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Both the Establishment and the Maintenance of Neuronal Polarity Require Active Mechanisms: Critical Roles of GSK-3β and Its Upstream Regulators

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Summary

Axon-dendrite polarity is a cardinal feature of neuronal morphology essential for information flow. Here we report a differential distribution of GSK-3β activity in the axon versus the dendrites. A constitutively active GSK-3β mutant inhibited axon formation, whereas multiple axons formed from a single neuron when GSK-3β activity was reduced by pharmacological inhibitors, a peptide inhibitor, or siRNAs. An active mechanism for maintaining neuronal polarity was revealed by the conversion of preexisting dendrites into axons upon GSK-3 inhibition. Biochemical and functional data show that the Akt kinase and the PTEN phosphatase are upstream of GSK-3β in determining neuronal polarity. Our results demonstrate that there are active mechanisms for maintaining as well as establishing neuronal polarity, indicate that GSK-3β relays signaling from Akt and PTEN to play critical roles in neuronal polarity, and suggest that application of GSK-3β inhibitors can be a novel approach to promote generation of new axons after neural injuries.

Introduction

The morphological polarity of neurons with crucial functional implications is an essential component of the neuron doctrine established by Cajal (1911). Typically, a neuron has a single axon and multiple dendrites. Dendrites usually receive signals, whereas the axon usually sends signals. Neuronal polarity is therefore important for information processing and ensures unidirectional signal flow in the nervous system or between the nervous and other systems. Work in the past two decades has shown that, in addition to the well-known morphological and functional polarities, there are also molecular and organelle differences between the axons and the dendrites, ranging from the preferential sorting of proteins and mRNAs to the differential distribution of polyribosomes (Caceres et al., 1986; Dotti and Banker, 1987; Goslin et al., 1988; Kleiman et al., 1990; Steward, 2002). How neuronal polarity forms remains an interesting and challenging question.

A well-established model for studying neuronal polarity is the pyramidal neurons from the mammalian hippocampus (Craig and Banker, 1994). Hippocampal neurons become polarized in successive steps (Banker and Cowan, 1977, 1979; Dotti and Banker, 1987). Shortly after culturing, a neuron extends lamellipodia around the soma (stage [st.] 1). It then extends several minor neurites in st. 2. At st. 3, one neurite is significantly longer and becomes the axon, whereas the others become dendrites. Experimental manipulations of neurite length can reset neurite competition (Dotti and Banker, 1987). Exposure of a neurite to extracellular adhesive molecules (Lein et al., 1992; Esch et al., 1999) or stimulation of a neurite by mechanical tension promotes neurite growth and favors axon formation (Lamoureux et al., 2002). Cytoskeleton dynamics has been implicated in breaking symmetry between stages 2 and 3 (Baas et al., 1988; Ahmad et al., 1994). Molecules affecting actin dynamics (Bradke and Dotti, 1999; but see also Ruthel and Hollenbeck, 2000) or microtubule (MT) dynamics such as the collapsin response mediator protein-2 (CRMP-2) (Inagaki et al., 2001; Fukata et al., 2002) regulate the formation of axon-dendrite polarity. The evolutionary conserved polarity complex composed of Par3/Par6/aPKC was first found in *C. elegans* for their roles in establishing the anterior-posterior polarity of the first blastomere. Par3/Par6, as well as two small GTPases CDC42 and Rap1B, have recently been implicated in the establishment of neuronal polarity in mammals (Shi et al., 2003; Nishimura et al., 2004b; Schwamborn and Puschel, 2004), though not in *Drosophila* (Rolls and Doe, 2004).

Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine (Ser)/threonine (Thr) kinase found ubiquitously in eukaryotes (Embi et al., 1980; Woodgett, 1990). Biochemically, GSK-3 has a high basal activity. Signaling pathways act by inhibiting GSK-3 activity. Phosphorylation of Ser at position 9 in GSK-3β inhibits the activities of GSK-3β. GSK-3 is now known to be important in many biological processes, ranging from the canonical Wnt signaling pathway, MT dynamics, to astrocyte migration (Doble and Woodgett, 2003; Etienne-Manneville and Hall, 2003).

In the course of our work on axon guidance and neuronal migration (Li et al., 1999; Wong et al., 2001; Liu et al., 2004), we considered the possibility that molecules implicated in directional guidance could be involved in the development of neuronal polarity. We have obtained evidence that GSK-3β played a regulatory role in establishing and maintaining the axon-dendrite polarity. We further provide evidence that Akt and PTEN function upstream of GSK-3β, while the accompanying paper provides evidence that GSK-3β directly inactivates CRMP-2 (Yoshimura et al., 2004 [this issue of *Cell*]). Taken together, these findings indicate that GSK-3β is a central regulator in a signaling pathway for neuronal polarity.

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Results

Polarized Distribution of a Phosphorylated Form of GSK-3β in Hippocampal Neurons

We used hippocampal neurons to investigate a potential role of GSK-3β in neuronal polarity. Hippocampal explants were isolated from embryonic day 18 (E18) rats and pyramidal neurons were cultured (Banker and Cowan, 1977; Dotti and Banker, 1987; Brewer et al., 1993).

To determine the distribution of GSK-3β in hippocampal neurons, we double-labeled neurons with an antibody against total GSK-3β and another one against GSK-3β phosphorylated at Ser 9 (referred to as pGSK-3β hereafter). GSK-3β was detected in all neurites, both in st. 2 nonpolarized neurons (Figure 1A, in green) and in st. 3 polarized neurons (Figure 1B, in green). Interestingly, although pGSK-3β is present in the tips of all neurites in st. 2 nonpolarized neurons (Figure 1A, in red and Figure 1C), there is more pGSK-3β present in the tips of axons than that in the tips of dendrites in st. 3 polarized neurons (Figure 1B, in red and Figure 1D). The ratio of pGSK-3β/GSK-3β in the tips of st. 3 axons was significantly higher than that in the tips of st. 3 dendrites (Figure 1E).

GSK-3β also has a tyrosine phosphorylation site at 216 (Tyr216) which increases GSK-3β activity (Hughes et al., 1993; Wang et al., 1994). No differential distribution of GSK-3β Tyr216 was detected in polarized hippocampal neurons (Figure 1G).

Inhibition of Axon Formation after Increasing GSK-3β Activity

To test for a functional role of GSK-3β in the formation of neuronal polarity, we used a constitutively active GSK-3β mutant S9A, in which Ser 9 was replaced with alanine. Dissociated hippocampal neurons were transfected with a plasmid for the green fluorescent protein (GFP) or cotransfected with a plasmid for GFP and another expressing GSK-3β S9A. Neurons were cultured for 5 days before analysis of the axon-dendrite polarity by the relative length of axons versus dendrites and by staining with multiple specific antibodies. MAP2 is a somatodendritic marker for all dendrites and the proximal part of axons (Caceres et al., 1984; Figure 2A), whereas the Tau-1, the anti-Synapsin I, and the anti-GAP43 antibodies recognize the distal part of the axon (Mandell and Banker, 1996; Figure 2A).

Control hippocampal neurons (transfected with GFP alone) developed normal polarity, with most of them having a single axon and multiple dendrites and a small percentage of neurons with either no or multiple axons (Figure 2C). GSK-3β S9A significantly inhibited neuronal polarity (Figure 2C). Only 7.6% of control neurons has no axons, whereas approximately 49% of neurons transfected with GSK-3β S9A has no axon ($p < 0.001$), as exemplified by the neuron in Figure 2B that had no axon, with MAP2 in the entire length of every neurite. Without an axon, the axonal markers were mislocalized to all neurites (Figure 2B). These neurites did not have recycling of synaptic vesicles (Figure 4E), a functional characteristic of axons.

GSK-3β S9A led to the absence of the axon but did not affect the number of dendrites (Figure 2D). Dendrites were significantly longer in neurons when axons were eliminated by GSK-3β S9A (Figure 2F), perhaps due to the redistribution of membrane and cytoplasmic materials to the dendrites in the absence of an axon.

Neither the wild-type GSK-3β nor a kinase-dead form of GSK-3β affected axon-dendrite polarity (Figures 2C and 2D). Wild-type GSK-3β reduced the length of axons (Figure 2E) but did not affect the number or the length of dendrites (Figure 2F).

