

ARTICLE

Received 16 Jul 2013 | Accepted 16 Sep 2013 | Published 29 Oct 2013

DOI: 10.1038/ncomms3628

Activity-dependent regulation of dendritic growth and maintenance by glycogen synthase kinase 3 β

Yanfang Rui^{1,2}, Kenneth R. Myers^{1,2}, Kuai Yu^{1,2}, Ariel Wise^{1,2}, Angel L. De Blas³,
H. Criss Hartzell^{1,2} & James Q. Zheng^{1,2}

Activity-dependent dendritic development represents a crucial step in brain development, but its underlying mechanisms remain to be fully elucidated. Here we report that glycogen synthase kinase 3 β (GSK3 β) regulates dendritic development in an activity-dependent manner. We find that GSK3 β in somatodendritic compartments of hippocampal neurons becomes highly phosphorylated at serine-9 upon synaptogenesis. This phosphorylation-dependent GSK3 β inhibition is mediated by neurotrophin signalling and is required for dendritic growth and arbourization. Elevation of GSK3 β activity leads to marked shrinkage of dendrites, whereas its inhibition enhances dendritic growth. We further show that these effects are mediated by GSK3 β regulation of surface GABA_A receptor levels via the scaffold protein gephyrin. GSK3 β activation leads to gephyrin phosphorylation to reduce surface GABA_A receptor clusters, resulting in neuronal hyperexcitability that causes dendrite shrinkage. These findings thus identify GSK3 β as a key player in activity-dependent regulation of dendritic development by targeting the excitatory-inhibitory balance of the neuron.

¹Department of Cell Biology and Neurology, Emory University School of Medicine, Atlanta, Georgia 30322, USA. ²Center for Neurodegenerative Diseases, Emory University School of Medicine, Atlanta, Georgia 30322, USA. ³Department of Physiology and Neurobiology, University of Connecticut, Storrs, Connecticut 06269, USA. Correspondence and requests for materials should be addressed to J.Q.Z. (email: james.zheng@emory.edu).

Elaborate dendritic arbours are a key feature of polarized neurons in the vertebrate brain, and they function in reception, integration and computation of various synaptic inputs¹. Proper growth, patterning and maintenance of dendritic arbours are essential for normal brain development and function. Intrinsic and extrinsic signals are known to work in concert to generate and maintain the distinct patterns of dendritic arbours of different neuronal types^{2–4}. Among them, neuronal activity has a crucial role in dendrite development and maintenance^{2,5–7}. However, the effects of neuronal activity on dendritic arbours appear to be complex and depend on the nature of the activity, its spatiotemporal patterns, the specific brain regions, as well as particular developmental stages^{2,5–7}. The mechanisms underlying activity-dependent effects on dendrites remain to be fully elucidated. Given that dendritic arbours are the primary determinant of circuitry wiring and function, it is no surprise that abnormalities in dendritic arbours are associated with a large number of neurological disorders^{8,9}. Therefore, there is immense interest in understanding the molecular and cellular mechanisms that govern and regulate dendritic development and maintenance.

Glycogen synthase kinase 3 β (GSK3 β) is a serine/threonine kinase that is involved in a wide array of neuronal functions, including Wnt/ β -catenin signalling, proteasomal degradation, microtubule dynamics, receptor trafficking, synaptic plasticity, neuronal polarity and axon growth^{10,11}. GSK3 β is highly expressed in the central nervous system, especially in the hippocampus during brain development¹². However, elevated GSK3 β activity has been linked to a number of neurological diseases^{13–18}. GSK3 β activity is regulated by phosphorylation: phosphorylation of tyrosine-216 leads to activation, whereas phosphorylation of serine-9 results in inhibition^{10,17}. In cells, inhibition by serine-9 phosphorylation is believed to be the primary mechanism of regulating GSK3 β activity. However, because GSK3 β determines the output of numerous signalling cascades, it remains a challenge to understand how GSK3 β generates diverse effects on neuronal development and function.

GSK3 β is known to have a crucial role in the establishment of neuronal polarity. Local inhibition of GSK3 β is required for the acquisition of axonal identity, whereas GSK3 β remains highly active in neighbouring minor (dendritic) processes^{19,20}. Here we investigated whether GSK3 β has a role in dendrite development and maintenance, after the establishment of neuronal polarity. We show that GSK3 β is inhibited by neurotrophin signalling in dendrites of cultured hippocampal neurons upon synaptogenesis. Importantly, such GSK3 β inhibition is essential for dendritic growth and stabilization. Finally, we identify GABA_A receptors (GABA_ARs) as the main target of GSK3 β in activity-dependent regulation of dendritic development. Our results reveal a novel role for GSK3 β in activity-dependent regulation of dendrite development and maintenance.

Results

Inhibition of GSK3 β promotes dendrite development. Hippocampal neurons in culture develop their axon-dendrite polarity within the first 5 days *in vitro* (DIV)²¹. Synaptic connections of cultured hippocampal neurons start to form around DIV7–9, peak around DIV11–14 and become relatively stable after 3 weeks^{22–24}. To study changes in GSK3 β activity at different developmental stages, we examined the level and distribution of phospho-serine-9-GSK3 β (pS9-GSK3 β) in cultured rat hippocampal neurons at DIV3, 9, 16 and 22. Cells were double labelled with a pan-specific antibody to determine the total levels of GSK3 β (Total-GSK3 β). To quantify changes in pS9-GSK3 β signals, background-subtracted images were normalized to Total-GSK3 β to generate ratiometric images depicting the pS9/Total-

GSK3 β intensity ratio. Consistent with previous studies¹⁹, we found that pS9-GSK3 β was highly concentrated at the tip of axons at DIV3 (arrows; Fig. 1a), whereas Total-GSK3 β signals were relatively uniform (Fig. 1a, see also Supplementary Fig. S1a).

When hippocampal neurons were examined under identical conditions at either DIV9 or DIV16, we found that pS9-GSK3 β levels were markedly increased in the somatodendritic region (Fig. 1a and Supplementary Fig. S1b). Quantitative analysis showed that the pS9/Total-GSK3 β ratio in the somatodendritic region at DIV9 is $188.69 \pm 49.61\%$ (mean \pm s.d., $n = 139$) of the DIV3 value, and even higher in the hippocampal neurons of later stages (Fig. 1b). We confirmed the increase in pS9 levels at DIV9 and 16 using western blot (Supplementary Fig. S2). Conversely, phospho-tyrosine-216 GSK3 β levels did not change over the same period (except at DIV22 when a small increase was observed). In addition, at DIV14–16, pS9-GSK3 β staining was more intense in dendrites than in axons (Supplementary Fig. S1c,d). As DIV9 corresponds to the early stage of synaptogenesis, our findings suggest that the increase in pS9-GSK3 β in dendrites may be associated with synapse formation.

Because neurotrophin signalling has been associated with synaptogenesis and dendritic development²⁵, we tested if it was involved in the elevation of pS9-GSK3 β levels in dendrites. We found that inhibition of Trk receptors by the selective inhibitor K252a (ref. 26), but not the ineffective analogue K252b, for 2 days (DIV14 to DIV16) resulted in a marked reduction in pS9-GSK3 β levels in the somatodendritic region of cultured hippocampal neurons ($54.97 \pm 8.93\%$ (mean \pm s.d., $n = 362$) of the control, Fig. 2a). Correspondingly, bath application of brain-derived neurotrophic factor (BDNF) to DIV14 hippocampal neurons for 2 days further elevated the pS9-GSK3 β level in the somatodendritic compartment to $127.99 \pm 26.39\%$ (mean \pm s.d., $n = 153$) of the control (Fig. 2a). These results suggest that neurotrophin signalling is involved in the elevation of dendritic pS9-GSK3 β levels.

To examine the effects of neurotrophin signalling on dendrites, we transfected DIV13 hippocampal neurons with EGFP constructs and imaged the same EGFP-expressing neurons at 1 and 5 days post transfection (referred to as +1 d and +5 d). Cells were treated with K252a or K252b immediately after the initial imaging for the next 4 days, followed by imaging on +5 d. We found that neurons treated with K252a exhibited reduced dendritic growth and shrinkage of some dendritic branches (red arrows, Fig. 2b). We quantified the total dendritic length and the number of dendritic branches on +1 d and +5 d and normalized these values (+5 d versus +1 d) to better illustrate the changes over the 4-day period. We found that neurons exposed to K252b, the ineffective compound, continued to extend their dendritic branches, resulting in an increase in the total dendritic length whereas the number of dendritic branches remained unchanged (mean \pm s.d.: $125.6 \pm 26.1\%$ and $92.9 \pm 10.1\%$ of the +1 d values, respectively, $n = 14$) (Fig. 2c). However, inhibition of Trk receptors by K252a caused a marked reduction in total dendritic length and branch number (mean \pm s.d.: $86.8 \pm 13.9\%$ and $80.9 \pm 16.0\%$ of the +1 d values, respectively, $n = 15$). Because K252a caused a substantial reduction in pS9-GSK3 β levels, we suspected that K252a-induced dendritic reduction might be mediated by GSK3 β activation. To test this possibility, we expressed GID5–6, an inhibitory peptide derived from the GSK3 β binding domain of Axin^{19,27}. We found that GID5–6 expression not only caused a marked increase in dendritic growth, but also abolished the dendritic reduction induced by K252a (Fig. 2c). To further confirm that GSK3 β mediated K252a-induced reduction in dendritic growth, we used small hairpin RNA (shRNA) to specifically knock down GSK3 β (U6-GSK-3 β HP1)²⁸. Immunostaining confirmed the effectiveness of GSK3 β knockdown by this

