A Novel Wnt5a-Frizzled4 Signaling Pathway Mediates Activity-Independent Dendrite Morphogenesis via the Distal PDZ Motif of Frizzled 4

Wen-Jie Bian,[1,2] Wan-Ying Miao,[1] Shun-Ji He,[1] Zong-Fang Wan,[1] Zhen-Ge Luo,[1] Xiang Yu[1]

[1] Institute of Neuroscience and State Key Laboratory of Neuroscience, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

[2] University of Chinese Academy of Sciences, Shanghai 200031, China

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ABSTRACT: **The morphology of the dendritic tree is critical to neuronal function and neural circuit wiring. Several Wnt family members have been demonstrated to play important roles in dendrite development. However, the Wnt receptors responsible for mediating this process remain largely elusive. Using primary hippocampal neuronal cultures as a model system, we report that Frizzled4 (Fzd4), a member of the Fzd family of Wnt receptors, specifically signals downstream of Wnt5a to promote dendrite branching and growth. Interestingly, the less conserved distal PDZ binding motif of Fzd4, and not its conserved proximal Dvl-interacting PDZ motif, is required for mediating this effect. We further showed that Dvl signaled parallel to and independent of Fzd4 in promoting dendrite growth. Unlike most previously described pathways, Wnt5a/Fzd4 signaling promoted dendrite development in an activity-independent and autocrine fashion. Together, these results provide the first identification of a Wnt receptor for regulating dendrite development in the mammalian system, and demonstrate a novel function of the distal PDZ motif of Fzd4 in dendrite morphogenesis, thereby expanding our knowledge of the complex roles of Wnt signaling in neural development.** © 2014 Wiley Periodicals, Inc. Develop Neurobiol 75: 805–822, 2015

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INTRODUCTION

The mammalian nervous system is comprised of an enormous number of morphologically complex and highly interconnected neurons. Information sent via the axonal terminals of upstream neurons reaches the dendrites of neurons via synaptic contacts. The morphology of the dendritic arbor thus plays a critical role in the processing and integration of information in the brain. Over the past several decades, a number of extracellular factors and intracellular signaling pathways have been shown to play critical roles in regulating dendrite morphogenesis (Wong and Ghosh, 2002; Cline and Haas, 2008; Urbanska et al., 2008; Jan and Jan, 2010; Arikkath, 2012; Koleske, 2013). Prominent among the extracellular secreted factors are neurotrophic factors such as brain-derived neurotrophic factor (BDNF), members of the Wnt and bone morphogenetic protein families, as well as Cpg15, Reelin, Semaphrins and Slits (Wong and

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Correspondence to: X. Yu (yuxiang@ion.ac.cn).

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Ghosh, 2002; Urbanska et al., 2008; Jan and Jan, 2010; Arikkath, 2012; Koleske, 2013).

Wnts are lipid-modified, secreted signaling molecules, highly conserved across species, from *Drosophila* and *C. elegans* to humans (Willert and Nusse, 2012). They have been shown to play critical roles in many fundamental developmental processes, including cell proliferation, cell fate determination, embryonic development, neurogenesis, organogenesis (Gordon and Nusse, 2006), and multiple aspects of neuronal development (Park and Shen, 2012; Salinas, 2012; Rosso and Inestrosa, 2013). Since the first identification of *Wnt1* over 30 years ago (Nusse and Varmus, 1982), the Wnt family has expanded to include 19 identified members in mammals. Receptors mediating Wnt signaling are equally complex. The seven-transmembrane Frizzled receptors (Fzds) were first demonstrated to function as Wnt receptors through direct binding and activation of downstream signaling (Bhanot et al., 1996; Janda et al., 2012). Fzd proteins, of which there are 10 family members in mammals, bind to Wnts through their large extracellular cysteine-rich domain (CRD), and have been shown to mediate both "canonical" and "noncanonical" Wnt signaling (Schulte, 2010; MacDonald and He, 2012; Niehrs, 2012). Canonical Wnt signaling is initiated by the binding of Wnt and Fzd, in a complex with co-receptor low density lipoprotein receptor-related protein (LRP). This leads to recruitment of Dishevelled (Dvl) and inhibition of the protein complex containing Axin, Glycogen synthase kinase-3, and Adenomatous Polyphonies Coli protein, resulting in β-catenin stabilization and activation of lymphoid enhancer factor (LEF) or T-cell factor (TCF)-dependent gene transcription (MacDonald and He, 2012; Niehrs, 2012). Noncanonical Wnt pathways include all Wnt signaling mechanisms that do not require β-catenin/LEF/TCF-dependent transcriptional regulation (van Amerongen et al., 2008; Schulte, 2010). The most well characterized noncanonical pathway is the planar cell polarity pathway, which is independent of LRP, β-catenin or LEF/TCF, but involves *Drosophila* Fzd, Dvl, Van Gogh, Flamingo, and other components (van Amerongen et al., 2008; van Amerongen, 2012). Another noncanonical Wnt pathway is the so-called Ca^{2+}, pathway which requires Ca^{2+}-dependent downstream effectors (van Amerongen et al., 2008; Schulte, 2010). Additional noncanonical Wnt pathways mediated by Fzds (Mathew et al., 2005; Ataman et al., 2006), as well as other Wnt receptors, including the related to tyrosine kinases (Ryks; Fradkin et al., 2010) and the receptor tyrosine kinase-like orphan receptors (Rors; Green et al., 2008) have also been identified.

Several Wnt signaling pathways, most of which noncanonical, have been shown to regulate dendrite development. Yu and Malenka found that elevating intracellular β-catenin level promoted dendrite growth in cultured hippocampal neurons via increased cadherin/catenin-dependent cell adhesion, and independent of LEF/TCF-dependent transcription. They further showed that neuronal activity promoted the secretion of a canonical Wnt from cultured neurons (Yu and Malenka, 2003). Rosso et al. showed that Wnt7b signaled via Dvl to activate Rac and Jun N-terminal kinase (JNK), and promoted dendritic arborization through regulation of cytoskeletal dynamics (Rosso et al., 2005). Wayman et al. identified *Wnt2* as an activity-dependent CREB-responsive gene that promoted dendritic arborization (Wayman et al., 2006). In *Drosophila*, Singh et al. found that Wnt signaling is required for dendritic refinement in the developing antennal lobe (Singh et al., 2010) while in *C. elegans*, Kirszenblat et al. showed that Wnt family member LIN-44 and Frizzled family member LIN-17 directed dendrite outgrowth of PQR oxygen sensory neurons (Kirszenblat et al., 2011). Together these results demonstrated that multiple Wnts and Wnt signal transduction pathways contributed to the regulation of dendrite morphogenesis across species. Yet, many questions remain unanswered. First, in the mammalian system, although Fzds are reported to be involved in many aspects of neuronal morphogenesis such as polarity, axonal growth, and synaptogenesis (Wang et al., 2006; Varela-Nallar et al., 2009; Sahores et al., 2010; Slater et al., 2013), it is still unknown which Wnt receptors mediate the effect of Wnts on dendrite morphogenesis. Furthermore, since Wnt expression can be upregulated by neural activity, do Wnts regulate dendrite development synergistically with or independently of activity? Finally, do Wnts function in an autocrine or paracrine fashion to regulate dendrite morphogenesis?

Here, using dissociated hippocampal neuronal cultures, we identified an autocrine and activity-independent role of Wnt5a in promoting dendrite arborization. We further showed that this effect is mediated by Fzd4. Interestingly, of the two PSD-95/Discs large/ZO-1 (PDZ)-binding motifs in Fzd4, it is the distal PDZ-binding motif located at the C-terminal end, rather than the proximal Dvl-interacting PDZ-binding motif, that is required for promoting dendritic branching and growth. We furthered show that PDZ scaffolds, including PSD95 and glutamate receptor interacting protein 1 (GRIP1), likely function as downstream effectors of Wnt5a/Fzd4 signaling during dendrite growth. Together, our results identify, for the first time, a specific Fzd

receptor for mediating the effect of Wnt on dendrite morphogenesis in the mammalian system, and uncover a novel signaling mechanism downstream of Wnt5a, mediated by the distal PDZ-binding motif of Fzd4.

METHODS

Hippocampal Neuronal Culture Preparation and Transfection

Primary dissociated hippocampal cultures were prepared from postnatal day 0 (P0) Sprague-Dawley rat pups as previously described (Yu and Malenka, 2003; Tan et al., 2010) and according to procedures approved by the Institutional Animal Care and Use Committee of the Institute of Neuroscience, Chinese Academy of Science. Briefly, dissociated hippocampal neurons were plated on matrigel- (BD Bioscience, San Jose, CA) or laminin- (Sigma-Aldrich, St. Louis, MO) coated glass coverslips (Assistant, Sondheim, Germany) at 50,000 cells/cm^2 or 1,500 cells/cm^2 (for isolated culture), in Neurobasal medium (Invitrogen, Carlsbad, CA) containing B-27 (Invitrogen, Carlsbad, CA), and 2 m*M* Glutamax-I (Invitrogen, Carlsbad, CA), and 2.5% FBS (HyClone, Logan, UT). On the third day *in vitro* (DIV 3), after a monolayer of astrocytes has covered the entire coverslip, cells were treated with the mitotic inhibitor 5-fluoro-2′-deoxyuridine (Sigma-Aldrich, St. Louis, MO). Calcium phosphate transfections were performed on DIV 6 using 2–4 μg of DNA per well (24-well plate; Corning, Corning, NY), and neurons were fixed on DIV 8 for morphological analysis. A plasmid encoding GFP or tdTomato (0.2–0.7 μg per well) was included in the transfection mixture to aid morphological visualization. Typically, 2 μg of DNA was added for overexpression assays while for rescue experiments, 3 μg of overexpression construct was added, together with 1 μg of RNAi DNA. More DNA was added in rescue experiments to determine the maximum extend of possible rescue. For all experiments using RNAi constructs, the empty pSuper vector was used as control, as previously described by others and us (Hoogenraad et al., 2005; Futai et al., 2007; Zhang et al., 2007; Tan et al., 2010).

