GSK3 signalling in neural development

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Abstract | Recent evidence suggests that glycogen synthase kinase 3 (GSK3) proteins and their upstream and downstream regulators have key roles in many fundamental processes during neurodevelopment. Disruption of GSK3 signalling adversely affects brain development and is associated with several neurodevelopmental disorders. Here, we discuss the mechanisms by which GSK3 activity is regulated in the nervous system and provide an overview of the recent advances in the understanding of how GSK3 signalling controls neurogenesis, neuronal polarization and axon growth during brain development. These recent advances suggest that GSK3 is a crucial node that mediates various cellular processes that are controlled by multiple signalling molecules — for example, disrupted in schizophrenia 1 (DISC1), partitioning defective homologue 3 (PAR3), PAR6 and Wnt proteins — that regulate neurodevelopment.

Mitotic spindle reorganization
Reorganization of microtubules and associated molecules during cell division to form a bipolar spindle between duplicated centrosome components. This leads to the segregation of chromosomes.

Leading process
A neuronal process that extends in the direction of neuronal migration before nuclear movement.

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doi:10.1038/nrn2870

Glycogen synthase kinase 3 (GSK3) proteins are serine/threonine kinases that were originally identified as key regulatory enzymes in glucose metabolism[1,2]. There are two isoforms, GSK3α and GSK3β, that are encoded by separate genes and that are 85% homologous to each other overall with 95% homology in the kinase domains[3]. In rodents and humans an alternative splice variant of GSK3β — GSK3β2 — has been reported. This variant contains a 13 amino acid insertion in an external loop near the catalytic domain[4]. In contrast to the ubiquitously expressed GSK3β1, GSK3β2 is expressed specifically in the nervous system, and the highest levels are found during development[4]. Interestingly, recent studies suggest that GSK3β2 plays a specific part in neuronal morphogenesis *in vitro*[5,6], and it remains to be determined whether different isoforms have specific functions *in vivo* during brain development. Since their discovery GSK3s have been shown to mediate various signalling pathways, among which the growth factor and Wnt signalling pathways are the most studied[7]. Consistent with the roles of growth factors and Wnt proteins in the nervous system, especially during neurodevelopment, emerging evidence points to GSK3s as key regulators in multiple neurodevelopmental processes. These processes include neurogenesis, neuronal migration, neuronal polarization and axon growth and guidance.

How do GSK3s regulate such a wide variety of developmental events? The answer may lie in the broad range of GSK3 substrates. Among GSK3 substrates are many transcription factors, such as cyclic AMP response element-binding protein (CREB)[8], the nuclear factor of activated T cells (Nfat) family of proteins[9,10], neurogenin 2 (REF. 11), SMAD1 (REF. 12), c-Jun[13] and β-catenin[14]. These transcription factors all play important parts in the regulation of gene expression throughout neurodevelopment. GSK3s regulate these transcription factors by controlling their protein levels, DNA binding activities and/or nuclear localization. In addition to gene expression, cell morphogenesis requires reorganization of the cytoskeleton and especially of the microtubules. GSK3s regulate the activity of several microtubule-associated proteins (MAPs)[15] and may therefore control mitotic spindle reorganization during cell division, coordinated movement of the leading process and soma during neuronal migration, and directed growth cone advancement during axon growth and guidance, all of which require coordinated control of microtubule dynamics.

Changes in GSK3 activity have been associated with many psychiatric and neurodegenerative diseases, such as Alzheimer's disease, schizophrenia and autism spectrum disorders, and it has become increasingly apparent that GSK3 might be a common therapeutic target for different classes of psychiatric drugs[16,17]. Indeed, lithium, which is a direct inhibitor of GSK3 (REF. 18), has been used in humans as a mood stabilizer for over 50 years[19]. The hypothesis that disturbances of brain development play a part in the aetiology of these disorders is further supported by the fact that several genetic susceptibility factors for psychiatric disorders have key roles in neurodevelopment. Intriguingly, many of the genes that are associated with schizophrenia encode proteins that are involved in GSK3 signalling — for example, disrupted in schizophrenia 1 (DISC1),

Leading edge
The region of a migrating cell that protrudes forward.

neuregulin 1 and frizzled 3 (REF. 20). Genes that are associated with autism spectrum disorders also encode proteins that are involved in the modulation of, or that are affected by, GSK3 activity. These proteins include phosphatase and tensin homologue (PTEN)[21], DISC1 (REF. 22), serotonin[23], tuberous sclerosis 1 (TSC1)–TSC2 complex[24] and adenomatous polyposis coli (APC)[25,26]. Therefore, a better understanding of the role of GSK3 in neurodevelopment could provide insights into the aetiology of these disorders and could possibly lead to a new library of potential therapeutic targets.

In this Review, we provide an overview of the involvement of GSK3 signalling in neurodevelopment with a particular emphasis on neurogenesis, neuronal polarization and axon growth. We also discuss the potential crosstalk between GSK3 signalling and other pathways that are implicated in these developmental steps.

Regulation of GSK3 activity in the CNS

GSK3 is an unusual kinase in the sense that it has a high basal activity in resting cells and is inactivated by upstream regulators in response to stimuli. Several signalling pathways regulate GSK3 activity (FIG. 1). Activation of the phosphatidylinositol 3-kinase (PI3K) pathway downstream of receptor tyrosine kinase signalling is thought to result in inactivation of GSK3 through phosphorylation of the amino-terminal serine residue (Ser9 in GSK3β and Ser21 in GSK3α). It is widely accepted that Akt is the major family that mediates this serine phosphorylation and the subsequent inactivation of GSK3 (REFS 27,28) (FIG. 1). However, mutant mice (GSK3α-Ser21Ala/GSK3β-Ser9Ala double knock-in mice) with GSK3s that cannot be phosphorylated at their N-termini developed normally with no overt phenotype in the nervous system (although insulin-induced activation of glycogen synthase was disrupted in the muscle)[29]. This suggests that alternative pathways might regulate GSK3 activity, and this may especially be the case in the developing nervous system. Indeed, a recent study[30] showed that p38 mitogen-activated protein kinase (p38MAPK) regulates the activity of GSK3β — but not GSK3α — by inducing an inhibitory phosphorylation on Ser389 (FIG. 1). However, p38MAPK-mediated phosphorylation is insufficient to provide a regulatory mechanism of GSK3 in the nervous system because both isoforms of GSK3 are required for normal development[31].

In the canonical Wnt pathway, Wnt-induced dissociation of GSK3 from its substrate β-catenin leads to the stabilization and activation of β-catenin. When the Wnt receptor is stimulated, GSK3 is recruited to the membrane, where PPPSPXS motifs in the Wnt co-receptor low-density-lipoprotein receptor-related protein 5/6 (LRP 5/6) associate with and inhibit GSK3 activity towards β-catenin[32,33] (FIG. 1). Regulation of GSK3 by LRP5/6 is achieved through protein–protein interaction, which sequesters GSK3 away from its substrate without affecting the kinase activity per se. Regulation of GSK3 activity towards a particular substrate rather than general inhibition of its kinase activity is also exemplified in a recent finding[22], which suggests that through physical interaction with GSK3, DISC1 prevents GSK3 from phosphorylating β-catenin.