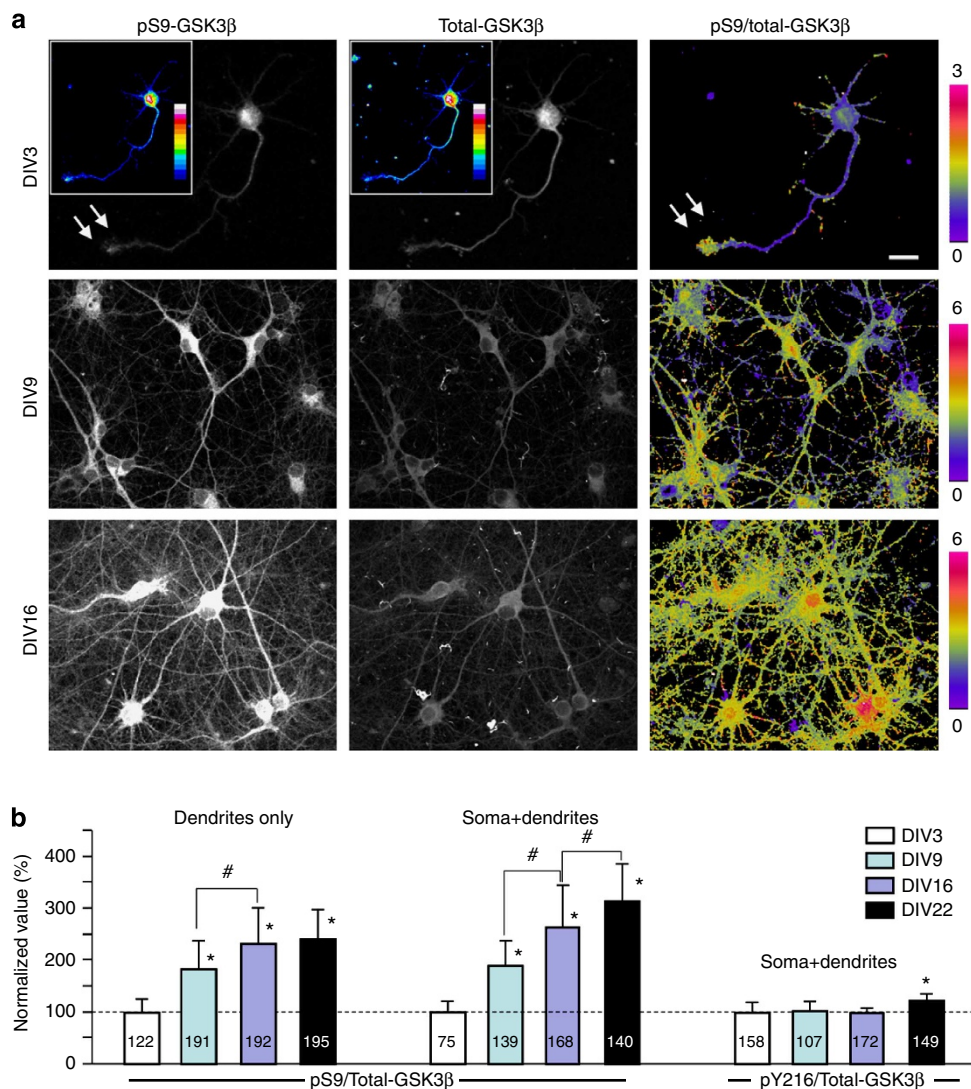


Figure 1 | Spatiotemporal patterns of pS9-GSK3 β in hippocampal neurons in culture. (a) Representative immunofluorescence images of hippocampal neurons from different culture days showing the spatial pattern of pS9-GSK3 β (left), total-GSK3 β (middle) and their ratios (right). The pS9/Total-GSK3 β ratio is depicted in pseudocolors. Colour insets in the DIV3 panels show the immunofluorescence in 16 pseudocolors for better illustration of the spatial distribution of pS9 and total-GSK3 β immunofluorescence. Arrows indicate the growth cone of an axon with a high level of pS9-GSK3 β fluorescence and pS9/Total-GSK3 β ratio. Scale bar, 10 μ m. **(b)** Bar graphs showing the quantitative results on the changes in the pS9/Total-GSK3 β ratio in the dendrites or soma + dendrites at different days in culture. All data are normalized to the DIV3 average. Numbers indicate the total number of cells examined (from at least three independent batches of culture). Error bars: s.d. * $P < 0.05$ (comparing with DIV3), # $P < 0.05$ (comparing with the indicated group) using Student's *t*-test.

shRNA, but not the control shRNA (XASH3HP) (Supplementary Fig. S3). To allow a sufficient time for shRNA-mediated knockdown of GSK3 β , we examined the cells +2 d and +6 d after transfection. We found that GSK3 β knockdown further enhanced the dendritic growth in this 4-day period in the presence of the ineffective K252b. Importantly, neurons expressing U6-GSK-3 β HP1 showed substantial dendritic growth even after 4-day exposure to K252a, whereas cells expressing the control shRNA exhibited marked reduction in dendritic branches (Fig. 2d). These results suggest that neurotrophin-dependent dendritic growth and arborization are mediated by GSK3 β inhibition in the somatodendritic region.

To better understand how GSK3 β activity regulates dendritic growth and arborization, we expressed a constitutively active GSK3 β mutant, in which serine-9 was replaced with alanine (S9A). To minimize any potential effects on neuronal polarity and axonal development, hippocampal neurons were transfected at

DIV13 with a plasmid encoding myc-tagged GSK3 β -S9A, together with a plasmid encoding EGFP for visualization. Immunostaining showed that neurons expressing GSK3 β -S9A exhibited a reduced level of pS9-GSK3 β , indicating overall elevated GSK3 β activity in these cells (Supplementary Fig. S4). The dendrites of control EGFP-expressing neurons showed continuous, but small growth between DIV14 and DIV18 (green arrows, Fig. 3a). However, hippocampal neurons expressing GSK3 β -S9A underwent progressive shrinkage of their dendritic branches over the same time period (red arrows, Fig. 3a). On the other hand, inhibition of GSK3 β by GID5-6 promoted dendritic growth (green arrows, Fig. 3a). Together, these results suggest that activation and inhibition of GSK3 β cause dendritic shrinkage and growth, respectively.

To quantify the effects of GSK3 β on dendritic growth, we performed Sholl analysis²⁹ on neurons 1, 3 and 5 days after transfection. GSK3 β -S9A expression significantly reduced the

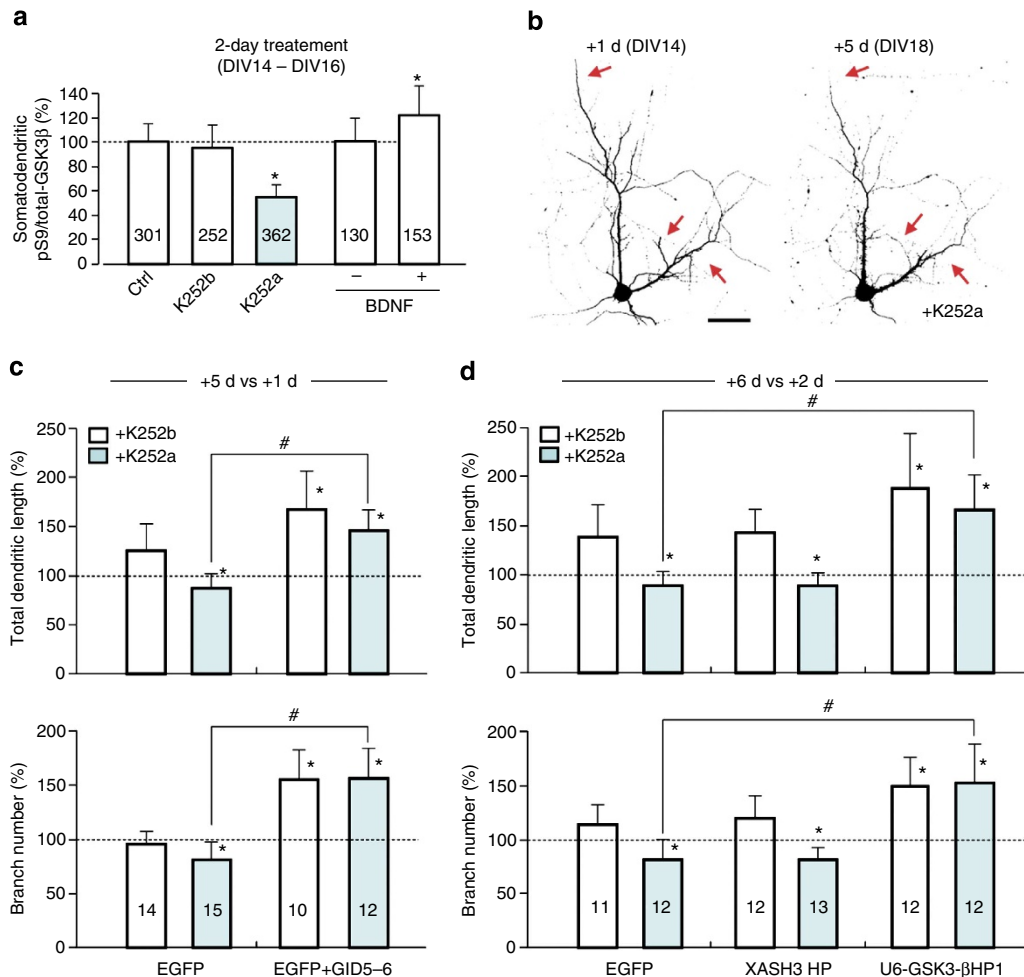


Figure 2 | Regulation of GSK3 β activity and dendritic growth by neurotrophic factors. (a) Bar graph summarizes the effects of 2-day bath application of K252a (200 nM), K252b (200 nM) or BDNF (100 ng ml⁻¹) on the pS9/Total-GSK3 β ratio in the somatodendritic region. Numbers indicate the number of cells examined. (b) Representative confocal images of hippocampal neurons expressing EGFP treated with K252a, at 1 and 5 days post transfection. Arrows indicate shrinking dendritic branches. Scale bar, 50 μ m. (c,d) Bar graphs show the normalized changes (+5 versus +1 d or +6 versus +2 d) in total dendritic length and branch number under different conditions. Numbers indicate the total number of cells examined (from at least three independent batches of culture). Error bars: s.d. * $P < 0.05$ comparing with the EGFP K252b treated group, # $P < 0.05$ comparing between the labelled groups (Student's *t*-test).

number of dendritic intersections between 20–100 μ m radii, whereas EGFP alone had no effect (line plots in Fig. 3a). Expression of the GSK3 β inhibitor GID5–6 resulted in a marked increase in the number of dendritic intersections. To compare the changes in dendrites across different groups, we summed the dendritic intersections within a radius of 10–200 μ m, as changes were mostly observed in this range. It is clear that 5 days after expression of GSK3 β -S9A, the total number of dendritic intersections was reduced to $74.45 \pm 3.16\%$ (mean \pm s.e.m., $n = 13$) (Fig. 3b). Similarly, the total length of dendritic branches was reduced substantially by GSK3 β -S9A expression, but not EGFP alone or wild-type GSK3 β (Fig. 3c). Conversely, expression of GID5–6 promoted dendritic growth. Moreover, co-expression of GID5–6 with GSK3 β -S9A effectively abolished S9A-induced dendritic reduction.

Because over activation of GSK3 β could induce neuronal apoptosis, we examined the viability of cells expressing GSK3 β -S9A. We used Hoechst 33342 dye to identify apoptotic cells, and propidium iodide (PI) to stain dead cells. Neurons transfected with either EGFP alone or EGFP and GSK3 β -S9A were live stained with Hoechst 33342 and PI 5 days post transfection.

We found that GSK3 β -S9A expression did not cause a significant increase in either cell apoptosis or cell death in our cultures (Supplementary Fig. S5a,b). To further exclude any non-specific effects from multi-day imaging, we also examined neurons after our five day imaging protocol, and found no effect on cell viability (Supplementary Fig. S5c).