For testing the efficiency of *Fzd4* RNAi against endogenous *Fzd4*, lenti-viruses carrying *Fzd4* RNAi sequence (same sequence as for pSuper construct described below; LV_*Fzd4* RNAi, GeneChem, Shanghai, China) or control lenti-viruses were added to culture medium on DIV 4 at a multiplicity of infection of 5 TU/cell. Infection efficiency was confirmed by GFP fluorescence, and cultures were harvested for quantitative real-time PCR on DIV 11.

For isolated culture, neurons were electroporated (Nucleofector II; Amaxa, Cologne, Germany) with *Wnt5a* overexpression and RNAi constructs at DIV 0, and plated on astrocyte-covered coverslips. Isolated cultures were fixed at DIV3 and stained for MAP2 to confirm that the neuron analyzed was isolated from other neurons.

For pharmacological treatments, 10 m*M* KCl and/or 20 μ*M* nifedipine (Nif, Tocris, UK) were added to culture medium 48 h before fixation or RNA extraction. For Wnt5a stimulation, purified recombinant human/mouse Wnt5a (R&D 645-WN-010, Minneapolis, MN) was added to culture medium 24 h before fixation.

DNA Constructs

The full length mouse *Fzd4* construct with extracellular HA tag was a gift of Prof. Xi He (Harvard Medical School; Wei et al., 2007). The coding sequence of mouse *Wnt5a*, mouse *Fzd4* (with HA tag at C-terminus) and rat *GRIP1* (gift of Dr. Richard Huganir, Johns Hopkins University; Dong et al., 1997) were subcloned into pCS2min, a modified version of pCS2 that overexpressed proteins at a high level and in an activity-independent fashion (Tan et al., 2010). *Fzd4ΔPDZ1+2*, *Fzd4ΔPDZ2*, and *Fzd4mPDZ1* were generated by, respectively, deleting Residues 499–537, 535–537 or making the point mutation (K to A) at Residue 499 of mouse *Fzd4* according to a previous study (Cong et al., 2004). We note that the mouse *Fzd4* used is resistant to degradation by rat *Fzd4* RNAi. *HA-CD8-Fzd4Ct* and *HA-CD8-Fzd4CtΔPDZ2* were generated by fusing the C-terminal intracellular domain of mouse Fzd4 (Residues 497–537, *Fzd4Ct*) or the Fzd4Ct with PDZ2 deleted (Residues 497–534, *Fzd4CtΔPDZ2*) to the transmembrane domain of rat CD8a (Residues 1–236); an HA tag was inserted immediately following the CD8a signal peptide (Residues 1–27).

PSD95 (GFP-tagged) was a gift of Prof. Robert Malenka (Stanford University; Regalado et al., 2006). RNAi resistant *Dvl* (*Dvl*^{r}) was generated by making the following silent point mutations T1670C, G1676C, G1685A, and C1688T. The *Wnt5a* RNAi, *Fzd4* RNAi, *Gpr177* RNAi, *Dvl* RNAi, *PSD95* RNAi, and *GRIP1* RNAi constructs targeting the following rat sequences were cloned into pSuper-GFP: *Wnt5a* RNAi-2: GCAAGCTGGTACAGGTCAA; *Wnt5a* RNAi-3: GCGTGGCTATGACCAGTTT; *Fzd4* RNAi: GCACCTCTCTTTACCTATT; *Gpr177* RNAi-1: GCATG AAGGTCGTCATTAT; *Gpr177* RNAi-2: GGAGAAGG ATCACCATGAT; *Gpr177* RNAi-3: GCACATGATGGAT CAACAT; *Dvl* RNAi: ATGTGGTGGACTGGCTGTA (Zhang et al., 2007); *PSD95* RNAi: GAGGCAGGTTCC ATCGTTC (Futai et al., 2007); *GRIP1* RNAi: GCCAA GAGTGTCCAACCTG (Hoogenraad et al., 2005). The *Dvl* RNAi was designed to target all three mammalian *Dvls*, *Dvl1*, nt 1669–1687; *Dvl2*, nt 1591–1609; and *Dvl3*, nt 1550–1618, and the efficiencies of *Dvl* RNAi, *PSD95* RNAi, and *GRIP1* RNAi were characterized in the original studies describing them (Hoogenraad et al., 2005; Futai et al., 2007; Zhang et al., 2007). The *Fzd4* RNAi was designed to specifically target rat *Fzd4*, without affecting the expression of mouse *Fzd4*, such that mouse *Fzd4* can be used for subsequent rescue experiments. LV_*Fzd4* RNAi was cloned into the GV118 lenti-viral vector, expressing shRNA under the U6 promoter and EGFP under the Ubiquitin promoter (GeneChem, Shanghai, China).

Immunocytochemistry, Image Acquisition, and Morphological Analysis

Cultured hippocampal neurons (DIV 3 for isolated culture experiments, DIV 15 for Fzd4 C-terminal variants immunostaining and DIV 8 for all others) were washed twice in phosphate-buffered saline (PBS) at 37°C and fixed for 20 min in 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO) in PBS at room temperature. Neurons were permeabilized in 0.1% Triton-X100 in PBS for 5 min, blocked with 3% bovine serum albumin (BSA; Calbiochem, San Diego, CA) in PBS for 1 h at 37°C and incubated with 3% BSA containing primary antibodies overnight at 4°C and correspondent secondary antibodies for 1 h at 37°C. The following antibodies were used: anti-HA (rabbit, Abcam ab9110-100, 1:5000, Cambridge, MA), anti-MAP2 (rabbit, Chemicon Ab5622, 1:500, Temecula, CA), goat anti-rabbit Alexa-Fluor 568 (A11036), and 647 (A21245) secondary antibodies (Invitrogen, 1:1000, Carlsbad, CA). Coverslips were mounted in Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA).

Z-stack images at 1 μm interval were acquired on a LSM5 Pascal laser scanning confocal microscope (Carl Zeiss, Jena, Germany) with a 40× oil immersion Neofluor objective (N.A. = 1.3, Carl Zeiss, Jena, Germany, for quantitative morphological analysis) at 1× optical zoom or with a 63× oil immersion Plan-Apochromat objective (N.A. = 1.4, Carl Zeiss, Jena, Germany, for immunocytochemistry) at 2× optical zoom. Pyramidal neurons were identified by their classical pyramidal morphology as visualized by GFP or tdTomato. Total dendritic branch tip number (TDBTN) was counted blindly from the projected Z-stacks using ImageProPlus (Media-Cybernetics, Silver Spring, MD). For total dendritic branch length (TDBL) analysis, dendritic branches were manually traced, and TDBL was measured using ImageProPlus. At DIV 8, hippocampal neurons have not yet developed dendritic spines, so protrusions longer than 3 μm were considered a dendritic branch tips.

Western Blot

P0, P7, or P14 mice were deeply anaesthetized with sodium pentobarbital, and forebrains, and hippocampi were dissected and homogenized in ice-cold HEPES-buffered sucrose (0.32 M sucrose, 4 m*M* HEPES, pH 7.4; Sigma-Aldrich, St. Louis, MO), containing freshly added protease inhibitor cocktail tablets (Roche, Mannheim, Germany). For RNAi efficiency test, plasmids were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and cells were lysed in RIPA containing protease inhibitor cocktail tablets (Roche, Mannheim, Germany) 48 h after transfection. Western blot was performed according to standard protocols, and results were quantified using ImageJ software (NIH Image). The following primary antibodies were used: Wnt5a (goat, R&D AF645, 1:500, Minneapolis, MN), GFP (rabbit, Invitrogen A11122, 1:1000, Carlsbad, CA), GAPDH (mouse, Kangcheng KC-5G4, 1:5000, Shanghai, China), and α-Tubulin (mouse, Sigma T6074, 1:20000, Sigma-Aldrich, St. Louis, MO).

Immunoprecipitation

Cultured hippocampal neurons (DIV 6) were lysed in IP buffer (P0013, Beyotime, Shanghai, China) containing protease inhibitor cocktail tablets (Roche, Mannheim, Germany) for 30 min at 4°C. After centrifugation at 10,000*g* for 10 min, the supernatant was incubated with 30 μL EZview Red Anti-HA Affinity Gel (E6779, Sigma-Aldrich, St. Louis, MO) overnight at 4°C. The sample was centrifuged at 7000*g* for 30 sec, the supernatant was discard, and the pellet was washed five times with 750 μL IP buffer. The bound proteins were then eluted and analyzed by immunoblotting with the following antibodies: HA (rabbit, Abcam ab9110, 1:1000), PSD95 (mouse, Neuromab 75-028, 1:3000), GAPDH (mouse, Kangcheng KC-5G4, 1:5000, Shanghai, China).

Reverse Transcription and Real-Time Quantitative PCR

DIV 8 cultured rat hippocampal neurons were washed twice in cold PBS, collected with a cell scrapper (Fisher, Pittsburgh, PA) and lysed in TRIzol (Invitrogen, Carlsbad, CA). For developmental expression of *Wnt5a* and *Fzd4*, P3, P7, or P14 mice were deeply anaesthetized, and forebrains and hippocampi were dissected and homogenized in TRIzol. Total RNA was extracted. Reverse transcription were performed using SuperScript™ III Reverse Transcriptase (Invitrogen, Carlsbad, CA), and the synthesized first-strand cDNA was subjected to real-time quantitative PCR (ABI Prism7000 System, Applied Biosciences, Foster City, CA), using *GAPDH* (for rat cultured neurons) or *β-tubulin* (for mouse cortex or hippocampus) as internal control. The primers used are listed below:

GAPDH, forward: CTGCCCAGAACATCATCCCT; reverse: TGAAGTCGCAGGAGACAACC.

Rat *Wnt5a*, forward: ATTGTGGATCAGTTCGTGT GCA; reverse: TCCCCCACTATTGCCAAACAT.

Rat *BDNF*, forward: AGCGTGTGTGACAGTATTAG CGAGT; reverse: CTATCTTCCCCTTTTAATGGTC AGT.

Rat *Fzd4*, forward: AGATGGGACCAAGACAGACA; reverse: ATCCACATGCCTGAAGTGAT.

β-tubulin, forward: CGACAATGAAGCCCTCTACG AC; reverse: ATGGTGGCAGACACAAGGTGGTTG.

Mouse *Wnt5a*, forward: CCCAGTCCGGACTACTG TGT; reverse: TTTGACATAGCAGCACCAGTG.