Formation of Multiple Axons after Inhibition of GSK-3 Activity by Pharmacological and Peptide Inhibitors and shRNAs

To further test for a role of GSK-3 in neuronal polarity, we used pharmacological inhibitors of GSK-3: SB216763, SB415286 (Coghlan et al., 2000), and lithium chloride (LiCl). Hippocampal neurons were treated with each inhibitor or the control vehicle dimethyl sulfoxide (DMSO) and were cultured for 5 days. Both SB216763 and SB415286 significantly decreased the number of neurons with a single axon but increased the number of neurons with multiple axons (Figures 3A and 3B). LiCl also caused the formation of multiple axons (Figure 3A), although not as efficiently as the SB compounds (Figure 3B). The total number of neurites per neuron was not significantly changed by SB216763, SB415286, or LiCl: they increased the number of axons and decreased the number of dendrites (Figure 3C), indicating the formation of axons at the expense of dendrites. Perhaps reflecting a limited amount of membrane and cytoplasmic materials during neurite outgrowth, the length of axons was shorter (Figure 3D) when multiple axons formed (all cases marked with (M) in Figure 3D). Shortening of axons was observed in all experiments, described later in this paper, when multiple axons formed. The length of dendrites was not affected by the pharmacological inhibitors (data not shown).

We then used GID5-6, a peptide inhibitor of GSK-3β derived from the GSK-3β interaction domain of axin (Hedgepeth et al., 1999). The control GID5-6LP contained a single amino acid mutation rendering it unable to interact with GSK-3β (Hedgepeth et al., 1999; Zhang et al., 2003). GID5-6 led to multiple axon formation, as evidenced by multiple markers (Figures 3E and 3F and Supplemental Figure S1A at http://www.cell.com/cgi/content/full/120/1/123/DC1/) and functional characterization by vesicle recycling (Figure 4F). The total number of neurites per neuron was not changed by GID5-6 (Figure 3G). Neurons transfected with GID5-6LP developed normal polarity (Figure 3F).

Finally, we used GSK-3βHP1 and GSK-3βHP2, two short hairpin RNA (shRNA) constructs designed specifically against GSK-3β (Yu et al., 2003). The control construct XASH3HP did not affect neuronal polarity (Figures 4B and 4C, upper panels). Either GSK-3βHP1 or GSK-3βHP2 led to the formation of multiple axons (see Figures 4A and 4B) at the expense of dendrites (Supplemental Figure S2A on the *Cell* website). When GSK-3β expression was examined (Figure 4C), reduction of GSK-3β expression correlated with multiple axon formation. Thus, GSK-3β expression was significantly reduced in GFP-positive neurons that formed multiple axons, GSK-3βHP1 (M) and GSK-3βHP2 (M), but not significantly reduced in those that had a single axon, GSK-3βHP1 (S) and GSK-3βHP2 (S).

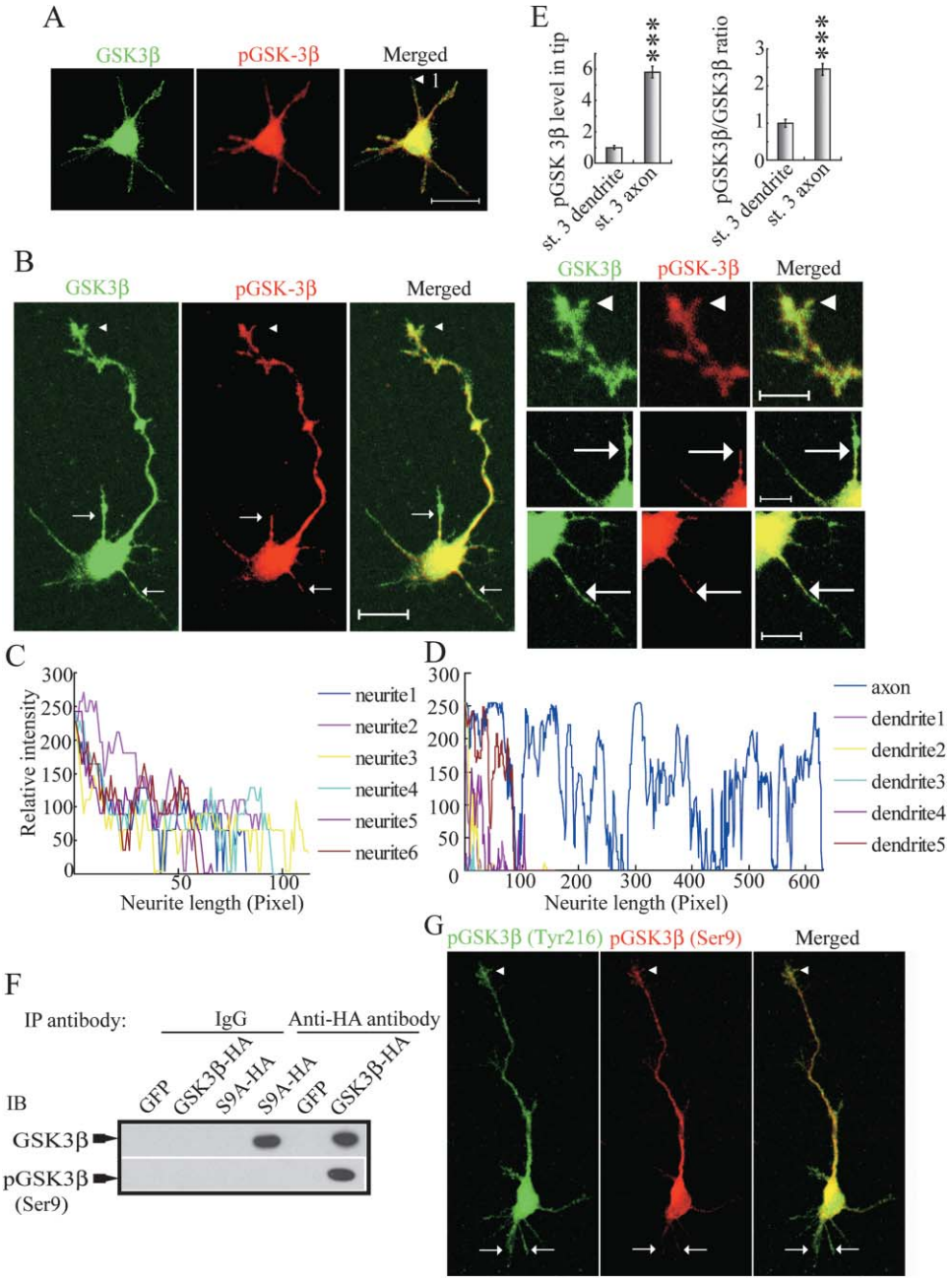

Figure 1. Distribution of GSK-3β and Phospho-GSK-3β (Ser 9) in Hippocampal Neurons

(A and B) Distribution of GSK-3β and pGSK-3β in st. 2 and st. 3 neurons, respectively. The scale bars are 20 μm in this paper unless specified otherwise. The small panels (scale bars of 5 μm) on the right part of (B) are higher magnification pictures of the tips of axons and dendrites.

(C and D) Fluorescence intensity after anti-pGSK-3β immunostaining of the st. 2 and st. 3 neurons.

(E) pGSK-3β and GSK-3β levels in neurite tips of st. 3 neurons. Left panel: the average level of pGSK-3β in dendrites was normalized as 1.0 ± 0.13 ($n = 42$); the relative level in axons is 5.8 ± 0.38 ($n = 16$; $p < 0.001$). Right panel: the average pGSK-3β/GSK-3β ratio in dendrites was normalized as 1.0 ± 0.11 ($n = 42$), while that in axons was 2.4 ± 0.16 ($n = 16$, $p < 0.001$).

(F) Specificity of anti-pGSK-3β (Ser 9) antibody, which does not recognize the GSK-3β S9A mutant transfected into HEK293 cells. IP: immunoprecipitation. IB: immunoblotting.

(G) Distribution of pGSK-3β (Tyr216) and pGSK-3β (Ser 9) in st. 3 neurons.

Functionality of Multiple Axons Induced by GSK-3β Inhibition

Although multiple axons are usually recognized by the relative length and expression of molecular markers, it is important to examine whether multiple axons are functional. A basic feature of functional axons is that they have active synaptic vesicle recycling which can be monitored by visualizing the FM4-64 dye uptake and release (Ryan et al., 1993). The FM dye was taken into the axon of control hippocampal neurons after stimulation by potassium (K^+) (Figure 4D), whereas little FM4-64 was taken into the somatodendritic region (Figure 4D).

