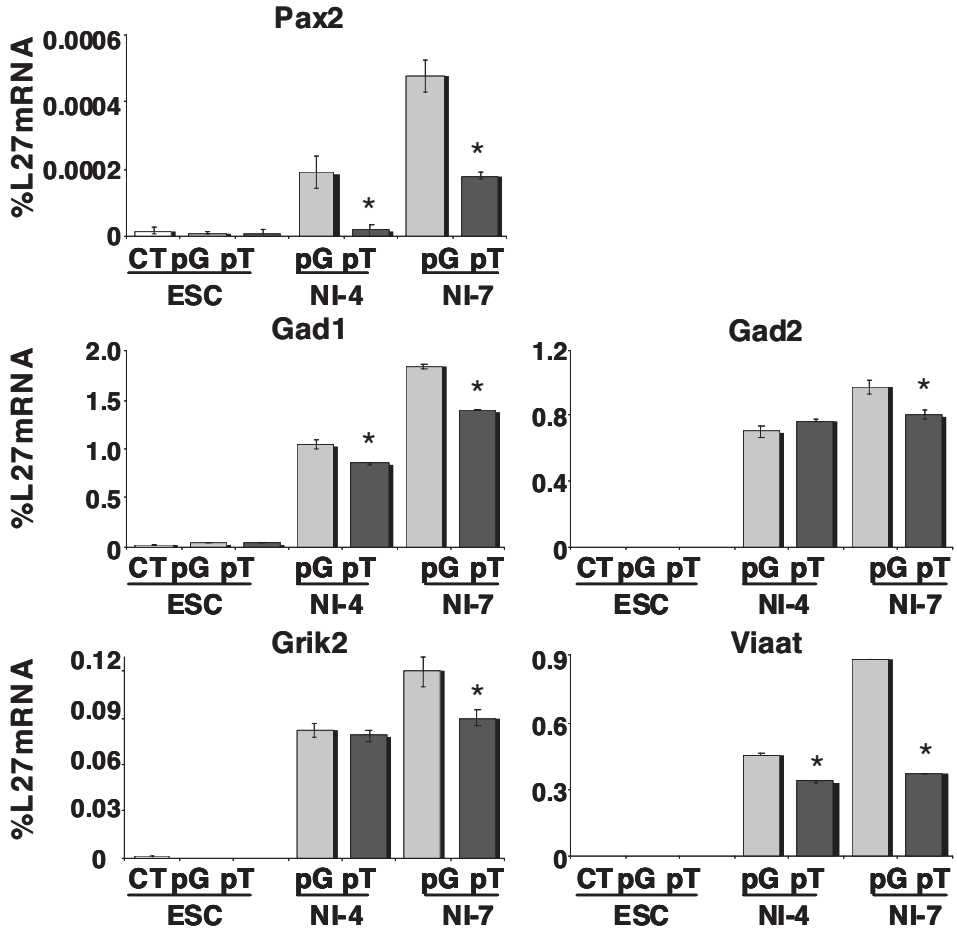

Fig. S4. Expression of GABAergic marker genes is suppressed in ES cell-derived neurons expressing Tlx3. Quantitative RT-PCR analysis for a transcription factor (*Pax2*), GABA synthesizing enzymes (*Gad1* and *Gad2*), a kainate receptor (*Grik2*) and a GABA transporter (*Viaat*) in ES cells or ES cell-derived neurons expressing the *Tlx3* expression vector (pT) in comparison with those expressing the control vector (pG). Undifferentiated ES cells (ESC) or ES-derived cells grown in a neural induction medium for 4 days (NI-4) or 7 days (NI-7). All of the GABAergic marker genes are absent or barely detectable in undifferentiated ES cells (ESC). Expression levels of these GABAergic genes increase with time after the start of neural induction. However, expression levels of these GABAergic genes in ES cell-derived neurons expressing pT were much lower than in those expressing pG. Neural induction day 4 (NI-4) or day 7 (NI-7). CT: untrasfected ES cells. *, $P \leq 0.05$.