Mouse *Fzd4*, forward: TGCTGGGTTGGGAGACG TGT; reverse: ACCACCTCTTGGGAGTGCGCTA.

Statistical Analysis

Statistical analysis was carried out using two-tailed Student's *t*-test (for sample pairs) or one-way ANOVA (for

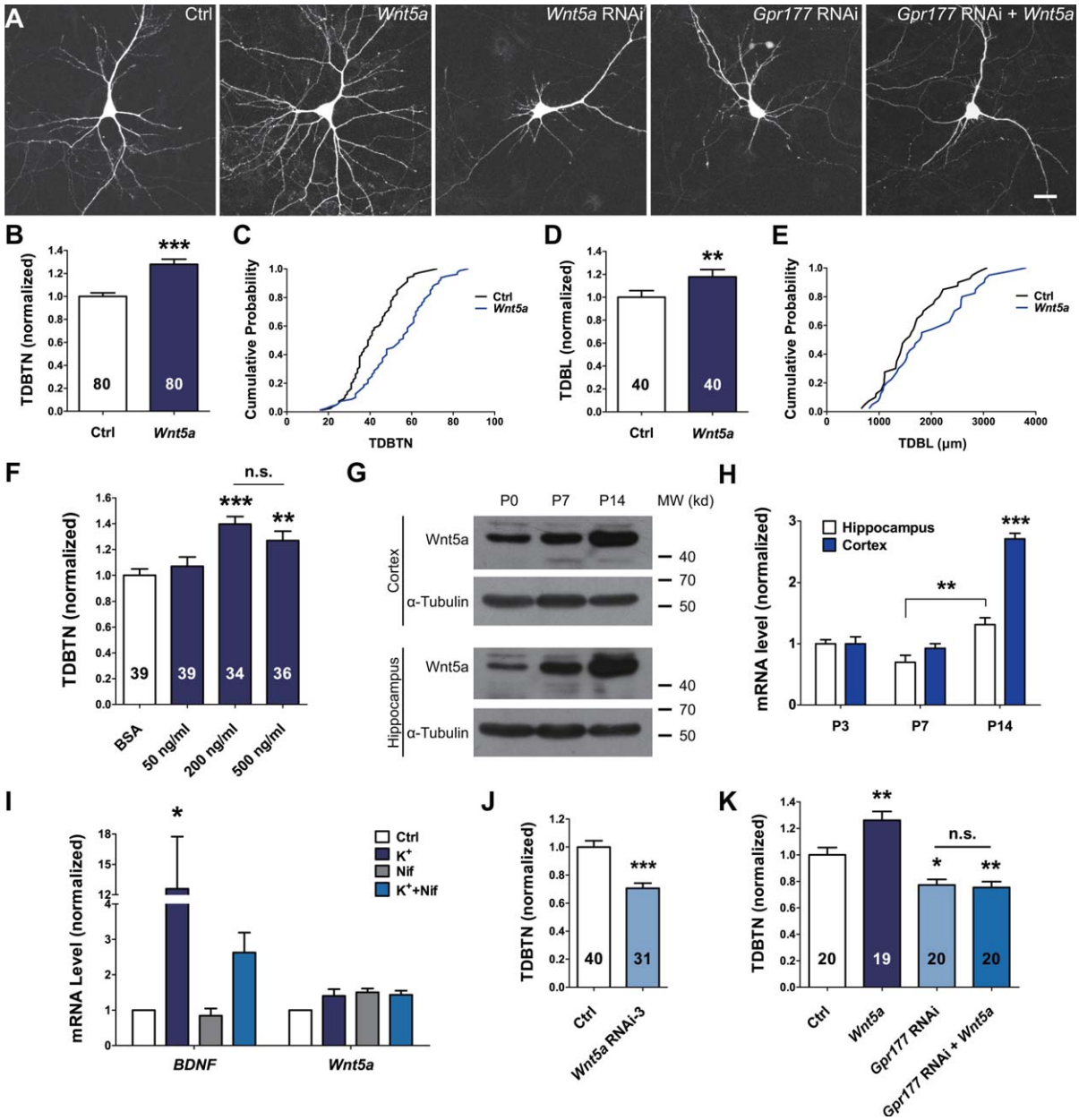

Figure 1 Wnt5a promoted dendrite morphogenesis. A. Representative images of DIV 8 rat hippocampal neurons, conditions as indicated. Scale bar: 20 μm. B–E. DIV 8 Neurons overexpressing *Wnt5a* had significantly higher TDBTN (B, $***p < 0.001$) and TDBL (D, $**p < 0.01$) as compared to control neurons expressing GFP only. Cumulative distributions of TDBTN (C, $***p < 0.001$) and TDBL (E, $*p = 0.047$) for data presented in B and D, respectively. F. Application of purified Wnt5a increased TDBTN in a dose-dependent manner. G and H. Western blots (G) and real-time qPCR results (H) showing significant increases in the expression level of *Wnt5a* in the cerebral cortex and hippocampus during the first two weeks of mouse development. I. Real-time qPCR results showing changes in the mRNA level of *BDNF* and *Wnt5a* in rat hippocampal neuronal cultures following activity manipulations. J. Lowering endogenous *Wnt5a* expression with *Wnt5a* RNAi-3 significantly reduced TDBTN at DIV 8. K. Interfering with Wnt5a surface delivery through *Gpr177* RNAi significantly reduced TDBTN and blocked the effect of *Wnt5a* overexpression at DIV 8. In this and all subsequent figures, error bars represent s.e.m., "*n*" as indicated inside bar graphs represents the number of neurons, and * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; n.s. is not significant, $p > 0.05$. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

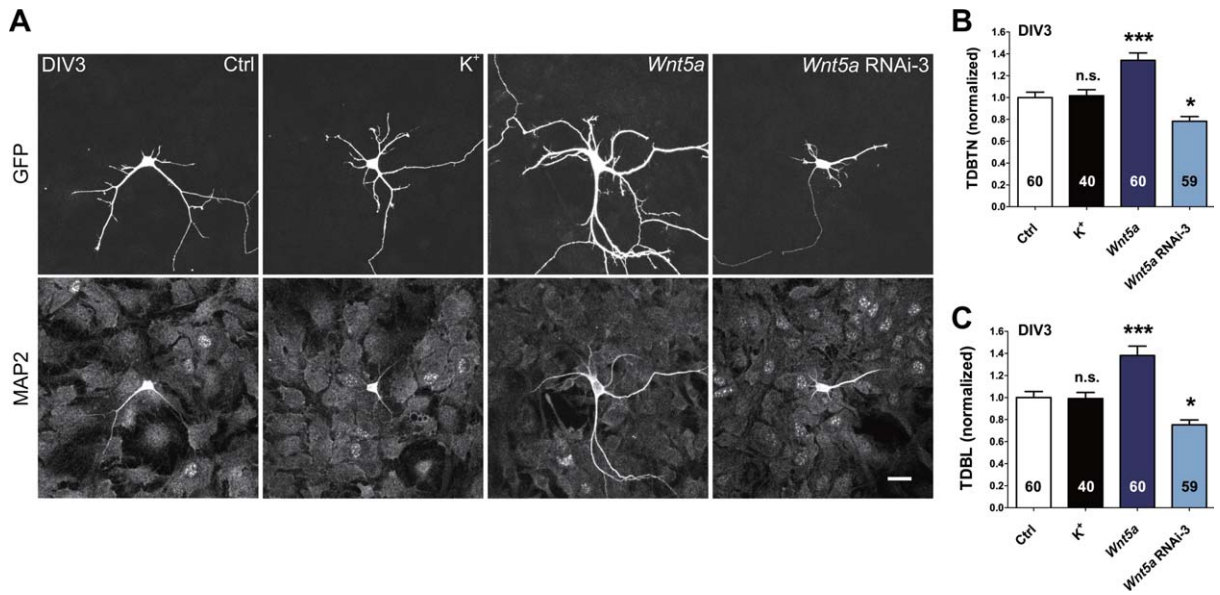

Figure 2 Wnt5a promoted dendrite growth independent of neural activity. A. Representative images of DIV 3 neurons from isolated neuronal cultures electroporated at the time of plating, conditions as indicated. MAP2 immunostaining was used to determine that the imaged neurons were truly isolated from other neurons. Scale bar: 20 μm. B and C. In DIV 3 neurons, *Wnt5a* overexpression significantly increased TDBTN and TDBL while *Wnt5a* RNAi significantly reduced these measurements, under conditions when high K^+ treatment had no significant effect. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

groups of three or more conditions) followed by Tukey's Multiple Comparison test. Results are shown as mean ± SEM, and "*n*" refers to the number of neurons (for morphological analysis) or the number of animals (for quantitative PCR and western blot). All conditions statistically different from control are indicated. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

RESULTS

Wnt5a Promoted Dendritic Branching and Growth Independent of Neuronal Activity

Previous studies have shown that the expression and/or secretion of canonical Wnts can be upregulated by neuronal activity (Yu and Malenka, 2003; Wayman et al., 2006), demonstrating a role of Wnt signaling in activity-dependent dendrite development. Is there a role of Wnts also in activity-independent dendrite growth? Here, we identified Wnt5a as such a molecule. In dissociated rat hippocampal neuronal cultures, Wnt5a significantly enhanced dendrite complexity either when transiently overexpressed from DIV 6 to 8 [Fig. 1(A–E)] or when applied as a purified protein for 24 h [Fig. 1(F)], as measured by increased TDBTN [Fig. 1(B, C, and F)] and TDBL [Fig. 1(D,E)]. Consistent with its role in promoting dendrite growth during early development, the mRNA and protein levels of Wnt5a increased significantly both in the cerebral cortex and in the hippocampus during the first two postnatal weeks [Fig. 1(G,H)], within the window of rapid dendrite growth and synapse formation in the rodent brain (Micheva and Beaulieu, 1996). Despite this developmental increase in expression, the mRNA level of *Wnt5a* was not regulated by neuronal activity: *Wnt5a* expression was not affected by depolarization-induced (K^+, 10 m*M* KCl) activity elevation or activity blockade with the L-type calcium channel antagonist nifedipine (Nif, 20 μ*M*), under conditions when significant change in the mRNA level of the activity-induced secreted protein *BDNF* was detected [Fig. 1(I)].