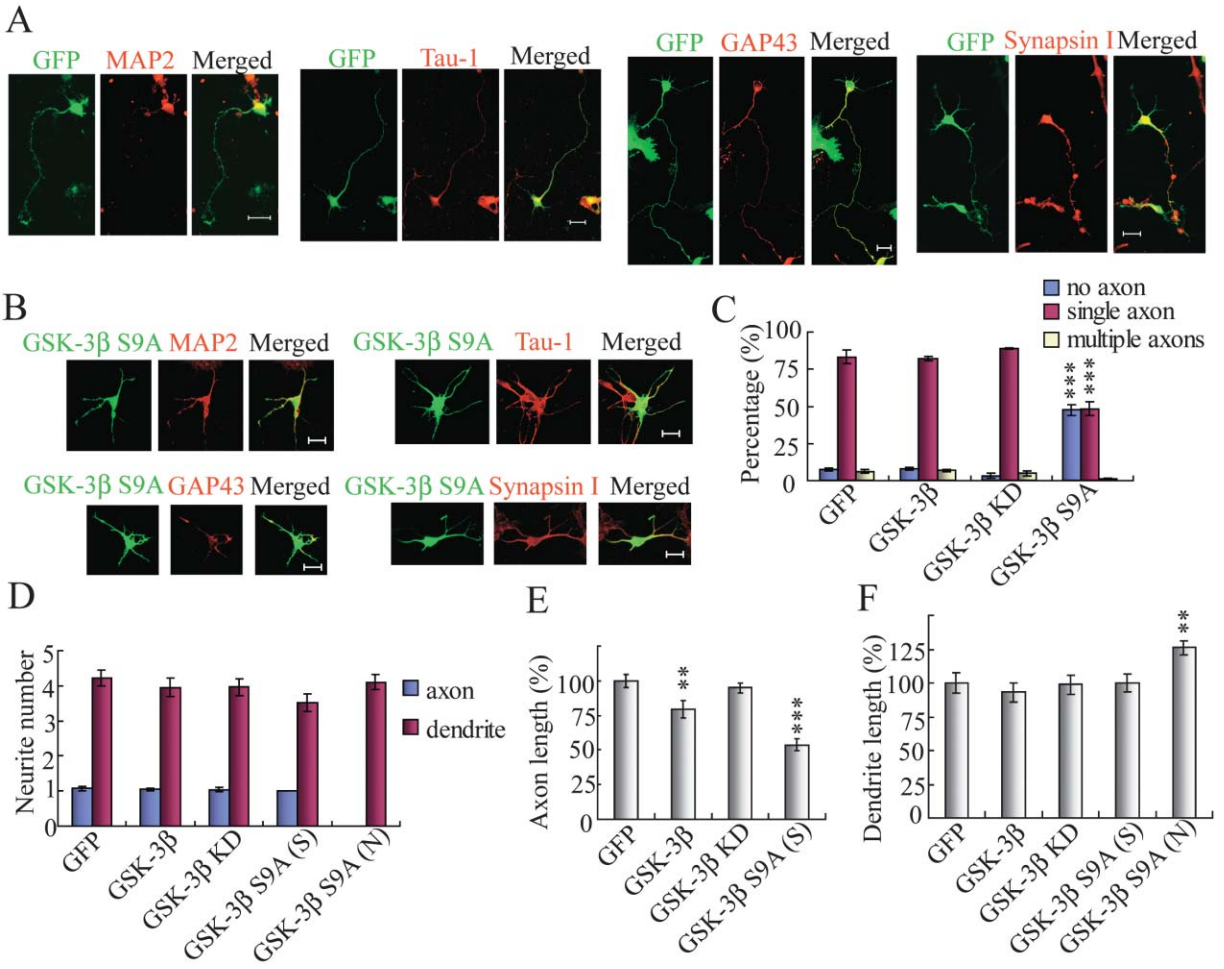

Figure 2. Inhibition of Axon Formation by a Constitutively Active Form of GSK-3β

(A) GFP-transfected neurons showing expression of the dendrite marker (MAP2) and axon markers (Tau-1, GAP43, and Synapsin I). MAP2 is localized in the soma, the dendrites, and the proximal part of the axon, while the axon markers are in the soma and the axon.

(B) Neurons transfected with GSK-3β S9A were stained with dendrite and axon markers. GSK-3β S9A transfected neurons lacked the axon, and axonal markers were mislocalized to all remaining neurites.

(C) Quantification of polarity defects. The p values of differences between control and GSK-3β S9A were: <0.001 in the percent of neurons with a single axon and <0.001 in those with no axons. Numbers were discussed in the text.

(D) Neurite numbers per neuron. GFP alone ($n = 56$): 1.07 ± 0.07 axon and 4.2 ± 0.22 dendrites. GSK-3β ($n = 48$): 1.04 ± 0.04 axon and 3.95 ± 0.27 dendrites. GSK-3β KD ($n = 54$): 1.04 ± 0.06 axon and 3.96 ± 0.24 dendrites. The numbers of axons and dendrites of neurons transfected with GFP and GSK-3β S9A were counted in two categories: (S) was neurons with single axons while (N) was neurons with no axons. Separate analysis should give a more accurate account of the effect of GSK-3β S9A because not all neurons positive for GFP were affected, most likely due to variations in GFP and GSK-3β S9A cotransfection efficiencies in different neurons.

(E) The average axon length of GFP-transfected neurons was normalized as 100%. The p values were <0.01 between the control and GSK-3β and <0.001 between the control and GSK-3β S9A (S).

(F) The average dendrite length of GFP-transfected neurons was normalized as 100%. The p value was <0.01 between the control and GSK-3β S9A (N). Neurons were separately analyzed as into those with a single axon (S), those with no axon (N), or those with multiple axons (M).

The GSK-3 inhibitor GID5-6 caused the formation of multiple axons positive for FM4-64 uptake after stimulation by 45 mM K^+ for 1 min (Figures 4F and 4G and Supplemental Figure S2B on the *Cell* website), whereas the dendrites did not take up the dye (Supplemental Figure S2B on the *Cell* website and Figure 4G). Vesicle recycling was observed: after the initial FM dye loading, treatment with 90 mM K^+ depleted it from two axons of a single GID5-6 transfected neuron (Figure 4F). Similar results were obtained with axons after GSK-3βHP2 transfection (data not shown). These results indicate that multiple axons induced by GSK-3β inhibition are functional in vesicle recycling.

We also examined FM dye uptake by neurites from neurons transfected with GSK-3β S9A with the same assay and found that neurites remaining after GSK-3β S9A transfection were negative in uptake (Figures 4E and 4G), confirming the earlier conclusion that GSK-3β S9A eliminated axons.

Role of GSK-3β in Establishing Neuronal Polarity

Establishment of neuronal polarity can be studied by examining the effect of manipulations before or up to st. 3, because most neurons reached st. 3 with axon-dendrite polarity established after culturing for 48 hr under the present conditions (Brewer et al., 1993). To study the role of GSK-3β in establishing neuronal polar-

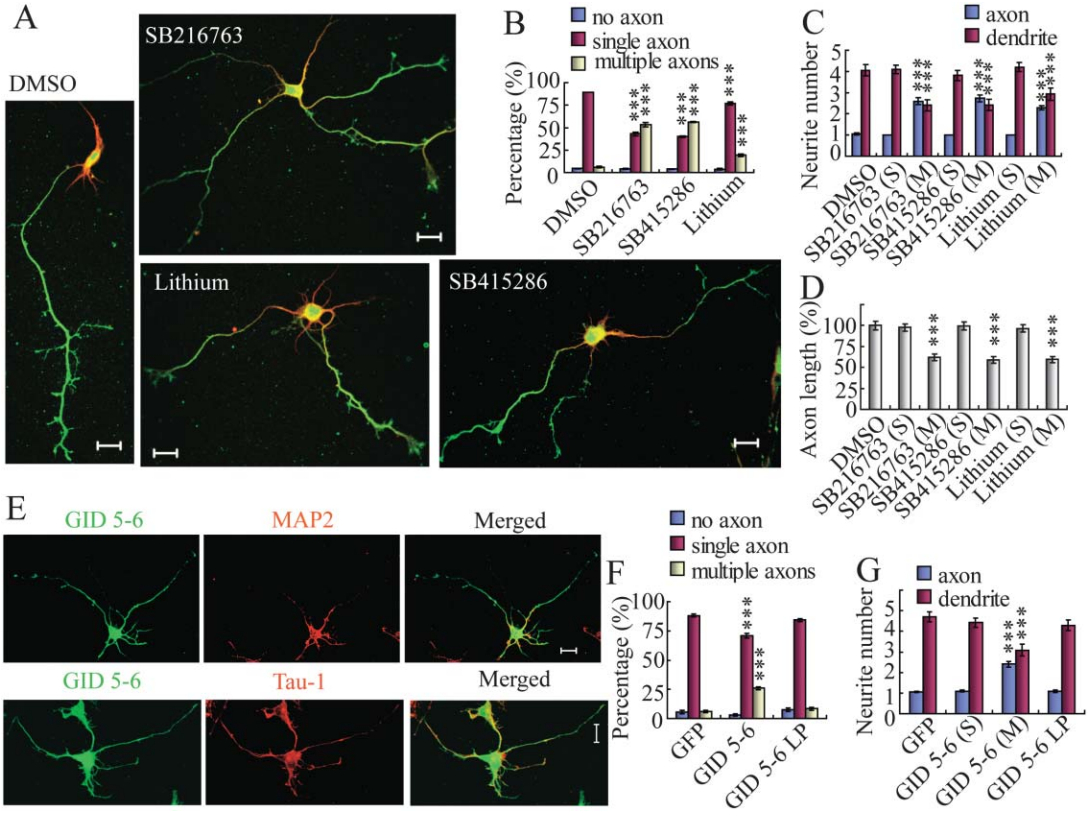

Figure 3. Formation of Multiple Axons upon GSK-3 Inhibition by Pharmacological and Peptide Inhibitors

(A) Neurons cultured in the presence of inhibitors (5 μM SB216763, 10 μM SB415286, or 1 mM LiCl) were examined for expression of Tau-1 (in green) and MAP2 (in red).