In response to Wnt[34] and growth factors[35] GSK3 activity is regulated by a conserved cell polarity pathway that involves cell division control protein 42 homologue (CDC42)–partitioning defective 3 (PAR3)–PAR6–protein kinase Cζ (PKCζ) (FIG. 1). The regulation of GSK3 activity by the Par complex was first described in directed cell migration[36]. Specifically, localized activation of the small GTPase CDC42 at the leading edge recruits the PAR3–PAR6–PKCζ complex, which interacts with GSK3β and this leads to GSK3β phosphorylation and inactivation[36]. However, PKCζ is unable to directly phosphorylate GSK3 (REF. 27). In fact, a study that used fibroblasts derived from GSK3α-Ser21Ala/GSK3β-Ser9Ala double knock-in mice[37] showed that GSK3 phosphorylation is not required for the PAR3–PAR6-mediated GSK3 inhibition. Instead, dishevelled (Dvl) and axin have been suggested to mediate GSK3 inhibition through this pathway by inducing a physical association between Dvl and PKCζ during directed cell migration (FIG. 1). Interestingly,

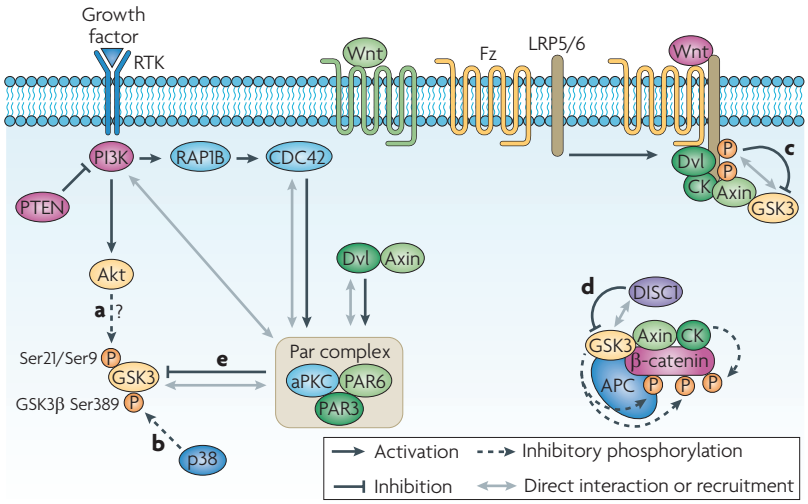

Figure 1 | **Proposed models of GSK3 inactivation.** On activation of the phosphatidylinositol 3-kinase (PI3K) pathway downstream of receptor tyrosine kinase (RTK) signalling, Akt is thought to be the major family of kinases that inactivates glycogen synthase kinase 3 (GSK3) through phosphorylation of the amino-terminal serine residue (Ser21 in GSK3α and Ser9 in GSK3β) (**a**). Phosphorylation of GSK3β (but not GSK3α) at Ser389 by p38 mitogen-activated protein kinase (p38MAPK) might be an alternative mechanism for inactivation[30] (**b**). GSK3 regulation in the Wnt pathway is distinct from the way by which RTK signalling inhibits GSK3. In the canonical Wnt pathway, Wnt signalling recruits the destruction complex to the membrane, and this leads to the phosphorylation of the cytoplasmic tail of low-density-lipoprotein receptor-related protein (LRP) 5/6 by casein kinase (CK) and GSK3β. Phosphorylated PPPSPXS motifs in the LRP5/6 intracellular domain directly inhibit GSK3's activity towards β-catenin (**c**)[32,33]. A recent study showed that in neuronal progenitor cells, disrupted in schizophrenia 1 (DISC1) directly interacts with GSK3 and prevents it from phosphorylating β-catenin[22] (**d**). In the Wnt[37] and the RTK signalling[35] pathways, GSK3 activity is also regulated by polarity proteins (**e**). GSK3 interacts with the partitioning defective homologue (PAR) complex — composed of PAR3, PAR6 and atypical protein kinase C (aPKC) — and so becomes inactivated. Although phosphorylation of GSK3 also occurs as a consequence of this interaction, GSK3 phosphorylation is not required for its inactivation[37]. CDC42, cell division control 42 homologue; Dvl, dishevelled; Fz, frizzled; PTEN, phosphatase and tensin homologue; RAP1B, Ras-related protein 1B.

Box 1 | **Current methods used to detect GSK3 activity**

The most widely used method to detect glycogen synthase kinase 3 (GSK3) activity is to examine Ser9 and Ser21 phosphorylation. However, results from GSK3α-Ser21Ala/GSK3β-Ser9Ala double knock-in mice[29] raise the question of whether this serine phosphorylation of GSK3 is the major regulatory mechanism in the CNS. More importantly, it is becoming increasingly apparent that changes in GSK3 activity are not always accompanied by changes in their phosphorylation status. Thus, serine phosphorylation of GSK3 might not be used as the sole indicator of GSK3 activity.

The second approach is the *in vitro* kinase assay. Endogenous GSK3 can be immuno-precipitated from cell lysates to detect its activity towards its substrates. However, because the interaction of GSK3 with its regulators in cells might be transient and because only a small percentage of total GSK3 in cells interacts with a particular regulator[118], it is possible that the changes in GSK3 activity are not preserved in the *in vitro* kinase assay or are less likely to be detected.

The third approach is to examine the tyrosine phosphorylation in GSK3 (Tyr279 in GSK3α and Tyr216 in GSK3β). This phosphorylation facilitates the activity of GSK3 by promoting substrate accessibility[119]. Although there is evidence for signal-dependent regulation of tyrosine phosphorylation[22,120,121], tyrosine phosphorylation of GSK3 is thought to occur through a post-translational and intramolecular autophosphorylation event[119,121], which in many cases is not dynamically regulated by external stimuli.

The last approach is to examine changes in the phosphorylation status of known GSK3 substrates. Because GSK3 might not have equal access to all potential substrates *in vivo* owing to subcellular localization, this method allows us to detect more physiologically relevant changes in GSK3 activity. However, when this method is applied to assess GSK3 activation or inactivation in a pathway that does not have known GSK3 substrates, one should consider multiple potential substrates that can represent a diverse family of proteins — for example, representing primed versus unprimed substrates with different subcellular localizations.

a recent study[38] shows that PAR3 directly binds PI3K and enhances its activity, and this provides an alternative explanation for the increased GSK3 phosphorylation downstream of PAR3. Together, these lines of evidence suggest protein–protein interaction as a recurrent theme in the regulation of GSK3 activity, which in many cases does not require changes in the phosphorylation status of GSK3 (BOX 1).

GSK3 signalling in neurogenesis

Recent evidence[22,39,40] suggests that GSK3 signalling is essential for coordinating the proliferation and differentiation of progenitor cells during brain development. As the developmental process of the mouse neocortex (FIG. 2) has been documented well, we will use it as a model system to discuss the role of GSK3 signalling in neural development.

GSK3 inactivation promotes progenitor proliferation. A recent study[39] revealed, through the selective deletion of both *Gsk3a* and *Gsk3b* in neural progenitors, that GSK3 signalling has a pivotal role in the developing nervous system. The double knockout mice had a substantially increased cortical surface area with a convoluted shape due to an over-expansion of the neural progenitor cells. However, the cortex was thinner than that of control littermates, which indicated a reduction in the number of neurons. Indeed, further analysis using various markers of progenitors, intermediate progenitor cells (IPCs) and postmitotic neurons showed that deletion of *Gsk3* genes markedly enhanced the proliferation of progenitor cells while suppressing neuronal differentiation. This indicates that spatiotemporal regulation of GSK3 activity is required for an appropriate transition from the proliferative to the neurogenic phase to occur during brain development.

Various signalling pathways have been identified as regulators of neural progenitor proliferation, including Wnt[41], sonic hedgehog (SHH)[42], fibroblast growth factor (FGF)[43] and the Notch signalling pathway[44]. Intriguingly, GSK3 is implicated in the regulation of each of these pathways. In the canonical Wnt pathway, inhibition of GSK3 is crucial for the stabilization and nuclear translocation of β-catenin that leads to the subsequent activation of the T cell factor 4 (TCF4)-dependent gene transcription. As discussed above, although the regulatory mechanism is not clear, GSK3 is a well-known downstream effector of PI3K, and this provides an explanation for the disruption of FGF signalling in brains from which *Gsk3* genes have been deleted[39]. The mechanism by which SHH signalling was elevated in *Gsk3*-deleted brains[39] is unclear. However, a study in *Drosophila melano gaster*[45] suggests that GSK3 controls the stability of key transcriptional effectors (CI/GLI proteins) in the hedgehog pathway in a similar manner to the way in which it regulates β-catenin in the Wnt pathway. There are contradicting results with respect to how GSK3 regulates Notch signalling. In one study Notch protein was stabilized by GSK3-mediated phosphorylation[46], whereas in another study the transcriptional activity of Notch was enhanced by inhibition of GSK3 (REF. 47). However, the fact that Notch signalling was hyperactivated in *Gsk3*-deleted brains[39] suggests that GSK3 negatively regulates Notch signalling in neural progenitors. Together, these lines of evidence suggest that GSK3 might function as a node molecule of multiple signalling pathways and thereby coordinate the proliferation and differentiation of neural progenitors.