To confirm the role of GSK3 β activity on dendrite growth and stability, we utilized organotypic hippocampal slice cultures. Biolistic gene gun transfection was used to deliver DNA constructs encoding EGFP, GSK3 β -S9A and GID5–6 into cells in slices. For simplicity, we only analysed the apical dendrites of pyramidal neurons and summarized changes in the length of apical dendritic branches in composite drawings, with red segments indicating shrinkage and green segments representing growth (Fig. 4a). For neurons expressing EGFP alone, small changes in both directions (growth and shrinkage) in apical dendritic branches were observed. This is likely due to the fact that the hippocampal slices were derived from postnatal rats and the neurons are more mature and stable in comparison with the dissociated embryonic cultures. Consistent with our findings in the dissociated neurons, we found that over an 8-day

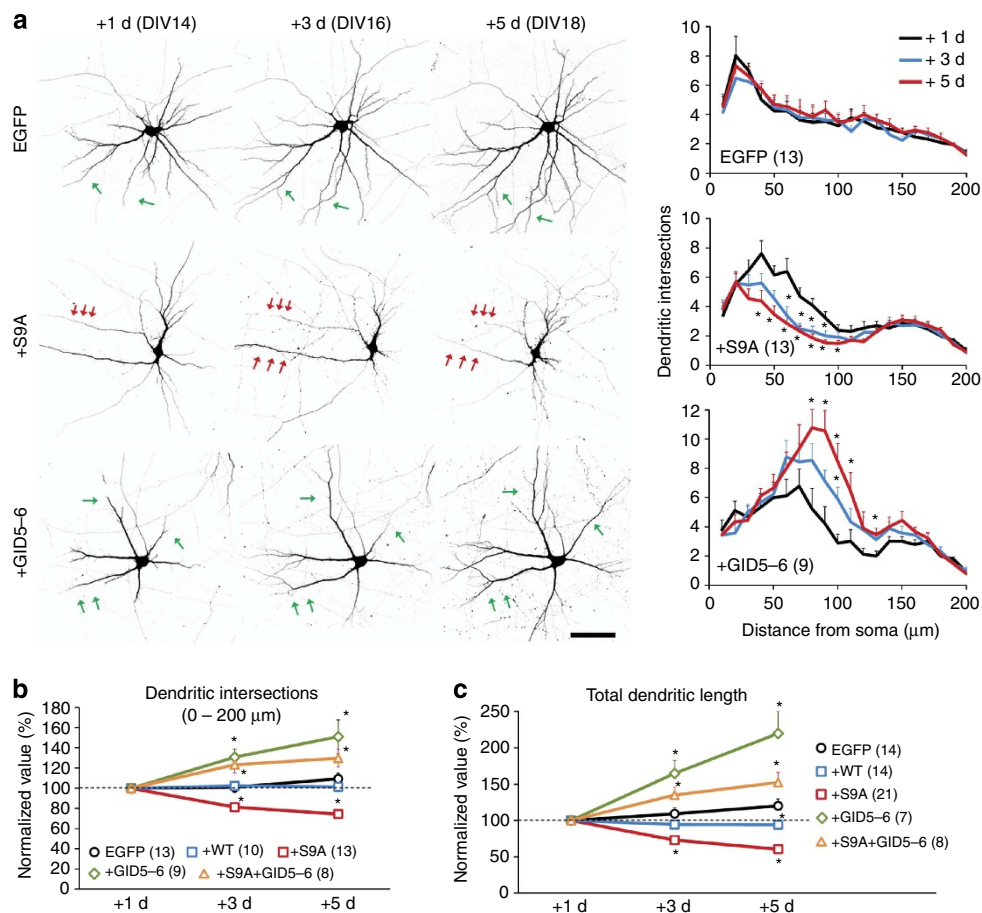


Figure 3 | Regulation of dendritic development by GSK3 β . (a) Representative confocal images of hippocampal neurons expressing EGFP alone, GSK3 β -S9A (+S9A) and GID5-6 (+GID5-6) at 1, 3 and 5 days after transfection. Images were inverted in grayscale for presentation. Red and green arrows indicate the shrinkage and growth of dendritic branches, respectively. Scale bar, 50 μ m. The line graphs on the right depict the results of Sholl analysis. * $P < 0.05$ (Student's t -test), comparing with +1 d. Numbers in brackets indicate the total number of cells examined (from at least three independent batches of culture). (b,c) Line graphs summarize the changes in dendritic intersections from the Sholl analysis **b** and the total dendritic length **c** at different days after transfection. * $P < 0.05$ (Student's t -test) comparing with the corresponding point of the EGFP group. For each condition, the data are normalized to the +1 day. Each measure was from at least three independent cultures. Numbers in brackets indicate the total number of cells examined. Error bars represent the s.e.m.

period, GSK3 β -S9A expression caused a substantial shrinkage of dendritic branches as highlighted by many red segments (Fig. 4a). Quantitative analysis confirmed that GSK3 β -S9A caused a marked reduction in both the length and number of dendritic branches (Fig. 4b). Comparing +10 d to +2 d, the dendritic length and branch number were reduced to $75.6 \pm 17.1\%$ and $82.7 \pm 15.2\%$ (mean \pm s.d., $n = 5$), respectively. However, expression of GID5-6 resulted in a small increase in both dendritic length and branch number (mean \pm s.d.: $108.4 \pm 8.6\%$ and $109.1 \pm 2.9\%$ of the +2 d values, respectively, $n = 3$). These data thus indicate that dendritic effects of GSK3 β are not an artifact of *in vitro* cell cultures.

We also confirmed the role of GSK3 β in dendritic growth by using two pharmacological inhibitors of GSK3 β . Bath application of either SB415286 (ref. 30) or CT99021 (ref. 31) promoted dendritic growth in EGFP-expressing cells, as well as alleviated GSK3 β -S9A-induced dendritic shrinkage (Supplementary Fig. S6a,b). Furthermore, we found that knockdown of GSK3 β by two different shRNAs (U6-GSK-3 β HP1 and U6-GSK-3 β HP2) largely enhanced dendrite extension, similar to the effect of GID5-6 expression (Supplementary Fig. S6c). Moreover, we observed that inhibition of PI-3 kinase by LY294002, which leads to GSK3 β activation¹⁹, resulted in pronounced dendritic shrinkage, similar

to the effect of GSK3 β -S9A expression (Supplementary Fig. S6d). All together, these results suggest that the inhibition of GSK3 β is required for normal dendritic growth and maintenance, whereas GSK3 β activation leads to dendritic shrinkage.

Neuronal activity mediates GSK3 β -induced dendritic shrinkage. We examined the role of neuronal activity in GSK3 β -S9A-induced dendrite shrinkage. Inhibition of neuronal activity by tetrodotoxin (TTX) was found to enhance dendritic growth and, importantly, attenuate the dendritic shrinkage induced by GSK3 β -S9A (Fig. 5). Inhibition of NMDA receptors or AMPA receptors by APV or CNQX, respectively, was also effective in blocking the dendritic shrinkage in GSK3 β -S9A-expressing neurons (Fig. 5). These results suggest that the excitatory neuronal activity underlies the dendritic shrinkage induced by GSK3 β activation.

To further test this hypothesis, we performed whole-cell voltage-clamp recording to examine how synaptic transmission is affected by GSK3 β -S9A expression. We first recorded the miniature excitatory synaptic currents (mEPSCs) and found no significant difference in the frequency or amplitude of mEPSCs among the EGFP, WT-GSK3 β , GSK3 β -S9A and

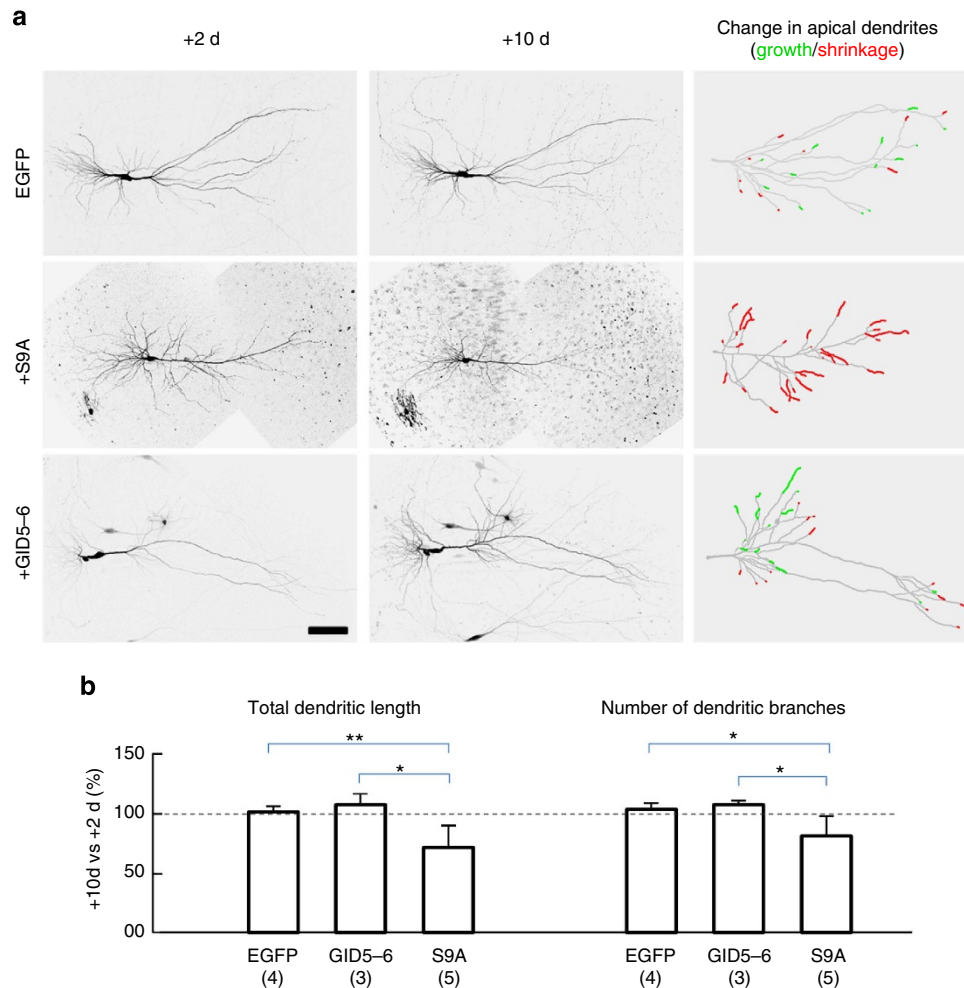


Figure 4 | Reduction in dendritic branches by GSK3 β activation in organotypic hippocampal slices. (a) Representative two-photon confocal images of CA3 hippocampal neurons expressing EGFP alone, EGFP with GSK3 β -S9A (S9A) and EGFP with GID5-6 (GID5-6) at 2 and 10 days after biolistic transfection. Images were inverted in grayscale for presentation. Composite tracings of the apical dendrites are shown on the right, with the red and green segments representing shrinkage and growth, respectively. Scale bar, 100 μ m. (b) Bar graphs show changes in total dendritic length and branch number as labelled. For each condition, data are normalized to the 2 day after transfection values. Numbers in brackets indicate the number of cells examined (from at least three independent batches of culture). Error bars: s.d. * P <0.05 and ** P <0.005 by Student's t -test.

