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Downregulation of Wnt/β-catenin signaling causes degeneration of hippocampal neurons *in vivo*

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Abstract

The possibility that the degeneration of hippocampal neurons can be caused by mis-regulation of Wnt/β-catenin signaling was tested. Downregulation of Wnt signaling by the inducible expression of Axin, ICAT, and dnTcf4E causes degeneration of hippocampal neurons, while upregulation of Wnt signaling by the inducible expression of Dvl and β-catenin has a negligible effect. Treatment with ICG-001, a small molecule known to inhibit Wnt signaling, causes degeneration of hippocampal neurons, while the treatment with a JNK specific inhibitor does not show any effect. The results from LDH and TUNEL assays suggest that degeneration occurs via apoptotic processes. Inhibition of Wnt signaling reduced IGF-1 expression and the addition of IGF-1 blocked degeneration, which suggests that downregulation of IGF-1/Akt signaling is partially responsible for the degeneration. Inducible expression of Axin in the hippocampal neurons isolated from Axin2P-rtTA/pBI-EGFP-Axin double transgenic mice also causes degeneration. Consistent with the findings, these mice had more neuronal cell death in hippocampus and had differences in contextual conditioning upon the inducible expression of Axin. In summary, our data strongly support the idea that downregulation of Wnt/β-catenin signaling causes degeneration of hippocampal neurons *in vivo* and may be a cause of neurodegenerative diseases related to an anxiety related response.

Keywords: Axin; Neurodegeneration; Tet-On inducible system; Transgenic mice; Wnt signaling

Wnt/β-catenin signaling participates in the regulation of embryonic development and in homeostasis in diverse biological processes during postnatal life (Clevers, 2006; Logan and Nusse, 2004). Therefore, mis-regulation of Wnt/β-catenin signaling is associated with diverse pathologies such as carcinogenesis, osteoporosis, and neurological disorders (MacDonald et al., 2009; White et al., 2007).

Wnt genes encode secreted glycoproteins and transduce signals via autocrine or paracrine ways. Wnts transduce signals by binding to the cell surface receptor, Frizzled, and to other coreceptors, such as LRP5/6. Binding of Wnts to receptors activates a downstream signaling cascade that includes Dvl, GSK-3β, adenomatous polyposis coli, and Axin, and leads to the accumulation of cytoplasmic β-catenin. The accumulated β-catenin enters the nucleus and forms a complex with Tcf/Lef transcription factors which controls expression of downstream target genes (MacDonald et al., 2009). About 100 target genes have been identified thus far (www.stanford.edu/~rnusse/pathways/targets.html).

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Mis-regulation of Wnt/β-catenin signaling is known to be involved in neurodegenerative disorders such as autism and Alzheimer's disease (AD) (De Ferrari and Moon, 2006; De Ferrari et al., 2007; Toledo et al., 2008). Previous studies showed that AD patients carrying a presenilin-1 mutation have very low levels of β-catenin (Zhang et al., 1998). In addition, genetic variation within the LRP6, whose product reduces the ability to enhance Tcf/Lef mediated reporter activity, is associated with late-onset Alzheimer's disease (De Ferrari et al., 2007). These data suggest that reduced Wnt/β-catenin signaling may be involved in the pathology of Alzheimer's disease (Toledo et al., 2008).

Because Wnt/β-catenin signaling controls expression of so many target genes, including genes that encode transcription factors and growth factors, we hypothesized that downregulation of Wnt/β-catenin signaling in hippocampal neurons (without any associated mutations in presenilin-1 or apolipoprotein E) resulted in neurodegeneration *in vivo*. To examine whether the degeneration of hippocampal neurons could be induced by the inhibition of Wnt/β-catenin signaling, we inhibited endogenous Wnt/β-catenin signaling in mouse primary hippocampal neurons using *in vitro*, *ex vivo,* and *in vivo* systems. To inhibit Wnt/β-catenin signaling, we mainly used the inducible expression of Axin by using the Tet-On inducible system (Lyu et al., 2003). All data from these different approaches suggested that downregulation of endogenous Wnt/β-catenin signaling can induce degeneration of hippocampal neurons. A fear conditioning test in transgenic mice, which showed enhanced cell death in the hippocampus by inducible expression of Axin, showed greater anxiety related response, which is the opposite of the behavioral defect shown in other mouse models for Alzheimer's disease (Dineley et al., 2002; Gong et al., 2004). Overall, our data strongly suggest that downregulation of Wnt/β-catenin signaling *in vivo* can cause degeneration of hippocampal neurons and results in a behavioral defect which may be involved in an anxiety related response.

1. Materials and methods

1.1. Mouse primary hippocampal neuronal cell culture and transfection

Hippocampus was isolated from ICR (Taconic, Germantown, NY) or transgenic mice embryos at embryonic Day 18.5 (E18.5) using Ca^{2+} and Mg^{2+} free Hanks' balanced salt solution. Isolated hippocampus were trypsinized and triturated with fire-polished Pasteur pipettes, then strained through 40-μm nylon mesh. These cells were cultured in Neurobasal medium with 2% B27 supplement, 0.5 mM L-glutamine, 25 μM L-Glutamate, and 1% antibiotics mixture. The same culture media without Glutamate or antibiotics was used for media changes after 4 d. One half of the medium was changed once per week for cultures maintained longer than 4 d. Calcium phosphate precipitation was used for transfections at low neuronal cell density (20,000/cm^2) (Kohrmann et al., 1999). A total of 8 μg of plasmid DNAs (Axin2P-rtTA: pBI vectors = 1: 1) per 6 well plate were used for transfection. Pictures were taken and used for analysis after the times indicated in figure legends.

1.2. Chemicals and indirect immunofluorescence analysis

Wnt/β-catenin signaling inhibitor (ICG-001, Emami et al., 2004) was kindly provided by the Choong Wae Pharma Corporation, Korea. Other reagents, such as JNK inhibitor (SP600125), PI3K inhibitor (LY294002), recombinant IGF-1, and DMSO, were purchased from Sigma. Hippocampal neurons were cultured on poly-D-lysine (0.05 mg/mL, MW 30,000 ~ 70,000) coated cover glasses for indirect immunofluorescence analysis. Two days after chemical treatments, hippocampal neurons were fixed with 4% paraformaldehyde and permeabilized with 0.4% Triton X-100 in PBS. Fixed neurons were blocked with 5% bovine serum albumin and then incubated with primary antibody against MAP2 (Sigma) for 1 h at room temperature. Secondary antibody conjugated with Rhodamine was purchased from KPL. Fluorescent images were captured with a Leica DM IRB microscope using a DC300 FX camera, acquired with IM1000 software and processed with Adobe Photoshop. Total length and tip number of neurites were measured from these images using IM1000 software.

1.3. LDH assay and apoptosis assay

The cytotoxicity of hippocampal neurons induced by the treatment of ICG-001 was measured by an LDH (Lactate Dehydrogenase) assay using CytoTox-One Homogenous Membrane Integrity Assay Kit (Promega) according to the manufacturer's protocol. Apoptosis was determined by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL) staining using ApopTag Red *in situ* Apoptosis Detection Kit (Chemicon) according to the manufacturer's protocol.

1.4. Reverse transcription-PCR (RT-PCR) and Real-time PCR analysis

Total RNA was isolated from mouse primary hippocampal neurons or hippocampus using TRI reagent (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was synthesized using ImProm™-Reverse Transcriptase (Promega) using poly T primers. Real-time quantitative PCR was performed using the iCycler MyiQ with SYBER Green detection system (Bio-Rad). The threshold cycle (Ct) value for each gene was normalized to the Ct value of GAPDH. Relative mRNA expression was calculated by the following formula: $2^{-\Delta\Delta Ct}$ where $\Delta Ct = Ct_{Axin2\ or\ IGF1} - Ct_{GAPDH}$ and $\Delta\Delta Ct_{DMSO} = \Delta Ct_{DMSO} - \Delta Ct_{ICG\text{-}001}$. The following primers were used for PCR: for *Axin2*, 5′-CAGGCAGATGAACCTGAAG-3′ and 5′-CCCCACTCCTCACATATTC-3′; for *IGF-1*, 5′-TTCACACCTCTTCTACCTGG-3′ and 5′-GTCTCCTCAGATCACAGCTC-3′.

1.5. Western blot analysis

Protein samples were prepared from mouse primary hippocampal neurons or mouse hippocampus. RIPA buffer (25 mM Tris–HCl at pH 8.0, 150 mM NaCl, 10% glycerol, 1% Igepal CA-630, 0.25% deoxycholic acid, 2 mM EDTA, 1 mM NaF, 50 mM glycerophosphate) was used for lysis and western blots were performed as described elsewhere (Lyu et al., 2003).