(B) Quantification of polarity defects. The p values were: <0.001 between the control and SB216763, <0.001 between the control and SB415286, and <0.01 between the control and LiCl.

(C) Numbers of neurites per neuron. (N): no axon. (S): single axon. (M): multiple axons. The p values of the difference were: <0.001 between DMSO and SB216763 (M), <0.001 between DMSO and SB415286 (M), and <0.001 between DMSO and LiCl (M).

(D) The average axon length of control (DMSO) neurons was normalized as 100%. The p values were: <0.001 between DMSO and SB216763 (M), <0.001 between DMSO and SB415286 (M), and <0.001 between DMSO and LiCl (M).

(E) Axon and dendrite markers in neurons transfected with GFP and GID5-6.

(F) Quantification of polarity defects. p values were: <0.001 between GFP and GID5-6 (single axon), <0.001 between GFP and GID5-6 (multiple axons), <0.001 between GID5-6 and GID5-6LP (single axon), and <0.001 between GID 5-6 and GID 5-6LP (multiple axons).

(G) (S): neurons with single axons. (M): neurons with multiple axons. The p values were <0.001 between the GFP control and GID5-6.

ity, neurons were treated with SB415286 for the first 24 hr and then removed. Neurons were cultured until day 6 (144 hr) before polarity examination. SB415286 caused the formation of multiple axons even when it was applied only for the first 24 hr (Figures 5A and 5B), indicating a role for GSK-3β in establishing axon-dendrite polarity.

We examined how GSK-3β inhibition affected the dynamics of neurite outgrowth by time lapse microscopy recording of st. 2 neurons (Figure 5E, upper panels and Figure 5D). SB415286 significantly reduced the time spent on retraction but increased the time spent both on pause and on growth (Figure 5E, lower panels and Figure 5D). The extent of the increase in pausing is less than that of the decrease in retraction. The net effect was promoting the formation of longer neurites, presumably axons.

Role of GSK-3 in Maintaining Neuronal Polarity

The availability of chemical inhibitors for GSK-3 allows us to test whether there is an active mechanism for maintaining neuronal polarity by applying them after the establishment of neuronal polarity.

Neurons were cultured for 3 days to reach st. 3 before being treated with SB415286 or the control DMSO for another 3 days (from 72 to 144 hr) and were fixed for immunostaining on day 6. The majority of neurons treated with DMSO at day 3 developed normal polarity by day 6 (Figure 5B). SB415286 treatment from 72 to 144 hr caused the formation of multiple axons (Figure 5A, lower panel and Figure 5B). It was almost as effective as treatment from day 0 to day 6 (Figure 5B). These results suggest that SB415286 could affect the maintenance of neuronal polarity.

To further examine the maintenance role of GSK-3, we recorded individual neurites before and after SB415286 treatment on day 3 and then followed them to day 6. As exemplified in Figure 5C, there was one long neurite on day 3 (marked with a white arrow) and three shorter neurites from the same neuron (marked with a white or a yellow arrowhead) (Figure 5C, left panel). On day 6, Tau-1 and MAP2 staining showed that the originally long neurite (marked by the white arrow) was indeed an axon (Figure 5C, right panel). Interestingly, an additional axon was formed on day 6 (its end marked with the red arrow

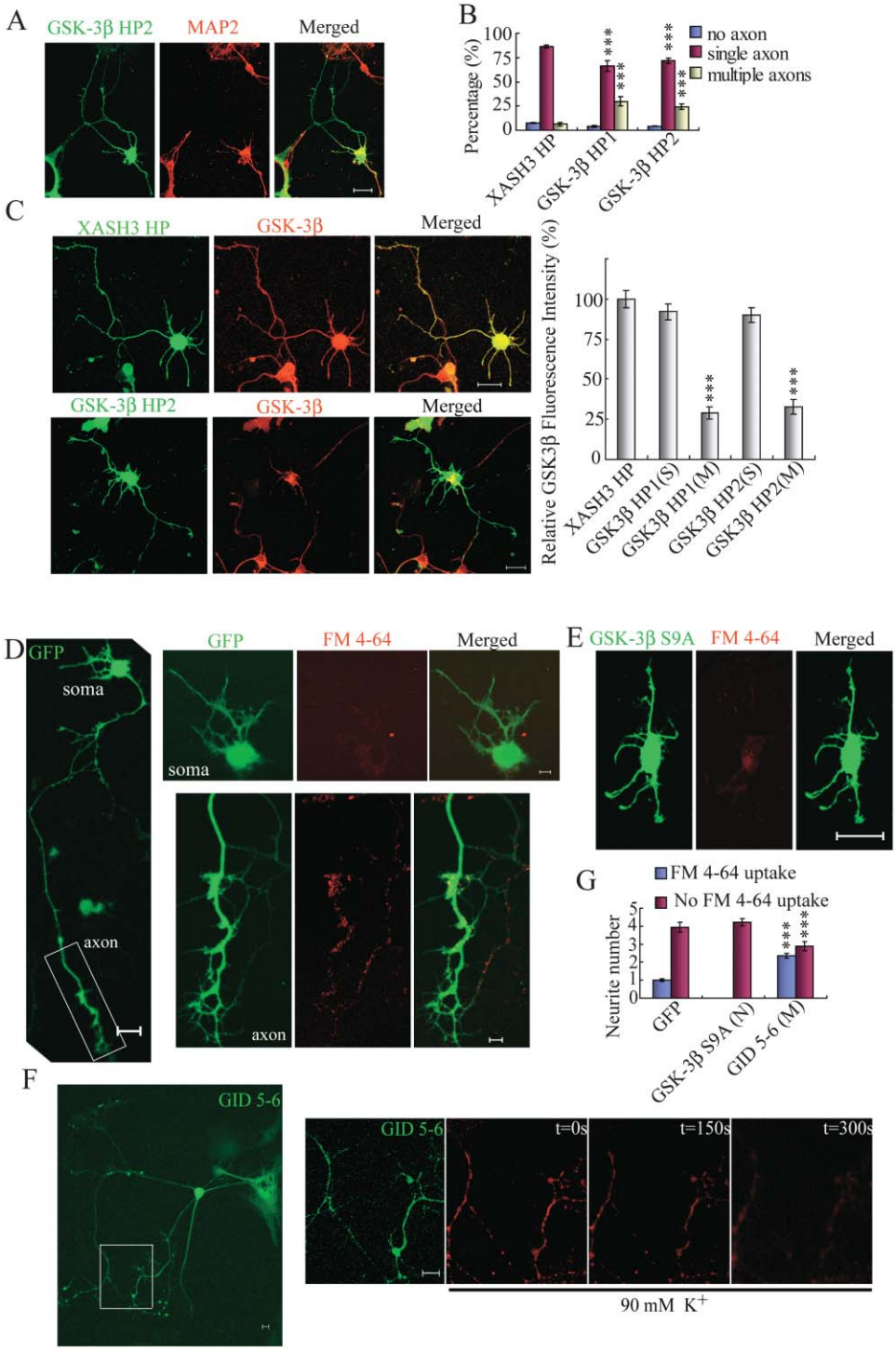

Figure 4. Effects of Specific siRNA Constructs on Hippocampal Neurons and Functional Characterization of Axons

(A) Localization of MAP2 in a neuron transfected with GFP and GSK-3βHP2.

(B) Quantification of polarity defects. The p values were: <0.001 between GSK-3βHP1 and XASH3HP and <0.001 between GSK-3βHP2 and XASH3HP.

(C) The average GSK-3β level in the neurites of control neurons (XASH3HP) was normalized as 100%. The p values were: <0.001 between the control and GSK-3β HP1 or HP2. (S): single axon. (M): multiple axons.

(D) A hippocampal neuron transfected with GFP was cultured for 7 days before being loaded with FM4-64 in 45 mM K^+ for 1 min. The neuron developed a single axon, which took up FM4-64. Boxed region in the left panel is shown in higher magnification in the lower right panels. The soma and dendrites had little uptake of FM4-64 (upper right panels).