In addition to its activity-independence, the effect of Wnt5a is also cell-autonomous. When high density neurons were transfected with the *Wnt5a* RNA interference (RNAi) construct to reduce endogenous Wnt5a level (Supporting Information Fig. S1A), the dendrite complexity of the transfected neurons (marked by co-transfected GFP) was significantly reduced, as compared to neurons transfected with the control RNAi construct [Fig. 1(A,J)], and can be fully rescued by application of exogenous Wnt5a protein (Supporting Information Fig. S1B). In these and

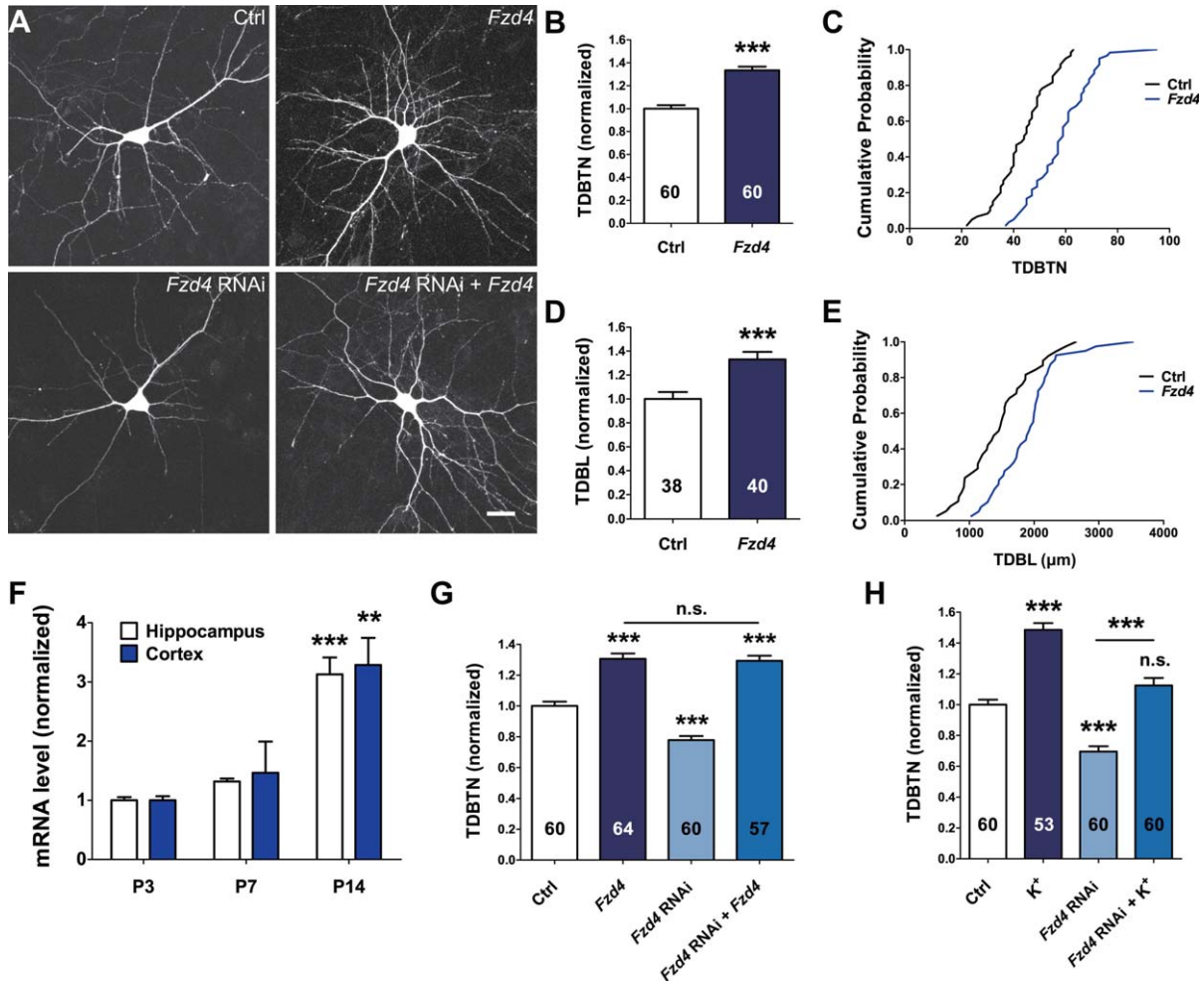

Figure 3 Frizzled4 promoted dendrite morphogenesis. A. Representative images of DIV 8 neurons, conditions as indicated. Scale bar: 20 μm. B–E. Neurons overexpressing mouse *Fzd4* had significantly higher TDBTN (B, $***p < 0.001$) and TDBL (D, $***p < 0.001$) at DIV 8. Cumulative distributions of TDBTN (C, $***p < 0.001$) and TDBL (E, $***p < 0.001$) for data presented in B and D, respectively. F. Real-time qPCR results showing significant increases in the mRNA level of *Fzd4* in the cerebral cortex and hippocampus during the first two weeks of mouse development. G. Rat *Fzd4* RNAi significantly reduced TDBTN, an effect completely rescued by overexpression of RNAi-resistant mouse *Fzd4*. H. High K^+ treatment increased TDBTN in control neurons and *Fzd4* RNAi neurons with similar magnitudes. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

subsequent experiments, neurons were transfected using the calcium phosphate method (Xia et al., 1996), which has the characteristics of low transfection efficiency (~1%), combined with a high rate of co-transfection (Washbourne and McAllister, 2002). Since we included the construct of interest at a much higher concentration (5–10 fold), as compared to the morphology marker GFP, we can be all but assured that the neurons analyzed for morphology co-expressed the construct of interest. To further investigate the cell-autonomous effect of *Wnt5a* on dendrite complexity, we lowered the endogenous level of G protein-coupled receptor 177 (Gpr177, also known as Wntless/Evi/Sprinter/Mig-14), a protein shown to be required for Wnt secretion (Banziger et al., 2006; Bartscherer et al., 2006; Fu et al., 2009). We found that *Gpr177* RNAi significantly reduced TDBTN ([Fig. 1(K)]; Supporting Information Fig. S1C, 3 different RNAi constructs) and completely blocked the effect of *Wnt5a* overexpression [Fig. 1(K)], supporting an autocrine role of Wnt5a in promoting dendrite arborization.

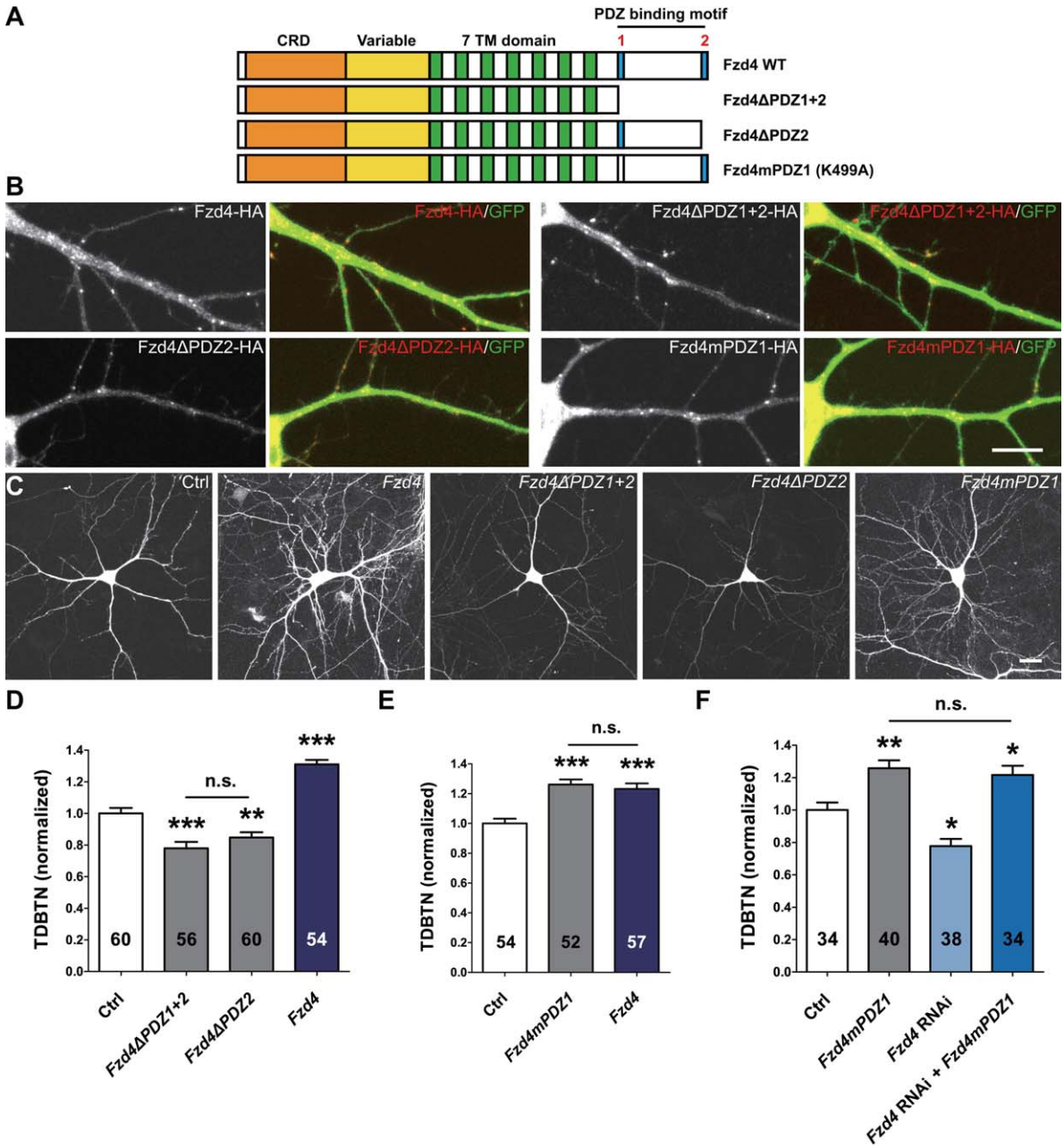

Figure 4 The distal, rather than proximal, PDZ-binding motif of Fzd4 promoted dendritic growth. A. Schematic of wildtype (WT) Fzd4 and its variants. Both proximal and distal PDZ binding motifs were deleted in Fzd4ΔPDZ1+2, only the distal motif was deleted in Fzd4ΔPDZ2 while in Fzd4mPDZ1, the critical lysine was mutated to alanine (K499A). Fzd4 domains included: CRD, variable domain, 7 transmembrane domain (7 TM) and PDZ binding motifs. B. Punctate staining pattern of WT Fzd4 and its variants revealed using antibody against HA tag. Scale bar: 10 μm. C. Representative images of DIV 8 neurons, conditions as indicated. Scale bar: 20 μm. D and E. Both *Fzd4ΔPDZ1+2* and *Fzd4ΔPDZ2* overexpression dominant negatively affected TDBTN (D), while *Fzd4mPDZ1* retained the capacity to increase TDBTN (E). F. *Fzd4mPDZ1* overexpression fully rescued the reduction in dendrite complexity induced by *Fzd4* RNAi.