1.6. Transgenic mice

Production of Axin2P-rtTA mice was described previously (Shakya et al., 2005). In brief, a DNA fragment encoding an improved form of rtTA (rtTA2^S-M2) (Urlinger et al., 2000) was inserted into a 5.6 kb DNA expression cassette containing the Axin2 promoter to generate the construct for transgenic mice (Jho et al., 2002). Generation of TRE-LacZ transgenic mice was described previously (Shakya et al., 2005). Mice were genotyped using PCR to determine the presence of the target genes. pBI-EGFP-Axin, which was described previously (Lyu et al., 2003), was used to generate transgenic mice. Dox (doxycycline, 2 mg/ml plus 50 mg/ml sucrose) was administrated orally in the drinking water for 4 to 8 weeks to induce target gene expression *in vivo* (Cawthorne et al., 2007). All mouse protocols were conducted in accordance with NIH guidelines and approved by the Animal Care and Use Committee at the University of Seoul.

1.7. H&E staining

Control (Ax2P-rtTA) mice and Ax2P-rtTA/TRE-Axin-EGFP mice, which were fed with doxycycline for 4 to 8 weeks in drinking water, were perfused with 4% paraformaldehyde. Sections of hippocampus were prepared for H&E staining using standard histochemical procedures.

1.8. Fear conditioning test

The cued and contextual fear-conditioning tasks were performed on Ax2P-rtTA/TRE-EGFP and Ax2P-rtTA/TRE-Axin-EGFP transgenic mice as described previously (Jeon et al., 2003). For conditional training, mice were placed in a shocker chamber for 4 min and 40 sec. An auditory cue was then delivered for 18 sec then a foot shock (0.45 mA) was delivered for 2 sec. This procedure was repeated two times with 1 min intervals. One day after training, the mice were placed in the same shocker chamber. We then measured the freezing time without stimulus to measure the ability to associate contextual features with a noxious stimulus (Contextual conditioning). For the cued conditioning test, one day after training, the trained mice were placed in a different chamber and allowed to habituate to the new environment for 3 min. The same auditory cues were delivered for 3 min and the freezing time was measured. Freezing behavior (defined as the absence of movements except for respiration) was measured by observers that were blind to genotype. Data analyses were performed using Student's *t*-test and two-way analysis of variance (ANOVA) with genotype and time. Errors bars mean SEM. The data were presented as mean ± SEM and were subjected to either two-way ANOVA or a *t*-test. $^*P < 0.05$ was considered statistically significant.

1.9. Statistical analysis

All statistical significances were tested using Student's *t*-test. Errors bars in graphs represent SEM or SD.

2. Results

2.1. Inhibition of Wnt/β-catenin signaling causes degeneration of hippocampal neurons

To test whether mis-regulation of Wnt/β-catenin signaling leads to neuro-degeneration, mouse primary hippocampal neurons were transiently transfected with Tet-On inducible vectors which can control Wnt/β-catenin signaling. Because the transfection efficiency of primary hippocampal neurons was very low (especially the primary hippocampal neurons cultured *in vitro* for more than 8 days; Supp. Fig. 1A), the bidirectional pBI vector which can express both GFP and a gene of interest was used to follow the fate of transfected hippocampal neurons (Fig. 1A). Canonical Wnt/β-catenin signaling seemed active in the primary hippocampal neurons cultured *in vitro*, because the expression of Venus, an improved version of YFP (Nagai et al., 2002), driven by the Tcf/Lef optimal promoter is very strong in hippocampal neurons cultured 7 days *in vitro* (DIV) (Supp. Fig. 1B). When pBI-EGFP, Dvl-1 or β-catenin was transfected into 9 DIV hippocampal neurons and their expression was induced by culturing with doxycycline for two more days, no obvious effects were detected (Fig. 1B). However, previous data suggested that ectopic expression of Dvl or β-catenin enhances dendritic arborization of hippocampal neurons (Rosso et al., 2005; Yu and Malenka, 2003; see Discussion). Interestingly, when Wnt/β-catenin signaling was inhibited by the inducible expression of Axin, it led to fragmentation of dendrites and caused degeneration of hippocampal neurons (Fig. 1B), while inducible expression of RGS domain deleted Axin (ΔRGS-Axin), which enhances Wnt/β-catenin signaling (Zeng et al., 1997), does not cause degeneration. Because the degeneration might be induced by the apoptosis caused by overexpression of Axin (Neo et al., 2000), we tested other components such as ICAT or the dominant negative form of Tcf4 (dnTcf4) which inhibit Wnt/β-catenin signaling (Lyu et al., 2003). Inducible expression of ICAT or dnTcf4 caused neurodegeneration, similar to Axin (Fig. 1B). Overall, these data strongly suggest that the inhibition of Wnt/β-catenin signaling causes degeneration of hippocampal neurons (Fig. 1B). Quantitative measures of total length of dendrites and total dendritic branching tip number (TDBTN) showed that inhibition of Wnt/β-catenin signaling resulted in degeneration of hippocampal neurons (Fig. 1C and D).

To confirm that inhibition of Wnt/β-catenin signaling resulted in degeneration of hippocampal neurons, ICG-001,

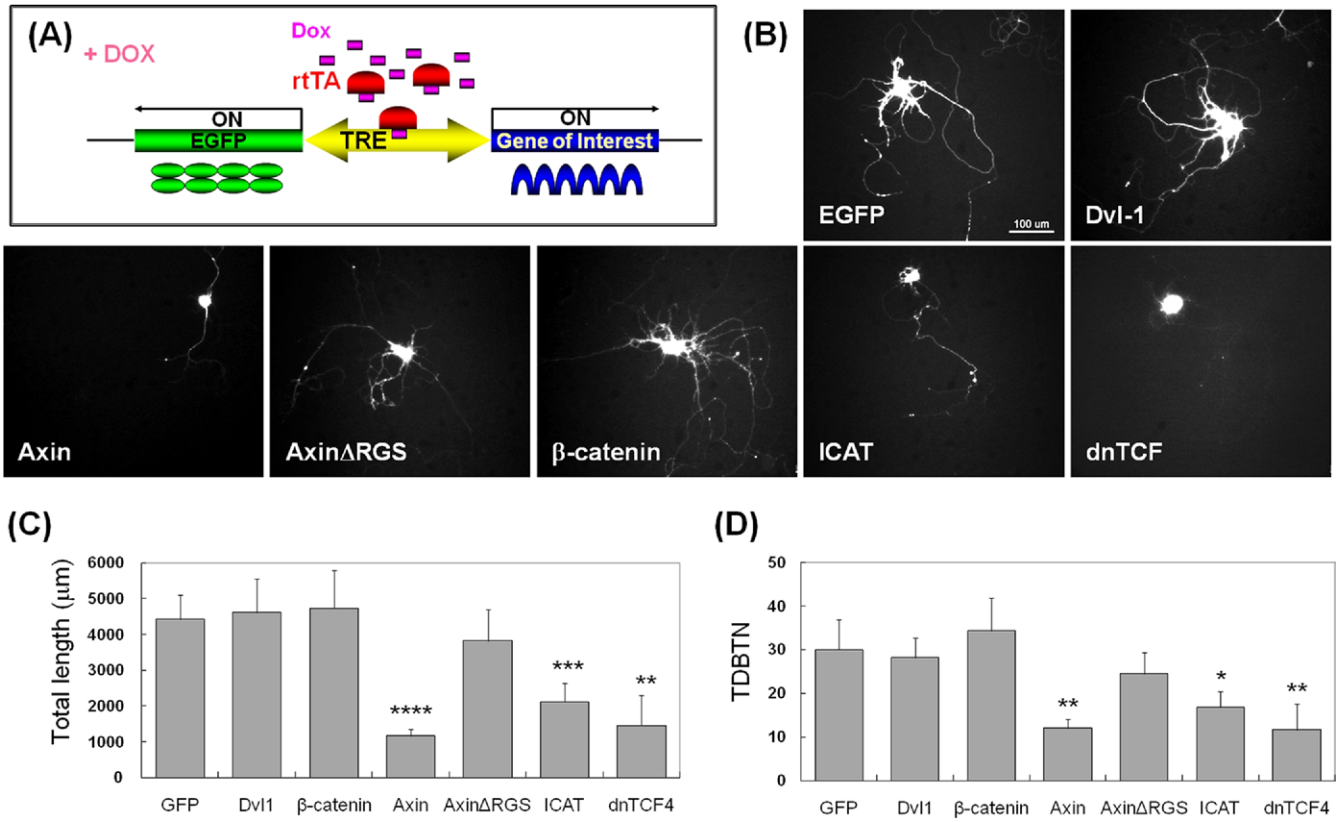


Fig. 1. Inhibition of Wnt/β-catenin signaling causes degeneration of mouse primary hippocampal neurons. (A) A schematic diagram for the Tet-On inducible system. (B) Mouse primary hippocampal neurons cultured *in vitro* for 9 days were transiently transfected with CMVP-rtTA2^{S}M2 and pBI vectors which encode cDNAs indicated in the figure (plus EGFP) and incubated for two more days after the addition of 500 ng/mL doxycycline. Degeneration of hippocampal neurons was observed when the expression of cDNAs that inhibit Wnt signaling was induced. Quantification of total length of dendrites (C) and total dendritic branching tip numbers (TDBTN) of the hippocampal neurons that were described above (D). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$, $n = 6$.