(E) A GSK-3β S9A transfected neuron took in little FM4-64 in their soma or any of the short neurites.

(F) A GID5-6 transfected neuron with multiple axons, which were positive in FM4-64 uptake (boxed region in the left panel). The

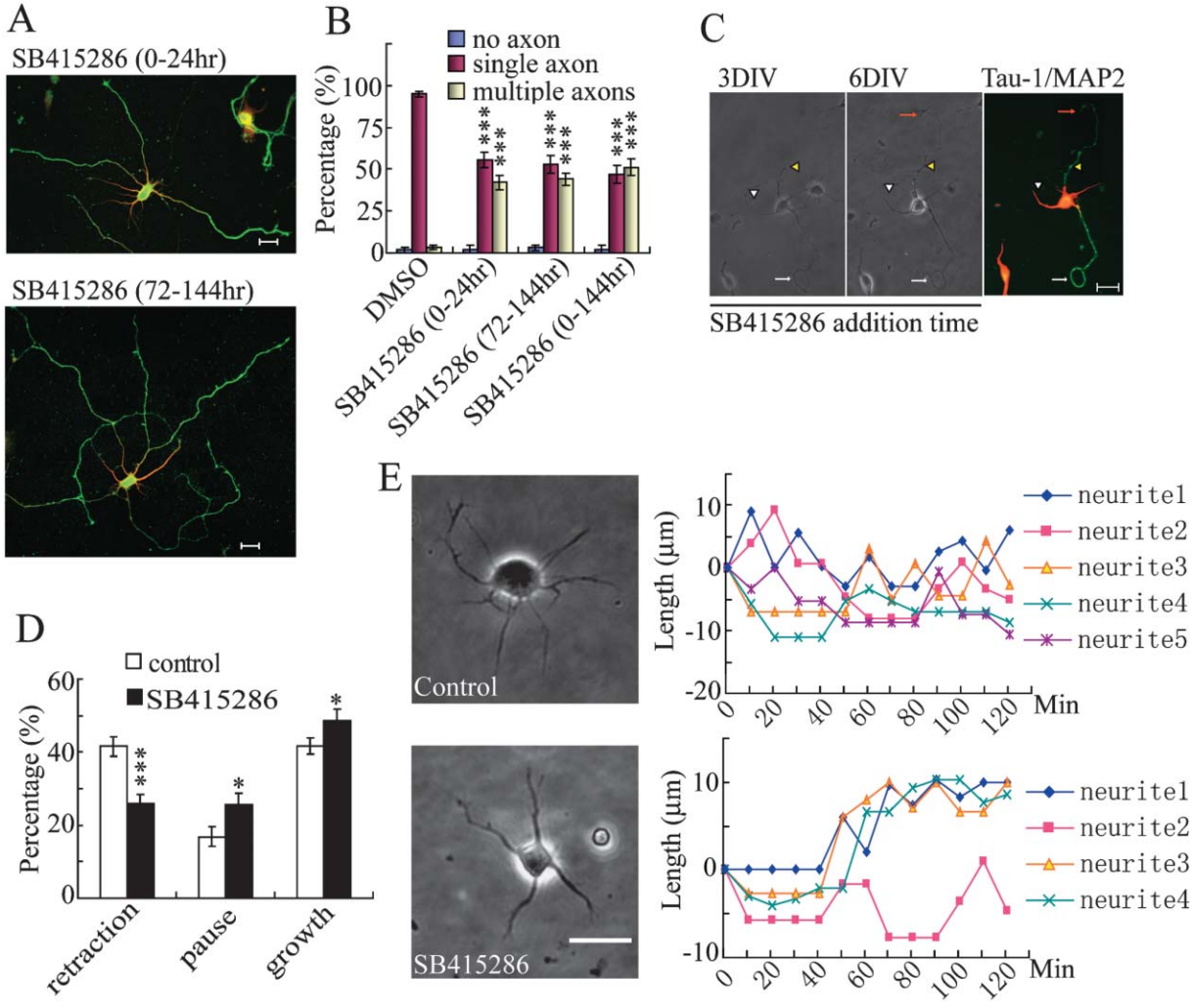

Figure 5. Roles of GSK-3β in Establishing and Maintaining Neuronal Polarity

(A) Neurons were treated with SB415286 from 0–24 hr, 72–144 hr, or 0–144 hr before being fixed for immunocytochemistry at day 6 (144 hr) with Tau-1 (in green) and MAP2 (in red).

(B) Quantification of polarity defects. The p values were: <0.001 between DMSO and SB415286 for 0–24, or 72–144, or 0–144 hr.

(C) Conversion of a preexisting dendrite into an axon by SB415286. The left panel shows a neuron on day 3, with one presumptive axon (a long neurite marked by a white arrow) and several dendrites (the tip of two dendrites marked by arrowheads). The middle and right panels show the same neuron on day 6. The long neurite indicated by the white arrow was indeed an axon (as shown by Tau-1 staining). One of the short neurites (indicated by the yellow arrowhead) in the leftmost panel on day 3 had grown by day 6 into an axon in the right panel. The red arrow points to the tip of the new axon.

(D) Neurite dynamics. St. 2 neurons were imaged by time lapse microscopy at an interval of 10 min for 2 hr. The % of time spent by each neurite on retraction, pause, or growth was recorded. The p values of differences between control and SB415286 were: retraction, <0.001; pause, <0.05; and growth, <0.05.

(E) Dynamics of neurites from a representative neuron treated with DMSO (upper panel) or SB415286 (lower panel).

in the middle and right panels of Figure 5C) from a neurite that was short on day 3 (its end marked with the yellow arrowhead in the left panel of Figure 5C). These results directly demonstrate that GSK inhibition can convert a preexisting dendrite into an axon, revealing that the potential for axon formation remains in the dendrites but is continuously suppressed by active GSK-3β.

Relationship of GSK-3β with Akt in the Formation of Neuronal Polarity

Ser 9 in GSK-3β can be regulated by multiple kinases (Cross et al., 1995; Fang et al., 2000; Doble and Woodgett, 2003), among which Akt is differentially localized in axons (Shi et al., 2003; Menager et al., 2004). To test for GSK-3β regulation by the candidate upstream

two axons boxed in the left panel are shown in higher magnification in the right panel: both were positive in FM dye uptake and release. Images were taken at the indicated time points before and after stimulation by 90 mM K^+. The scale bars are 10 μm.

(G) Quantification of neurites, with the axon defined as neurites positive in FM4-64 uptake, and dendrites as neurites negative in dye uptake. The p values were: <0.001 between GFP and GID5-6 (M). (N): no axon. (M): multiple axons.