The role of GSK3 signalling in neurogenesis is further supported by two recent studies[22,40] in which DISC1 or PAR3 — upstream regulators of GSK3 — were manipulated in neural progenitors. Ectopic expression of DISC1 or PAR3 increased progenitor proliferation and suppressed neuronal differentiation, and this is reminiscent of *Gsk3* knockout mice. One study[22] showed that DISC1 directly interacts with GSK3 — this seems to prevent GSK3 from phosphorylating β-catenin — and

that overexpression of DISC1 leads to the activation of canonical Wnt signalling. The other study[40] did not directly test the involvement of GSK3 but showed that overexpression of PAR3 led to the activation of Notch signalling. This was also observed in *Gsk3* knockout mice[39]. As Wnt and Notch signalling independently promote the proliferation of progenitors in *Gsk3* knockout mice[39], it could be suggested that DISC1 and PAR3 regulate GSK3 activity in the Wnt and the Notch pathway, respectively, and that this allows pathway-specific control of GSK3. These lines of evidence suggest that inhibition of GSK3 signalling, through the deletion of *Gsk3*s[39] or the overexpression of their negative regulators[22,40], promotes the proliferation of progenitor cells while suppressing their differentiation into neurons.

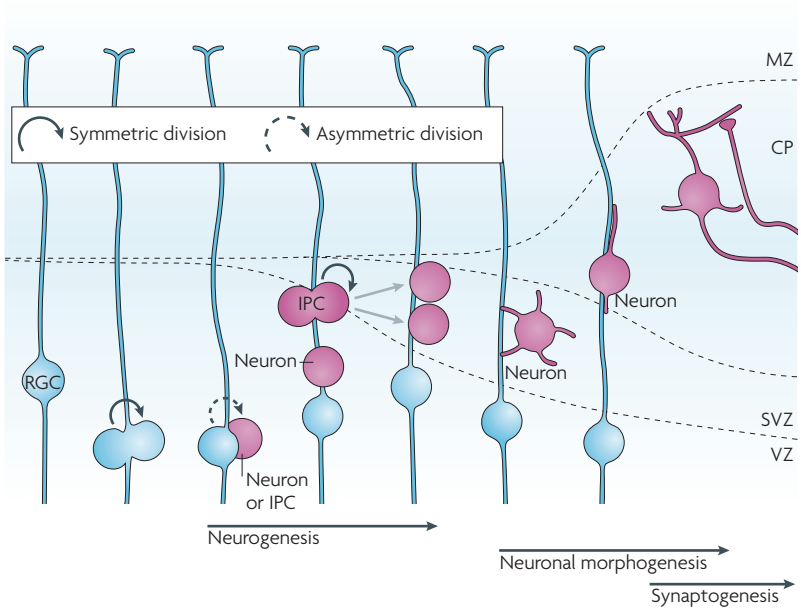

Figure 2 | **Neural development of the mammalian neocortex.** The formation of neural circuits during development involves the coordination of multiple cellular events, which can be divided into three steps. The first step is neurogenesis, during which a cohort of neural progenitors undergoes cycles of proliferation and differentiation to generate neurons in a highly regulated manner. The second step is neuronal morphogenesis, during which differentiated neurons migrate towards their final destination. During migration these neurons polarize to form axons and dendrites, and these then grow to reach their specific target fields under the control of a myriad of guidance cues. The third step is synaptogenesis, during which axons cease to grow and form synapses with their innervating targets. Radial glial cells (RGCs) are progenitors that generate the majority of neurons in the developing neocortex, directly or indirectly, by undergoing two phases of coordinated cell division. In the first — proliferative — phase, which occurs early in development, the RGC mainly divide symmetrically in the ventricular zone (VZ) to generate two similar progenitor cells, which can further self-renew to expand the progenitor pool. In the second — neurogenic — phase, most of the RGC divide asymmetrically to generate one self-renewing progenitor and one post-mitotic neuron or one intermediate progenitor cell (IPC). The IPCs do not self-renew but migrate to the subventricular zone (SVZ) and divide symmetrically to form neurons. At the end of neurogenesis some radial glial cells undergo a terminal symmetrical division to generate two neurons (not shown). Asymmetric division as well as the transition of progenitors from symmetric to asymmetric division are tightly regulated to produce the final total number of neurons. Perturbation in any of these processes can cause defects in neurogenesis and cortical development. CP, cortical plate; MZ, marginal zone.

GSK3 activation promotes neuronal differentiation. Kim *et al*[39] found that phosphorylation levels of c-Myc and β-catenin (targets of GSK3) are increased at later stages in development (when progenitor proliferation is subsiding and neuronal differentiation predominates). As c-Myc[48] and β-catenin are pro-proliferation factors, and as phosphorylation by GSK3 leads to their degradation, this finding suggests that activation of GSK3 promotes neuronal differentiation. This idea is supported by a study in which *Disc1* knockdown in neural progenitors was shown to cause premature neuronal differentiation at the expense of the size of the progenitor pool[22]. When GSK3β signalling was examined the levels of phosphorylated β-catenin and tyrosine-phosphorylated GSK3β, which is associated with increased kinase activity (BOX 1), were increased[22]. Moreover, it was shown that pharmacological inhibition of GSK3 prevented the progenitor proliferation defects that are induced by *Disc1* knockdown[22]. These results suggest that the premature differentiation observed in *Disc1*-depleted cells might have been due to activation of GSK3 signalling, although future studies of *Disc1* knockout mice are needed to confirm this conclusion. Depletion of PAR3 also increases the neuronal population with a concomitant decrease in the number of progenitor cells[40]. Future studies are required to determine whether this defect is also attributed to GSK3 activation.

As mentioned above, GSK3 has high basal activity in resting cells and signalling is usually initiated by GSK3 inactivation in response to extracellular factors. However, lysophosphatidic acid (LPA) seems to increase GSK3 activity above basal levels, and this activation is mediated in part by the small GTPase RhoA[49]. Consistent with the idea that GSK3 activation promotes neuronal differentiation, treatment of *ex vivo* cultured cortical hemispheres with LPA promoted terminal mitosis of neural progenitors, which led to the differentiation of neural progenitors into neurons[50]. Moreover, a recent study[51] showed that LFC, a guanine nucleotide exchange factor of RhoA, promotes neuronal differentiation. Knocking down *Lfc*, which inhibits Rho activation, increased the pool of radial glial progenitors and prevented neurogenesis, whereas genetic silencing of *Tctex1*, a negative regulator of *Lfc*, had the opposite effect[51]. Although these results are consistent with the hypothesis that activation and inactivation of GSK3 promote neuronal differentiation and progenitor proliferation, respectively, it remains to be determined whether these effects are indeed mediated by regulation of GSK3 activity.

The conventional view is that GSK3 is constitutively active in resting cells. If this is the case, neural progenitors would undergo neuronal differentiation without any additional stimuli. However, a recent study[52] showed that an extracellular cue — retinoic acid derived from forebrain meninges — is required for neuronal differentiation. Interestingly, it has been shown that retinoic acid decreases Ser9 phosphorylation of GSK3β[53] and increases GSK3 expression[5], raising the intriguing possibility that retinoic acid signalling leads to the elevation of GSK3 activity above basal levels

Asymmetrical division
Division of cells that leads to the production of two daughter cells with different developmental potentials.

Centrosome
An organelle that controls the organization of microtubules and regulates cell-cycle progression.

Centriole
One of two perpendicular structures in the centrosome that are composed of a ring of nine microtubules.

Ventricular zone
A region in the brain next to the ventricles that is composed of neuroepithelial cells that generate neuronal and glial cells.

Microtubule minus end-anchoring proteins
A family of proteins that specifically bind to the microtubule minus ends and function to anchor the microtubule to specific cellular structures, such as the centriole.

to control neurogenesis. This hypothesis is supported by the finding that PAR3 was significantly upregulated in neural progenitors in the absence of retinoic acid[52]. Collectively, these studies provide some indirect evidence that GSK3 activation might also be regulated by extracellular cues during neuronal differentiation. The most direct way to test whether an increase in GSK3 activity promotes neuronal differentiation would be to overexpress GSK3 in the developing brain. Unfortunately the currently available *Gsk3* transgenic mice overexpress *Gsk3b* under the control of the *Thy1* promoter, which is specifically expressed in postmitotic neurons[54]. This renders these mice unsuitable for examining the role of GSK3 in neurogenesis. A mouse model that overexpresses GSK3 specifically in neural progenitors is needed to test this idea.