a small molecule which inhibits Wnt/β-catenin signaling, was used (Fig. 2, (Emami et al., 2004)). When 9 DIV hippocampal neurons were treated with ICG-001, the dendrites became thinner and fragmented (Fig. 2A). However, treatment with SP600125 (a JNK inhibitor which was shown to inhibit dendritic arborization of hippocampal neurons; Rosso et al., 2005) or DMSO (used for dissolving ICG-001 and SP600125) did not have any significant effect (Fig. 2A). To measure the degeneration of hippocampal neurons by ICG-001 more quantitatively, a LDH assay, which can estimate the viability of a population of cells by measuring integrity of cell membrane (Decker and Lohmann-Matthes, 1988), was performed. The culture media for hippocampal neurons treated with ICG-001 was significantly higher in LDH activity (Fig. 2B). In addition, we found that treatment with ICG-001 enhances the number of positive neurons in TUNEL staining (Supp. Fig. 2), which suggests that degeneration occurs via apoptotic processes. Overall, the degeneration of hippocampal neurons due to inhibition of Wnt/β-catenin signaling, either by inducible expression of genes or by treatment with a specific inhibitor, strongly suggests that tight regulation of endogenous levels of Wnt/β-catenin signaling is necessary for the maintenance and survival of hippocampal neurons.

We also tested whether inhibition of Wnt/β-catenin signaling has any effect on dendritic arborization of hippocampal neurons (Fig. 3 and Supp. Fig. 3). Four DIV hippocampal neurons had shorter and fewer dendrites than 9 DIV hippocampal neurons (Supp. Fig. 1A). When Wnt/β-catenin signaling was inhibited in 4 DIV hippocampal neurons either by inducible expression of genes that inhibit Wnt/β-catenin signaling or by treatment with ICG-001, both the total length and tip number of dendrites was dramatically reduced (Fig. 3 and Supp. Fig. 3). The LDH activity in the culture media for hippocampal neurons treated with ICG-001 was higher than the control (Supp. Fig. 3B), which suggests that the death of hippocampal neurons is caused by the inhibition of Wnt/β-catenin signaling.

2.2. *Reduced IGF signaling is partially responsible for degeneration of hippocampal neurons caused by the inhibition of Wnt/β-catenin signaling*

Insulin like growth factor-1 (IGF-1) signaling is mediated via the activation of PI3K and Akt signaling and is

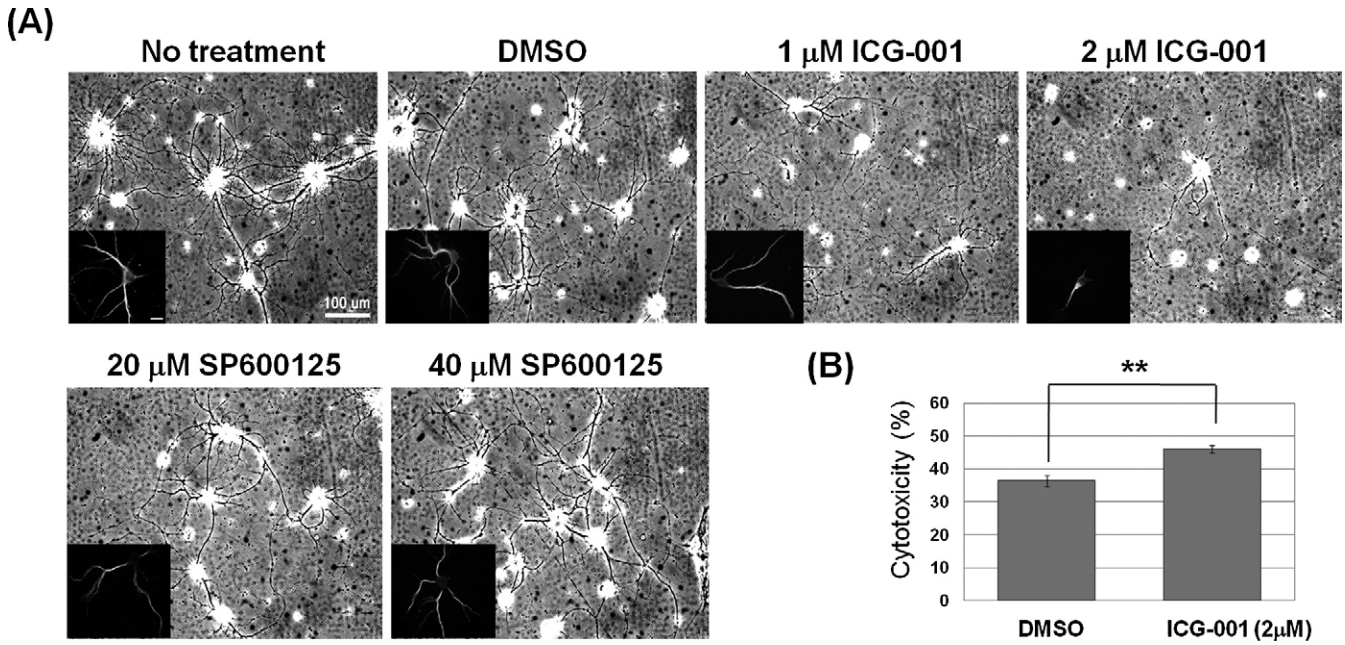


Fig. 2. Treatment with ICG-001 causes degeneration of mouse primary hippocampal neurons. (A) Mouse primary hippocampal neurons cultured *in vitro* for 9 days were incubated with the treatments indicated in the figure for two more days. Inlets; cells was stained with antibody for MAP2 to enhance visualization of neuronal degeneration. (B) Nine DIV hippocampal neurons were incubated with DMSO or 2 μM ICG-001 for 2 days and LDH assays were performed. **, $p < 0.01$.

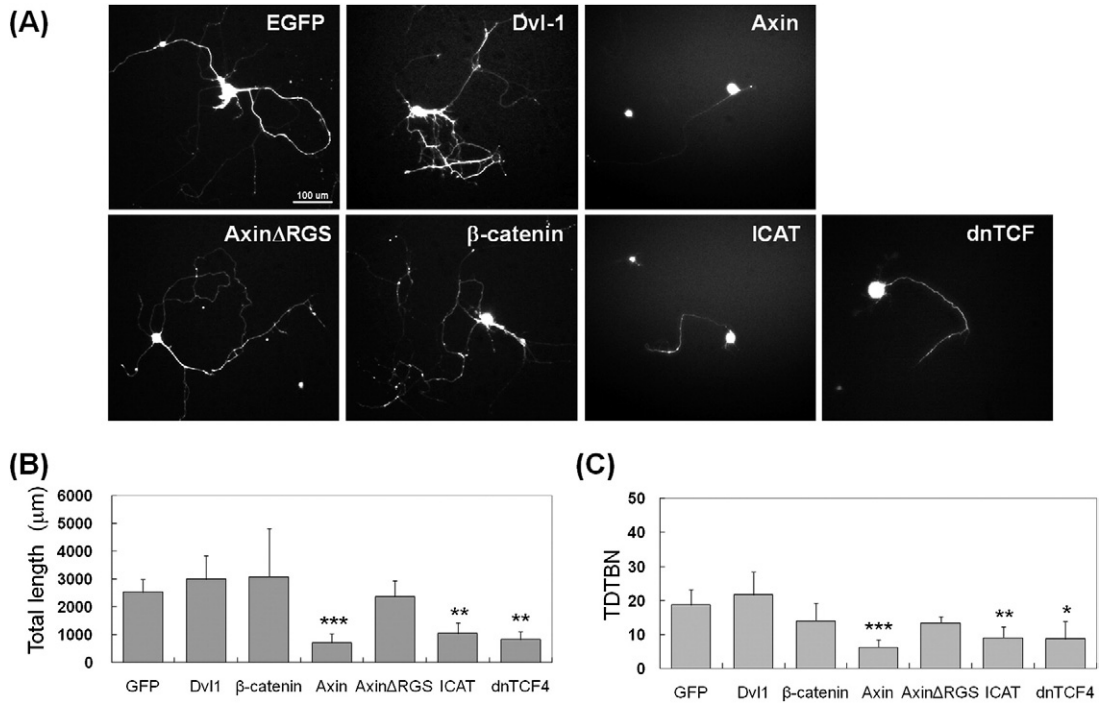


Fig. 3. Inhibition of Wnt/β-catenin signaling blocks the dendritic arborization of primary hippocampal neurons. (A) Mouse primary hippocampal neurons cultured *in vitro* for 4 days were transiently transfected with CMVP-rtTA2^{S}M2 and pBI vectors which encode the cDNAs indicated in the figure and incubated for two more days after the addition of 500 ng/mL doxycycline. Dendritic arborization of hippocampal neurons were severely blocked when the expression of cDNAs that inhibit Wnt signaling were induced. Quantification of total length of dendrites (B) and total dendritic branching tip numbers (TDBTN) of the hippocampal neurons that were described above (C). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, $n = 6$.

