Axon development involves spatial-temporal cytoskeletal reorganization. However, how the cytoskeleton remodeling is modulated by extracellular cues is unclear. Here, we report a role of Wnt/Ca\textsuperscript{2+} signaling in regulating actin and growth cone dynamics. We found that treatment of cultured cortical neurons with Wnt5a, a non-canonical Wnt, either globally or locally, caused an increase in the activity of calpain, a calcium-dependent protease responsible for the cleavage of several actin binding proteins, including spectrin. Treatment with Wnt5a promoted growth cone advance, as well as axonal growth, and these effects were prevented by chelating intracellular calcium, inhibition or down-regulation of calpain, or blockade of spectrin cleavage by competitive peptides. Interestingly, both Wnt5a and activated calpain were found to be mainly distributed in the axon-rich intermediate zone of neocortex. Down-regulating calpain expression interfered with the growth of callosal axons \textit{in vivo}. Thus, Wnt5a serves as a physiological cue to stimulate localized axonal activity, which in turn promotes growth cone advance and axonal growth.

Wingless-type murine mammary tumor virus integration site (Wnt) family proteins regulate many aspects of neuronal development, including neuronal positioning, axon and dendrite development, and synaptogenesis (1). Canonical Wnts, such as Wnt1, -3a, and -8, by binding to Frizzled (Fz) receptors and co-receptor LRP-5 or -6, cause inhibition of GSK3 and prevent phosphorylation and degradation of β-catenin; the rise of β-catenin in the nucleus regulates target gene expression (2, 3). Some other Wnts, such as Wnt5a and -11, cannot induce β-catenin signaling; rather, in some contexts, they even exert an antagonizing effect on β-catenin signaling (4–8). Interestingly, Wnt5a has been shown to enhance intracellular calcium transients in zebrafish or \textit{Xenopus} embryos (9) and modulates calcium-sensitive proteins (10, 11). The role of Wnt signaling in neuronal polarity and axonal growth is just beginning to be understood. For example, Wnt signaling has been reported to orient anterior-posterior axon outgrowth in \textit{Caenorhabditis elegans} (12, 13) and anterior-posterior guidance of spinal cord commissural axons (14). However, the mechanism of Wnt action in regulating axon development is not clear.

Calpain, a family of intracellular cystine proteases, can be activated at various pathological or physiological conditions, including ischemic or traumatic neuronal insults (15, 16), NMDA receptor-mediated excitotoxicity or synaptic plasticity (17–20), or exposure to extracellular factors, such as brain-derived neurotrophic factor (BDNF) or epidermal growth factor (EGF) (21–23). In cultured \textit{Aplysia} neurons, localized and transient elevations of intracellular calcium and activation of calpain induce growth cone formation from intact neurons or injury sites after axotomy (24–26), presumably via the cleavage and removal of submembrane cytoskeleton protein spectrin (26). In line with this notion, depolymerization of the actin cytoskeleton by pharmacological treatments has been shown to promote axon development in cultured hippocampal neurons (27–30). Thus, it is believed that spatial instability of actin structures at the growth cone is important for neuronal polarization and axonal growth (31). However, it remains unclear how this actin instability is induced by physiological cues during axon development.

Here we demonstrate a role of Wnt5a in regulating calpain activity during axonal growth. We found that treatment of cultured primary neurons with Wnt5a, but not Wnt3a, causes an increase in calpain activity, leading to spectrin cleavage. Importantly, local application of Wnt5a causes localized activation of calpain in developing axons of polarizing neurons. Furthermore, treatment with Wnt5a enhanced forward movement of growth cones, and this effect depended on intracellular calcium and calpain activity. This study demonstrates a novel role of Wnt/Ca\textsuperscript{2+} signaling in cytoskeleton regulation and axon development.

**EXPERIMENTAL PROCEDURES**

**Constructs**—

M-calpain was subcloned into pEGFP-N1 (Clontech) in frame with EGF\textsuperscript{3} at the HindIII and KpnI sites. The siRNA targeting sequence was as follows: si-mCalpn, 5′-...
GAATGGCGATTTCTGCATC-3′; si-Scrambled, 5′-GCT-GATGCGCATCTAGAT-3′. Double-stranded oligonucleotides were subcloned into BglII- and HindIII-digested pSUPER (OligoEngine, Seattle, WA) to generate construct for si-mCalpn or si-Scrambled.