GSK3 controls neurogenesis by coordinated regulation of protein degradation and microtubule reorganization. Emerging evidence suggests that GSK3 regulates the stability of a wide range of proteins via activation of the ubiquitin-proteasome system (UPS)[55,56]. Thus, one plausible mechanism by which GSK3 could regulate neurogenesis is through controlling the levels of molecules that are involved in neurogenesis. Prominent candidates are transcriptional regulators, such as β-catenin in the Wnt pathway, GLI in the SHH pathway and c-Myc in the FGF pathway[39,45,48], all of which control progenitor proliferation through regulation of gene transcription. This hypothesis is supported by the finding that during mitotic cell division GSK3-phosphorylated β-catenin, which is destined for degradation, is inherited by only one daughter cell[57]. Thus, it is possible that during asymmetrical division of radial glial cells, two daughter cells have different GSK3 activity because of asymmetrical segregation of upstream GSK3 regulators (for example, PAR3). The daughter cell with lower GSK3 activity will accumulate β-catenin (and perhaps other pro-proliferative proteins) and thus remains a progenitor. Conversely, the daughter cell with higher GSK3 activity will degrade these proteins through activation of the UPS and then differentiate into either a neuron or an IPC (FIG. 3).

In addition to proteins that regulate gene transcription, GSK3 could regulate other molecules that are important for controlling proliferation or differentiation. A recent study[58] has shown that during asymmetrical division of radial glial progenitors, daughter cells that inherit the centrosome containing the new centriole migrate away from the ventricular zone (VZ) and differentiate into neurons. By contrast, daughter cells that inherit the centrosome containing the original, mature centriole retain the capacity to self-renew and remain in the VZ. Removal of ninein, which is mainly localized in the mature centriole, disrupts the asymmetric segregation of the centrosome and promotes neuronal differentiation. Intriguingly, ninein is a substrate of GSK3 (REFS 59–61) and ninein levels are regulated by the UPS[62]. Therefore, during asymmetric division ninein would be accumulated in the daughter cell with low GSK3 activity. As ninein is a microtubule minus end-anchoring protein that is involved in the formation of astral microtubules (which anchor cells to their niches) daughter cells with ninein in their centrosome are more likely to be anchored to the apical surface of VZ and inherit a progenitor fate. However, daughter cells with high GSK3 activity will have little ninein in their centrosome. This will lead to their detachment from the VZ surface and their differentiation into neurons or IPCs.

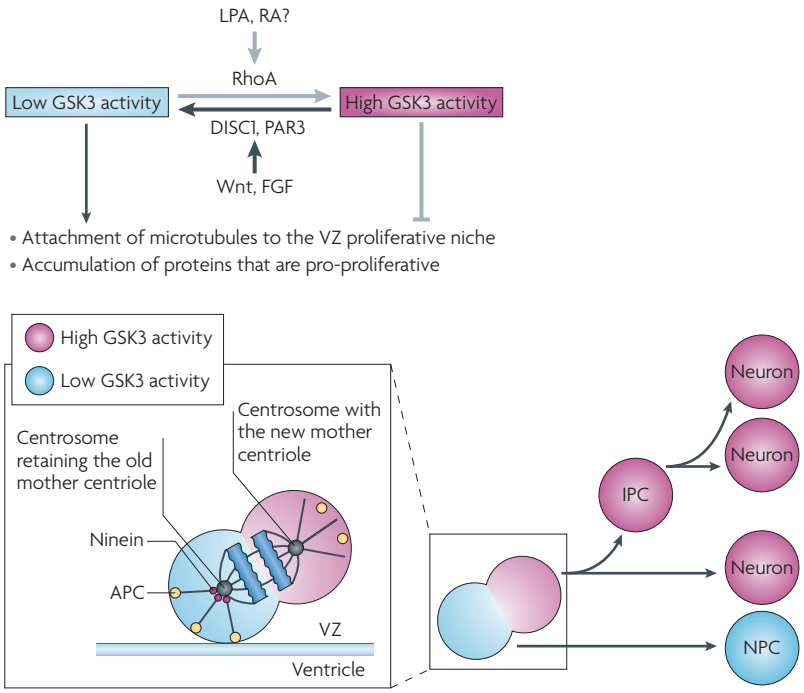

Figure 3 | **Proposed model for the role of GSK3 signalling during neurogenesis.** During asymmetric division of radial glial cells the daughter cells may have different glycogen synthase kinase 3 (GSK3) activities due to asymmetric inheritance of upstream regulators (for example, partitioning defective 3 (PAR3)[36], disrupted in schizophrenia (DISC1)[22] or Rho[49]) under the control of extracellular factors (for example, the Wnt family, the fibroblast growth factor (Fgf) family, lysophosphatidic acid (LPA)[50] and retinoic acid (RA)[52]) (top part). The daughter cell with lower GSK3 activity (shown in blue) accumulates pro-proliferation factors[39], such as β-catenin, GLI and c-Myc. The microtubule-associated proteins adenomatosis polyposis coli (APC) and ninein organize the astral microtubules to attach the cell to the ventricular zone (VZ) surface, and the cell adopts a progenitor fate (becoming a neural progenitor cell (NPC)). Conversely, the daughter cell with higher GSK3 activity (shown in purple) removes pro-proliferative proteins and other proteins that are involved in microtubule assembly, presumably through ubiquitin–proteasome system-mediated protein degradation. In addition, APC is unable to bind to the astral microtubules, which disrupts their function. As a result, the cell detaches from the VZ surface and becomes a neuron or an intermediate progenitor cell (IPC).