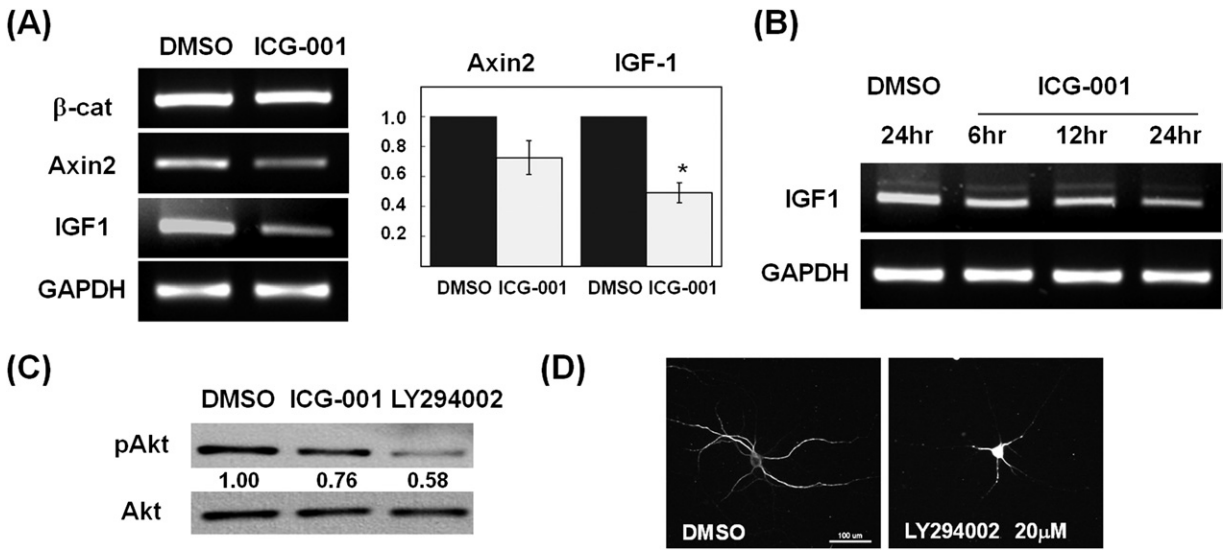


Fig. 4. Inhibition of Wnt/β-catenin signaling in the hippocampal neurons results in reduced expression of *IGF-1* and activity of Akt. (A) Nine DIV hippocampal neurons were incubated with DMSO or 2 μM ICG-001 for 24 h and RT-PCR and real-time PCR were performed with the primers indicated in the figures. *, $p < 0.05$, $n = 3$. (B) RT-PCR analysis showed reduced expression of *IGF-1* by the treatment with ICG-001 in a time-dependent manner. (C) Western blot analysis using the indicated antibodies with the lysates from the hippocampal neurons, which were treated with DMSO, 2 μM ICG-001 or 20 μM LY294002 for 24 h from 9 DIV. The level of phosphorylated Akt was normalized to total Akt level and expressed as fold-decrease over untreated neurons. (D) The neurons, which were incubated with DMSO or 20 μM LY294002 for 24 h from 9 DIV, were immunostained with antibody for MAP2.

essential for dendritic growth (Cheng et al., 2003; Sinha et al., 2005). It was shown that the expression of IGF-1 can be regulated by Wnt/β-catenin signaling (Longo et al., 2002). We hypothesized that the degeneration of hippocampal neurons by inhibition of Wnt/β-catenin signaling might be due to reduction of IGF signaling. To test this hypothesis, 9 DIV hippocampal neurons were treated with ICG-001 for 24 h and the level of mRNAs was measured by RT-PCR and real-time PCR (Fig. 4A). Levels of both Axin2 (a target gene of Wnt/β-catenin signaling) and IGF-1 were reproducibly reduced in RT-PCR analysis, while the difference in the level of Axin2 was not statistically significant in real-time PCR analysis (Fig. 4A). Especially the level of IGF-1 declined in a time-dependent manner (Fig. 4B). The reduced level of the active form of Akt (phosphorylated Akt) in 9 DIV hippocampal neurons treated with 1 μM ICG-001 for 24 h suggests that inhibition of Wnt/β-catenin signaling resulted in the reduction of Akt signaling (Fig. 4C). Treatment of LY294002 (an inhibitor of PI3-kinase) led to degeneration of hippocampal neurons and to a reduction in the level of Akt phosphorylation (Fig. 4C and D; Jones et al., 2003).

Because we hypothesized that inhibition of Wnt/β-catenin signaling led to reduction in the level of IGF-1 and caused degeneration of hippocampal neurons, we tested whether the addition of IGF-1 can block the degeneration induced by treatment with ICG-001. We also thought that IGF-1 might not block the effect of LY294002, because LY294002 inhibits signaling downstream of IGF-1. Consistent with our expectation, the degeneration induced by ICG-001 was partially blocked by treatment with IGF-1, but not with LY294002 (Fig. 5A). The quantitative analysis of total length of dendrites and total dendritic branching tip numbers also showed that IGF-1 partially blocks the effects induced by ICG-001 (Fig. 5B and C). It is still possible that the blocking of degeneration is due solely to the enhanced growth induced by IGF-1, which is unrelated to rescuing the effect caused by the inhibition of Wnt signaling. Overall, our data suggest that the reduction of IGF-1 signaling by the inhibition of Wnt/β-catenin signaling is partially responsible for the degeneration of hippocampal neurons (Supp. Fig. 3C).

2.3. Inducible expression of Axin in vivo causes degeneration of hippocampal neurons

To test degeneration of hippocampal neurons *in vivo* occurs when Wnt/β-catenin signaling is inhibited, double transgenic mice which have both rtTA2^{S}M2 controlled with Axin2 promoter (Axin2P-rtTA) and pBI-EGFP-Axin were used (Shakya et al., 2005; Yu et al., 2007). Using these mice, we took two different approaches (Supp. Fig. 4A). First, we tested whether degeneration of hippocampal neurons can be induced in an *ex-vivo* system. Because hippocampal neurons cultured *in vitro* expressed Axin2 (Supp. Fig. 4B), Axin2P-rtTA was used for the expression of rtTA in hippocampal neurons. After mating two transgenic mice (one with Axin2P-rtTA and the other with pBI-EGFP-Axin), hippocampal neurons were isolated from each embryo and genotyped. The siblings had four different genotypes (Fig. 6). None of the hippocampal neurons from the four different genotypes showed any clear difference until 8 days *in vitro* culture (Fig. 6). However, when hippocampal