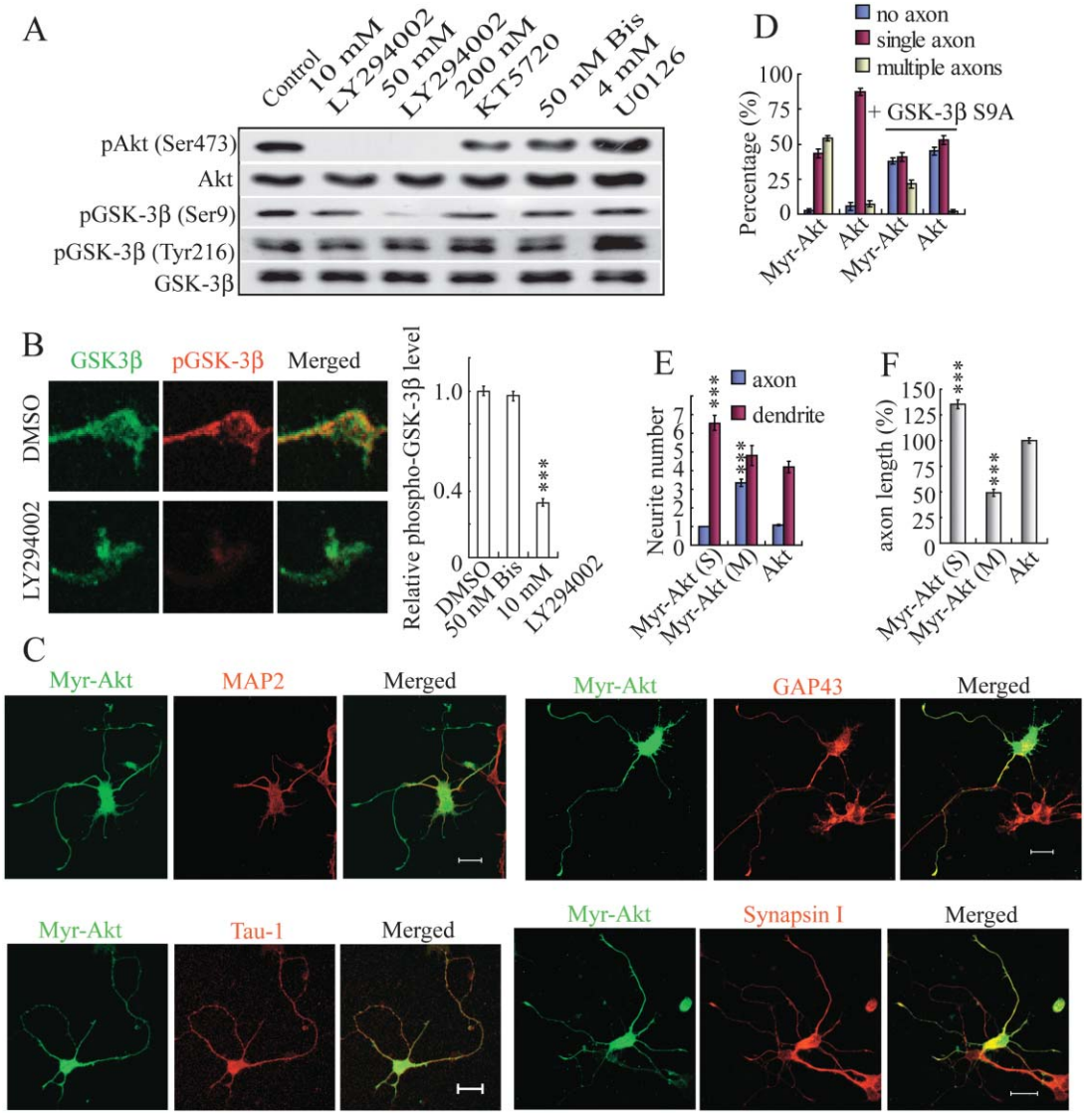

Figure 6. Control of GSK-3β Phosphorylation and Neuronal Polarity by Akt

(A) Inhibition of GSK-3β (Ser 9) phosphorylation by the PI3K inhibitor LY294000, but not by the PKA inhibitor KT5720, the PKC inhibitor Bis, or the MAPK inhibitor U0126. Neurons were cultured for 24 hr before being treated with the inhibitors for 1hr.

(B) The relative pGSK-3β/GSK-3β ratios in growth cones. The p value in the difference was <0.001 between DMSO and LY294000.

(C) A hippocampal neuron transfected by Myr-Akt developed multiple axons.

(D) Quantification of polarity defects. The p values were: <0.001 between Akt and Myr-Akt and <0.001 between Myr-Akt and Myr-Akt+ GSK-3β S9A.

(E) Neurite number per neuron. (S): neurons with a single axon. (M): neurons with multiple axons. The p values were: <0.001 between Akt and Myr-Akt (M) in axon numbers and <0.001 between Akt and Myr-Akt (S) in dendrite numbers.

(F) The average axon length was normalized to 100% for Akt. The p values were: <0.001 between Akt and Myr-Akt (S) and <0.001 between Akt and Myr-Akt (M).

molecules, we used the same inhibitors for PI3K as those used previously for studies of neuronal polarity (Shi et al., 2003). LY294002 significantly inhibited Akt phosphorylation at Ser 473 and GSK-3β phosphorylation at Ser 9 (Figure 6A). LY294002 did not affect GSK-3β phosphorylation at Tyr216. In addition to the biochemical evidences, we also examined the distribution of GSK-3β and pGSK-3β. LY294002 reduced the ratio of pGSK-3β over GSK-3β (Figure 6B). aPKC is another candidate GSK-3β regulator (Etienne-Manneville and Hall, 2003; Shi et al., 2003), but GSK-3β phosphorylation was not affected by the aPKC inhibitor bisindolylmaleimide I (Bis), or by the MAPK inhibitor U0126 or the PKA inhibitor KT5720 (Figure 6A).

To test for the functional significance of Akt, we tried to examine the effect of inhibiting Akt by using shRNAs, but they caused neuronal death (Yu et al., 2003). We investigated the effect of increasing Akt activity on neuronal polarity by using Myr-Akt, a constitutively active Akt derived by fusion with the myristoylation signal of Src (Ramaswamy et al., 1999). The wild-type Akt did not affect neuronal polarity (Figure 6D), whereas Myr-Akt

caused the formation of multiple axons (Figures 6C and 6D).

Because GSK-3β S9A should not be phosphorylated by Akt after the substation of Ser 9, we could test for the relationship between Akt and GSK-3β by cotransfection of GSK-3β S9A and Myr-Akt. GSK-3β S9A could partially, but not completely, reverse the effect of Myr-Akt on the formation of multiple axons. These results are consistent with the idea of Akt being upstream of GSK-3β. The partial reversal could result from either incomplete overlap in GSK-3β S9A and Myr-Akt transfection or the possibility that GSK-3β constitutes a part, but not all, of the output for Akt.

Detailed examination indicates that Akt played multiple roles in axon and dendrite development. Myr-Akt increased the number of neurites per neuron both in neurons forming multiple axons (Myr-Akt [M]), and in neurons with single axons (Myr-Akt [S]) (Figure 6E). Myr-Akt increased both the numbers of axons and dendrites from the normal total of five neurites to eight. When the number of axons was increased in Myr-Akt (M) neurons, axon length was shorter than normal (Figure 6F), perhaps due to limited materials for an increased number of axons. When the number of axons was not increased in Myr-Akt (S) neurons, the single axon was longer than normal (Figure 6F), indicating that Akt promotes axon outgrowth. The length of dendrites was not affected by Myr-Akt (data not shown).

Only the effect of increasing axon number is shared between Akt activation and GSK-3β inactivation. GSK-3β is therefore only one downstream component of Akt, specifically mediating the function of Akt in axon-dendrite polarity, but not in neurite number or axon length.

Functional Relationship between GSK-3β and PTEN

The phosphatase and tensin homolog deleted on chromosome 10 (PTEN) is a lipid and protein phosphatase that functions opposite to PI3K by dephosphorylating the lipid product of PI3K, phosphatidylinositol 3,4,5-trisphosphate (PIP_3) (Maehama and Dixon, 1998). We examined the role of PTEN further and characterized the functional relationship of PTEN and GSK-3β. Consistent with the earlier finding (Shi et al., 2003), neuronal polarity was lost upon PTEN overexpression (Figure 7). Detailed analysis indicated that PTEN transfection inhibited axon formation (Figures 7A and 7D and Supplemental Figure S3 on the *Cell* website) without affecting dendrite formation (Figure 7E). The inactive PTEN control (PTEN G129R) did not affect neuronal polarity.

To investigate the role of endogenous PTEN, we used PTEN siRNA, a small inhibitor RNA (siRNA) construct designed specifically for PTEN (Ning et al., 2004), and PTEN SsiRNA, a scrambled control. PTEN siRNA, but not PTEN SsiRNA, reduced PTEN protein levels in hippocampal neurons (Figure 7F). PTEN siRNA increased the number of axons (Figures 7C and 7D and Supplemental Figure S3 on the *Cell* website) at the expense of dendrites (Figure 7E). When the level of PTEN protein was examined (Figure 7F), the level of PTEN was reduced by more than 50% in PTEN siRNA (M) neurons with multiple axons, whereas PTEN was reduced less than 20% in PTEN siRNA (S) neurons with single axons. Significant reduction of PTEN expression therefore correlated with the formation of multiple axons.

To test for a functional relationship between PTEN and GSK-3β, we did two kinds of experiments. The first was to transfect neurons with PTEN and also treat them with SB415286. SB415286 almost completely reversed the effect of PTEN overexpression on axon-dendrite polarity, resulting in multiple axons (Figures 7B and 7D) at the expense of dendrites (Figure 7E). The second kind of experiment was to cotransfect PTEN siRNA and GSK-3β S9A. The effect of PTEN siRNA on multiple axon formation could be inhibited by GSK-3β S9A (Figure 7D). These results indicate that GSK-3β is downstream of PTEN in axon-dendrite polarity formation because GSK-3β manipulations dominate over PTEN manipulations.

One role of PTEN does not seem to be downstream of GSK-3β. When neurons were cotransfected with PTEN siRNA and GSK-3β S9A, GSK-3β S9A could not eliminate axons (Figure 7D, last three bars), suggesting that either PTEN siRNA can antagonize the effect of GSK-3β S9A in axon formation or that GSK-3β S9A has to act in a PTEN-dependent pathway to inhibit axon formation. In the latter scenario, GSK-3β activation could be upstream of PTEN in inhibiting axon formation.