Biochemical Analysis—Rat brains were homogenized in lysis buffer (50 mM Tris-HCl at pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate) containing protease inhibitors, followed by centrifugation (15,700 relative centrifugal force (rcf) for 1 h at 4 °C). Cultured cortical neurons were lysed in the lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Nonidet P-40, 1 mM EDTA, and protease inhibitors). Brain homogenates or cell lysates (30–100 μg) were size-fractionated by electrophoresis on 6 or 10% SDS-PAGE, followed by immunoblotting with the indicated antibodies. Antibodies used were spectrin (MAB1622 (1:1000), Millipore), β-actin (MAB1501 (1:5000), Millipore), GFP (A11122 (1:1000), Invitrogen). Antibody against cleaved spectrin was generated by immunizing rabbit with the peptide with the sequence of CQQQEVY, which was fused with carrier protein keyhole limpet hemocyanin (KLH), and affinity-purified. The secondary antibody used was goat anti-rabbit IgG (AP132P, Millipore) or goat anti-mouse IgG coupled to HRP (AP124P, Millipore).

Neuronal Culture, Transfection, and Treatments—Dissociated hippocampal or cortical neurons were isolated from E18 rats as described previously (32) and transfected by electroporation using the Amaza Nucleofector device. In some experiments, testing plasmids were co-transfected with EGFP or RFP at a ratio of 3:1. Control or transfected neurons were plated onto coverslips coated with poly-D-lysine (0.3 mg/ml). After culturing for 4 h, media were changed to neuronal culture media (neurobasal media containing 1% glutamate and 2% B27). In some instances, neurons at DIV2 or -3 were treated with Wnt5a (100 ng/ml) or Wnt3a (100 ng/ml), without or with pretreatment with various reagents (e.g. 30 μM BAPTA-AM for 30 min, 40 μM calpeptin for 1.5 h, or 20 μM TAT fusion peptides for 1.5 h). Upon application of Wnts, 10-min movies with intervals of 10 s were captured with LSM 510 (excitation, 420–445 nm; emission, 460–510 nm), a YFP filter cube (excitation, 520–560 nm), and a CFP filter cube (excitation, 420–445 nm; emission, 460–510 nm). Images were recorded simultaneously by the Cascade 512B CCD (Roper Scientific) with the Macro Manager software. The ratio of YFP to CFP, as determined by the ImageJ after transcardial perfusion. After dehydration, fetal brains were cryopreserved in optimum cutting temperature (OCT) compound (Sakura). Coronal cryostat sections were cut at 30-μm thickness on a freezing microtome and immediately processed for immunostaining by a three-step free floating protocol (at 4 °C). Antibodies used were Wnt5a (AF645 (1:100) R&D), Smi-312 (1:1000, Covance), or cleaved spectrin (1:100).

In Utero Electroporation—In utero electroporation was performed as described previously (33). Briefly, uterine horns of pregnant rats at E16 were exposed, and 1–2 μl of plasmid solution was injected into the lateral ventricle of the embryonic brain with a fine glass micropipette. Embryos were then clamped between 5-mm diameter tweezers-type disc electrodes (CUY650-5, Tokiwa Science) and were given five electrical pulses (55 V, 100 ms) at an interval of 1 s (electroporator ECM830, BTX). Uterine horns were then placed back into the abdominal cavity to allow the embryos to continue development.

Time Lapse Imaging—Cortical neurons were co-transfected with testing plasmids and RFP and were plated onto the coverslips coated with poly-D-lysine (0.3 mg/ml). At DIV2, cells were imaged under a Zeiss LSM510 confocal microscope with a 40× objective lens. Under some conditions, neurons were treated with Wnt5a (100 ng/ml) or Wnt3a (100 ng/ml), without or with pretreatment with various reagents (e.g. 30 μM BAPTA-AM for 30 min, 40 μM calpeptin for 1.5 h, or 20 μM TAT fusion peptides for 1.5 h). Upon application of Wnts, 10-min movies with intervals of 10 s were captured with LSM software to record the growth cone behavior. For each growth cone, the distance between the tip of the longest filopodia and the center of the original growth cone at the indicated time point was measured using IPP software. Average values of 10 neurons at each time point upon Wnt treatments were illustrated to reflect growth cone dynamics.