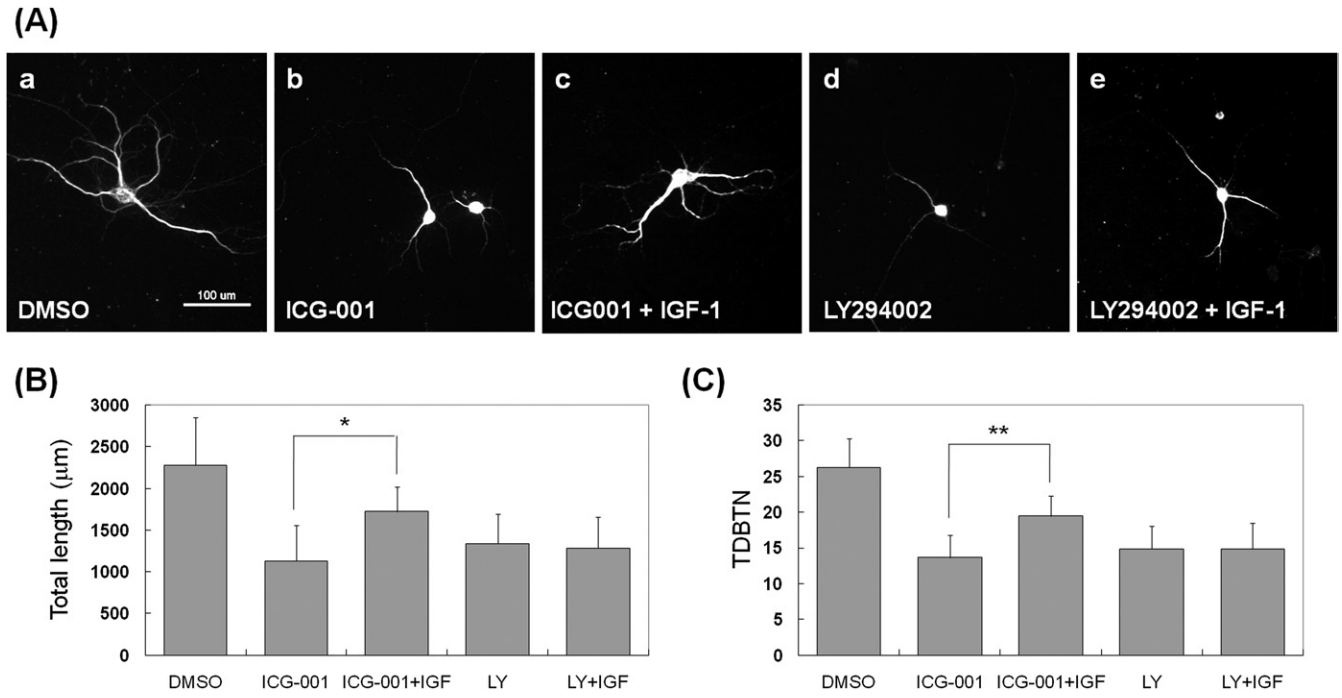
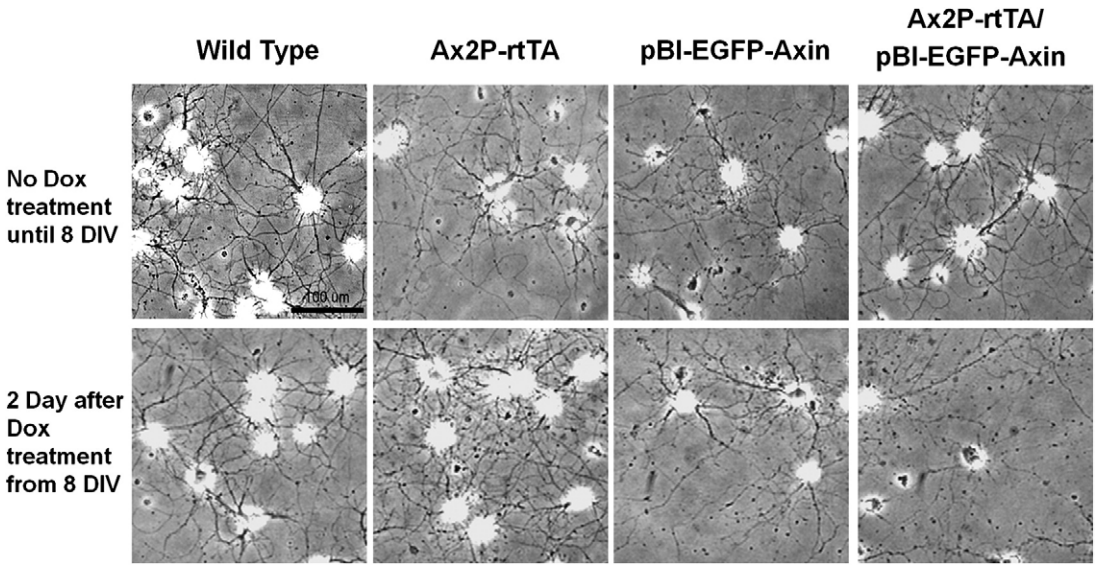


Fig. 5. Addition of IGF-1 partially blocks the degeneration of hippocampal neurons caused by the treatment with ICG-001. (A) Nine DIV hippocampal neurons were cultured with the following treatments for two more days and then immunostained with antibody for MAP2; a: DMSO; b:, 2 μM ICG-001; c: 2 μM ICG-001 + 100 ng IGF-1; d: 20 μM LY294002; e: 20 μM LY294002 + 100 ng IGF-1. Quantification of total length of dendrites (B) and total dendritic branching tip numbers (TDBTN) of the hippocampal neurons that were described above (C). *, $p < 0.05$; **, $p < 0.01$, $n = 9$.

neurons were treated with doxycycline for 2 days the hippocampal neurons containing both Axin2P-rtTA and pBI-EGFP-Axin showed drastic degeneration, while the other hippocampal neurons did not show any effect. These data strongly suggest that tight control of Wnt/β-catenin signaling is necessary for the survival of hippocampal neurons.

Before examining degeneration of hippocampal neurons *in vivo*, we tested whether the inducible expression of target



AQ: 1 Fig. 6. Hippocampal neurons isolated from transgenic mice containing both Axin2P-rtTA and pBI-EGFP/Axin displayed degeneration after inducible expression of Axin. Transgenic mice containing Axin2P-rtTA or pBI-EGFP-Axin were mated with each other and hippocampal neurons isolated from the individual embryos at 18.5 dpc and cultured *in vitro* for 8 days. Each embryo was genotyped and the genotypes of hippocampal neurons are marked in the figure. Doxycycline was added to the culture media and the hippocampal neurons were incubated for two more days.

genes occurs in the hippocampal area. The *in situ* hybridization data (available at www.brain-map.org/) show that Axin2 is expressed in adult mouse hippocampus. Axin2P-rtTA transgenic mice were mated with TRE-LacZ transgenic mice and double transgenic mice containing both Axin2P-rtTA and TRE-LacZ were obtained (Supp. Fig. 5). Transgenic mice containing Axin2P-rtTA or Axin2P-rtTA/TRE-LacZ were given water containing 2 mg/mL doxycycline for 4 weeks. β-galactosidase activity and expression of *LacZ* in the hippocampus were then measured (Supp. Fig. 5A and B, respectively). Both β-galactosidase activity and expression of *LacZ* were observed only in Axin2P-rtTA/TRE-LacZ double transgenic mice. These results suggest that the expression of target genes can be induced in the hippocampus by using Axin2P-rtTA (Supp. Fig. 5).

Transgenic mice (approx. 1 yr old) containing Axin2P-rtTA alone or both Axin2P-rtTA and pBI-EGFP-Axin were given water containing doxycycline for 4 to 8 weeks. Degeneration of hippocampal neurons was then examined. The mice which were administered doxycycline appeared to be normal. However, significantly increased cell death was identified in the hippocampal area of transgenic mice containing both Axin2P-rtTA and pBI-EGFP-Axin (Fig. 7A). Western analysis using hippocampus lysates from these transgenic mice and RT-PCR analysis using primers specific to myc-epitope tag and Axin showed expression of ectopically expressed Axin (Fig. 7B and C). However, the level of ectopically expressed Axin was extremely low, which might have been due to the very low stability of Axin and/or low Axin2 promoter activity *in vivo*. Consistent with data shown in hippocampal neurons cultured *in vitro* (Fig. 4A and B), inhibition of Wnt/β-catenin signaling by the inducible expression of Axin resulted in the reduced expression of *Axin2* and *IGF-1* (Fig. 7D). Overall, our data suggest that reduction of Wnt/β-catenin signaling can cause degeneration of hippocampal neurons *in vivo* and it may be a cause of neurodegenerative diseases.

2.4. Inhibition of Wnt/β-catenin signaling by inducible expression of Axin caused differences in contextual conditioning

Because we saw much more cell death in the hippocampus of transgenic mice which had ectopic expression of Axin, we expected to see neurological differences. To test this possibility, fear conditioning analysis was performed (Fig. 8). Transgenic mice (about 1 yr old) containing Axin2P-rtTA and pBI-EGFP-Axin and control mice containing Axin2P-rtTA and pBI-EGFP were given water with doxycycline for 4 weeks. Both types of transgenic mice showed similar initial freezing time to a noxious unconditional food shock stimulus, which suggests that they do not have any motor impairment (Fig. 8A). In addition, there was no difference in the ability of the transgenic mice to learn and remember an association between foot shock stimuli and an auditory cue (cued conditioning; Fig. 8B). However, the freezing time to measure the ability of the transgenic mice to remember an association between foot shock stimuli and an environmental contexture significantly increased (contextual conditioning; **, $p < 0.01$;