As an additional assay for the activity-independence and cell autonomy aspects of the Wnt5a effect, we employed the isolated culture system that we previously described (Tan et al., 2010). In this assay, neurons were plated at very low density such that they were completely isolated from one

another, and dendrite morphology was assayed at the early developmental stage of DIV 3. Under these conditions, depolarization-induced activity elevation had no effect on dendrite growth (Fig. 2; Tan et al., 2010). *Wnt5a* overexpression from the time of plating by electroporation, conversely, significantly increased both TDBL and TDBTN while downregulation of endogenous Wnt5a level by RNAi significantly reduced dendrite arborization (Fig. 2). Despite being a secreted molecule, *Wnt5a*, because of its lipid modification, can only travel via membrane-based contacts (Willert and Nusse, 2012). Thus, the increase or reduction in dendrite growth observed in these isolated neurons can only result from alterations in the *Wnt5a* level of the analyzed neuron. These results further demonstrate the activity-independence and autocrine effects of Wn5a on dendrite growth.

Fzd4 Promoted Dendrite Arborization in a Manner Similar to Wnt5a

We next asked which receptor mediated the Wnt5a effect. Fzds are the most prominent Wnt receptors, and extensive evidence in non-neuronal systems have shown that Wnt5a can bind to Fzd4 and activate its downstream signaling (Sheldahl et al., 1999; Umbhauer et al., 2000; Chen et al., 2003; Mikels and Nusse, 2006). We thus generated a mouse *Fzd4* construct containing an HA epitope tag at the C-terminus and found that its overexpression from DIV 6 to 8 significantly increased both TDBL and TDBTN [Fig. 3(A–E)]. The order of magnitude of changes in dendrite arborization was similar for *Wnt5a* and *Fzd4* (TDBTN, $28.0 \pm 4.3\%$ in *Wnt5a*, [Fig. 1(B)] and $33.5 \pm 3.1\%$ in *Fzd4*, [Fig. 3(B)]; TDBL, $17.7 \pm 6.4\%$ in *Wnt5a*, [Fig. 1(D)] and $33.0 \pm 6.3\%$ in *Fzd4*, [Fig. 3(D)]). For both proteins, overexpression resulted in a shift of the entire distribution to the right [Figs. 1(C,E) and 3(C,E)], suggesting increased dendrite complexity across the entire neuronal population. Consistent with the developmental pattern of *Wnt5a* expression *in vivo*, *Fzd4* mRNA level in mouse hippocampus and cerebral cortex also showed a significant developmental increase during the first two postnatal weeks [Fig. 3(F)].

Complementing the overexpression results, *Fzd4* RNAi significantly reduced dendrite complexity ([Fig. 3(G)]; Supporting Information Fig. S1D, *E* for efficiency of *Fzd4* RNAi constructs against both endogenous and over-expressed *Fzd4*). The effect of *Fzd4* RNAi was completely rescued by overexpression of mouse *Fzd4* (*Fzd4*), which is resistant to RNAi targeting rat *Fzd4* [Fig. 3(G)], demonstrating specificity of the RNAi construct. Consistent with the activity-independence of Wnt5a signaling, high K^+-induced depolarization elicited an increase of dendrite complexity in *Fzd4* RNAi neurons, similar in magnitude to its effect on control neurons ([Fig. 3(H)], 48.5% for Ctrl vs. K^+, and 61.6% for *Fzd4* RNAi vs. *Fzd4* RNAi + K^+). Together, the above results demonstrate a role for endogenous Fzd4 in promoting dendrite arborization, in a pathway independent of neural activity and resembling the effect of Wnt5a.

The Distal Rather Than Proximal PDZ-Binding Motif of Fzd4 Regulates Dendrite Morphology

Fzd4 has been shown to mediate both canonical and noncanonical signaling downstream of Wnt5a (Umbhauer et al., 2000; Chen et al., 2003; Mikels and Nusse, 2006) via its C-terminal intracellular domain (Schulte, 2010; MacDonald and He, 2012; Niehrs, 2012). Dvl, a critical mediator of both canonical and noncanonical Wnt signaling (Kikuchi et al., 2011; Niehrs, 2012), binds to Fzd through the highly conserved juxtamembrane PDZ-binding motif KTxxxW located in the intracellular C-terminal domain of Fzd (Chen et al., 2003; Schulte, 2010; MacDonald and He, 2012; Niehrs, 2012). In addition to this Dvl-interacting proximal PDZ-binding motif (PDZ1 in [Fig. 4(A)]), Fzd4 also contains a distal C-terminal ETxV PDZ-binding motif (PDZ2 in [Fig. 4(A)]) that has been shown to interact with PDZ proteins including post-synaptic density protein 95 (PSD95) and GRIP1 (Hering and Sheng, 2002; Schulte and Bryja, 2007). To examine the functions of the Dvl-interacting proximal (PDZ1) and PSD95-interacting distal (PDZ2) PDZ-binding motifs in Fzd4 signaling during dendrite development, we generated a series of *Fzd4* constructs deleting or mutating these PDZ-binding motifs [Fig. 4(A)]. When overexpressed in cultured neurons, all mutant Fzd4 proteins displayed punctate subcellular distribution and dendritic localization indistinguishable from wildtype Fzd4, as shown by immunostaining for the C-terminal HA-tag [Fig. 4(B)].

With regard to changes in dendritic morphology, we found that overexpression of *Fzd4ΔPDZ1+2*, in which both PDZ-binding motifs of Fzd4 were deleted, had a dominant negative effect on dendrite arborization, reducing TDBTN by approximately 20% [Fig. 4(C,D)]. Interestingly, this phenotype was mimicked by overexpression of *Fzd4ΔPDZ2*, in which only the distal PDZ binding motif was deleted [Fig. 4(C,D)]. The reduction in TBDTN was similar

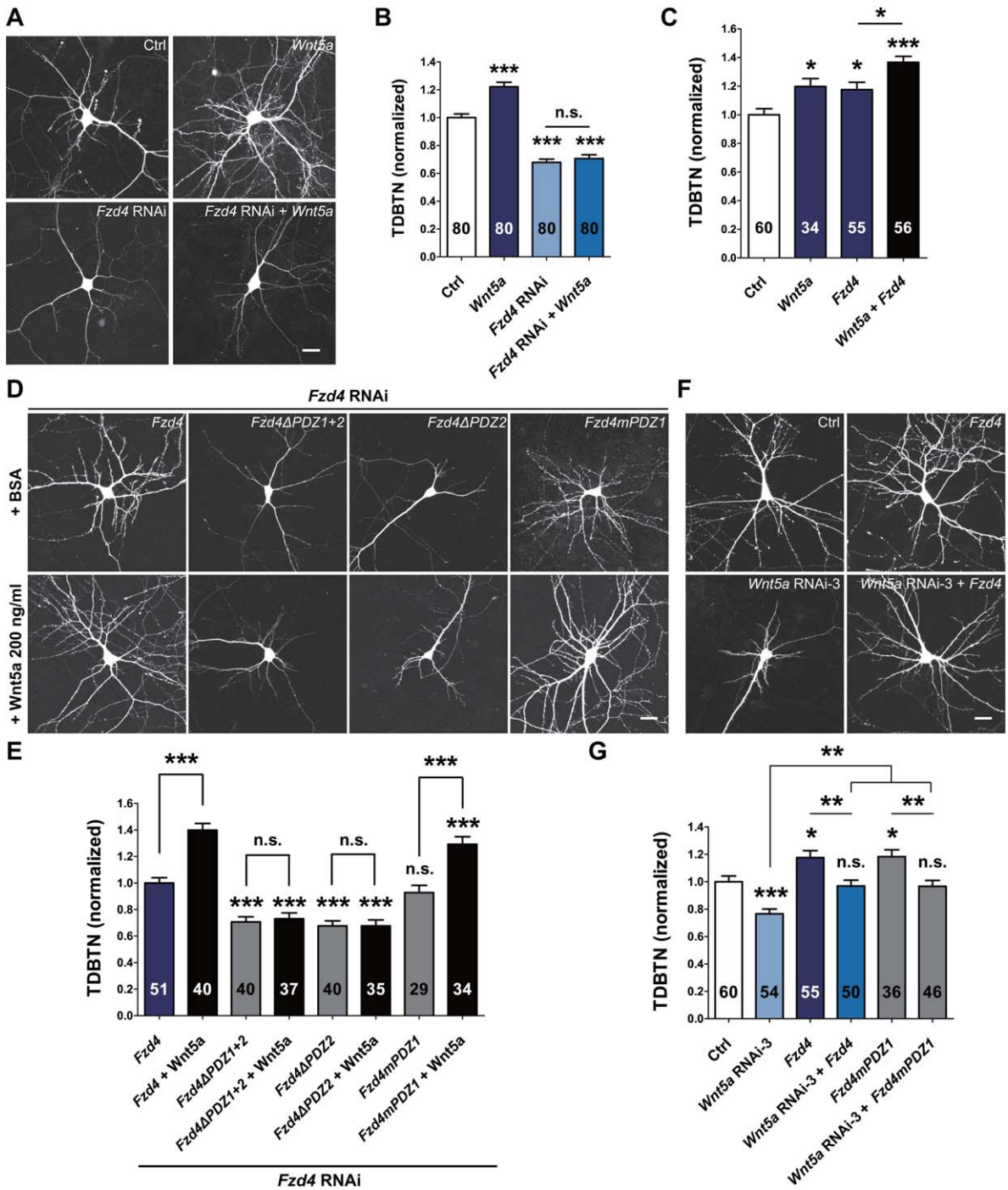

Figure 5 Fzd4 functioned as Wnt5a receptor in regulating dendrite morphology. A, D, and F. Representative images of DIV 8 neurons, conditions as indicated. Scale bar: 20 μm. B. *Fzd4* RNAi effectively blocked the increase in TDBTN induced by *Wnt5a* overexpression. C. Co-overexpression of *Wnt5a* and *Fzd4* produced a further increase in TDBTN. E. Expression of *Fzd4* or *Fzd4mPDZ1* in *Fzd4* RNAi neurons restored basal TDBTN level, and endowed the neurons with the ability to grow more dendrites on stimulation with 200 ng/mL Wnt5a. In contrast, expression of *Fzd4ΔPDZ1+2* or *Fzd4ΔPDZ2* blocked the effect of Wnt5a stimulation. G. Overexpression of *Fzd4* or *Fzd4mPDZ1* partially rescued the reduction in TDBTN induced by *Wnt5a* RNAi. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