Fluorescence Resonance Energy Transfer (FRET) Assay—A linker sequence encoding a peptide identical to the calpain cleavage site of αII-spectrin flanked by glycine and serine residues (GSGSGQQVEYGMPRDGS) (34) was used to adjoin EYFP and ECFP sequences, generating a FRET probe designated as pYSC. Cortical neurons were transfected with pYSC, followed by local application of Wnt5a or Wnt3a (200 ng/ml) with a micropipette that had a tip opening of ~1 μm, which was 100 μm away from the center of the growth cone. The pressure was applied to the pipette with an electrically gated pressure application system (PM8000-B eight-channel pressure injector, WPI). Immediately after application of Wnts, growth cones were imaged on a Nikon Ti microscope equipped with a mercury lamp using an APO TIRF 100× oil objective (numerical aperture 1.49). Images were acquired using three different fluorescence filter cubes: a CFP filter set (excitation, 420–445 nm; emission, 460–510 nm), a YFP filter set (excitation, 490–500 nm; emission, 520–560 nm), and a FRET filter set (excitation, 420–445 nm; emission, 520–560 nm). Images were recorded simultaneously by the Cascade 512B CCD (Roper Scientific) with the Macro Manager software. The ratio of YFP to CFP, as determined by the ImageJ.
software and RifRET plugin, represents the FRET signal, which is inversely correlated with the calpain activity.

RESULTS

Wnt5a Increases Calpain Activity and Induces Localized Cleavage of Spectrin in Developing Axons—Wnt5a has been shown to promote neuronal polarization when overexpressed in cultured hippocampal neurons (35). We determined distribution of Wnt5a in the rat brain. Interestingly, Wnt5a was found to be enriched in the intermediate zone (IZ) of the rat neocortex, which is enriched with traversing callosal axons positive for axonal marker Smi-312 (Fig. 1A), coinciding with the notion that Wnt5a promotes axon development. Given the role of Wnt5a in triggering calcium transients in various cell types, we determined whether Wnt5a activates calpain. Among the numerous calpain substrates, spectrin, the main protein component of the cell membrane skeleton, exhibits high sensitivity to calpain cleavage (36–38). αII-spectrin, which is mainly located in the axonal membrane and plays a crucial role in synaptic transmission (39), can be specifically cleaved by calpain, resulting in two proteolytic fragments of similar electrophoretic mobility, ~136 and ~148 kDa, respectively (38, 40). Thus, the levels of cleaved fragment are usually used to reveal calpain activity. We found that treatment with Wnt5a, but not Wnt3a, caused a progressive increase in the level of cleaved spectrin in cultured cortical neurons (Fig. 1, B and C), and this increase was prevented by pretreatment with BAPTA-AM, a selective chelator of intracellular calcium store, or calpain inhibitor calpeptin (Fig. 1D). Thus, Wnt5a-induced cleavage of spectrin depends on calpain activity.

To determine regional distribution of calpain activity in the brain, we generated an antibody that was specific for calpain-mediated spectrin degradation product (Fig. 2A). This antibody specifically recognized cleaved ~136-kDa fragment but not full-length spectrin (Fig. 2B). Next, we used this antibody to determine regional distribution of calpain activity in the rat brain. Interestingly, cleaved spectrin was found in both the cortical plate and the axon-rich IZ regions (Fig. 2D). These results suggest a correlation between calpain activation and axon development.

To monitor calpain activity in cultured neurons, we generated a construct by linking YFP and CFP coding sequences with the calpain cleavage site of αII-spectrin (Fig. 3A), designated as pYSC (where YSC represents YFP-spectrin-CFP), following the method described in a previous report (34). Under resting conditions without calpain activation, this fusion protein (YSC) was assumed to exhibit FRET. Upon calpain activation, proteolysis of linking region was speculated to