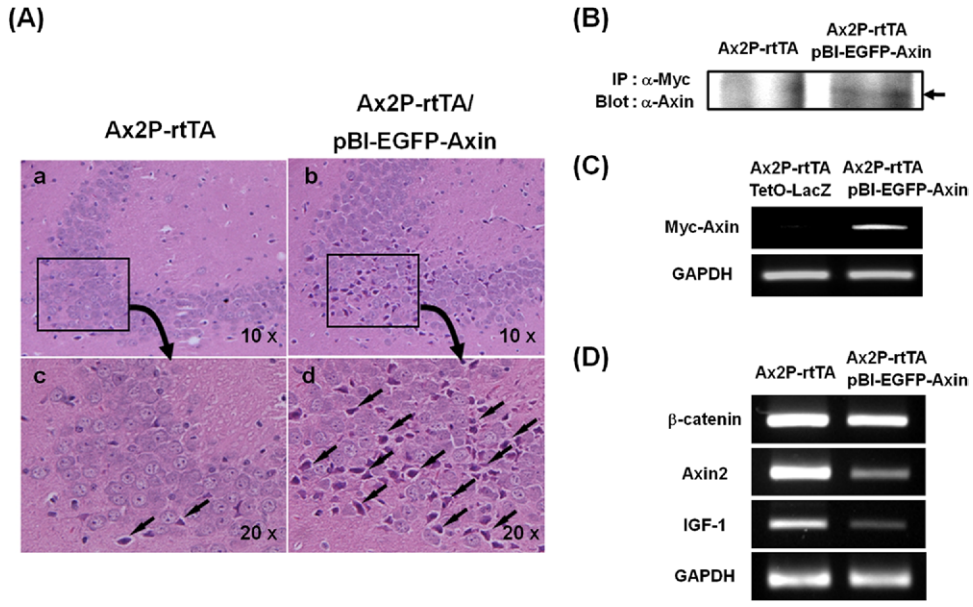


Fig. 7. Inducible expression of Axin caused degeneration of hippocampal neurons *in vivo* and reduced the expression of *IGF-1*. (A) H&E staining of the hippocampal area of Axin2P-rtTA/pBI-EGFP-Axin transgenic mice showed increased number of dead cells. Small arrows indicate dead cells. (B) Cell lysates of hippocampus from the transgenic mice were immunoprecipitated with anti-myc antibody and blotted with anti-axin antibody to detect inducibly expressed myc-epitope tagged Axin. Myc-Axin band is marked with arrow. (C) RT-PCR analysis with the RNAs from the transgenic mice indicated in the figure. (D) RT-PCR analysis with the primers indicated in the figure.

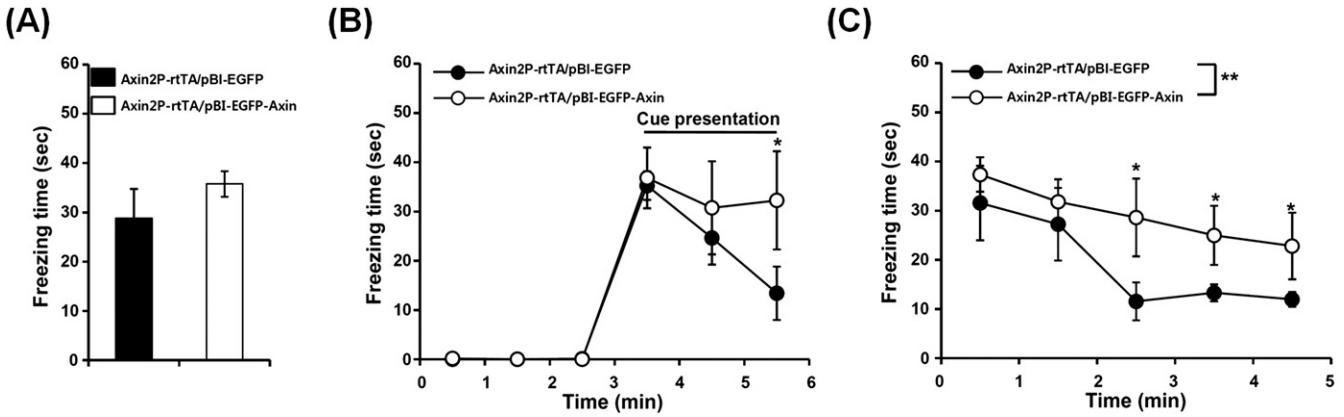


Fig. 8. Inhibition of Wnt/β-catenin signaling in hippocampus caused differences in contextual conditioning. (A) Initial freezing time of Axin2P-rtTA/pBI-EGFP (control, $n = 4$) and Axin2P-rtTA/pBI-EGFP-Axin (Tg, $n = 4$) transgenic mice which were given doxycycline in water for 4 weeks. (B) Measurement of freezing time for the cued conditioning. (C) Measurement of freezing time for the contextual conditioning. All values represent the mean ± SEM. Student's *t*-test (*) and two-way ANOVA (**); *, $p < 0.05$.

Fig. 8C). Although we do not know why the transgenic mice (which have more neuronal cell death in the hippocampus) showed increased freezing time, it is interesting to mention that the hippocampus is responsible for contextual conditioning while the amygdala is responsible for cued conditioning (Kim and Fanselow, 1992; Maren et al., 1996). Lower expression of Axin2 is observed in the amygdala than in the hippocampus (www.brain-map.org/). Overall, these data suggest that downregulation of Wnt signaling in hippocampus may disturb the anxiety-related response (see Discussion).

3. Discussion

Wnt/β-catenin signaling plays a pivotal role in the regulation of developmental processes and in the maintenance of homeostasis in postnatal life by controlling expression of large sets of genes (www.stanford.edu/~rnusse/pathways/targets.html). Recently, it was shown that Tcf3, which is a terminal effector of Wnt/β-catenin signaling, occupies > 1,000 promoters in mouse embryonic stem cells (Cole et al., 2008). Based on these findings, we hypothesized that downregulation of Wnt/β-catenin signaling in the hippocampus disturbs the expression of target genes and leads to degeneration of hippocampal neurons which may be a cause of human neurodegenerative diseases.

In this report, the following approaches have been used to show that downregulation of Wnt/β-catenin signaling causes degeneration of hippocampal neurons. First, inducible expression of transiently transfected genes in mouse primary hippocampal neurons. Second, incubation of hippocampal neurons with a specific inhibitor of Wnt/β-catenin signaling. Third, treatment with doxycycline to induce expression of Axin in the primary hippocampal neurons isolated from transgenic mice containing Axin2P-rtTA and pBI-EGFP-Axin. Fourth, identification of cell death in the hippocampal area of Axin2P-rtTA/pBI-EGFP-Axin transgenic mice. Data from all these approaches suggests that downregulation of Wnt/β-catenin signaling causes degeneration of mature hippocampal neurons.

Our data showed that ectopic expression of Dvl-1 or β-catenin did not enhance dendritic arborization, contrary to two previous studies (Rosso et al., 2005; Yu and Malenka, 2003). The following are possible reasons for this discrepancy. Yu and Malenka used rat hippocampal neurons and a stabilized form of β-catenin (β-cat*), and observed a correlation between the level of β-catenin and dendritic arborization. Because we used wild type β-catenin, which has much lower activity than β-cat*, the level of inducibly expressed β-catenin in our system may not have been sufficient to enhance dendritic arborization. In the second paper (Rosso et al., 2005) the authors used rat hippocampal neurons at 2 DIV for the expression of Dvl-1 while we tested mouse hippocampal neurons at 4 and 9 DIV. It may be possible that the time is critical for the enhancement of dendritic development by the ectopic expression of Dvl-1. Ectopic expression of Dvl-1 at 2 DIV might have stronger effects than at 4 or 9 DIV.

Because overexpression of Axin can induce apoptosis via activation of JNK signaling (Neo et al., 2000), we were concerned that the degeneration of hippocampal neurons we observed after ectopic expression of Axin was not caused by the inhibition of Wnt/β-catenin signaling but by JNK mediated apoptosis. Although we cannot completely exclude the latter possibility, our data strongly support the former because inhibition of Wnt/β-catenin signaling by ectopic expression of ICAT or dominant negative form of Tcf4 and by treatment with ICG-001 caused degeneration of hippocampal neurons (Figs 1 and 2).

When we examined the expression of several Wnt signaling components in mouse primary hippocampal neurons and in adult mouse hippocampus by RT-PCR, the expression of various Wnts, Frizzleds, LRP 5/6, Dvls, β-catenin

and Axin were observed (data not shown.). In addition, a strong expression of optimal Tcf binding promoter driven Venus suggests that Wnt/β-catenin signaling is active in hippocampal neurons (Supp. Fig. 1). Because hippocampal neurons in control culture conditions do not degenerate, but they do when Wnt signaling is inhibited, we think that Wnt signaling works in an autocrine manner. Adult hippocampal neurogenesis is regulated by internal growth factors, such as BDNF, VEGF and IGF-1, and by external stimuli (Lee and Son, 2009). We showed that the reduced level of IGF-1 by the inhibition of Wnt/β-catenin signaling is partially responsible for the degeneration of mature hippocampal neurons (Figs 4 and 5). Our data suggest that the internal growth factor(s) regulated by Wnt/β-catenin signaling may play important roles in the survival of mature hippocampal neurons.

Recently, an association between variants of LRP6, a coreceptor of Wnt, and late-onset Alzheimer's disease (AD) has been identified (De Ferrari et al., 2007). The authors suggested that altered Wnt/β-catenin signaling is involved in Alzheimer's disease. Our data and published data suggest that upregulation of Wnt/β-catenin signaling might provide a new approach for the treatment of neurodegenerative diseases and anxiety in humans. Although the therapeutic potential of lithium (which stabilizes β-catenin by inhibition of GSK-3β and is a clinically used drug for the treatment of depression) for the inhibition of AD is still controversial (Hampel et al., 2009; Leyhe et al., 2009), it will be very interesting to see if small molecules which specifically activate Wnt/β-catenin signaling can be developed, and to test their therapeutic potential for neurodegenerative diseases.