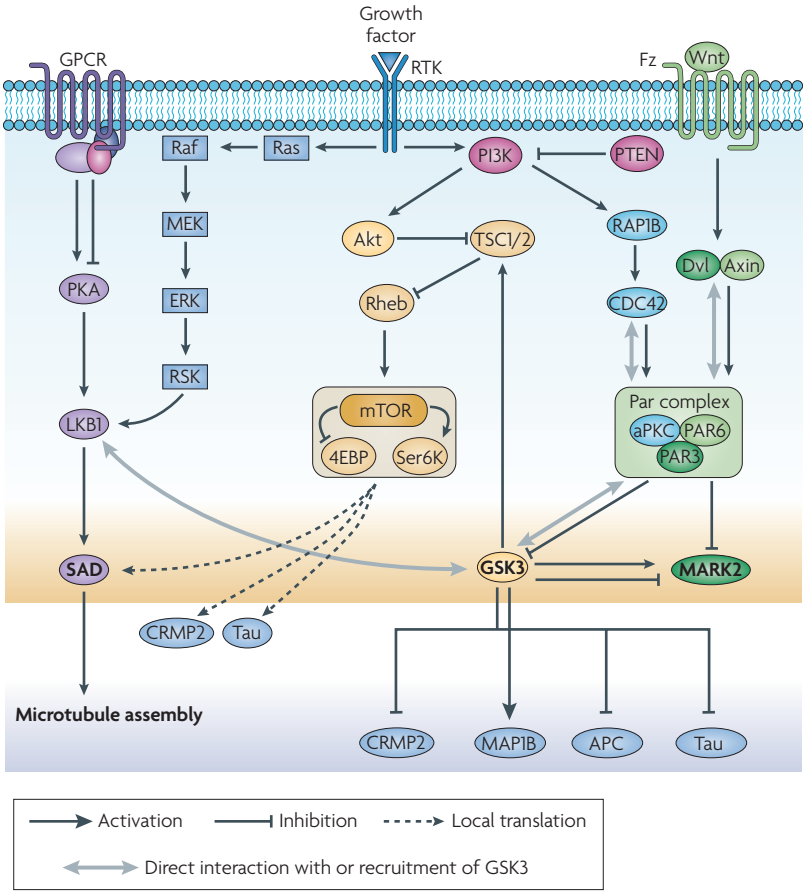

Figure 4 | **GSK3 in the regulation of neuronal polarization.** Local activation of phosphatidylinositol 3-kinase (PI3K) at the tip of the nascent axon is thought to function as a landmark for the induction of neuronal polarization. PI3K-mediated production of phosphatidylinositol-3,4,5-trisphosphate (PIP3) leads to the activation of RAS-related protein 1B (RAP1B) and Akt. RAP1B activates cell division control protein 42 homologue (CDC42), which recruits and activates the partitioning defective homologue 3 (PAR3) –PAR6–atypical protein kinase C (aPKC) complex. This leads to the inactivation of glycogen synthase kinase 3 (GSK3)[36,37]. Microtubule affinity-regulating kinase 2 (MARK2) has also been suggested to function downstream of the Par complex to control neuronal polarization[68]. aPKC in the Par complex associates with dishevelled (Dvl) and mediates the Wnt5a-induced differentiation of axons[94]. Activation of Akt induces inhibitory phosphorylation of tuberous sclerosis 2 (TSC2) by which the GTPase activating protein (GAP) activity of TSC2 towards Rheb is reduced, subsequently leading to the activation of mammalian target of rapamycin (mTOR) signalling. Although phosphorylation of TSC2 by Akt inhibits TSC1 and TSC2 activity (TSC1/2), phosphorylation of TSC2 by GSK3 has an opposite effect[107]. Thus, inhibition of GSK3 downstream of PI3K or Wnt signalling would reduce GSK3-dependent stimulatory phosphorylation of TSC2 and thereby increase Rheb-GTP levels. This leads to the activation of mTOR-mediated translation. Activation of mTOR signalling in the axon induces local translation of the GSK3 substrates collapsin response mediator protein 2 (CRMP2) and Tau[106]. Activation of the serine/threonine protein kinase LKB1 by protein kinase A (PKA) or ribosomal Ser6 kinase (RSK) leads to the activation of SAD kinases, which then phosphorylate microtubule-associated proteins (MAPs), such as Tau[67,72]. The MAPs that are phosphorylated in response to LKB1–SAD kinase signalling to control neuronal polarization remain to be determined. A study in *Xenopus laevis* shows that LKB1/ XEEK1 (XEEK1 is the *X. laevis* orthologue of LKB1) physically associates with GSK3 and regulates its activity[99]. Through the pathways described here, GSK3, MARK2 and SAD control microtubule assembly and dynamics by regulating the phosphorylation status of several MAPs. APC, adenomatosis polyposis coli; Dvl, dishevelled; 4EBP, eukaryotic translation initiation factor 4E-binding protein; ERK, extracellular signal regulated kinase; Fz, frizzled; GPCR, G protein-coupled receptor; MAP1B, microtubule-associated protein 1b; MEK, mitogen-activated protein kinase; PTEN, phosphatase and tensin homologue; RTK, receptor tyrosine kinase; S6K, protein S6 kinase.

Another major class of GSK3 substrates consists of MAPs, which control several aspects of microtubule dynamics. APC is not only a component of the Wnt pathway that regulates β-catenin stability but is also a MAP that belongs to a family of microtubule plus end-binding proteins (also known as microtubule plus end tracking proteins (+TIPs))[63]. When GSK3 activity is inhibited, APC binds to a microtubule plus end and there it anchors the spindle microtubules to the kinetochore or the astral microtubules to the cell cortex, both of which are important for (symmetric and asymmetric) cell division. APC plays a key part in asymmetric stem cell division by anchoring one daughter cell in a specific niche so that it retains self-renewal capacity[64]. In the Wnt signalling pathway APC interacts with GSK3 to degrade β-catenin, and, similar to *Gsk3* deletion, loss of *Apc* leads to stabilization of β-catenin and the subsequent activation of TCF4 (REF. 65). However, in contrast to *Gsk3* knockout, removal of *Apc* in progenitors results in reduced rather than increased proliferation, and this is probably due to the role of APC in regulating microtubules during cell division.

In summary, GSK3 signalling is emerging as a key regulator of neurogenesis. Through its role in the regulation of protein stability and microtubule assembly, GSK3 signalling coordinates proliferation and differentiation (FIG. 3).

GSK3 signalling in neuronal polarization

Studies in cultured hippocampal and cortical neurons have identified a number of molecules that are involved in neuronal polarization. These proteins — which include PI3K, the Akt family, GSK3, the small GTPases RAS-related C3 botulinum toxin substrate 1 (RAC1) and CDC42, and the Par polarity complex[66–76] — might act in distinct neuronal populations at specific stages of embryonic development, but it is also possible that they form a network that controls neural development in a coordinated manner. In this regard, GSK3 is an appealing candidate 'coordinator' because it can respond to and integrate upstream signals, and downstream it can phosphorylate various substrates that are involved in a wide array of cellular activities[77]. Below, we discuss the evidence for a role of GSK3 signalling in neuronal polarization and explore the potential crosstalk of GSK3 signalling with other pathways that are involved in polarization (FIG. 4).

GSK3 regulates neuronal polarization by controlling microtubule dynamics. Several lines of evidence point to GSK3 as an essential regulator of neuronal polarity. Studies in hippocampal neurons show that the inactive form of GSK3β (which is phosphorylated at Ser9) is located at the tip of each neurite before polarization, but when neurons begin to polarize and one of these neurites develops into the axon, phospho-GSK3β becomes concentrated at the axonal tip. This suggests that maintaining local inactivation of GSK3β at the nascent axon is crucial for polarization[69,73,76]. Indeed, global inhibition of GSK3 by small-molecule inhibitors or knocking down of *Gsk3b* induces the formation of multiple axons, whereas overexpressing GSK3β-Ser9Ala prevents axon

formation[69]. Furthermore, local inhibition of GSK3 can convert dendritic processes into axons in already polarized neurons[69], which suggests that localized inactivation of GSK3 is required for both the establishment and maintenance of neuronal polarity[69,76]. To date, it is not clear how GSK3 inactivity becomes limited to one neurite during neuronal polarization.

Neuronal polarization requires major reorganization of the growth cone cytoskeleton. It has been proposed that a loose actin network at the tip of the future axon enables microtubules to selectively engorge into the axonal growth cone and so create the platform for subsequent axon elongation[78–82]. A recent study[83] showed that microtubule stabilization in one neurite precedes axon formation and that changing microtubule dynamics is sufficient to alter axon and dendrite specification[83]. This points to the idea that regulation of local microtubule stability and dynamics has an instructive role in neuronal polarization[84].

Several substrates of GSK3 are involved in neuronal polarization by regulating microtubule dynamics. These include collapsin response mediator protein 2 (CRMP2), APC, Tau and microtubule-associated protein 1B (MAP1B). Binding of CRMP2 to tubulin dimers, which is abolished by GSK3β phosphorylation[76], promotes microtubule polymerization[85]. CRMP2 is enriched in the nascent axon and overexpression of CRMP2 is sufficient to induce the formation of multiple axons[86]. In hippocampal neurons the microtubule +TIP protein APC localizes at the tip of immature neurites and later becomes enriched in the growth cone of the nascent axon[87]. Similar to CRMP2, microtubule-binding activity of APC is abrogated by GSK3β phosphorylation[88]. Thus, GSK3β inactivation in the nascent axon promotes the association between APC and microtubule plus ends and thereby stabilizes the growing ends of axonal microtubules to support axon extension. As with CRMP2 and APC, Tau phosphorylation by GSK3β abolishes its binding to microtubules and thus impairs microtubule assembly[89]. GSK3 phosphorylation of MAP1B maintains microtubules in a dynamic state[90]. However, the lack of polarity defect in *Tau/Map1b* double knockout mice suggests that Tau and MAP1B mainly function to regulate axon growth rather than neuronal polarization[91].