FIGURE 1. Wnt5a increases calpain activity in cultured primary neurons. A, neocortex of E18 rat was immunostained for Wnt5a and axonal marker Smi-312. n.c., negative control. Scale bar, 400 μm. B and C, cultured cortical neurons at DIV4 were treated with 100 ng/ml Wnt5a (B) or Wnt3a (C) for the indicated time. Cell lysates were subjected to immunoblot with the indicated antibody. D, cultured cortical neurons at DIV4 were treated with 100 ng/ml Wnt5a for 30 min, without or with pretreatment with BAPTA-AM (30 μM, 30 min) or calpeptin (40 μM, 1.5 h). Cell lysates were subjected to immunoblot with the indicated antibodies.
cause diminution in FRET signals; thus, the changes of FRET signal could be used to reflect calpain activity (Fig. 3A). First, we determined the responsiveness of the FRET construct to Wnt5a in cultured cortical neurons transfected with pYSC. We found that treatment with Wnt5a, but not Wnt3a, resulted in an increase in the level of monomeric fluorophores (Fig. 3, B and C), indicating the proteolysis of YSC fusion proteins and the activation of calpain. In contrast, the calpain-resistant construct with scrambled sequence linking YFP and CFP exhibited no responsiveness to Wnt5a treatment (Fig. 3D). This induced YSC cleavage happened as early as 5 min upon Wnt5a treatment (Fig. 3B) and requires intracellular calcium and calpain because BAPTA-AM or calpeptin treatment prevented accumulation of monomeric fluorophores (Fig. 3E, lanes 4 and 5). To further test the specificity of Wnt5a in inducing spectrin cleavage, cortical neurons transfected with pYSC were treated with cell-penetrating peptides composed of the TAT sequence derived from the transactivator of transcription of human immunodeficiency virus and the calpain cleavage site of spectrin or filamin A, hereafter referred to as TAT-S or TAT-F. We found that these peptides also prevented the accumulation
Wnt5a Activates Calpain

Thus, Wnt5a activates calpain, and YSC is a reliable tool to monitor calpain activity in situ.

Next, local calpain activity was determined in real-time imaging experiments in cultures of dissociated cortical neurons, whose axonal growth cones were locally administered with Wnt5a or -3a. Images were collected before and after treatment. We found that local application of Wnt5a to the growth cone of a pYSC-expressing neuron caused a decrease in the relative proportions of FRET-based fluorescence to donor fluorescence, whereas application of Wnt3a had no effect on FRET signals (Fig. 4, A and C). The effect of Wnt5a on FRET signals of YSC could be blocked promptly by the addition of Wnt5a antibodies (Fig. 4, A and C). Thus, Wnt5a, but not Wnt3a, causes activation of calpain in developing axons.

Requirement of Calpain for Wnt5a-induced Axonal Growth—Given the role of Wnt5a in evoking the fluctuations of intracellular calcium (7, 9, 11, 41) and in stimulating calpain activity shown above, we investigated the role of calpain in axonal growth. We found that Wnt5a treatment caused a marked increase in axon length (Fig. 5, A, B, and E). Interestingly, this Wnt5a-induced axonal growth was prevented by pretreatment with BAPTA-AM or calpeptin (Fig. 5B). This result suggests the involvement of calpain in Wnt5a-induced axonal growth. To exclude potential side effects of synthetic inhibitors, we took the approach of small interference RNA (siRNA) to down-regulate the expression of calpain. Among the various types of calpains, m-calpain plays a specific role in regulating fibroblast membrane protrusion and is required for the proteolysis of the cytoskeletal and focal adhesion proteins FAK, paxillin, spectrin, or talin (42). Thus, we chose to down-regulate m-calpain.

The siRNA construct against m-calpain (si-mCalpn) was effective in down-regulating the expression of co-transfected m-calpain-GFP in HEK293 cells (Fig. 5C) and endogenous m-calpain in cultured hippocampal neurons (Fig. 5D), whereas vehicle vector pSUPER or control siRNA construct (si-Scrambled) had no knockdown effect. We found that transfection with si-mCalpn, but not si-Scrambled, prevented the Wnt5a axonal growth-promoting effect (Fig. 5E). Thus, the role of Wnt5a in promoting axonal growth requires appropriate levels of m-calpain.