GID5-6-expressing neurons (Fig. 6a). Surprisingly, however, both the frequency and amplitude of miniature inhibitory synaptic currents (mIPSCs) were significantly reduced in the GSK3 β -S9A-expressing neurons in comparison with EGFP-expressing neurons (P <0.005, paired Student's t -test, n =9). These results suggest that GSK3 β -S9A expression in postsynaptic neurons did not affect the excitatory input, but instead reduced the strength of the inhibitory input these neurons received. As the overall activity of a neuron is controlled by a balance of excitatory and inhibitory inputs, a reduction in inhibitory strength should lead to an increase in overall neuronal excitability even in the absence of any changes in excitatory input. For simplicity, we tested this notion by measuring spontaneous postsynaptic currents (sPSCs) in the absence of TTX and other channel blockers to allow action potential-dependent neurotransmission. It should be noted that sPSCs represent integrated currents from both excitatory and inhibitory inputs. Moreover, it is well established that dendritic membrane expresses voltage-gated sodium and calcium channels and cannot be effectively voltage-clamped by the somatic clamping technique^{32,33}. Therefore, sPSCs likely also contain contributions from voltage-gated channels on the dendritic surface. We found that GSK3 β -S9A-expressing neurons

displayed a higher frequency of sPSCs than neighbouring non-transfected neurons in the same network (Fig. 6b). These results support the notion that activated GSK3 β reduces the inhibitory signalling to the neuron, leading to an increase in overall neuronal excitability and dendritic shrinkage.

Dendritic shrinkage arises from reduced GABA_AR expression.

GABAergic synapses represent a major class of inhibitory synapses for hippocampal neurons³⁴. We found that expression of GSK3 β -S9A for 3–5 days led to a reduction in surface GABA_AR levels compared with control neurons expressing EGFP (Fig. 7a). Conversely, expression of GID5-6 resulted in an increase in surface GABA_ARs. Quantification shows that expression of GSK3 β -S9A and GID5-6 markedly reduced and increased the number and size of surface GABA_AR puncta, respectively (Fig. 7c,d). In contrast, surface staining of the AMPA receptor subunit, GluA1, was unchanged under identical conditions (Fig. 7b,e,f). These findings support our electrophysiology data, and suggest that GABA receptors of inhibitory synapses, but not glutamate receptors of excitatory synapses, are the target of dendritic GSK3 β activity.

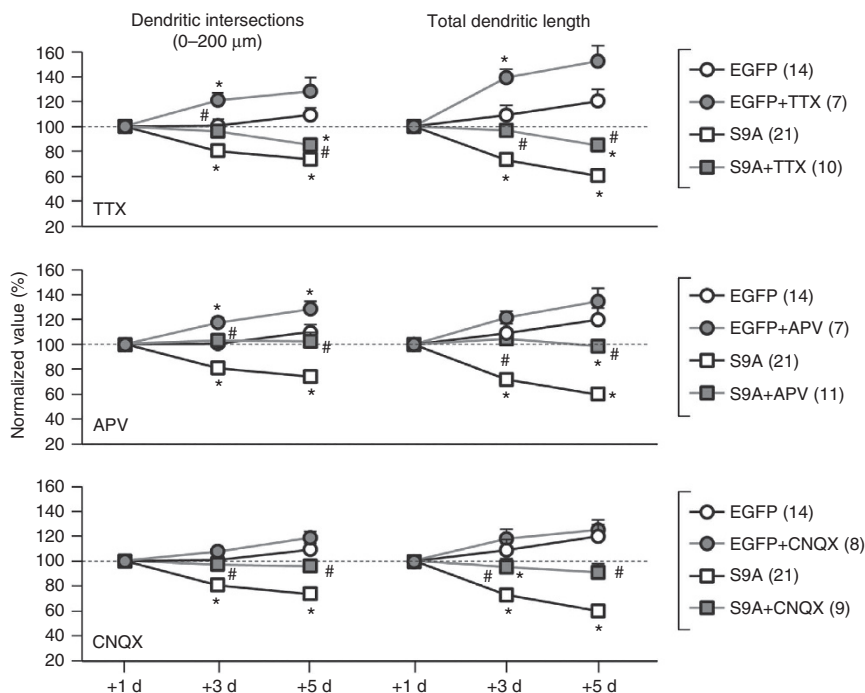


Figure 5 | Activity dependence of dendrite shrinkage induced by GSK3 β -S9A expression. Number of dendritic intersections between 10–200 μm radii and total dendritic length are shown as normalized to the +1 day values. Numbers in brackets indicate the total number of cells examined for each group (from at least three independent batches of culture). Error bars: s.e.m. * $P < 0.05$ comparing with the corresponding point of the EGFP group (Student's t -test). # $P < 0.05$ between GSK3 β -S9A and GSK3 β -S9A + inhibitors (TTX, APV or CNQX) groups (Student's t -test).

To verify that our GABA_AR staining faithfully reported surface GABA_AR clusters, we stained surface GABA_AR clusters in neurons expressing EGFP-gephyrin, a scaffold protein that mediates GABA_AR surface clustering. Our data show that $93.3 \pm 4.6\%$ (mean \pm s.e.m., $n = 12$) of EGFP-gephyrin puncta colocalized with surface GABA_AR puncta, indicating that the staining reliably detects surface GABA_AR clusters (Fig. 7g,h). Consistent with our GABA_AR staining, we found that neurons expressing GSK3 β -S9A caused a reduction in the density and size of EGFP-gephyrin puncta in dendrites, whereas GID5–6 increased the density of EGFP-gephyrin puncta, but not their size (Fig. 7i,j). We also stained surface GluA1 receptors in neurons expressing EGFP-gephyrin to further confirm that GSK3 β -S9A does not affect surface GluA1 in the same dendritic branches (Supplementary Fig. S7). These data are consistent with the electrophysiological recordings, and suggest that GSK3 β activation selectively targets inhibitory synapses by reducing surface GABA_ARs.

To determine if reduced GABAergic activity is the cause of GSK3 β activation-induced dendritic shrinkage, we examined dendritic growth in the presence of GABA_AR agonists or antagonists. We found that the GABA_AR agonist muscimol significantly increased dendritic growth (Fig. 8a), whereas the antagonist picrotoxin caused dendritic shrinkage (Fig. 8b). Interestingly, muscimol was unable to attenuate the dendritic shrinkage induced by GSK3 β -S9A expression, suggesting that the number of surface GABA_ARs is the determinant factor. We next performed knockdown experiments using shRNAs against the 3'-untranslated region (UTR) of the GABA_AR $\gamma 2$ subunit ($\gamma 2$ UTR), or a scrambled control shRNA ($\gamma 2$ UTR3m)³⁵. Hippocampal neurons were cotransfected with the shRNA and EGFP constructs on DIV13, and fixed 5 days later. We found that $\gamma 2$ UTR resulted in a marked reduction of surface GABA_AR puncta and gephyrin clusters³⁵, whereas $\gamma 2$ UTR3m had no effect (Fig. 8c). Importantly, $\gamma 2$ knockdown resulted in marked

dendritic shrinkage, similar to that of GSK3 β -S9A expression, whereas the control shRNA had no effect (Fig. 8d). Quantitative analysis showed that GABA_AR knockdown resulted in a decline in dendritic complexity, similar to that of GSK3 β -S9A expression. Expression of the GSK3 β inhibitor peptide GID5–6, however, was unable to rescue the dendritic shrinkage induced by GABA_AR knockdown, suggesting that GSK3 β acts upstream of GABA_ARs. Together, these findings suggest that GSK3 β inhibition is required for the presence of GABA_ARs on the neuronal cell surface.

Serine-270 of gephyrin was identified as a target of GSK3 β , and shown to modulate gephyrin clustering and GABAergic synaptic transmission³⁶. We thus expressed a phosphorylation defective S270A-gephyrin mutant and a phospho-mimetic S270E-gephyrin mutant. We found that expression of the S270A mutant slightly increased dendrite growth, whereas expression of the S270E mutant slightly decreased dendrite growth (Fig. 8e). Importantly, however, co-expression of the S270A-gephyrin mutant with GSK3 β -S9A, but not wild-type gephyrin, was able to attenuate GSK3 β -S9A-induced dendrite shrinkage (Fig. 8f). These findings suggest that GSK3 β controls dendritic growth by regulating surface GABA_AR clustering through phosphorylation of gephyrin at serine-270.

Discussion

The highly elaborate dendritic arbours of a neuron host a large number of synapses that are organized in spatial domains for distinct synaptic inputs³⁷. Their proper development and maintenance are crucial for the reception, integration and computation of synaptic signals that encode spatial and temporal information. It is well established that genetic programming, neuronal activity and extracellular factors work in concert to shape dendritic arbourization patterns^{3,4}. Synaptic activity is also believed to have an important role in the stabilization of dendritic