It was also shown that fear conditioning is impaired in AD mouse models (Dineley et al., 2002; Gong et al., 2004). Because we found that downregulation of Wnt/β-catenin signaling caused neuronal cell death in the mouse hippocampus (Fig. 7A), we expected that the transgenic mice would show reduced freezing time in contextual fear conditioning tests, as shown in APP/PS1 transgenic mice (Gong et al., 2004). However, the mice did not show increased freezing time in contextual conditioning tests (Fig. 8C). Although further study is needed to understand why these transgenic mice showed enhanced contextual conditioning, the death of hippocampal neurons via inhibition of Wnt/β-catenin signaling seems to cause greater anxiety-related responses when mice are confronted with aversive contextual cues. This may be a reason why elderly people with dementia show greater anxiety (Gottfries, 1998). Recent work showed that deregulation of Wnt signaling is linked with cell senescence, although one group showed that reduced Wnt signaling (Ye et al., 2007) while other two groups claimed that increased Wnt signaling is involved in cellular senescence (Brack et al., 2007; Liu et al., 2007) (Brack et al., 2007; Liu et al., 2007). Axin2P-rtTA/pBI-EGFP-Axin transgenic mice may be a valuable animal model for neurodegenerative diseases of elderly people who show greater anxiety-related responses.

Disclosure statement

The authors report no real or potential conflicts of interest.

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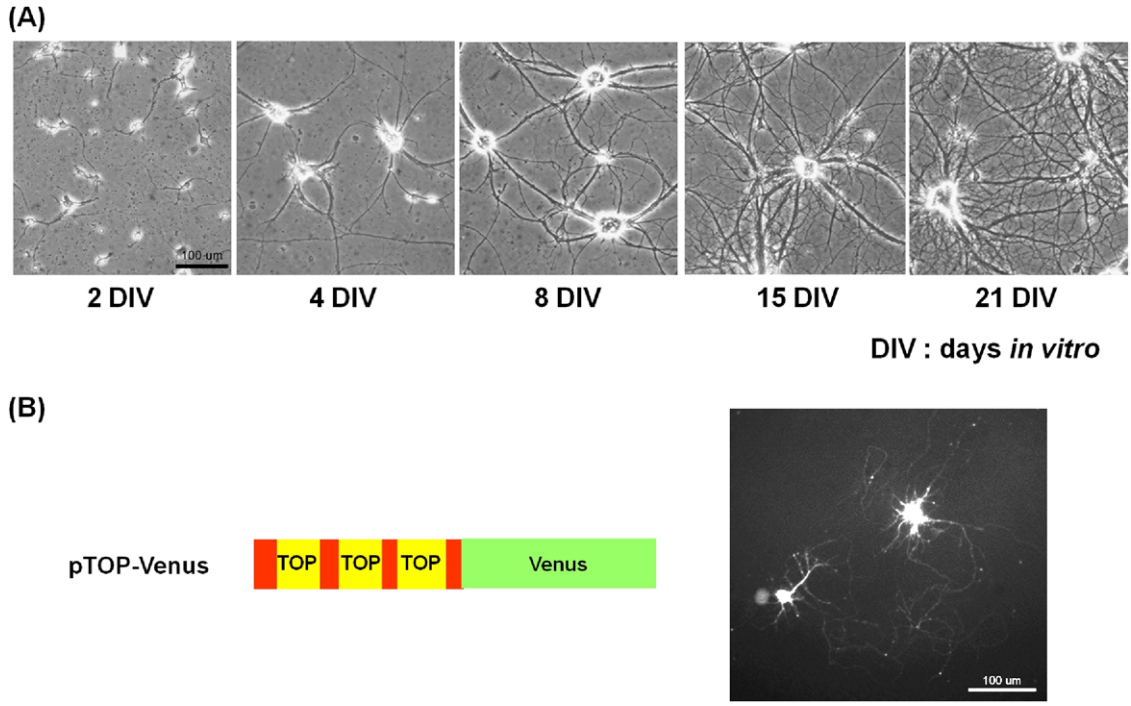
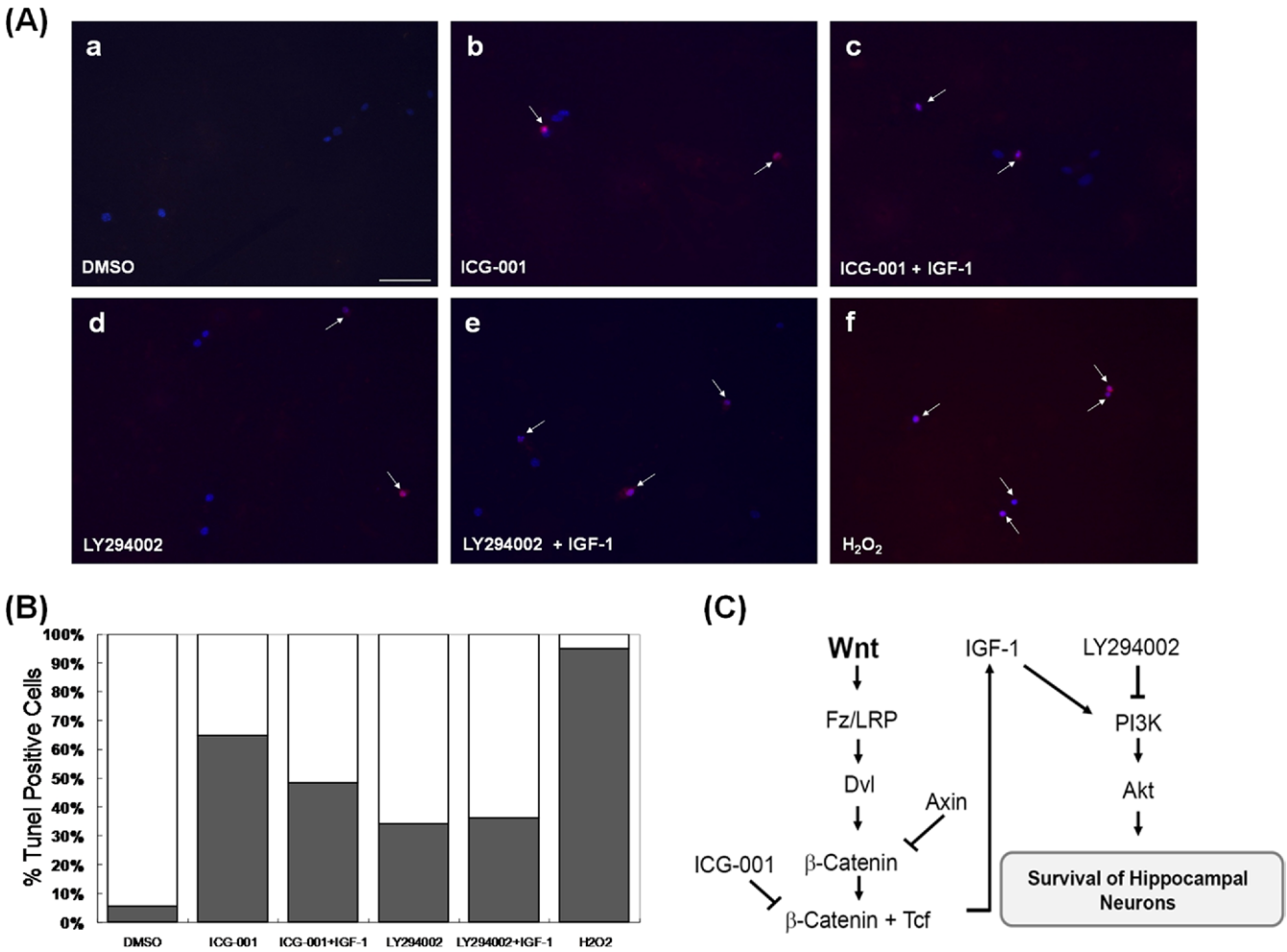
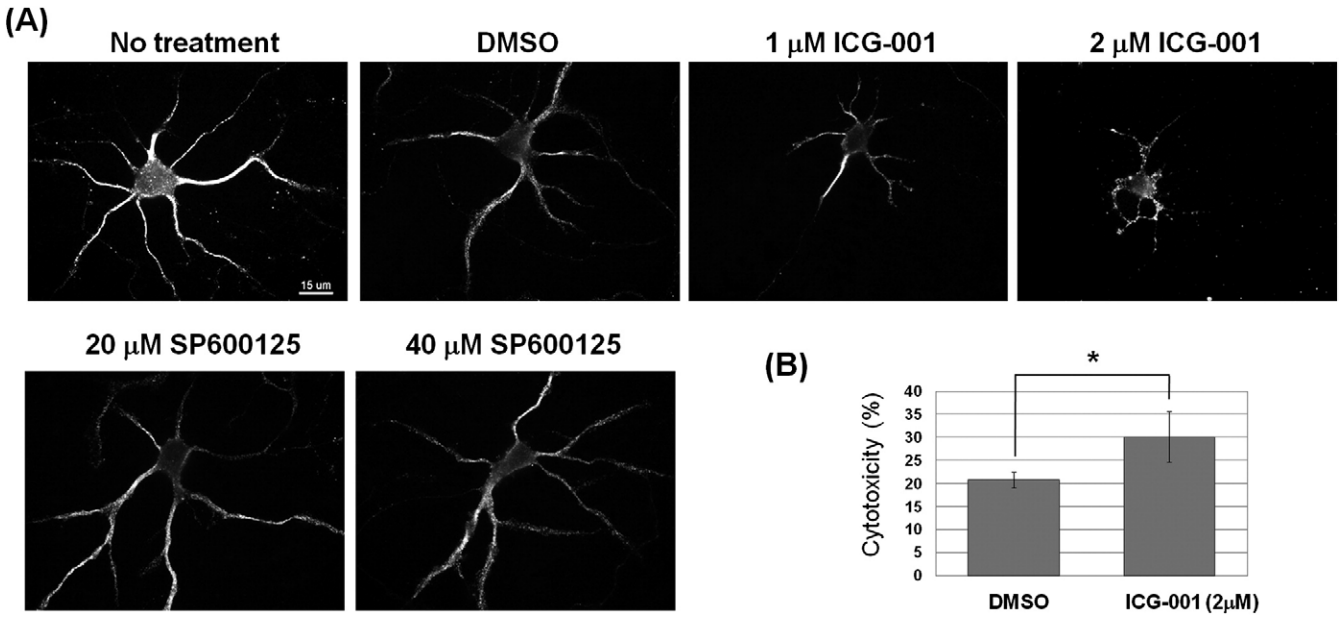