FIGURE 3. Wnt5a activation of calpain in cultured primary neurons. A, FRET construct generated by linking YFP and CFP sequences with calpain cleavage site of spectrin. B and C, cortical neurons were transfected with the construct encoding YFP-spectrin-CFP (pYSC) and treated with 100 ng/ml Wnt5a (B) or Wnt3a (C) at DIV4 for the indicated time. Cell lysates were subject to immunoblot with anti-GFP antibody, which recognizes YSC (YFP-spectrin-CFP) fusion protein as well as monomeric fluorophores. β-Actin was probed as a loading control. D, cortical neurons were transfected with calpain-resistant YFP-scrambled-CFP (YFP-Scram-CFP) construct, followed by treatment with Wnt5a (100 ng/ml) for the indicated time. Cell lysates were subject to immunoblot as described above. E, pYSC-transfected neurons were pretreated with BAPTA-AM (30 μm, 30 min), calpeptin (40 μm, 1.5 h), or TAT fusion peptides (TAT-S or TAT-F, 20 μm, 1.5 h) and then treated with Wnt5a (100 ng/ml) for 30 min. Cell lysates were subjected to immunoblot as shown above.
Calpain Is Required for Wnt5a-induced Growth Cone Advance—The long term extension of neurites is believed to rely on the transport of vesicles along microtubules, and the short term cellular events that initiate the growth cone formation may be regulated by actin dynamics. Given the relation between calpain activity and growth cone formation (26) and the activation of calpain by Wnt5a, we investigated the role of Wnt5a in regulating short term growth cone behavior. Cultured cortical neurons were transfected with RFP plasmid, and the behavior of the growth cone was recorded by the usage of time lapse video fluorescence microscopy. We found that treatment with Wnt5a promoted growth cone motility within a short period of time (Fig. 6, A and B), suggesting enhanced actin dynamics triggered by Wnt5a. In line with this notion, treatment with Wnt5a markedly enhanced forward movement by the growth cone with the fastest advancing speed (Fig. 6C). However, Wnt3a, which was incapable of activating calpain (Figs. 1C and 3C), had a mild but not significant effect on growth cone advance (Fig. 6C). Thus, Wnt5a has a specific role in regulating growth cone behavior.

Next, we determined whether calpain is involved in Wnt5a-regulated growth cone behavior. Cultured cortical neurons at

**FIGURE 4.** Wnt5a activation of calpain in developing axons. A and B, cortical neurons transfected with the construct encoding YFP-spectrin-CFP (A) or YFP-scrambled-CFP (B) were locally applied with 200 ng/µl Wnt3a or Wnt5a or vehicle PBS (control; Ctrl), at 100 µm away from axonal growth cones. FRET signals were captured at the indicated time and calibrated, as shown by pseudocolors (bar on the right). Scale bar, 2 µm. C and D, changes in the FRET signal during the 30-min period of exposure to Wnts or control buffer. Data are presented as means ± S.E. (n = 18 for control, n = 18 for Wnt3a, n = 12 for Wnt5a in C; n = 10 for control, n = 10 for Wnt5a in D).
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Inhibition or down-regulation of calpain suppresses the axon outgrowth-promoting effect of Wnt5a. A and B, hippocampal neurons were transfected with GFP plasmids before plating, followed by treatment with 100 ng/ml Wnt5a, 3 h after plating, without or with pretreatment using BAPTA-AM (30 μM, 20 min) or calpeptin (40 μM). Neurons were stained with axonal marker Smi-312 (A). Axon length was quantitatively analyzed with values in the control group normalized as 1.0 (B). Data are presented as means ± S.E. of three experiments. ***, p < 0.001. C, HEK293 cells were transfected with m-calpain-GFP, together with pSUPER, or plasmid encoding m-calpain-siRNA (si-mCalpn) or scrambled sequence (si-Scrambled) at a ratio of 1:4. Co-transfection with pEGFP was used as control (Ctrl). Levels of m-calpain-GFP and pEGFP- or pSUPER-encoded GFP were probed using GFP antibody, with β-actin as a loading control. D, effect of siRNA on endogenous m-calpain in cultured hippocampal neurons at DIV2. Arrows, neuron with m-calpain down-regulated. E, hippocampal neurons were transfected with pSUPER plasmid or plasmid encoding si-mCalpn or si-Scrambled, followed by the treatment with Wnt5a. After staining with Smi-312 antibody, axon length from different treatment groups was quantitatively analyzed, with values from the control group normalized as 1.0. Data are presented as means ± S.E. of three experiments. ***, p < 0.001. Scale bar, 20 μm.