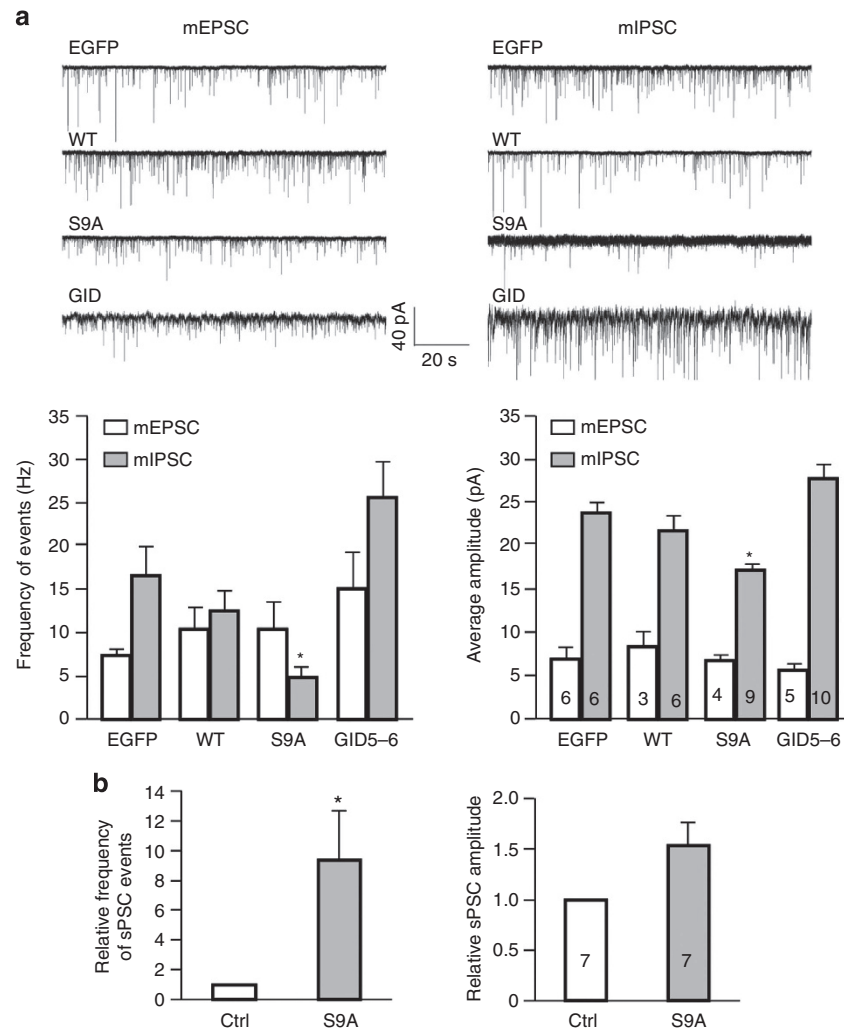


Figure 6 | Reduction of inhibitory synaptic strength by GSK3 β activation. Whole-cell patch-clamp recordings showing the changes in postsynaptic currents in hippocampal neurons expressing EGFP, EGFP with wild-type GSK3 β (WT), EGFP with GSK3 β -S9A (S9A), and EGFP with GID5-6. Cells were transfected at DIV13 and recordings were performed between DIV17-19. **(a)** Measurements of the miniature excitatory and inhibitory postsynaptic currents. Sample traces of mEPSC and mIPSC are shown on the top and the quantified frequencies and amplitudes are shown in the bar graphs below. mIPSCs are inward at -80 mV because $E_{Cl} = 0$ mV. $*P < 0.05$ comparing with the EGFP group (Student's *t*-test). **(b)** Bar graphs show the spontaneous postsynaptic currents (sPSCs) in neurons expressing GSK3 β -S9A and normalized to neighbouring, non-transfected neurons (Ctrl). $*P < 0.05$ comparing with the control group (Student's *t*-test). Numbers indicate the total number of cells examined for each group (from at least three independent batches of culture). Error bars: s.e.m.

arbours. Individual dendritic arbours are often maintained for up to decades, and their disruption has been observed in many neurological diseases^{8,9}. Although activity-dependent dendritic growth and stabilization represent a key step of dendrite development, the molecular and cellular mechanisms that translate neuronal activities into distinct dendritic outputs currently remain unknown. In this study, we have identified an important role for GSK3 β in activity-dependent dendrite growth and stabilization. Our findings reveal an intriguing mechanism in which GSK3 β targets the surface levels of GABA_ARs to regulate the balance of neuronal excitation and inhibition to control dendritic growth. Importantly, we show that activation of GSK3 β results in a reduction of GABAergic signalling via phosphorylation of the scaffold protein gephyrin, leading to dendrite atrophy. Our results thus provide important insights into the mechanisms of activity-dependent dendrite development and maintenance.

GSK3 β is known for its role in axonal specification during development¹⁹. It remains unclear, however, how spatially

restricted GSK3 β phosphorylation is achieved for polarity formation. Growth factors have been suggested to locally activate PI-3 kinase and Akt/PKB to inhibit GSK3 β by phosphorylation¹⁰. Previous studies have shown that expression of two neurotrophins, BDNF and NT-3, are elevated upon synaptogenesis, and that both neurotrophins have a role in dendrite development²⁵. Furthermore, a BDNF knockout mouse displayed reduced dendritic arborization, supporting a role for neurotrophins in dendrite development³⁸. In this study, we show that GSK3 β serine-9 phosphorylation in somatodendritic regions was substantially elevated at DIV9 and later stages, likely in correspondence with synapse formation and maturation. This increase in serine-9 phosphorylation is at least partly a result of neurotrophin signalling, as inhibition of Trk receptors by K252a substantially reduced the pS9-GSK3 β level in dendrites, whereas exogenous BDNF treatment increased pS9-GSK3 β levels. Whether other members of the neurotrophin family are involved in GSK3 β serine-9 phosphorylation remains to be determined. Nonetheless, inhibition of Trk receptors resulted in a

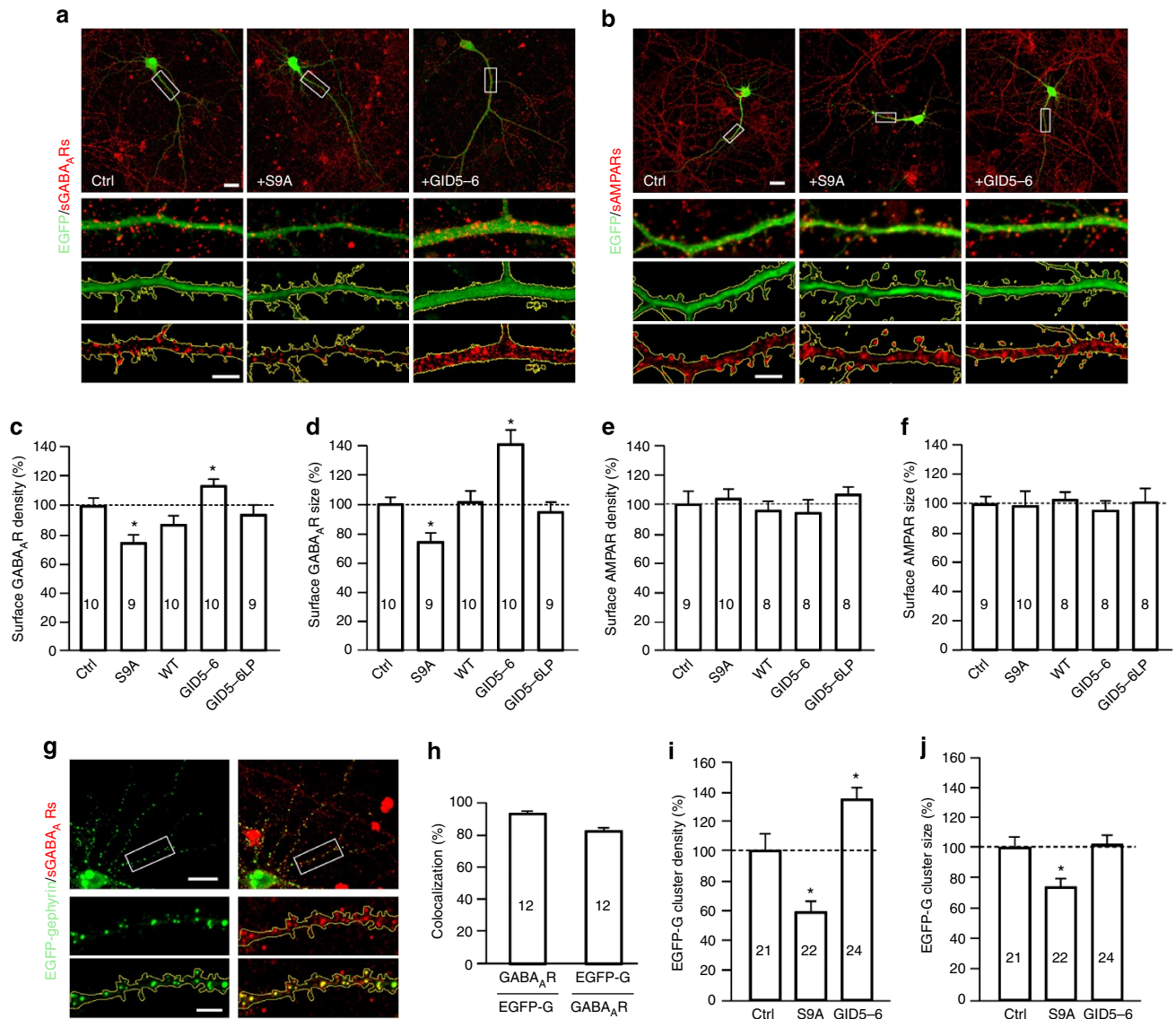


Figure 7 | Regulation of surface GABA_A receptors by GSK3 β activation. (a,b) Representative images show surface GABA_ARs by γ 2 staining (a) and AMPARs by GluA1 labelling (b) on dendrites of neurons expressing EGFP (Ctrl), GSK3 β -S9A (+S9A) and GID5-6 (+GID5-6) at DIV16 (+3 d after transfection). Dendritic regions enclosed by the white rectangles in the top panels are shown in magnified views below. For each cell, three high magnification panels are shown: colour merged (green: EGFP, red: surface receptors), EGFP with yellow outline of the dendritic segment, and surface GABA_A receptors with regions outside of dendritic segment masked using EGFP fluorescence. Scale bar in top panels, 20 μ m, lower panels, 5 μ m. (c,d) Bar graphs show changes in density and size of surface GABA_AR puncta normalized to the EGFP group (Ctrl). (e,f) Bar graphs show the normalized changes in density and size of AMPAR puncta. (g) Representative images show the co-localization of EGFP-gephyrin clusters with surface GABA_ARs. Magnified panels are shown below. Scale bars are 20 μ m and 5 μ m for the top and bottom panels, respectively. (h) Bar graph depicts incidents of co-localization between EGFP-gephyrin (EGFP-G) and surface GABA_ARs. The first bar indicates the percentage of EGFP-G puncta colocalized with GABA_AR clusters, and the second represents the percentage of GABA_AR clusters localized with EGFP-G puncta on the same branch. (i,j) Bar graphs depict the changes in density and size of EGFP-gephyrin puncta in dendrites at DIV16 (+3 after transfection). Numbers indicate the total number of cells examined for each group (from at least three independent batches of culture). Error bars: s.e.m. * P <0.05 compared with EGFP-G (Ctrl) by Student's t -test.

reduction in dendritic arborization, which was blocked by inhibition or knockdown of GSK3 β . These results connect neurotrophin signalling to GSK3 β in regulating dendritic growth. It is conceivable that elevated expression and secretion of neurotrophic factors upon synaptogenesis elicit S9 phosphorylation of GSK3 β in dendrites, which in turn enhances dendrite development.