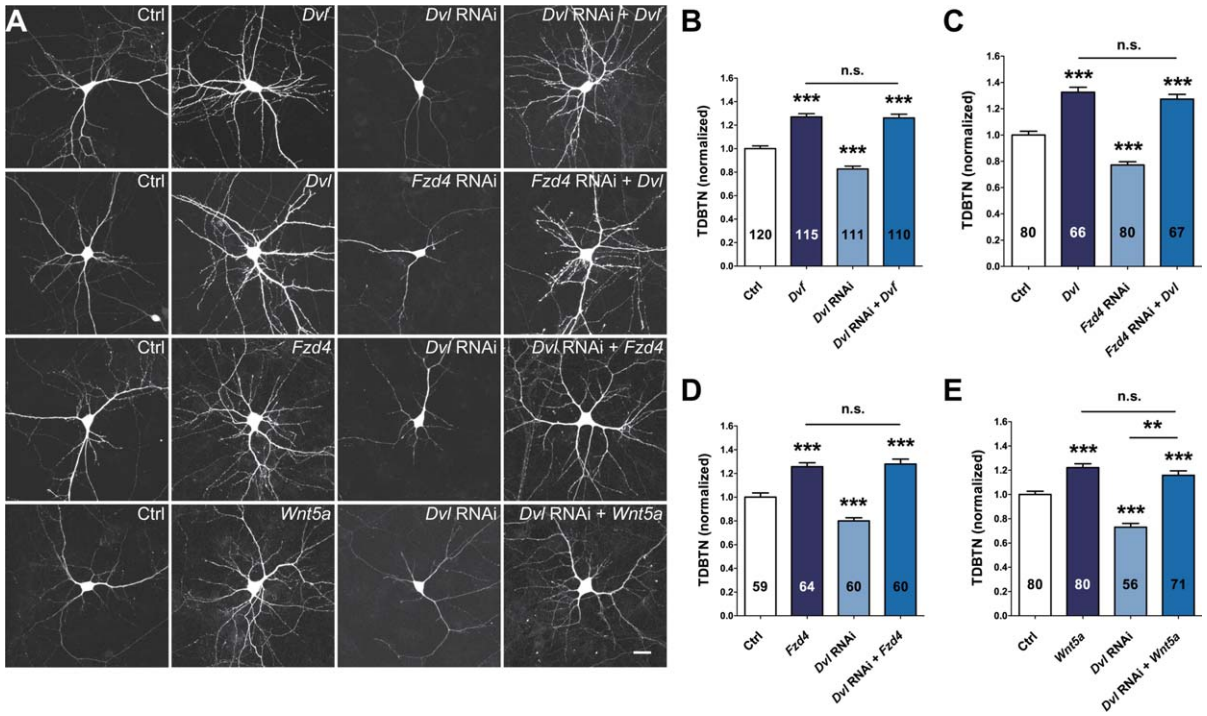

Figure 6 Wnt5a/Fzd4 signaling acted in parallel with Dvl to promote dendrite development. A. Representative images of DIV 8 neurons, conditions as indicated. Scale bar: 20 μm. B. Overexpression of *Dvl* increased TDBTN, whereas *Dvl* RNAi reduced TDBTN, a phenotype fully rescued by co-expression with RNAi resistant *Dvl* (*Dvl*^{r}). C. *Dvl* overexpression fully rescued the TDBTN phenotype of *Fzd4* RNAi. D. *Fzd4* overexpression fully rescued the TDBTN phenotype of *Dvl* RNAi. E. *Wnt5a* overexpression fully rescued the TDBTN reduction induced by *Dvl* RNAi. The data presented for Ctrl and *Wnt5a* in this figure are the same as those in Figure 5(B), because all experiments for Figures 5(B) and 6(E) were carried out with the same batches of neurons. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

in magnitude for neurons overexpressing *Fzd4ΔPDZ1+2* or overexpressing *Fzd4ΔPDZ2* [Fig. 4(D), 0.78 ± 0.04 in *Fzd4ΔPDZ1+2* and 0.85 ± 0.03 in *Fzd4ΔPDZ2*, $p > 0.05$], suggesting that PDZ2 is the main PDZ-binding motif in Fzd4 responsible for promoting dendrite morphogenesis. Consistent with these observations, when the critical amino acid residue in the proximal PDZ-binding motif was mutated (K499A, [Fig. 4(A)]) in *Fzd4mPDZ1*, to disrupt the interaction with Dvl (Umbhauer et al., 2000; Cong et al., 2004), its overexpression still elicited a significant increase in TDBTN, to a level indistinguishable from overexpression of wildtype *Fzd4* ([Fig. 4(C,E)], *Fzd4mPDZ1*: 1.26 ± 0.03; *Fzd4*: 1.23 ± 0.04, $p > 0.05$). Furthermore, *Fzd4mPDZ1* overexpression fully rescued the reduction in TDBTN induced by *Fzd4* RNAi [Fig. 4(F)]. Together these results demonstrate that the distal PDZ2 motif of Fzd4, rather than the proximal Dvl-interacting PDZ1 motif, is the key domain for signaling downstream of Fzd4 to promote dendrite morphogenesis.

Fzd4 Functions as the Receptor of Wnt5a to Promote Dendrite Morphogenesis via Its Distal PDZ2 Motif

Given the resemblance of the effects of Wnt5a and Fzd4 on dendrite growth, their similar developmental expression profile and previously demonstrated direct binding (Mikels and Nusse, 2006), we asked whether Fzd4 functioned as the receptor of Wnt5a in mediating its dendrite promoting effect. To this end, we overexpressed *Wnt5a* in neurons in which endogenous *Fzd4* is knocked-down using *Fzd4* RNAi. We surmised that if Wnt5a activates receptors other than Fzd4 to elicit its dendrite promoting effect, there should be at least a partial rescue of the TDBTN reduction induced by *Fzd4* RNAi. Instead, we found that *Fzd4* RNAi completely blocked the effect of *Wnt5a* overexpression in promoting dendrite growth [Fig. 5(A,B)], demonstrating that Fzd4 is likely the sole or most important receptor responsible for mediating the effects of Wnt5a in promoting dendrite development. This result