DIV2 were pretreated with 30 μM BAPTA-AM, 40 μM calpeptin, or vehicle DMSO before exposure to Wnt5a (100 ng/ml). We found that the role of Wnt5a in stimulating growth cone forward movement was abolished by pretreatment with BAPTA-AM or calpeptin (Fig. 7, A, C, and E), suggesting the involvement of intracellular calcium and calpain in induced growth cone dynamics. Interestingly, pretreatment with TAT-S or TAT-F peptides, which was shown to prevent Wnt5a-induced cleavage of spectrin (Fig. 3E), also abolished the effect of Wnt5a on growth cone advance (Fig. 7, A, C, and E). This result suggests that calpain-mediated cleavage of actin-binding proteins, such as spectrin, may be involved in growth cone dynamics. Furthermore, down-regulation of m-calpain with si-mCalpn prevented the growth cone-stimulating effect of Wnt5a (Fig. 7, B, D, and F). Taken together, all of these results suggest that Wnt5a-Ca^{2+} signaling acts to activate calpain, leading to the truncation of actin-binding proteins and enhanced growth cone dynamics, which are essential for axonal growth.

Requirement of Calpain for Axonal Growth in Vivo—Having investigated the role of calpain in axonal growth in vitro, we next determined the role of calpain in vivo. The cerebral cortex is made up of six cellular layers, with neurons at each layer sending an axon ventrally toward the IZ. Once the axons reach the IZ, some of them turn toward the midline and form the corpus callosum, the largest fiber tract in the brain that connects neurons in the left and right cerebral hemispheres. The major projections across the corpus callosum are derived from neurons in layers 2/3 and 5 (43–45). Given the activation of calpain in IZ regions (Fig. 2C) and the role of calpain in mediating the effect of Wnt5a on axonal growth in cultured primary neurons, we determined the role of calpain in callosal axonal growth in vivo. We took advantage of the in utero electroporation technique to deliver expression constructs into rat cortical neurons (33). Vehicle vector pSUPER or that encoding si-mCalpn was co-injected with pCAG-EYFP plasmid into the lateral ventricle of the E16 rat brain, followed by electroporation into a subpopulation of neuronal progenitor cells.
At P4, brains were sectioned and stained with Hoechst to mark cortical layers. We found numerous callosal axons in the control group had crossed midline along the corpus callosum (Fig. 8A, top panels). However, down-regulation of m-calpain markedly interfered with callosal axonal growth (Fig. 8A, bottom panels), as reflected by decreased axon length of si-mCalpn-transfected neurons in comparison with control axons (Fig. 8B). Thus, m-calpain is required for axonal growth in vivo.

**DISCUSSION**

As an important second messenger in the cell, calcium has been shown to play important roles in controlling growth cone behavior (46, 47). Calpain, as a calcium sensor, regulates actin remodeling during cell spreading (48) and has been shown to be associated with growth cone formation (24, 26). Here, we report a role of calpain in Wnt5a-induced axonal growth. We found that Wnt5a activates calpain, which causes the truncation of actin-binding protein spectrin at the growth cone. Inhibition or down-regulation of calpain or interference with calpain-mediated spectrin cleavage prevented the promoting effects of Wnt5a on growth cone advance and axonal growth. We thus propose that Wnt5a-induced localized activation of calpain results in truncation of actin-binding proteins, such as spectrin, and causes transient dissociation of cytoskeletal components, leading to spatial-temporal actin dynamics; these processes allow growth cone advance and axonal growth (Fig. 9).

The axonal growth cone is composed of a central region filled with organelles and microtubules and a peripheral, highly dynamic, actin-rich region composed of lamellipodia and filopodia (49–51). The future axon shows enhanced growth cone dynamics and actin turnover, whereas the future dendrites show stable actin cytoskeleton (30, 31). Pharmacological depolymerization of the actin cytoskeleton is sufficient to transform dendrites into axons (27–30), whereas microtubule assembly is also actively involved in neuronal polarization (52). Nevertheless, the direct regulation of cytoskeleton structures by physiological cues is not clear.