The functional consequence of increased GSK3 β inhibition via serine-9 phosphorylation in the somatodendritic region appears to enable dendritic growth and stabilization. We found that activation of GSK3 β causes dendrite atrophy, whereas inhibition

results in enhanced extension of dendritic branches. It should be noted that these studies do not preclude a similar role for the GSK3 β homologue, GSK3 α , in dendritic development and maintenance. The kinase domains of both GSK3 α and GSK3 β are highly similar, and there are numerous contexts where both have been demonstrated to be at least partially functionally redundant^{39,40}. Our data show that GSK3 β targets the surface levels of GABA_A receptors to selectively modulate the balance between excitatory and inhibitory inputs on the dendrites. By tilting the balance to increased neuronal excitation, dendrite shrinkage was favored. Consistent with this result,

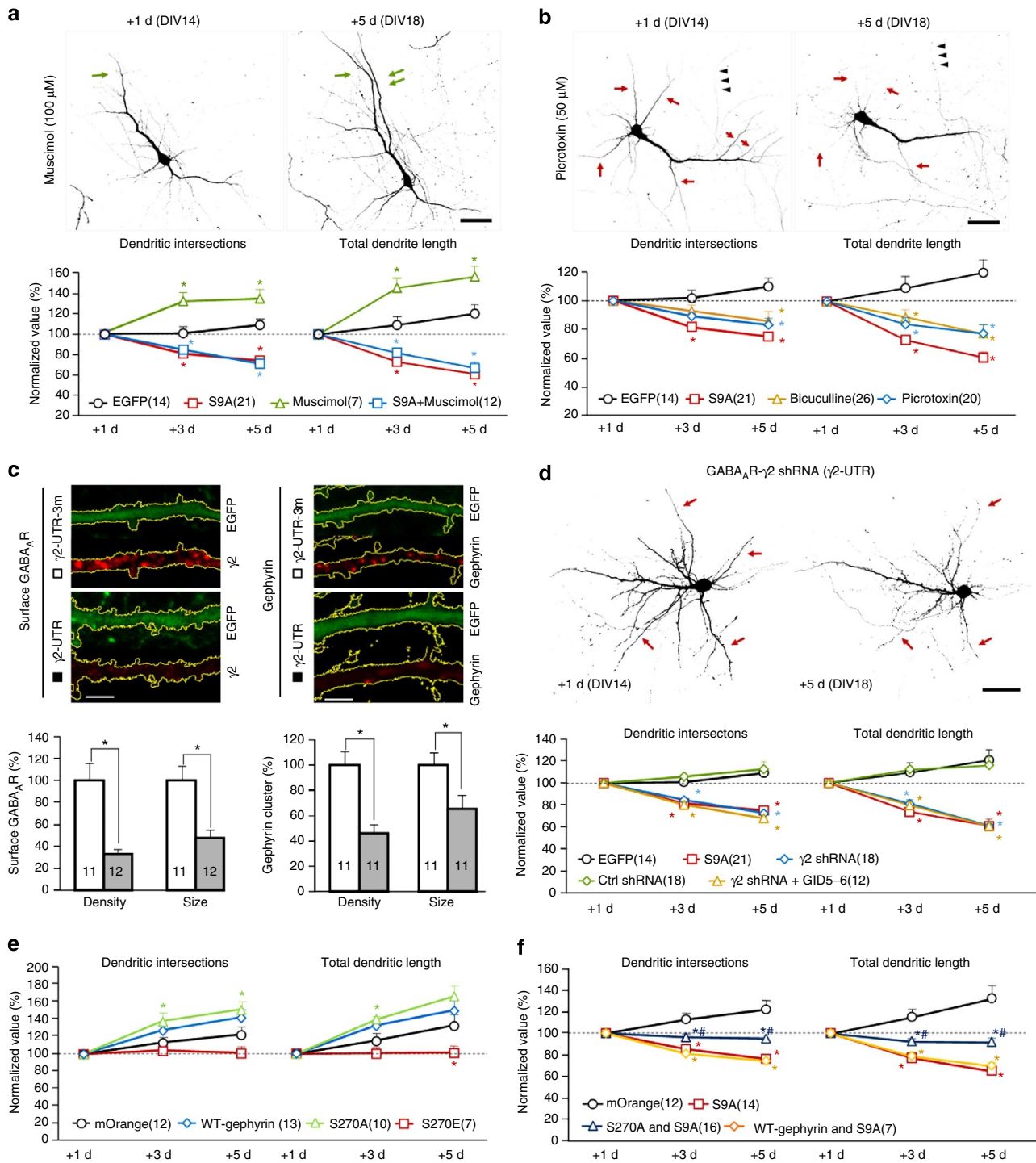


Figure 8 | Effects of GABA_AR manipulations on dendritic development. (a,b) Effects of GABA_AR agonist and antagonist on dendrites. Hippocampal neurons were transfected on DIV 13, and treated with either Muscimol (GABA_AR agonist) (a) or Picrotoxin and bicuculline (GABA_AR antagonists) (b) after initial imaging on DIV 14 (+1 d). Representative images of EGFP-expressing neurons are shown on the top. Green and red arrows indicate the growth and shrinkage of dendritic branches, respectively. Normalized number of dendritic intersections and total length are shown at the bottom. Scale bars, 50 μ m. **P* < 0.05 comparing with the corresponding point of EGFP-expressing cells (Student's *t*-test). (c,d) Effects of GABA_AR knockdown on dendrite development. (c) Neurons transfected with shRNAs were identified by EGFP fluorescence. Representative confocal images of EGFP fluorescence and immunofluorescence (of GABA_AR or gephyrin) are shown on top. Scale bars = 5 μ m. The bar graphs show the cluster size and density of surface GABA_AR (left) and cytoplasmic gephyrin (right) in cells expressing the shRNA (γ 2-UTR) or the control shRNA (γ 2-UTR-3m). **P* < 0.01, Student's *t*-test. (d) GABA_AR knockdown results in dendritic shrinkage. Representative images of EGFP-expressing neurons are shown at the top. Scale bar, 50 μ m. Normalized number of dendritic intersections and total length are shown at the bottom. For each group, all data are normalized to the +1 day. **P* < 0.05 comparing with the corresponding point of the control (EGFP, Student's *t*-test). (e,f) Effects of co-expression of wild-type gephyrin, S270A-gephyrin and S270E-gephyrin, without (e) or with (f) GSK3 β -S9A on dendrites. The line graphs show the number of dendritic intersections and total dendritic length. **P* < 0.05 comparing to the corresponding point of the mOrange group (Student's *t*-test). #*P* < 0.05 comparing with the corresponding point of GSK3 β -S9A group (Student's *t*-test). The data are normalized to +1 d. Numbers in brackets indicate the number of cells examined for each group (from at least three independent batches of culture). Error bars: s.e.m.

hyperexcitability from chronic inhibition of GABAergic transmission was found to reduce dendritic length of CA1 hippocampal pyramidal neurons in slice culture⁴¹. Therefore, a balancing act of excitatory and inhibitory activities appears to be a key component of activity-dependent regulation of dendritic development and maintenance.

Our conclusion that GSK3 β mainly targets the surface expression of GABA_A receptors to affect dendritic branches is based on several lines of experiments: (1) electrophysiology data showed a substantial reduction of mIPSCs by GSK3 β -S9A expression, without effects on mEPSCs; (2) quantitative immunofluorescence showed the reduction of surface GABA_ARs without changes in AMAPRs; (3) GABA_AR antagonists mimicked the dendritic shrinkage induced by GSK3 β -S9A; (4) knockdown of GABA_ARs reproduced the dendritic shrinkage phenotype; (5) gephyrin S270A mutant could attenuate GSK3 β -S9A-induced dendritic shrinkage. It should be noted that inhibition of GSK3 β by GID5-6 was unable to attenuate the dendrite shrinkage induced by GABA_AR knockdown, supporting the notion that GSK3 β acts upstream of GABA_ARs. The finding that GABA_AR agonist muscimol was unable to rescue the effects induced by GSK3 β -S9A expression suggests that further activation of the reduced number of GABA_ARs may not be sufficient to restore the strength of inhibitory inputs. Previous studies have shown that GSK3 β phosphorylates gephyrin, a key scaffolding protein for the localization and accumulation of GABA_ARs at the postsynaptic surface of inhibitory synapses^{36,42}. It is plausible that gephyrin is involved in the GSK3 β regulation of dendritic development through GABA_ARs. Indeed, we found that gephyrin puncta in dendrites were substantially reduced and increased by GSK3 β -S9A and GID5-6 expression, respectively (Fig. 7i,j). We further confirmed that gephyrin is involved in GSK3 β -S9A-induced dendritic shrinkage as S270A-gephyrin mutant, which cannot be phosphorylated by GSK3 β , blocked the dendritic reduction by GSK3 β -S9A.

Dendrite atrophies have been observed in a wide range of neurological disorders ranging from Alzheimer's disease to Schizophrenia⁸. Dendrite abnormality is the strongest correlate with mental retardation³. However, the mechanisms leading to the alteration of dendritic arbours in specific diseases remain to be elucidated. Interestingly, both aberrantly elevated GSK3 β activity and reduced GABAergic synaptic signalling have been associated with many, if not all of the neurological disorders, in which dendrite atrophy was observed, including Fragile X syndrome and FMR1 (refs 43–50). In addition, several signalling pathways upstream of GSK3 β , including Akt and DISC1, have also been implicated in a large number of neurological disorders^{10,15,16,18,51}. The findings in this study provide evidence that GSK3 β activity, GABAergic signalling, and dendritic development and maintenance are ultimately connected. Alterations in this intricately connected pathway may lead to dendritic defects. Aberrantly elevated GSK3 β activity could disrupt GABAergic signalling and alter the excitatory–inhibitory balance, resulting in dendritic atrophy and synapse loss. Therefore, the GSK3 β –GABA_AR–dendrite pathway may represent an important link that contributes to the structural and functional abnormality in brain disorders.

Methods

DNA constructs and chemical reagents. DNA constructs encoding wild-type GSK3 β , GSK3 β -S9A, Axin GID5-6, Axin GID5-6LP, XASH3HP, U6-GSK-3 β HP1 and U6-GSK-3 β HP2 were generously provided by Dr Yi Rao at Peking University, China¹⁹. pEGFP-C1 was purchased from Clontech. The small shRNA (γ 2 UTR): 5'-GGUGAGAUUCAGCGAAUAAGACCCCUA-3' targeted a sequence of the 3'UTR of the rat γ 2 subunit (nucleotides 1,467–1,491); the control small shRNA (γ 2 UTR3m): 5'-GGUGACAUACCGAAUAAGACCCCUA-3' was generated by introducing three point mutations in the sense and antisense arms of the

corresponding shRNA³⁵. EGFP-gephyrin was from rat gephyrin P1 variant cloned using EcoRI–KpnI sites and inserted to the carboxy-terminal of pEGFP-C2 construct. S270A-gephyrin and S270E-gephyrin mutants were made by mutating serine-270 to alanine or glutamate by a site-directed mutagenesis.