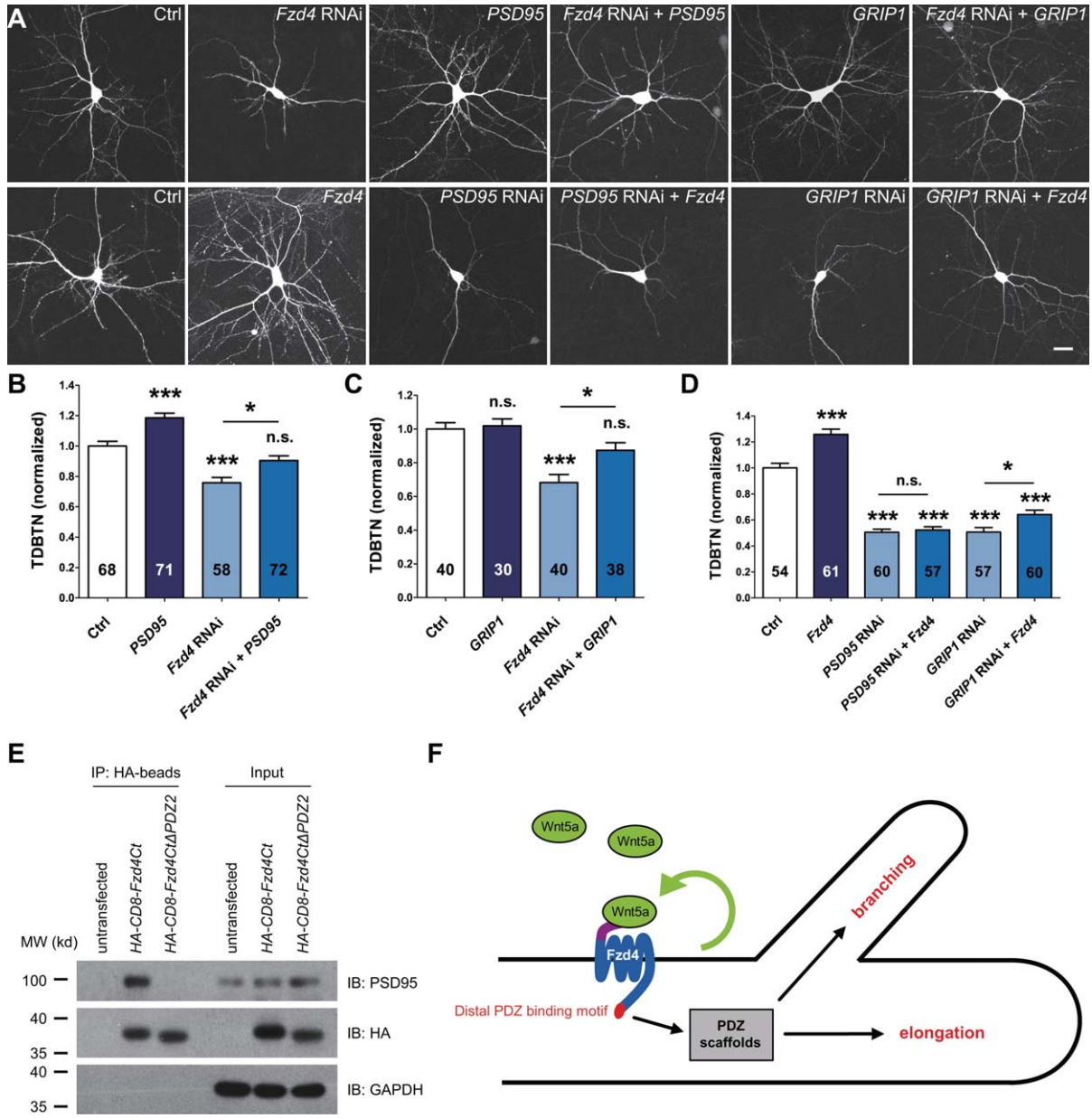

Figure 7 PDZ scaffolds PSD95 and GRIP1 likely serve as downstream effectors of Fzd4. A. Representative images of DIV 8 neurons, conditions as indicated. Scale bar: 20 μm. B. Overexpression of *PSD95* significantly increased TDBTN and partially rescued the effect of *Fzd4* RNAi. C. Overexpression of *GRIP1* alone did not significantly affect TDBTN, but partially rescued the effect of *Fzd4* RNAi. D. *Fzd4* overexpression failed to rescue the reduction in TDBTN induced by either *PSD95* RNAi or *GRIP1* RNAi. E. Immunoprecipitation results showing interaction of PSD95 with HA-CD8-Fzd4Ct but not HA-CD8-Fzd4CtΔPDZ2. F. A working model of Wnt5a/Fzd4 signaling in the regulation of activity-independent dendrite morphogenesis.

was further supported by the observation that when *Wnt5a* and *Fzd4* were concurrently overexpressed in the same neuron, the magnitude of the increase in TDBTN was approximately twice that of overexpressing either molecule alone, demonstrating additive effects between these molecules [Fig. 5(C)].

Does Wnt5a/Fzd4 signaling rely on the function of the distal PDZ2 motif of Fzd4? More specifically, could *Fzd4ΔPDZ1+2* and *Fzd4ΔPDZ2*, but not *Fzd4mPDZ1*, block the effect of Wnt5a? To address this question without interference from endogenous Fzd4, we performed a gene replacement assay, in

which we knocked down endogenous *Fzd4* using RNAi, and at the same time overexpressed wildtype mouse *Fzd4* or its mutants, with or without additional Wnt5a stimulation [Fig. 5(D,E)]. Consistent with our previous results, application of 200 ng/mL Wnt5a significantly increased TDBTN in neurons expressing wildtype *Fzd4* or *Fzd4mPDZ1* while those expressing *Fzd4ΔPDZ1+2* and *Fzd4ΔPDZ2* had less dendrites at basal condition and did not respond to Wnt5a stimulation [Fig. 5(D,E)]. Thus, the PDZ2 motif of Fzd4 is required for mediating the effects of Wnt5a in promoting dendrite development.

In another set of experiment, overexpression of *Fzd4* rescued the reduction in TDBTN induced by *Wnt5a* RNAi back to control level, but significantly below that of *Fzd4* overexpression alone [Fig. 5(F,G)]. The effects of *Fzd4mPDZ1* overexpression were similar [Fig. 5(F,G)]. This partial rescue is consistent with a ligand/receptor interaction between Fzd4 and Wnt5a, in that Fzd4 seems to require a certain amount of Wnt5a to be fully activated and functional (please also see Discussion section).

Wnt5a/Fzd4-Dependent Regulation of Dendrite Growth Did Not Solely Rely on Dvl Function

Since the Dvl-interacting PDZ1 motif of Fzd4 is not required for mediating the effect of Fzd4 on promoting dendrite growth, an immediately arising question is whether Dvl is required for Wnt5a/Fzd4-dependent dendrite growth. We first assayed whether *Dvl* overexpression can promote dendrite arborization. *Dvl* overexpression significantly increased TDBTN [Fig. 6(A,B)], consistent with previous report (Rosso et al., 2005), and fully rescued the effect of *Dvl* RNAi [Fig. 6(A,B)], which was designed to knock-down all three endogenous *Dvls* (Zhang et al., 2007). *Dvl* overexpression also completely rescued the reduction in dendritic complexity induced by *Fzd4* RNAi, to a level significantly above control and indistinguishable from *Dvl* overexpression alone ([Fig. 6(A,C)], 1.273 ± 0.036 in *Fzd4* RNAi + *Dvl* vs. 1.326 ± 0.037 in *Dvl*, $p < 0.001$ vs. Ctrl, $p > 0.05$ vs. *Dvl*). This result is consistent with Dvl either functioning downstream of or in parallel to Fzd4 in promoting dendrite growth. To distinguish between these possibilities, we performed the reverse rescue experiment, overexpressing *Fzd4* while knocking down endogenous *Dvl*. If Wnt5a/Fzd4 functioned via mediators additional to Dvl, we would expect *Fzd4* overexpression to also fully rescue the effect of *Dvl* RNAi. Conversely, if Dvl signaled exclusively downstream of Fzd4, we would expect the reverse rescue to not work or to have partial effect. The results showed that *Fzd4* overexpression completely rescued the reduction in TDBTN induced by *Dvl* RNAi [Fig. 6(A,D)]. *Dvl* RNAi also did not prevent the increase in TDBTN induced by *Wnt5a* overexpression [Fig. 6(E)]. Together with important role of the Fzd4 PDZ2 motif described in Figures 4 and 5, these results suggest that an additional Dvl-independent pathway functions downstream of Wnt5a/Fzd4 to promote dendrite growth, likely via the PDZ2 motif of Fzd4. (please also see Discussion section).

PDZ Scaffolds PSD95 and GRIP1 Likely Serve as Downstream Effectors of Fzd4

Having shown that Wnt5a/Fzd4 signaling promoted dendrite development via the distal PDZ binding motif of Fzd4, we next asked what downstream effector might mediate this signaling. PDZ scaffolds including PSD95 and GRIP1 have been shown to directly interact with the C-terminal distal PDZ-binding motif of Fzd4 (PDZ2 in [Fig. 4(A)]; Hering and Sheng, 2002; Ataman et al., 2006; Schulte and Bryja, 2007), thus we tested these proteins first. We surmised that if PSD95 and/or GRIP1 functioned downstream of Fzd4 to promote dendrite morphogenesis, they would partially or completely rescue the effect of *Fzd4* RNAi. Indeed, we found that both *PSD95* and *GRIP1* overexpression significantly rescued the reduction in TDBTN induced by *Fzd4* RNAi [Fig. 7(A–C)], to a level indistinguishable from control. *PSD95* also significantly increased TDBTN [Fig. 7(A,B)] when overexpressed alone while *GRIP1* overexpression had no significant effect [Fig. 7(A,C)].

Since the above results did not distinguish between downstream or parallel signaling by PSD95/GRIP1, we also performed the reverse rescue experiment, expressing *Fzd4* in the present of *PSD95* and *GRIP1* RNAi (Hoogenraad et al., 2005; Futai et al., 2007). Both *PSD95* RNAi and *GRIP* RNAi significantly reduced TDBTN [Fig. 7(A,D)], more dramatically than *Wnt5a* RNAi or *Fzd4* RNAi [Figs. 1(J) and 3(G)]. The *GRIP1* RNAi effect is consistent with previous report (Hoogenraad et al., 2005). *Fzd4* overexpression did not significantly affect the dendrite reducing effect of *PSD95* RNAi ([Fig. 7(A,D)], 0.505 ± 0.024 in *PSD95* RNAi vs. 0.523 ± 0.025 in *PSD95* RNAi + *Fzd4*, $p > 0.05$), and only slightly increased TDBTN when co-expressed with *GRIP1* RNAi ([Fig. 7(A,D)], 0.506 ± 0.035 in *GRIP1* RNAi vs. 0.642 ± 0.034 in *GRIP1* RNAi + *Fzd4*, $p < 0.05$). In both cases, neurons co-expressing *Fzd4* with *PSD95* RNAi or *GRIP* RNAi had significantly less

dendrites as compared to control neurons or those overexpressing *Fzd4* alone [Fig. 7(D)]. Furthermore, our immunoprecipitation results using lysates from cultured neurons demonstrated a strong interaction between PSD95 and the full length C-terminus of Fzd4 (HA-CD8-Fzd4Ct), but not the C-terminus lacking PDZ2 (HA-CD8-Fzd4CtΔPDZ2, [Fig. 7(E)]). These results, in combination with those of the previous experiment, suggest that PDZ scaffolds including PSD95 and GRIP1 are important downstream mediators of Wnt5a-Fzd4 signaling during dendrite morphogenesis.

DISCUSSION

Dendrite morphogenesis is a delicate process under the control of numerous molecular pathways and is critical to the formation of proper dendritic territory and neural connectivity. Here, we identified a novel Wnt5a/Fzd4 signaling pathway that contributes to the regulation of this important developmental process. As summarized in Figure 7(F), Wnt5a is released during early development in an activity-independent fashion, and cell-autonomously activated Fzd4 through its N-terminal CRD domain. This signal is then transduced downstream via the distal PDZ-binding motif (PDZ2) of Fzd4, through interactions with PDZ scaffolds such as PSD95 and GRIP1, to promote dendritic branching and growth, and thus contributing to the shaping and patterning of the dendritic tree.