Discussion

Our results have revealed that active mechanisms are required to maintain as well as establish the axon-dendrite polarity of mammalian neurons and that GSK-3β plays roles in both establishing and maintaining neuronal polarity. These results provide insights into the molecular basis of neuronal polarity determination. We propose a model in Figure 8 which is consistent with our results as well as those from the accompanying paper (Yoshimura et al., 2004) and previous papers (Inagaki et al., 2001; Fukata et al., 2002; Shi et al., 2003; Menager et al., 2004).

GSK-3β activity is of central importance. There is no significant difference in GSK-3β activity in the neurites of nonpolarized neurons. However, after the transition from st. 2 nonpolarized neurons to st. 3 polarized neurons, GSK-3β activity is higher in dendrites than in axons, which is critical for determining axon-dendrite polarity. GSK-3β is regulated by Akt, which is in turn regulated by PI3K and PTEN, perhaps through $PI(3,4,5)P_3$ and a kinase that phosphorylates Akt. Downstream of GSK-3β are CRMP2 and others such as Tau and MAP1B. There are no data at the moment to place Par3/Par6, adenomatous polyposis coli (APC) (Shi et al., 2003, 2004), or CDC42 and Rap1B in this pathway (Schwamborn and Puschel, 2004).

Roles of GSK-3β in Establishing and Maintaining Neuronal Polarity

The conclusion that GSK-3β plays critical roles in both establishing and maintaining neuronal polarity is based on observations of pGSK-3β distribution and experimental manipulations of GSK-3β activity.

In st. 2 neurons, GSK-3β inactivation (as indicated by phosphorylation at Ser 9) is similar in all neurites. In st. 3 neurons, there is more inactive GSK-3β in axons than that in dendrites. A constitutive active GSK-3β inhibited axon formation but did not affect dendrite formation,

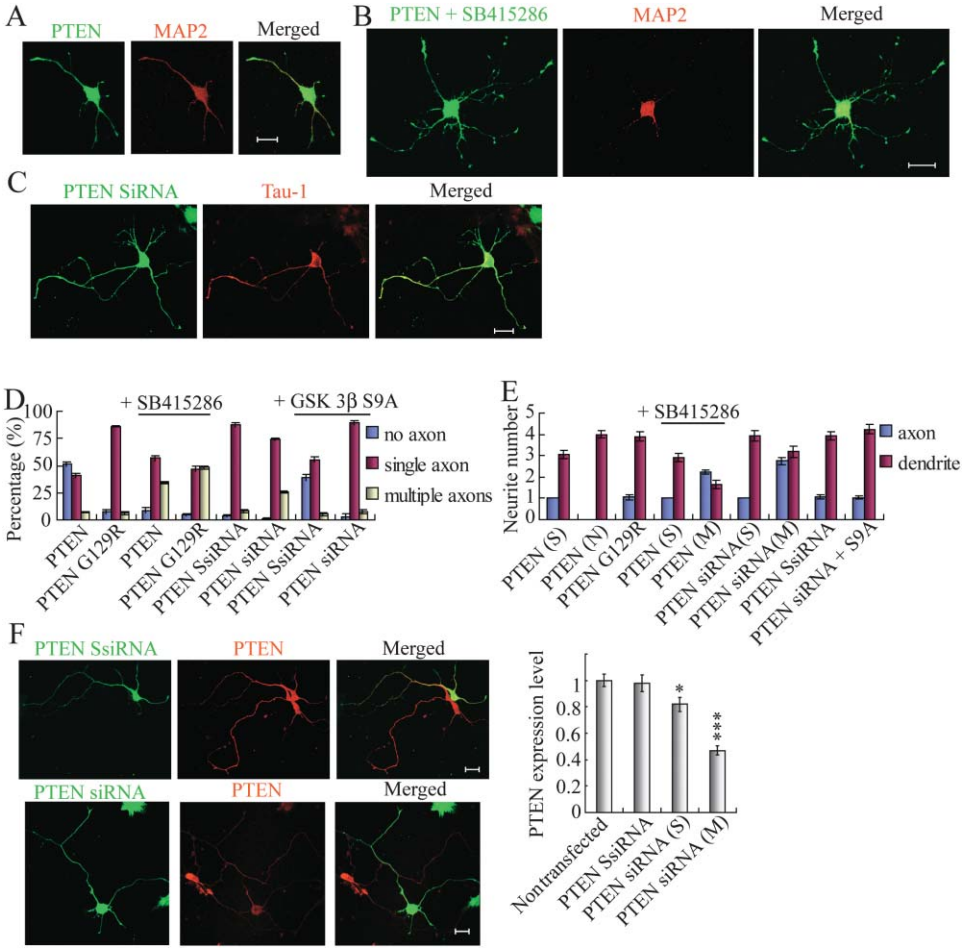

Figure 7. Effects of PTEN and PTEN siRNA on Neuronal Polarity

(A) A neuron transfected with PTEN developed no axons.

(B) A neuron transfected with PTEN and treated with GSK-3β inhibitor SB415286 developed multiple axons.

(C) A neuron transfected with PTEN siRNA with multiple axons.

(D) Quantification of polarity defects. The p values were: <0.001 between PTEN and PTEN G129R, <0.001 between PTEN and PTEN+SB415286, <0.001 between PTEN SsiRNA and PTEN siRNA, and <0.001 between PTEN siRNA and PTEN siRNA+GSK-3β S9A.

(E) Neurite number per neuron. (S): neurons with a single axon. (N): neurons with no axon. (M): neurons with multiple axons. The p values were: <0.01 between PTEN and PTEN G129R in dendrite numbers, <0.05 between PTEN siRNA (M) and PTEN SsiRNA in dendrite numbers, <0.001 between PTEN siRNA (M) and PTEN SsiRNA in axon numbers, and < 0.001 between PTEN siRNA (M) and PTEN siRNA+ GSK-3β S9A in axon numbers.

(F) PTEN expression levels in the cell body were measured and normalized as 1 for the control untransfected neurons. (S): neurons with single axons. (M): neurons with multiple axons. The p values were: <0.001 between the control and PTEN siRNA (M) and <0.05 between the control and PTEN siRNA (S).

indicating that GSK-3β inactivation is necessary for axon formation. Inhibition of GSK-3β activity by three different methods (pharmacological inhibitors, a peptide inhibitor, and shRNAs) all resulted in multiple axon formation with concomitant reduction of dendrite formation, indicating that GSK-3β inhibition is sufficient for axon formation. There is a recent report that AR-A014418 and LiCl, two pharmacological inhibitors of GSK-3β, led to loss of axon formation (Shi et al., 2004), which were different from effects reported here and in the accompanying paper (Yoshimura et al., 2004). These apparent differences might be due to differences in the concentrations of LiCl used in different labs (5 mM in Shi et al. [2004], 1 mM in this paper, and 2 mM in Yoshimura et al. [2004]) and in the length of culture (48 hr in Shi et al. [2004] and 5 days in this paper). Culturing for longer time makes it easier for identification with multiple markers and functional testing. The shRNA constructs provide strong evidence for the formation of multiple axons upon GSK-3β inhibition.

One model is that, during the transition from st. 2 to st. 3, GSK-3β is activated in all neurites except one. Activation of GSK-3β in several neurites causes them to become dendrites, leaving one neurite with a lower level of active GSK-3β to form the axon. The alternative model is that GSK-3β is inactivated in axons during the transition from st. 2 to st. 3.

Our conclusion that GSK-3β is involved in maintaining neuronal polarity is based on the findings that preexisting dendrites from day 3 neurons can be converted

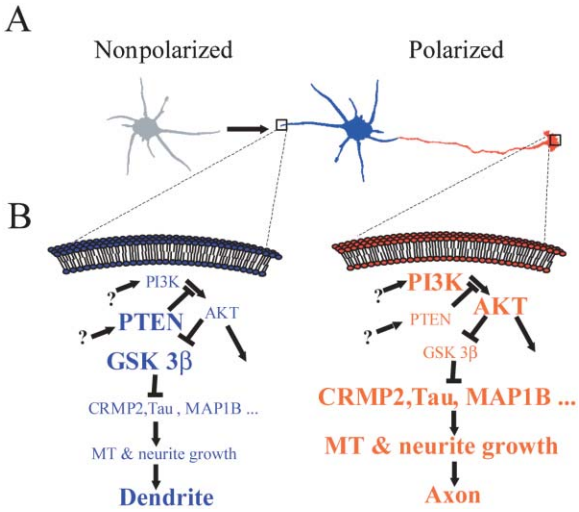

Figure 8. A Model for a Signaling Pathway in Determining Axon-Dendrite Polarity

(A) A neuron developing from the nonpolarized st. 2 to the polarized st. 3.