We used the following antibodies and chemicals for our study: mouse anti-MAP2 (Sigma; 1:250), rabbit anti-MAP2 (EMD Millipore; 1:2,000), mouse anti-total-GSK3 β (Abcam; 1:500), rabbit anti-pS9-GSK3 β (Cell Signaling; 1:50), mouse anti-pY216-GSK3 β (BD Biosciences; 1:200), rabbit anti-pY216-GSK3 β (Abcam; 1:200), rabbit anti-GABA_AR- γ 2 (EMD Millipore; 1:300), rabbit anti-GluR1 (EMD Millipore; 1:50), mouse anti-gephyrin (Synaptic system; 1:300), mouse anti-c-myc (Santa Cruz; 1:1,000) and mouse anti-HA (Santa Cruz; 1:200). SB415286, Muscimol was from Tocris; Ly294002 was from EMD Millipore; CT99021 was from Selleckchem; K252a, K252b, TTX, APV, CNQX, Bicucullin and picrotoxin were from Sigma; Hoechst 33342 was from Invitrogen and propidium iodide was from Abcam.

Neuronal culture and transfection. Sprague–Dawley timed-pregnant adult rats (8–10 weeks) were purchased from Charles River Laboratories. Primary hippocampal neurons were prepared from embryonic day 18 rat embryos and plated on 25-mm coverslips or 35-mm glass bottom dishes pretreated with 0.1 mg ml⁻¹ poly-D-lysine (EMD Millipore) at a density of approximately 350,000 cells per dish⁵². Neurons were plated and maintained in Neurobasal medium supplemented with B-27 and GlutaMax (Invitrogen). Cells were transfected with a Calphos calcium phosphate transfection kit (Clontech) on DIV13 and imaged at different days after transfection. Each experiment was repeated at least three times from independent batches of cultures. All the experiments were carried out in accordance to the guidelines of US National Institutes of Health for animal use and were approved by the Institutional Animal Care and Use Committee of Emory University.

Live-cell imaging. For multi-day live-cell imaging of dendrite development, neurons cultured on glass-bottomed dishes were changed from normal culture medium to phenol-red free neurobasal medium and sealed with Parafilm. The sealed dish was placed on the microscope stage and housed in a temperature controlled chamber (Warner Instruments, New Haven, CT) at ~35 °C. Cells were imaged using a Nikon C1 laser-scanning confocal system based on a Nikon inverted microscope (TE300), with a 40 \times N.A.1.3 Plan Fluor oil immersion objective. To image all of the dendritic branches at different focusing planes of a dendritic segment, z-stacks of images of EGFP-expressing neurons were acquired and converted to 2-dimensional images by maximal intensity projection. After imaging, the dish was unsealed, changed back to the original neurobasal medium, and returned to the CO₂ incubator for further culture and imaging at later time points.

Organotypic hippocampal slice culture and imaging. Hippocampal slice cultures were prepared from postnatal day 6 (P6) Sprague–Dawley rat pups (mixed sex) as previously described⁵³. Briefly, rat pups were anesthetized and decapitated according to institutional guidelines, and then hippocampi were removed and transversely sliced (400- μ m thick) using a McIlwain Tissue Chopper (Ted Pella, Inc). Slices were transferred onto 0.4 μ m Millicell cell culture membrane inserts (Millipore) and cultured for 2 weeks. Biolistic transfection of neurons in hippocampal slices was then performed using a Helios gene gun (Bio-Rad)⁵⁴. Cell imaging was performed using a two-photon FV1000 laser-scanning confocal (Olympus) 48 h after transfection, and again 10 days after transfection. CA3 pyramidal neurons expressing EGFP were randomly chosen by location and morphology. Maximal intensity projections of z-stacks were used to generate two-dimensional images for analysis. Tracing and quantification of dendrites were done using the Simple Neurite Tracer ImageJ plugin⁵⁵.

Electrophysiology. Whole-cell patch-clamp recordings were performed using EPC7 amplifier (HEKA) as described previously⁵². Patch pipettes were pulled from borosilicate glass and fire polished (4–6 M Ω). The recording chamber was continuously perfused with HEPES buffered recording solution (128 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM HEPES, 30 mM glucose, pH 7.3 adjusted with NaOH and osmolarity at 310–320 mOsm l⁻¹). The pipette solution contained 147 mM KCl, 2 mM KH₂PO₄, 5 mM Tris-HCl, 2 mM EGTA, 10 mM HEPES, 4 mM Mg-ATP, 0.5 mM Na₂GTP, pH 7.3 adjusted with KOH, and osmolarity at 310–320 mOsm (ref. 1). The membrane potential was clamped at –80 mV. Data were acquired using pClamp 9 software, sampled at 5 kHz and filtered at 1 kHz. For mEPSC recordings, 0.5 μ M TTX and 20 μ M bicuculline (or 100 μ M picrotoxin) were added to block action potentials and GABA_A receptors. For mIPSC recording, 0.5 μ M TTX, 50 μ M APV and 20 μ M CNQX were added to block action potentials and NMDA and AMPA receptors. Here mIPSCs were inward at –80 mV as the reversal potential for Cl⁻ was 0 mV.

For recording of spontaneous PSCs (sPSCs), the pipette solution contained 140 mM K-gluconate, 2 mM MgCl₂, 1 mM CaCl₂, 11 mM EGTA, 10 mM HEPES, 2 mM Mg-ATP, 0.5 mM Na₂GTP, pH 7.3 adjusted with N-methyl-D-glucamine, and osmolarity at 308 mOsm l⁻¹. The cells were in normal recording buffer without channel blockers added. As a result, the reversal potential for Cl⁻ was –79 mV. The cell was voltage clamped at –70 mV for recording sPSCs. Off-line data

analysis of sPSC was performed using Clampfit 9.0 software (Molecular Devices). Data were expressed as mean \pm standard error of the mean. Paired Student's *t*-test was used for testing statistical significance, unless otherwise stated.

Analysis of dendrites. We used the maximal intensity projection of z-stacks of images to generate the two-dimensional images for analysis. Tracing and quantification of dendrites were done using ImageJ with the NeuronJ plugin⁵⁶. Only neurons showing intact dendritic arborisation were analysed and only branches over 10 μ m in length were quantified. Sholl analysis was performed using ImageJ with the Sholl analysis plugin developed by the Ghosh Lab (<http://biology.ucsd.edu/labs/ghosh/software>) with a 10- μ m interval in radius between concentric circles.

Immunostaining. For immunostaining of the total GSK3 β and phospho-GSK3 β (pS9 and pY216), neurons were fixed with 4% (w/v) paraformaldehyde containing 4% (w/v) sucrose in phosphate-buffered saline (PBS) for 20 min at room temperature. Fixed neurons were washed with PBS and blocked with 2% BSA (w/v) in cytoskeleton buffer (10 mM MES (pH 6.1), 138 M KCl, 3 mM MgCl₂, 2 mM EGTA)⁵⁷ containing 0.1% (v/v) Triton X-100 at room temperature for 1 h. Then cells were incubated with primary antibodies overnight at 4 °C, followed by 1-h labelling with fluorescent secondary antibodies at room temperature. Both primary antibodies and secondary antibodies were diluted with the blocking buffer.

For fluorescent staining of surface GABA_A receptors and AMPAR receptors, anti- γ 2 subunit of GABA_ARs and anti-GluA1 subunit of AMPARs were incubated with live cells in Krebs-Ringer's saline (KRB: 150 mM NaCl, 5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4) for 20 min at 37 °C. The cells were then washed with PBS and fixed with 4% paraformaldehyde PBS for 20 min, followed by labelling with appropriate secondary antibodies in 1% BSA (w/v) for 45 min.

For all other staining, neurons were fixed for 20 min with 4% (w/v) paraformaldehyde and permeabilized with 0.1% (w/v) Triton X-100 in PBS. After blocking with 3% (w/v) BSA in PBS for 1 h, cells were incubated with primary antibodies in blocking buffer overnight at 4 °C, followed by 1-h labelling with fluorescent secondary antibodies in 1% BSA (w/v) in PBS at room temperature.

Cell death and viability assays. Hippocampal neurons transfected with EGFP or GSK3 β -S9A were incubated with 10 μ M Hoechst 33342 dye and 5 μ M PI in Krebs-Ringer's buffer (KRB) at 37 °C for 15 min. Afterwards, the cells were washed three times with KRB buffer and imaged immediately.

Western blotting. Hippocampal neurons at DIV3, DIV9 and DIV16 under the same culture condition were collected, respectively. Cells were lysed using a lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM ethylene diamine tetra-acetic acid (EDTA), 0.5% NP-40, 0.25% Na deoxycholate and 1 mM dithiothreitol (DTT), supplemented with 1 mM phenylmethylsulfonyl fluoride, protein inhibitor cocktail (Sigma, 1:100), 5 mM NaF and 2 mM Na₃VO₄. Bradford assay was used to quantify the total protein volume in each sample and 10 μ g samples were added to NuPage sample buffer (Invitrogen) with 50 mM DTT and heated at 85 °C for 5 min. Samples were loaded and fractionated by SDS-PAGE on 10% Tris-glycine gel (Invitrogen) and subsequently transferred to nitrocellulose membranes for blotting. Antibodies used: mouse anti-GSK3 β (Abcam, 1:2,000) and rabbit anti-pS9-GSK3 β (Cell Signalling, 1:1,000).

References

- Hausser, M., Spruston, N. & Stuart, G. J. Diversity and dynamics of dendritic signaling. *Science* **290**, 739–744 (2000).
- McAllister, A. K. Cellular and molecular mechanisms of dendrite growth. *Cerebr. Cortex* **10**, 963–973 (2000).
- Parrish, J. Z., Emoto, K., Kim, M. D. & Jan, Y. N. Mechanisms that regulate establishment, maintenance, and remodeling of dendritic fields. *Annu. Rev. Neurosci.* **30**, 399–423 (2007).
- Gao, F. B. Molecular and cellular mechanisms of dendritic morphogenesis. *Curr. Opin. Neurobiol.* **17**, 525–532 (2007).
- Cline, H. T. Dendritic arbor development and synaptogenesis. *Curr. Opin. Neurobiol.* **11**, 118–126 (2001).
- Chen, Y. & Ghosh, A. Regulation of dendritic development by neuronal activity. *J. Neurobiol.* **64**, 4–10 (2005).
- Wong, R. O. & Ghosh, A. Activity-dependent regulation of dendritic growth and patterning. *Nat. Rev. Neurosci.* **3**, 803–812 (2002).
- Kulkarni, V. A. & Firestein, B. L. The dendritic tree and brain disorders. *Mol. Cell Neurosci.* **50**, 10–20 (2012).
- Lin, Y. C. & Koleske, A. J. Mechanisms of synapse and dendrite maintenance and their disruption in psychiatric and neurodegenerative disorders. *Annu. Rev. Neurosci.* **33**, 349–378 (2010).
- Hur, E. M. & Zhou, F. Q. GSK3 signalling in neural development. *Nat. Rev. Neurosci.* **11**, 539–551 (2010).