Activity-Independent and Cell-Autonomous Regulation of Dendritic Morphogenesis by Wnt5a

Among the many mechanisms that govern the development of dendrites, those regulated by neural activity have always been extensively investigated. Neural activity has been shown to promote dendrite arborization through regulation of cytoskeletal dynamics, cell adhesion, transcription, and secretion of extracellular factors (Wong and Ghosh, 2002; Cline and Haas, 2008; Urbanska et al., 2008; Jan and Jan, 2010; Arikkath, 2012; Koleske, 2013). Yet, the ability of the dendritic arbors to elaborate under sensory deprivation (Tieman et al., 1995; Scott et al., 2003) or when neurons are cultured in isolation (Montague and Friedlander, 1989; Montague and Friedlander, 1991) suggests that activity-independent mechanisms also contribute significantly to dendrite development. Consistently, Kossel et al. showed that contact with living axons *per se*, rather than synaptic transmission or neuronal excitability, affected dendrite branching most significantly, whereas neural activity mainly regulate spine morphology (Kossel et al., 1997). Additionally, Verhage et al. showed that *Munc18* knockout mice, in which neurotransmitter release was completely absent, had normal formation of brain layers and of axonal fibers, as well as assembly of synaptic structures (Verhage et al., 2000), consistent with relatively normal dendrite development in these animals.

The above observations all point to the presence of additional, activity-independent regulators of dendrite development. Here, we identified Wnt5a as such a regulator [Figs. 1, 2, and 7(F)]. *Wnt5a* mRNA and protein levels were highly upregulated during the first two postnatal weeks *in vivo* [Fig. 1(G,H)] and significantly promoted dendrite growth during the first three days of postnatal neuronal development (Fig. 2), within the time window when neuronal activity and contact-dependent cell adhesion had negligible effects (Tan et al., 2010). We thus surmise that Wnt5a-dependent dendrite growth is particularly important during early development, prior to the formation of synaptic contacts and the emergence of neuronal activity.

Fzd4 Serves as the Wnt5a Receptor for Promoting Dendrite Morphogenesis

In spite of the number of studies focusing on the function of Wnt ligands in the developing nervous system (Mathew et al., 2005; Rosso et al., 2005; Ataman et al., 2006; Wayman et al., 2006; Farias et al., 2009; Fradkin et al., 2010; Singh et al., 2010; Kirszenblat et al., 2011), the Wnt receptor required for regulating dendrite development in the mammalian system had yet to be determined. Here, we identified Fzd4 as such a receptor, specifically for mediating the activity-independent effects of Wnt5a on dendrite morphogenesis (Figs. 3 and 5).

The physical interaction between Wnt5a and Fzd4 has been well characterized in a previous study in HEK293 cells (Mikels and Nusse, 2006), and the function of this ligand/receptor pair has been reported in multiple non-neuronal systems (Sheldahl et al., 1999; Umbhauer et al., 2000; Chen et al., 2003; Mikels and Nusse, 2006). Here, we focused on the functional interaction of these molecules in the neuronal system. The results of our epistatic analyses between Wnt5a and Fzd4, and their additive effect on co-expression (Fig. 5), strongly suggested that Fzd4 serves as the Wnt5a receptor for regulating activity-independent dendrite growth.

The question of whether endogenous *Wnt5a* or *Fzd4* is expressed in excess of the other is somewhat

difficult to address, albeit very interesting. Based on our results, we would like to propose that under basal conditions, endogenous Wnt5a can be divided into two subgroups: Fzd4-accessible Wnt5a and Fzd4-inaccessible Wnt5a; similarly, endogenous Fzd4 can also be divided into Wnt5a-accessible and inaccessible subgroups. On *Wnt5a* overexpression, Fzd4 previously inaccessible to Wnt5a now becomes accessible and binds Wnt5a to initiate downstream signaling, and *vice versa* for *Fzd4* overexpression. Thus, overexpression of only one of the molecules would lead to increased binding of Wnt5a and Fzd4, promoting dendrite growth to some extent (typically less than 30% in our experiments). Co-expression of both molecules would produce an additive effect, by increasing both the level of available Wnt5a and Fzd4, thus resulting in a further increase in TDBTN [Fig. 5(C)]. Importantly, the increase in binding induced by one member of the ligand/receptor pair is restricted by the other, since the effect of *Wnt5a* overexpression is completely abolished by either *Fzd4* RNAi or replacing endogenous *Fzd4* with *Fzd4ΔPDZ2* [Fig. 5(B,E)]. Similarly, overexpression of *Fzd4* only partially rescued the reduction in TDBTN in neurons expressing *Wnt5a* RNAi, even when additional amount of rescue plasmids were added [Fig. 5(G)]. These results suggest that endogenous Wnt5a and Fzd4 are in a "balanced" state, a state optimal for regulation by and interaction with other factors or signaling pathways.

Wnt5a/Fzd4 Signaling Regulates Dendrite Morphogenesis via the PDZ2 Motif of Fzd4

Interestingly, Wnt5a/Fzd4-dependent regulation of dendrite morphogenesis does not solely rely on Dvl (Fig. 6). Due to its ability to recruit downstream effectors to membrane-bound Fzd, Dvl plays critical roles in both canonical and noncanonical Wnt pathways (Chen et al., 2003; van Amerongen et al., 2008; Schulte, 2010; MacDonald and He, 2012; Niehrs, 2012; van Amerongen, 2012). In sharp contrast to the complete inability of *Wnt5a* overexpression to rescue *Fzd4* RNAi [Fig. 5(B)], overexpression of either *Wnt5a* or *Fzd4* fully rescued the dendrite reducing effect of *Dvl* RNAi [Fig. 6(A, D, and E)]. We further found that mutation of the Dvl-interacting motif in Fzd4 did not interfere with its ability to promote dendrite morphogenesis and to respond to Wnt5a stimulation [Figs. 4(E) and 5(E)]. Together, these results suggest that the dendrite promoting effect of Wnt5a/Fzd4 relies more dominantly on the distal PDZ2 motif, probably through its interactions with additional mediators. Involvement of Dvl in this process cannot be ruled out, as we used RNAi-mediated knockdown to reduce endogenous Dvl, rather than complete knockout of the protein.

Although many previous studies have investigated the function of the proximal PDZ1 motif of Fzd receptors (Chen et al., 2003; Schulte, 2010; MacDonald and He, 2012; Niehrs, 2012), little is known about the function of the distal PDZ2 motif. Unlike the highly conserved PDZ1 motif, the distal PDZ2 motif is only present in a subset of Fzds (Hering and Sheng, 2002; Schulte and Bryja, 2007). Here, we first identify the function of this PDZ2 motif as the domain through which Fzd4 mediates Wnt5a signaling to promote dendrite morphogenesis in an activity-independent manner. Putting our results together with those of previous studies, there are to date at least three identified Wnt-dependent pathways regulating dendrite morphogenesis: (1) regulation of activity-dependent dendrite growth by Wnt2 and/or other classical Wnts (Yu and Malenka, 2003; Wayman et al., 2006); (2) Dvl-Rac-JNK-dependent regulation of dendrite development downstream of Wnt7b (Rosso et al., 2005); (3) activity-independent Wnt5a/Fzd4 signaling although PDZ2 motif of Fzd4 as identified here. We propose that Wnts regulate dendrite morphogenesis through multiple signaling pathways, using different Wnt receptors and through distinct subdomains of the receptor, depending on the specific Wnt and Wnt receptor expressed, as well as the presence/level of other signaling components of the pathway.

The Role of PDZ Scaffolds in Mediating Dendrite Morphogenesis

What downstream factors mediate Wnt5a/Fzd4 signaling through interaction with the distal PDZ binding motif (PDZ2) of Fzd4? Our results suggested that PDZ scaffolds including PSD95 and GRIP1 are likely involved. The Fzd4 PDZ2 motif, which is necessary and sufficient for promoting Wnt5a-dependent dendrite morphogenesis (Figs. 4 and 5), has been reported to interact with PDZ scaffolds (Hering and Sheng, 2002). *Fzd4* overexpression failed to rescue the dendrite reducing effect of *PSD95* or *GRIP1* RNAi while overexpression of *PSD95* or *GRIP1* rescued the effect of *Fzd4* RNAi at least back to control level (Fig. 7), consistent with PSD95 and/or GRIP1 functioning as downstream effectors of Fzd4. The ability of *PSD95/GRIP1* overexpression to only partially rescue *Fzd4* RNAi may be due to the requirement for the co-presence of Fzd and PSD95/GRIP1 to form a complex on Wnt5a-dependent activation of

Fzd4, with Fzd4 responsible for recruiting PSD95/GRIP1 to the membrane. Since the effect of *PSD95* or *GRIP1* RNAi was more dramatic than that of *Fzd4* or *Wnt5a* RNAi, it is likely that PDZ scaffolds functioned downstream of multiple PDZ-binding proteins. Consistently, a previous study (Hoogenraad et al., 2005) identified EphB2-GRIP1 interaction through the distal PDZ-binding motif of EphB2 as important regulator of dendrite growth.

We note that while our *GRIP1* RNAi results are consistent with previous report (Hoogenraad et al., 2005), those of *PSD95* RNAi are seemingly contradictory to previous reports (Charych et al., 2006; Chen and Firestein, 2007). These differences are likely due to differences in culture conditions and the timing of the experiments. Specifically, the cell density in Firestein laboratory neuronal culture was approximately three times that of ours. Perhaps more importantly, their experiments were performed at a later developmental stage (DIV 12–17), where synaptogenesis is at its peak, and the well-established role of PSD95 as an excitatory synapse scaffold is likely to be dominant. In contrast, in our study, we mainly focused on an earlier stage (DIV 3–8), when synaptogenesis is relatively low and dendrite growth is rapid, as we are specifically interested in activity-independent dendrite growth in this study. Thus, the differences in the results are likely due to the different functions of PSD95 at the two developmental time points, mostly promoting dendrite growth downstream of Fzd4 and other PDZ-binding proteins early on, and promoting the formation and stabilization of excitatory synapses at later stages of development.

In summary, we propose that multiple transmembrane proteins containing PDZ-binding motifs likely function to recruit PDZ scaffolds to the cell membrane to promote dendrite morphogenesis. This process is initiated during very early neuronal development, before the formation of synapses and the emergence of neuronal activity. These PDZ scaffolds along the membrane, in addition to promoting dendrite growth, could be rapidly recruited to post-synaptic densities at the onset of synaptogenesis. In other words, the Wnt5a-Fzd4 signaling pathway, together with other signaling pathways that can recruit PDZ scaffolds, likely form the linker seamlessly joining activity-independent and activity-dependent pathways regulating dendrite morphogenesis and neural circuit formation.

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