GSK3 signalling and the PI3K–Akt pathway. Local activation of PI3K and accumulation of phosphatidylinositol-3,4,5-trisphosphate (PIP3) at the tip of the nascent axon is thought to act as a landmark for the induction of neuronal polarization. Several studies[69,73,76] have proposed a model of neuronal polarization in which local activation of the PI3K–Akt pathway leads to the inactivation of GSK3β by inducing Ser9 phosphorylation. This model is supported by the finding that the myristoylated, constitutively active form of Akt (Myr-Akt), but not the wild-type, induces the formation of multiple axons — a process that is partially blocked by overexpression of GSK3β-Ser9Ala. Moreover, ectopic expression of PTEN (a phosphatase that counteracts the actions of PI3K) prevented axon formation, and this effect was reversed by a GSK3 inhibitor[69]. Conversely, knocking down *Pten* induced the formation of multiple axons, which was prevented by expression of GSK3β-Ser9A. This led the authors to conclude that GSK3β acts downstream of PI3K–Akt[69]. However, neurons from GSK3α-Ser21Ala/GSK3β-Ser9Ala double knock-in mice develop normal polarity *in vivo* and *in vitro*[92]. Importantly, these neurons still form multiple axons when treated with various GSK3 inhibitors. These results confirm that regulation of GSK3 activity is crucial for neuronal polarization, but suggest that there might be alternative mechanisms for GSK3 inactivation. Similarly, the finding that expression of Myr-Akt induces multiple axons suggests that there is an alternative pathway downstream of Akt for the regulation of polarization.

GSK3 signalling and the PAR3–PAR6–PKCζ polarity pathway. The PAR3–PAR6–PKCζ complex is required for axon–dendrite specification of hippocampal neurons. Before polarization, PAR3 and PAR6 are localized at the tips of all processes but later they become selectively enriched in the nascent axon and in the developing growth cone[74,93]. This is consistent with the roles of PAR3 and PAR6 as negative regulators of GSK3. Localized inhibition of GSK3 at the nascent axon is also required for the polarized transport and proper localization of PAR3 (REF. 73), suggesting that there is feedback regulation between PAR3 and GSK3.

Similar to the mechanism (mentioned above) that controls directed cell migration through the PAR3–PAR6–GSK3 pathway[37], in hippocampal neurons Dvl becomes enriched at the tip of the axon in response to Wnt5a and induces axon formation through activation of PKCζ in the PAR3–PAR6–PKCζ complex[94]. In these neurons, *Dvl* overexpression leads to the production of multiple axons but does not affect GSK3β-Ser9 phosphorylation, and Dvl is still able to induce multiple axon formation in the presence of GSK3β-Ser9Ala[94]. Based on these results the authors concluded that this pathway might not involve GSK3β, but might employ microtubule affinity-regulating kinase 2 (MARK2) as an alternative downstream effector to control microtubule dynamics[94]. In this model, activation of PKCζ in response to Wnt5a–Dvl leads to the inhibitory phosphorylation of MARK2, which then causes dephosphorylation of MAPs, such as Tau (FIG. 4). Consistent with this model, an earlier observation showed that knocking down *Mark2* decreased Tau phosphorylation and induced the formation of multiple axons, whereas ectopic expression of MARK2 increased Tau phosphorylation and blocked axon growth[68]. However, based on the remarkable similarity of the factors (Wnt5a–Dvl–PKCζ) that are involved in neuronal polarization[94] and cell migration[37], it could be suggested that GSK3 inactivation has a role downstream of Wnt–Dvl–PKCζ to control neuronal polarization as well. Interestingly, in *Drosophila*, MARK acts as a priming kinase, triggering subsequent Tau phosphorylation by GSK3β[95]. This suggests that inactivation of MARK2 and GSK3β at the axonal tip together

Microtubule plus end-binding proteins
A family of microtubule-binding proteins that specifically track the growing ends (plus ends) of microtubules and function to stabilize microtubules or to mediate the interactions between microtubules and other cellular structures.

Microtubule plus end
The more quickly polymerizing end of microtubule polymers.

Kinetochore
A specialized condensed region on the chromosome to which the spindle fibres attach during cell division.

Cell cortex
A cytoplasmic region beneath the plasma membrane that functions as a mechanical support of the plasma membrane.

Myristoylation
An irreversible protein modification that covalently attaches a myristoyl group, using an amide bond, to the α-amino group of an amino-terminal amino acid of a nascent polypeptide.

reduces Tau phosphorylation downstream of the PAR3–PAR6–PKCζ complex. The situation is further complicated by the findings that GSK3β can directly activate[96] as well as inhibit[97] MARK2 through phosphorylation. The exact roles of MARK2 and GSK3β, and their interplay during neuronal polarization remain to be fully defined (FIG. 4).

GSK3 signalling and the LKB1–SAD pathway. The SAD kinases SADA and SADB (also known as BRSK2 and BRSK1, respectively), which are expressed in the developing nervous system, contain a kinase domain that is related to that of *Caenorhabditis elegans* PAR-1 and its vertebrate orthologues, MARK1–MARK4 (REF. 70). In the brains of $SadA^{-/-}SadB^{-/-}$ (*Sad*-null) mice, segregation of neuronal subtypes to sublayers within the cortical plate was disordered and major axonal tracts were missing, which suggests that SAD kinases are required for neural development *in vivo*. When cultured *in vitro*, hippocampal neurons from *Sad*-null mice developed processes of equivalent lengths, each of which containing dephosphorylated Tau (labelled by Tau1), which is often used as an axonal marker, and MAP2, which is normally enriched in dendrites[70]. In the brains of *Sad*-null mice, Tau1 was found not only in the axon-rich intermediate zone but also in the cortical plate where dendrites elaborate[70], which suggests that SAD kinases play a pivotal part in neuronal polarization.

Similarly, neurons lacking *Lkb1*, the gene that encodes the serine/threonine kinase LKB1 and that is the orthologue of abnormal embryonic partitioning of cytoplasm (*par-4*) in *C. elegans*, developed neurites of equivalent length that contain dephosphorylated Tau and MAP2 (REFS 67,72). In the cortices of *Lkb1* knockout mice levels of phosphorylated SADA and SADB were greatly reduced, and biochemical experiments revealed that LKB1 phosphorylated SAD kinases[67]. Phosphorylated forms of LKB1 and SAD kinases were found to be concentrated in axons, and LKB1-mediated SAD phosphorylation was correlated with Tau phosphorylation[67,72]. Thus, once LKB1 is activated in axons, it activates SAD kinases, which then phosphorylate MAPs such as Tau. Although it seems contradictory that SAD kinases are active in axons, which generally have low levels of phosphorylated Tau[98], these studies[66,67,70,72] provide convincing evidence that LKB1 and SAD kinases are required for neuronal polarization *in vivo*. Interestingly, when *Lkb1* was knocked down by *in utero* electroporation, Ser9-phosphorylated GSK3β was not detectable at the tip of any neurites in cortical neurons despite the uniform distribution of GSK3β[66], which suggests that GSK3β could be a downstream target of LKB1 (FIG. 4). To date, it remains unclear whether the defects in neuronal polarization observed in *Lkb1*-depleted neurons involve GSK3 signalling. It is, however, interesting to note that XEEK1 (the *Xenopus* orthologue of LKB1) regulates GSK3 activity and physically associates with GSK3β and atypical PKC (aPKC) *in vivo*[99]. Future studies should examine whether LKB1 regulates axonal specification by inhibiting GSK3β in addition to activating SAD kinases in the mammalian nervous system.

GSK3 signalling and the TSC–mTOR pathway. Mutations in *TSC1* and *TSC2* cause tuberous sclerosis, a disease characterized by tumour predisposition and neurological abnormalities that include epilepsy, mental retardation and autism[100]. TSC1 and TSC2 form a heterodimer that negatively regulates mammalian target of rapamycin (mTOR) signalling[101]. On activation of the PI3K–Akt pathway, Akt induces inhibitory phosphorylation of TSC2, by which its GTPase activating protein (GAP) activity towards Rheb is reduced, subsequently leading to the activation of mTOR kinase (FIG. 4). mTOR then phosphorylates translational regulators, such as ribosomal S6 kinase (S6K) and eukaryotic translation initiation factor 4E-binding protein (4EBP), and thereby induces protein synthesis[102–104]. It has been suggested that inactivation of the TSC1–TSC2 complex and subsequent mTOR activation in a single neurite regulates axon formation by inducing the translation of polarity proteins such as SAD kinases[105]. In support of this idea, phosphorylated forms of S6K and 4EBP are enriched in the axon during polarization, which suggests local activation of mTOR signalling[105,106]. Together with the elevation of SAD kinase activity by LKB1 (REF. 67) that is mentioned above, the control of SAD protein levels by TSC–mTOR signalling might have a role in axon specification. Because GSK3α-Ser21Ala/GSK3β-Ser9Ala double knock-in mice show normal neuronal polarization[92], Akt phosphorylation of TSC2 has been suggested as an alternative mechanism to convey PI3K–Akt signalling.