- Kim, L. & Kimmel, A. R. GSK3 at the edge: regulation of developmental specification and cell polarization. *Curr. Drug. Targets* **7**, 1411–1419 (2006).
- Leroy, K. & Brion, J. P. Developmental expression and localization of glycogen synthase kinase-3beta in rat brain. *J. Chem. Neuroanat.* **16**, 279–293 (1999).
- Bhat, R. V. & Budd, S. L. GSK3beta signalling: casting a wide net in Alzheimer's disease. *Neurosignals* **11**, 251–261 (2002).
- Kaytor, M. D. & Orr, H. T. The GSK3 beta signaling cascade and neurodegenerative disease. *Curr. Opin. Neurobiol.* **12**, 275–278 (2002).
- Bhat, R. V., Budd Haerberlein, S. L. & Avila, J. Glycogen synthase kinase 3: a drug target for CNS therapies. *J. Neurochem.* **89**, 1313–1317 (2004).
- Kozlovsky, N., Belmaker, R. H. & Agam, G. GSK-3 and the neurodevelopmental hypothesis of schizophrenia. *Eur. Neuropsychopharmacol.* **12**, 13–25 (2002).
- Grimes, C. A. & Jope, R. S. The multifaceted roles of glycogen synthase kinase 3beta in cellular signaling. *Prog. Neurobiol.* **65**, 391–426 (2001).
- Beaulieu, J. M., Gainetdinov, R. R. & Caron, M. G. Akt/GSK3 signaling in the action of psychotropic drugs. *Annu. Rev. Pharmacol. Toxicol.* **49**, 327–347 (2009).
- Jiang, H., Guo, W., Liang, X. & Rao, Y. Both the establishment and the maintenance of neuronal polarity require active mechanisms: critical roles of GSK-3beta and its upstream regulators. *Cell* **120**, 123–135 (2005).
- Yoshimura, T. *et al.* GSK-3 beta regulates phosphorylation of CRMP-2 and neuronal polarity. *Cell* **120**, 137–149 (2005).
- Dotti, C. G., Sullivan, C. A. & Banker, G. A. The establishment of polarity by hippocampal neurons in culture. *J. Neurosci.* **8**, 1454–1468 (1988).
- Grabrucker, A., Vaida, B., Bockmann, J. & Boeckers, T. M. Synaptogenesis of hippocampal neurons in primary cell culture. *Cell Tissue. Res.* **338**, 333–341 (2009).
- Ziv, N. E. & Smith, S. J. Evidence for a role of dendritic filopodia in synaptogenesis and spine formation. *Neuron* **17**, 91–102 (1996).
- Friedman, H. V., Bresler, T., Garner, C. C. & Ziv, N. E. Assembly of new individual excitatory synapses: time course and temporal order of synaptic molecule recruitment. *Neuron* **27**, 57–69 (2000).
- Dijkhuizen, P. A. & Ghosh, A. Regulation of dendritic growth by calcium and neurotrophin signaling. *Prog. Brain. Res.* **147**, 15–27 (2005).
- Tapley, P., Lamballe, F. & Barbacid, M. K252a is a selective inhibitor of the tyrosine protein kinase activity of the trk family of oncogenes and neurotrophin receptors. *Oncogene* **7**, 371–381 (1992).
- Hedgepeth, C. M., Dearnoff, M. A., Rankin, K. & Klein, P. S. Regulation of glycogen synthase kinase 3beta and downstream Wnt signaling by axin. *Mol. Cell Biol.* **19**, 7147–7157 (1999).
- Yu, J. Y., Taylor, J., DeRuiter, S. L., Vojtek, A. B. & Turner, D. L. Simultaneous inhibition of GSK3 alpha and GSK3 beta using hairpin siRNA expression vectors. *Mol. Ther.* **7**, 228–236 (2003).
- Sholl, D. A. Dendritic organization in the neurons of the visual and motor cortices of the cat. *J. Anat.* **87**, 387–406 (1953).
- Coghlan, M. P. *et al.* Selective small molecule inhibitors of glycogen synthase kinase-3 modulate glycogen metabolism and gene transcription. *Chem. Biol.* **7**, 793–803 (2000).
- Peineau, S. *et al.* A systematic investigation of the protein kinases involved in NMDA receptor-dependent LTD: evidence for a role of GSK-3 but not other serine/threonine kinases. *Mol. Brain* **2**, 22 (2009).
- Magee, J. C. & Johnston, D. Synaptic activation of voltage-gated channels in the dendrites of hippocampal pyramidal neurons. *Science* **268**, 301–304 (1995).
- Williams, S. R. & Mitchell, S. J. Direct measurement of somatic voltage clamp errors in central neurons. *Nat. Neurosci.* **11**, 790–798 (2008).
- Luscher, B., Fuchs, T. & Kilpatrick, C. L. GABAA receptor trafficking-mediated plasticity of inhibitory synapses. *Neuron* **70**, 385–409 (2011).
- Li, R. W. *et al.* Disruption of postsynaptic GABA(A) receptor clusters leads to decreased GABAergic innervation of pyramidal neurons. *J. Neurochem.* **95**, 756–770 (2005).
- Tyagarajan, S. K. *et al.* Regulation of GABAergic synapse formation and plasticity by GSK3beta-dependent phosphorylation of gephyrin. *Proc. Natl. Acad. Sci. USA* **108**, 379–384 (2011).
- Spruston, N. Pyramidal neurons: dendritic structure and synaptic integration. *Nat. Rev. Neurosci.* **9**, 206–221 (2008).
- Gorski, J. A., Zeiler, S. R., Tamowski, S. & Jones, K. R. Brain-derived neurotrophic factor is required for the maintenance of cortical dendrites. *J. Neurosci.* **23**, 6856–6865 (2003).
- Doble, B. W. & Woodgett, J. R. GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* **116**, 1175–1186 (2003).
- Doble, B. W., Patel, S., Wood, G. A., Kockeritz, L. K. & Woodgett, J. R. Functional redundancy of GSK-3 alpha and GSK-3 beta in Wnt/beta-catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev. Cell* **12**, 957–971 (2007).

41. Nishimura, M., Owens, J. & Swann, J. W. Effects of chronic network hyperexcitability on the growth of hippocampal dendrites. *Neurobiol. Dis.* **29**, 267–277 (2008).
42. Fritschy, J. M., Harvey, R. J. & Schwarz, G. Gephyrin: where do we stand, where do we go? *Trends Neurosci.* **31**, 257–264 (2008).
43. Rudelli, R. D. *et al.* Adult fragile X syndrome. Clinico-neuropathologic findings. *Acta. Neuropathol.* **67**, 289–295 (1985).
44. Berman, R. F., Murray, K. D., Arque, G., Hunsaker, M. R. & Wenzel, H. J. Abnormal dendrite and spine morphology in primary visual cortex in the CGG knock-in mouse model of the fragile X premutation. *Epilepsia* **53**(Suppl 1): 150–160 (2012).
45. Min, W. W. *et al.* Elevated glycogen synthase kinase-3 activity in Fragile X mice: key metabolic regulator with evidence for treatment potential. *Neuropharmacology* **56**, 463–472 (2009).
46. Mines, M. A., Yuskaitis, C. J., King, M. K., Beurel, E. & Jope, R. S. GSK3 influences social preference and anxiety-related behaviors during social interaction in a mouse model of fragile X syndrome and autism. *PLoS One* **5**, e9706 (2010).
47. Guo, W. *et al.* Inhibition of GSK3beta improves hippocampus-dependent learning and rescues neurogenesis in a mouse model of fragile X syndrome. *Hum. Mol. Genet.* **21**, 681–691 (2012).
48. Fatemi, S. H., Reutiman, T. J., Folsom, T. D. & Thuras, P. D. GABA(A) receptor downregulation in brains of subjects with autism. *J. Autism. Dev. Disord.* **39**, 223–230 (2009).
49. Centonze, D. *et al.* Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome. *Biol. Psychiatry.* **63**, 963–973 (2008).
50. Olmos-Serrano, J. L. *et al.* Huntsman MM. Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. *J. Neurosci.* **30**, 9929–9938 (2010).
51. Mao, Y. *et al.* Disrupted in schizophrenia 1 regulates neuronal progenitor proliferation via modulation of GSK3beta/beta-catenin signaling. *Cell* **136**, 1017–1031 (2009).
52. Rui, Y., Gu, J., Yu, K., Hartzell, H. C. & Zheng, J. Q. Inhibition of AMPA receptor trafficking at hippocampal synapses by beta-amyloid oligomers: the mitochondrial contribution. *Mol. Brain* **3**, 10 (2010).
53. Fuller, L. & Dailey, M. E. Preparation of rodent hippocampal slice cultures. *CSH Protoc.* doi:10.1101/pdb.prot4848 (2007).
54. Woods, G. & Zito, K. Preparation of gene gun bullets and biolistic transfection of neurons in slice culture. *J. Vis. Exp.* e675 (2008).
55. Longair, M. H., Baker, D. A. & Armstrong, J. D. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. *Bioinformatics.* **27**, 2453–2454 (2011).
56. Meijering, E. *et al.* Design and validation of a tool for neurite tracing and analysis in fluorescence microscopy images. *Cytometry. A.* **58**, 167–176 (2004).
57. Zhou, F. Q., Zhou, J., Dedhar, S., Wu, Y. H. & Snider, W. D. NGF-induced axon growth is mediated by localized inactivation of GSK-3 β and functions of the microtubule plus end binding protein APC. *Neuron* **42**, 897–912 (2004).

Acknowledgements

This research was supported in part by grants from the National Institutes of Health to JQZ (GM083889, GM084363, and HD023315), HCH (GM60448 and EY114852), and ALDB (NS038752). The work also received support from a pilot grant from Emory Alzheimer's Disease Resource Center (ADRC P50 AG025688), a NINDS core facilities grant (P30NS055077) to the Neuronal Imaging Core of Emory Neuroscience, and a postdoctoral fellowship from the Ellison Medical Foundation/American Federation for Aging Research to Y.R.

Author contributions

Y.R. designed and performed a majority of the experiments in this study. K.R.M. acquired the data involving brain slices, K.Y. and H.C.H. contributed the electrophysiology data, A.W. made the gephyrin mutations, A.L.D.B. helped with experiments concerning GABA_AR knockdown and discussion, H.C.H. provided invaluable input to the manuscript, J.Q.Z. designed, oversaw and wrote the manuscript with Y.R. We would also like to thank other members of Zheng lab for their insightful discussion of the project and technical help.

Additional information

Supplementary Information accompanies this paper at <http://www.nature.com/naturecommunications>

Competing financial interests: The authors declare no competing financial interests.

Reprints and permission information is available online at <http://npg.nature.com/reprintsandpermissions/>

How to cite this article: Rui, Y. *et al.* Activity-dependent regulation of dendritic growth and maintenance by glycogen synthase kinase 3 β . *Nat. Commun.* **4**:2628 doi: 10.1038/ncomms3628 (2013).