(B) Diagrams illustrate relative activities of molecules in the pathway: the left panel for the dendrites and the right panel for the axon. GSK-3 activity is higher in dendrites than in axons. Akt activity is higher in axons than in dendrites. Differences of activities of endogenous PI3K and PTEN were inferred from results from manipulating PI3K and PTEN without direct detection.

into axons upon GSK-3β inhibition. This conclusion is also consistent with the finding of preferential pGSK-3β localization in axon tips of neurons at stages later than 2 and 3 (Zhou et al., 2004). This indicates that the potential to form axons persists in dendrites and that the presence of active GSK-3β in the dendrites suppresses their axon formation potential.

It is not clear whether there is a default fate for neurites. Dendrites have been assumed to be the default since there are normally more dendrites from each neuron and they grow slower. On the other hand, the effect of GSK-3β inhibition on dendrites is more consistent with axons as the default fate, which has to be actively and continuously suppressed by GSK-3β. It should also be noted that, while there are several ways to make neurites which normally do not form axons to become the extra axons, so far there is no manipulation that can convert axons into dendrites.

Regulation of GSK-3β in Hippocampal Neurons

GSK-3β has a high level of basal activity, and its regulation usually results from inactivation by upstream signals (Doble and Woodgett, 2003). Phosphorylation at Ser 9 is a major means to inactivate GSK-3β. Our results with Akt and PTEN indicate that both are upstream of GSK-3β in determining neuronal polarity. Akt functions to inhibit GSK-3β whereas PTEN functions to activate GSK-3β. Our results also provide evidence that Akt plays more roles than neuronal polarity because its activation could also increase the number of dendrites and the length of axons, neither of which could be caused by GSK-3β inactivation. They indicate that these roles of Akt are mediated by downstream components other than GSK-3β, which only mediates the role of Akt in neuronal polarity.

GSK-3β manipulations predominate over PTEN manipulations on neuronal polarity, indicating that PTEN is upstream of GSK-3β in polarity formation. One effect of PTEN seems to be downstream of GSK-3β: activation of GSK-3β in GSK-3β S9A could inhibit axon formation, but this effect was blocked if PTEN was inhibited by PTEN siRNA. It is also possible that there is a feedback loop between PTEN and GSK-3β.

GSK-3β Regulation of Its Targets

There are multiple downstream targets for GSK-3β. Some downstream targets are involved in controlling cytoskeleton dynamics. The accompanying paper shows that CRMP-2 is a direct target of GSK-3β and phosphorylation of CRMP-2 decreases its tubulin binding activity (Yoshimura et al., 2004).

GSK-3β can also phosphorylate other MT-associated proteins such as MAP1B (Goold et al., 1999; Lucas et al., 1998), tau (Hanger et al., 1992), and APC. Phosphorylation by GSK-3β inhibits the ability of tau and MAP1B to bind MT and thereby inhibits MT assembly. Hippocampal neurons isolated from mice lacking both tau and MAP1B showed strong defects in neuron polarity: they could form dendrites but not axons (Takei et al., 2000). Tau and MAP1B may therefore also be downstream of GSK-3β: GSK-3β inactivation in axonal tips would allow tau and MAP1B to be active and promote MT assembly, thus favoring axon elongation. In Alzheimer's disease, tau is highly phosphorylated in the brain by aberrantly activated GSK-3β (Hanger et al., 1992). It will be interesting to test the significance of axon defects resulting from activated GSK-3β in the pathogenesis of Alzheimer's disease.

GSK-3β phosphorylation of its substrates usually requires prior phosphorylation (priming) by other kinases. One priming kinase is casein kinaseIα for GSK-3β phosphorylation of β-catenin (Liu et al., 2002; Cho and Johnson, 2003) and another is PAR-1 for GSK-3β phosphorylation of tau in *Drosophila* (Nishimura et al., 2004a). A mammalian Par-1 homolog MARK phosphorylates multiple MT-associated proteins including tau and triggers MT disassembly (Drewes et al., 1997). It will be interesting to investigate the role of MARK(s) in determining neuronal polarity.

Generation of New Axons from Preexisting Dendrites and Its Implications

Current approaches to promote recovery of connectivity after neural injuries aim to regenerate damaged axons or facilitate sprouting from damaged axons. The ability to convert dendrites into axons by molecular inhibitors suggests that it will be worthwhile to explore the approach to generate new axons from dendrites on adult neurons with damaged axons.

Before the therapeutic application can be tested, it will be necessary to investigate the feasibility of using GSK inhibitors to convert dendrites into axons in vivo and in adult animals.

The availability of pharmacological inhibitors of GSK-3β as well as inhibitors based on DNA and siRNA provide a range of potential therapeutic reagents for future tests. GSK-3β inhibition therefore offers a novel approach that may be used to induce axon conversion

from dendrites, and, if applied selectively to redundant or nonessential dendrites, may help generate new axons to promote functional recovery after neural injuries.

Experimental Procedures

Materials

We used the following antibodies and chemicals: Tau-1 (Chemicon), anti-MAP2 (Chemicon), anti-GSK3β (Chemicon), anti-phospho-GSK3β (Ser 9) (Cell Signaling), anti-phospho-GSK3β (Y-216) (BD Bioscience Pharmingen), anti-phospho-Akt (Ser 473) (Cell Signaling), anti-Akt (Cell Signaling), anti-β-catenin (Upstate), anti-GAP43 (Novus Biologicals), anti-PTEN (Cell Signaling), anti-Synapsin I (Chemicon), SB216763 and SB415286 (Tocris), LiCl (Sigma), Bisindolylmaleimide I (Calbiochem), LY294002 (Calbiochem), U0126 (Calbiochem), Roscovitine (Calbiochem), and KT5720 (Calbiochem).

DNA Constructs

Axin GID5-6/pCS2MT, Axin GID5-6LP/pCS2MT, and GSK-3β S9A/pCS2 were generously provided by P.S. Klein (Hedgepeth et al., 1999; Zhang et al., 2003). shRNA constructs including U6-XASH3-HP, U6-GSK3β-HP1, and U6-GSK3β-HP2 were generous gifts from D.L. Turner (Yu et al., 2003). HA-PTEN/pSG5L, HA-PTEN G129R/pSG5L, Myr-HA-Akt/pLNCX, and HA-Akt/pLNCX were generous gifts from W.R. Sellers (Ramaswamy et al., 1999). GSK KD was generously provided by Kozo Kaibuchi. SiRNApten-GFP and SsiRNApten-GFP were from Q. Wan (Ning et al., 2004). GSK-3β wild-type and mutants were generously provided by G. Johnson (Cho and Johnson, 2003), and the wild-type GSK-3β was provided by J. Woodgett (Woodgett, 1990).

Neuronal Cultures

Hippocampal explants isolated from E18 rat embryo were digested with 0.1% trypsin for 30 min at 37°C, followed by trituration with pipettes in the plating medium (DMEM with 10% fetal bovine serum). Dissociated neurons were plated onto coverslips coated with poly-D-lysine at a density of 100–200 neurons/mm^2. After culturing for 4 hr, media were changed into neuronal culture media (neurobasal medium supplemented with 2% B27). For Western analysis, all inhibitors were added 1 hr before lysis. Transfection of neurons was carried out immediately after dissociation using the Amaxa Nucleofector device. Testing plasmids were cotransfected with GFP at a ratio of 3:1. Numbers in the figures were ± SD.

FM Dye Recycling

FM4-64 experiments were performed on LSM510 Zeiss Axiovert 200 m inverted microscope with 40× oil objectives. Neurons were stained in 10 μM FM4-64 and solution with 45 mM K^+ for 1 min and washed with solution containing 3 mM K^+ for 15 min. After collecting FM fluorescent images, neurons were subjected to destaining in 90 mM potassium solution for 5 min. All solutions contained 10 mM kynuracid to prevent recurrent action potentials.

Western Analysis

Cells were lysed in lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 1% nonident P-40, 0.25% sodium deoxycholate, 1% sodium dodecylsulfate [SDS], 2 mM EGTA, 1 mM sodium orthovanadate, 1 mM sodium fluoride [NaF], 1mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin A, 1 μg/ml aprotinin, 1 μg/ml leupeptin). Samples for electrophoresis were solubilized with the loading buffer (2.5 mM Tris [pH 6.8], 25% glycerol, 2% SDS, 5% 2-mercaptoethanol, 0.05% bromophenol blue), heated to 100°C for 5 min, and resolved by SDS-PAGE using 10% acrylamide. For Western blots, equal amounts of samples were loaded into discontinuous gels (10% acrylamide tested under reducing conditions). Proteins were transferred to PVDF membrane for 1.5 hr at 100V, blocked with 5% BSA for 1 hr at room temperature. Membranes were then incubated with the primary antibodies in blocking solutions at 4°C overnight before detection with HRP-conjugated secondary antibodies. Chemiluminescence was detected with ECL solution.

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