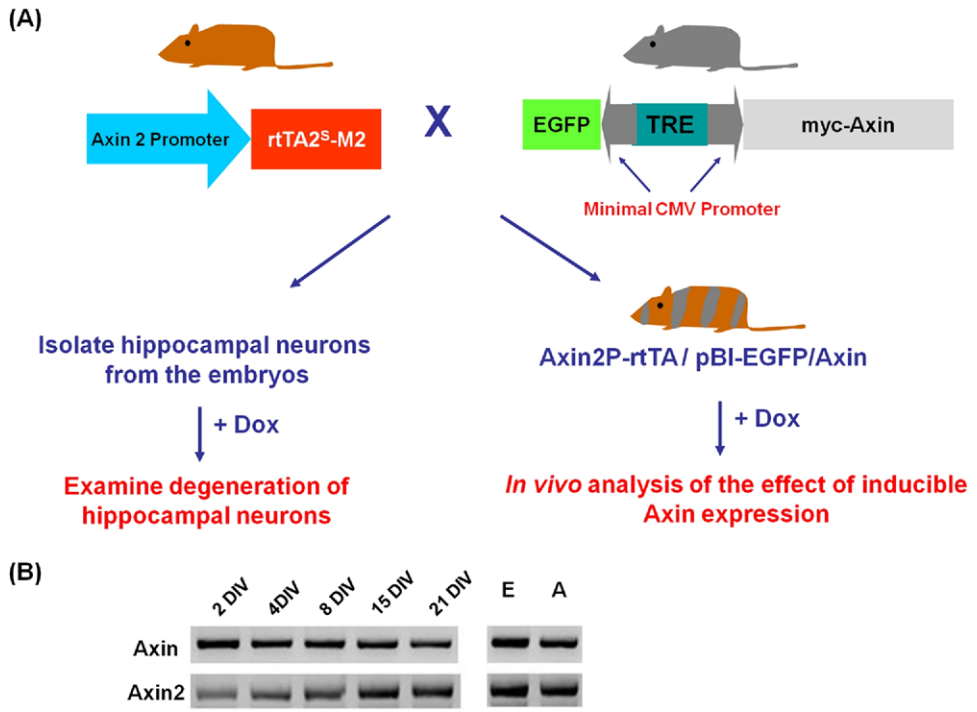
Supplementary Fig. 1. Wnt/β-catenin signaling is active in mouse primary hippocampal neurons cultured *in vitro*. (A) *In vitro* culture of mouse primary hippocampal neurons isolated from E18.5 days. (B) Schematic diagram of pTOP-Venus (left panel). Seven DIV hippocampal neurons were transfected with pTOP-Venus and the expression of Venus was examined after 2 days (right panel).



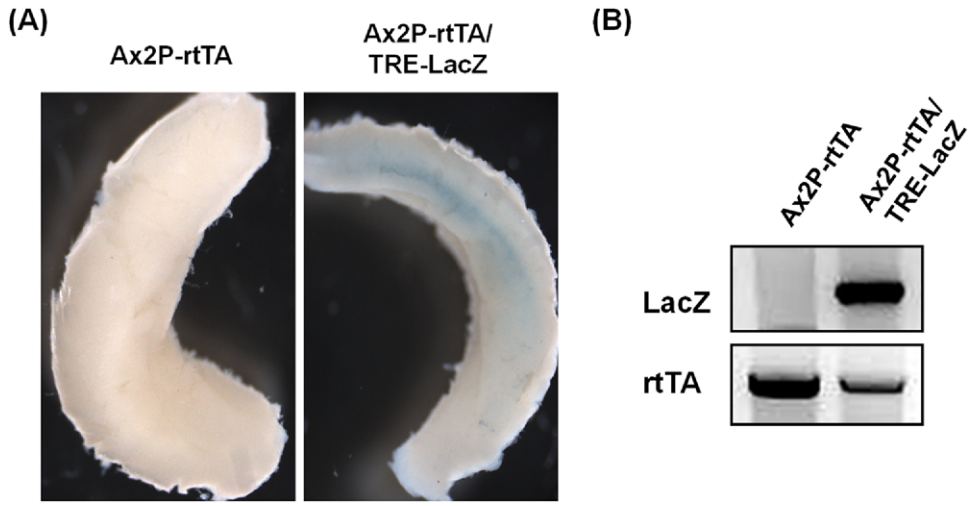
Supplementary Fig. 2. Apoptosis of hippocampal neurons induced by the inhibition of Wnt/β-catenin signaling was partially rescued by the addition of IGF-1. (A) Nine DIV hippocampal neurons were cultured with the following treatments for 2 days and TUNEL assay was performed. a: DMSO; b: 2 μM ICG-001; c: 2 μM ICG-001 + 100 ng IGF-1; d: 20 μM LY294002; e: 20 μM LY294002 + 100 ng IGF-1; f: 2 mM H_2O_2. Scale bar, 100 m. (B) The percentage of TUNEL positive cells observed ($n > 35$). (C) Schematic diagram for signaling involved in the survival of hippocampal neurons.



Supplementary Fig. 3. Treatment with ICG-001 blocks dendritic arborization of mouse primary hippocampal neurons. (A) Mouse primary hippocampal neurons cultured *in vitro* for 4 days were incubated with the following treatments for two more days; a: no treatment, b: DMSO; c: 1 μM ICG-001; d: 2 μM ICG-001; e: 20 μM SP600125; f: 40 μM SP600125. Hippocampal neurons were stained with antibody for MAP2 to enhance visualization of the blocking of dendritic arborization. (B) Four DIV hippocampal neurons were incubated with DMSO or 2 μM ICG-001 for 2 days and LDH assays were performed. *, $p < 0.05$.



Supplementary Fig. 4. Schematic diagram for the experiments performed using Axin2P-rtTA and pBI-EGFP-Axin transgenic mice. (A) Axin2P-rtTA transgenic mice were mated with pBI-EGFP-Axin transgenic mice. The degeneration of primary hippocampal neurons which were isolated from the embryos produced by the above mating was examined after the addition of 500 ng/mL doxycycline to the culture media (left panel). Transgenic mice (approximately 1 yr old) containing Axin2P-rtTA/pBI-EGFP or Axin2P-rtTA/pBI-EGFP-Axin were given doxycycline in water for 4 to 8 weeks and *in vivo* analysis and behavioral testing were performed. (B) RT-PCR analysis showed the expression of Axin2 in the primary hippocampal neurons cultured *in vitro* for the different periods indicated in the figure. E and A, RT-PCR analysis using RNAs isolated from the hippocampus of embryos or adult mice, respectively.



Supplementary Fig. 5. Inducible expression of LacZ in the hippocampal area of Axin2P-rtTA/TRE-LacZ transgenic mice. (A) Axin2P-rtTA transgenic mice were mated with TRE-LacZ transgenic mice. Axin2P-rtTA or Axin2P-rtTA/TRE-LacZ transgenic mice (approximately 1 yr old) were given doxycycline in water and LacZ staining was performed on their hippocampus. Axin2P-rtTA/TRE-LacZ transgenic mice showed positive LacZ staining in the hippocampus. (B) RT-PCR analysis showed that *LacZ* is expressed in the hippocampus of Axin2P-rtTA/TRE-LacZ transgenic mice.