In addition to the well identified canonical pathway, accumulating lines of evidence suggest that Wnt signaling also regulates cytoskeleton organization. For example, Wnt3 or Wnt7a, via Dvl, modulates microtubule dynamics during axon remodeling through both JNK activation and GSK3 inhibition (1, 53–55). Interestingly, Wnt signaling proteins Axin and Dvl bind directly to microtubules (54, 55). Our previous report showed that Wnt5a, by the activation of aPKC and the inhibition of microtubule affinity-regulating kinase 2 (MARK2), promotes axon development (35). Wnt5a has been shown to cause calcium elevation in various cellular contexts (6, 9, 11), including cultured cortical neurons shown recently (41). Given the regulatory role of Ca^{2+} in growth cone motility, axonal elongation, and guidance (56, 57), we investigated Wnt/Ca^{2+} signaling in axonal growth.

**FIGURE 6.** Effect of Wnt5a on growth cone behavior. A, DIV2 cortical neurons transfected with RFP plasmids were exposed to Wnt3a or Wnt5a. Time lapse images show the dynamics of the growth cone of the longest neurite. Frames show the fluorescence images of RFP at 2-min intervals. Dashed lines refer to the center of original growth cones before treatments. Scale bar, 5 μm. B, growth cone dynamics in different groups. Data are shown as means ± S.E. from at least 10 neurons. S.E. values were omitted for greater clarity. C, growth cone advance after 10-min treatment. *, p < 0.05, analysis of variance with Tukey’s test. Error bars, S.E.
Calpain, by partially truncating a variety of cytoskeletal proteins, is believed to be involved in regulating cell shape and motility (48, 58, 59) and, importantly, growth cone formation (26). Upon Wnt5a treatment, calpain-mediated specific cleavage of the actin-binding protein spectrin was increased, and particularly, this cleavage was observed in the developing axon (Fig. 4). Inhibition or down-regulation of calpain abolished the effect of Wnt5a on growth cone advance as well as axonal growth (Figs. 5 and 7). The rapid stimulation effect of Wnt5a on growth cone advance was prevented by the intracellular calcium chelator BAPTA-AM or the calpain inhibitor calpeptin and, importantly, by blocking spectrin cleavage using competitive peptides (Fig. 7). In line with the role of calpain in mediating axonal growth, remarkable activation of calpain, as reflected by cleaved spectrin, was observed in axon-rich IZ regions of the neocortex (Fig. 2C). Thus, localized activation of calpain, without causing cell death, may lead to cytoskeleton remodeling that is important for axonal growth.

A number of studies have identified deleterious roles of calpain in neurotoxic insults ranging from ischemia to Alzheimer disease (60–62). In addition, neuronal activity triggered by γ-burst stimulation or N-Methyl-D-aspartic acid (NMDA)
receptor stimulation activates calpain, which has been implicated in long-term potentiation (LTP) induction (34, 63, 64) or activity-dependent gene expression (20). Moreover, it is widely believed that calpain, by partially truncating various cytoskeletal proteins, also plays a role in regulating cell shape and motility (65). In addition to Wnt5a shown in this study, calpain can be activated by several other extracellular factors, such as BDNF and EGF (22, 23, 66, 67), and these activations were recently suggested to be calcium-independent (21). Our observation that BAPTA-AM blocks Wnt5a-induced calpain activation, as evidenced by the accumulation of a selective calpain-mediated spectrin breakdown product or the cleavage of the spectrin motif in the FRET probe, suggests a Ca\(^2\)\+‐dependent mechanism of calpain activation by Wnt5a.

Axonal growth can be stimulated by multiple extracellular factors, including neurotrophic factors, such as NGF and BDNF, and Wnt family proteins, such as Wnt5a. Multiple intracellular signaling pathways may mediate the role of these factors. Given the activation of calpain by BDNF in long-term potentiation induction (23) and the recently observed axon initiation effect of BDNF (68), it would be of interest to investigate whether calpain is also involved in BDNF-induced axon development. Axonal growth requires multiple intracellular steps, mainly actin dynamics at the growth cone, microtubule assembly at the neurite shaft, and, it is generally believed, membrane trafficking. Our demonstration that Wnt5a activation of calpain promotes actin dynamics during axonal growth, along with our previous observation that Wnt5a promotes neuronal polarization through microtubule regulation, suggests the intriguing possibility that an extracellular factor is capable of coordinating multiple intracellular mechanisms in regulating neuronal morphogenesis.

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