Notably, a direct interaction between TSC–mTOR signalling and GSK3 has been reported in the regulation of cell growth. Inoki *et al.*[107] showed that inhibition of GSK3 is sufficient to activate mTOR-dependent protein translation through regulation of TSC2 phosphorylation. It is therefore plausible that suppression of GSK3 activity in the axonal growth cone has a role in the activation of local translational machinery that is used in the synthesis of key proteins for axonogenesis. Interestingly, mTOR activation in the axon leads to local translation of *Crmp2* and *Tau* genes, which are substrates of GSK3[106]. It will be of particular interest to investigate whether the suppression of GSK3 activity at the tip of the axon controls neuronal development by regulating local translation of polarity genes — including its own substrates — as well as directly controlling microtubule dynamics by regulating MAP phosphorylation.

A role of GSK3 activation in dendrites? Local inhibition of GSK3 in the axon indicates higher GSK3 activity in dendrites. Does active GSK3 in dendrites play a part in polarization? Local protein degradation through activation of the UPS is a possible mechanism for the control of asymmetrical accumulation of polarity proteins. Inhibition of the UPS with MG132 or lactacystin before or after the establishment of neuronal polarity leads to the formation of multiple axons[108], which suggests that UPS activation is required for the establishment and maintenance of neuronal polarity. Interestingly, Akt, PAR3 and aPKC are ubiquitylated at early stages of polarization before they accumulate at axonal tips[108].

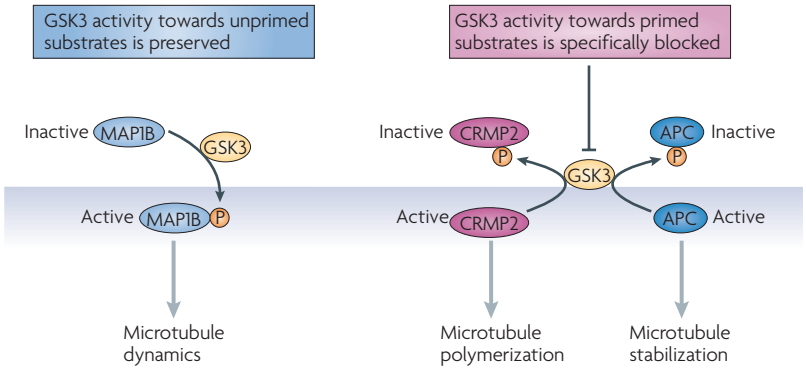

Figure 5 | **Differential regulation of GSK3 substrates during axon growth.** During rapid axon extension, glycogen synthase kinase 3 (GSK3) activity in the growth cone seems to be precisely controlled, so that GSK3 activity towards primed substrates is specifically blocked while its activity towards unprimed substrates is preserved[31]. Inhibition of GSK3 activity towards collapsin response mediator protein 2 (CRMP2) and adenomatosis polyposis coli (APC) allows CRMP2 and APC to remain active and bind microtubules, thereby increasing microtubule polymerization and stability[76,85]. By contrast, GSK3 activity towards microtubule-associated protein 1B (MAP1B) is preserved in the growth cone. Phosphorylation (activation) of MAP1B maintains microtubules in a dynamic state, and this is essential for axon growth[15,112]. In this way, GSK3 can coordinate essential properties of axonal microtubules to ensure optimal microtubule assembly in axons.

UPS inhibition restores the presence of Akt in all neurites and this restoration is accompanied by multiple axon formation. This suggests that Akt is a target of the UPS for local degradation in dendrites[108]. UPS inhibition does not seem to alter the protein level of GSK3β but an increase in GSK3β-Ser9 phosphorylation has been observed[108], suggesting changes in its activity. A recent study[55] indicates that GSK3β regulates proteolytic degradation of a much broader range of proteins than was previously appreciated[55]. It will be interesting to investigate whether GSK3 activation in dendrites is involved in the regulation of local protein degradation that contributes to the redistribution of key molecules during polarization.

GSK3 signalling in axon outgrowth

It is generally accepted that coordinated regulation of local axon assembly at the growth cone and of gene transcription in the neuronal soma is required for efficient axon growth[109]. In this section, we will discuss evidence for GSK3 signalling in these processes.

Differential regulation of GSK3 substrates to control local axon assembly. Local inhibition of GSK3 signalling is essential for promoting microtubule assembly at the growth cone. However, in other studies, inhibition of GSK3 has been found to block axon growth. To reconcile these contradictory data, Kim *et al.*[31] suggested a model in which inhibition of GSK3 can both enhance and prevent axon growth depending on the substrates that are involved. An interesting feature of GSK3s is that many substrates of GSK3s require phosphorylation by a distinct kinase — an event known as priming — before they can be phosphorylated by GSK3s (primed substrates), although GSK3s can also phosphorylate some substrates without priming (unprimed substrates). Among GSK3 substrates that regulate microtubule assembly at the growth cone, APC and CRMP2 are primed substrates, and GSK3 phosphorylation abrogates their microtubule-binding affinity[76,88]. Consistent with local inhibition of GSK3 in the distal axon, dephosphorylated forms of CRMP2 and APC are enriched in the growth cone, and this promotes axon formation and mediates neurotrophin-induced axon growth[76,110]. Inhibition of GSK3 activity specifically towards primed (but not unprimed) substrates (using a GSK3β–Arg96Ala construct)[28,111] results in reduced CRMP2 phosphorylation and increased axon outgrowth[31]. By contrast, MAP1B is an unprimed substrate that can be phosphorylated by GSK3 on Ser1260 and Thr1265 without priming[90]. Phosphorylation of MAP1B at these sites renders microtubules more dynamic, so that they can efficiently probe the intracellular space and respond to extracellular signals, and these activities are essential for axon growth[15,112]. Therefore, to ensure efficient axon growth, GSK3 activity should be precisely controlled so that its activity towards one subset of substrates is specifically blocked while its activity towards others is preserved (FIG. 5). Indeed, phosphorylated MAP1B is enriched at the distal ends of growing axons[90], where dephosphorylated forms of APC and CRMP2 are concentrated.

These findings have led to a working model in which preferential suppression of GSK3 activity towards primed substrates promotes axon growth, whereas inhibition towards unprimed substrates blocks axon growth[31] (FIG. 5). Identification of additional GSK3 substrates that are enriched in the growth cone will help to test this hypothesis and to better understand how GSK3 signalling controls local axon assembly. Moreover, further studies are required to elucidate the molecular mechanisms by which GSK3 activity towards different substrates can be differentially regulated.

Transcriptional control of axon outgrowth by GSK3 signalling. In addition to stimulating the tips of axons for cytoskeletal regulation, GSK3 has been implicated in the transcriptional control of axon growth through regulation of β-catenin and the NFAT family of transcription factors. In the canonical Wnt pathway, Wnt3a induces axon growth from developing sensory neurons through accumulation of β-catenin and subsequent activation of TCF4 (REF. 113) (FIG. 6), providing evidence for a role of GSK3–β-catenin signalling in axon growth. GSK3 could also play a part in the transcriptional control of neurotrophin-induced axon growth by phosphorylating CREB and NFAT proteins. CREB is a well-established transcription factor downstream of neurotrophins, and *Creb*-null mice display impaired axon growth from sensory and sympathetic ganglion neurons[114]. Neurotrophins (and other stimuli) lead to the activation of CREB kinases, which phosphorylate CREB on the transcriptional regulatory site Ser133, thereby recruiting the

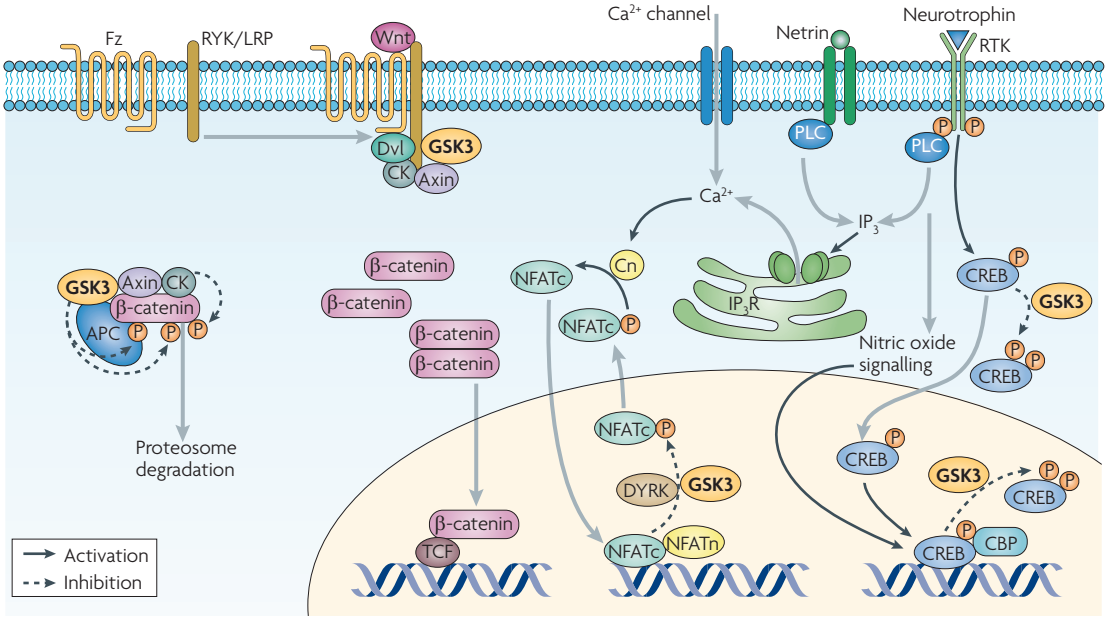

Figure 6 | **Potential roles of GSK3 in the transcriptional regulation of axon growth.** In the absence of Wnt, the transcriptional co-activator β-catenin is phosphorylated and thereby inhibited by glycogen synthase kinase 3 (GSK3) in the destruction complex. This tags β-catenin for proteasomal degradation. Wnt promotes the association of frizzled (Fz) receptor and the co-receptor low-density lipoprotein receptor-related protein (LRP) or RYK[113], which leads to recruitment of the cytoplasmic protein dishevelled (Dvl) and the destruction complex to the membrane. The consequent inhibition of GSK3 activity results in the accumulation of hypophosphorylated β-catenin, which then can enter the nucleus and promote T cell factor (TCF; also known as lymphoid enhancer-binding factor)-mediated gene transcription. Another family of transcription factors that is involved in axon growth is nuclear factor of activated T cells (NFAT) proteins. Neurotrophins and netrins require calcineurin (Cn)–NFAT signalling to induce axon growth[117]. A rise in intracellular Ca^{2+} induced by neurotrophins, netrins or calcium channels activates the serine/threonine phosphatase Cn, which dephosphorylates (and thereby activates) NFATc proteins. This triggers the translocation of NFATc to the nucleus, where it forms complexes with NFATn to induce gene transcription. NFATc proteins are rapidly excluded from the nucleus through sequential inhibitory phosphorylation, first by a priming kinase, such as protein kinase A (not shown) and dual-specificity tyrosine-phosphorylation regulated kinase 1A (DYRK1A) and then by GSK3 (REF. 9). Because NFAT phosphorylation by GSK3 inhibits its DNA-binding activity[10] and is required for its nuclear export[9], GSK3 could have a role in NFAT-mediated gene transcription downstream of neurotrophin and/or netrin signalling. Cyclic AMP response element binding protein (CREB) is a well-established transcription factor that mediates neurotrophin-induced axon growth[114]. Neurotrophins and other stimuli lead to the phosphorylation of CREB at Ser133, which activates CREB and allows it to bind to the transcriptional co-activator CREB-binding protein (CBP). In developing neurons, DNA-binding activity of CREB is tightly controlled through nitric oxide signalling, as opposed to the prevailing view that CREB is constitutively bound to promoter sequences[116]. Similar to NFATc, GSK3 phosphorylation of CREB is inhibitory and prevents CREB from binding to DNA[8]. Because GSK3 is found in the cytoplasm and the nucleus, CREB phosphorylation by GSK3 can occur in both of these places. However, it should be noted that GSK3 regulation of NFAT and CREB has not been directly tested in the context of axon growth. APC, adenomatous polyposis coli; CK, casein kinase; IP_3, inositol-1,4,5-trisphosphate; IP_3R, IP_3 receptor; PLC, phospholipase C; RTK, receptor tyrosine kinase.

transcriptional co-activator CREB-binding protein (CBP) and so promoting the assembly of basal transcriptional complex[115]. In developing neurons the DNA-binding activity of CREB is tightly controlled by extracellular stimuli via nitric oxide-dependent signalling, which contrasts with the prevailing view that CREB is constitutively bound to promoter sequences[116]. As CREB phosphorylation by GSK3 abrogates its DNA-binding activity[8], it is plausible that GSK3 and nitric oxide signalling work in concert to control the duration and intensity of CREB-dependent transcription (FIG. 6). Neurotrophins and netrins also induce transcription of genes that are essential for axon growth by triggering Ca^{2+}/calcineurin-dependent nuclear translocation of NFAT family of proteins, and mice that lack calcineurin–NFAT signalling display profound defects in sensory axon projection and commissural axon growth[117]. Because NFAT phosphorylation by GSK3 inhibits its DNA-binding activity[10] and is required for nuclear export[9], GSK3 is likely to have a role in NFAT-mediated gene transcription during axon growth (FIG. 6). Given that many other transcription factors are modulated by GSK3, additional evidence for the transcriptional control of axon growth by GSK3 signalling awaits to be determined.

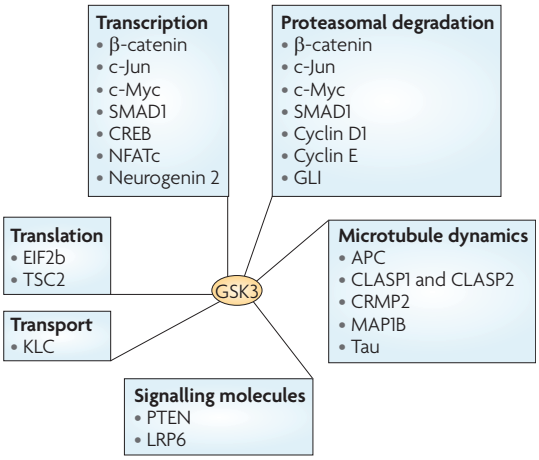

Figure 7 | **Representative substrates of GSK3 that are implicated in neural development.** Regulation of neural development by glycogen synthase kinase 3 (GSK3) is achieved through the phosphorylation of proteins that control key steps in neurodevelopment. GSK3 phosphorylates several transcription factors, such as β-catentin[122], c-Jun[123], c-Myc[48], SMAD1 (REF. 12), cyclic AMP response element binding protein (CREB)[8], nuclear factor of activated T cells (NFATc)[9] and neurogenin 2 (REF. 11). Many of these transcription factors undergo proteasomal degradation after GSK3 phosphorylation. GSK3 phosphorylation mediates proteasomal targeting and degradation of other proteins as well — for example, cyclin D1 (REF. 124) and cyclin E[125]. The most well-characterized substrates of GSK3 that are involved in neural development are microtubule-associated proteins that control neuronal polarization and axon growth. These substrates include adenomatosis polyposis coli (APC)[88], CLIP-associated protein 1 (CLASP1) and CLASP2 (REF. 126), collapsin response mediator protein 2 (CRMP2)[76], microtubule-associated protein 1B (MAP1B)[127] and Tau. Although CLASP is enriched in the growth cone and mediates Slit-induced axon repulsion during neural development in *Drosophila*[128], the localization and role of CLASP during neuronal polarization and/or axon growth in the mammalian nervous system remains to be determined. Signalling molecules, such as phosphatase and tensin homologue (PTEN)[129] and Wnt co-receptor low-density lipoprotein receptor-related protein 6 (LRP6), are also phosphorylated by GSK3. Other GSK3 substrates are kinesin light chain (KLC), which regulates selective transport[130], and essential components of the translational machinery, such as eukaryotic initiation factor 2B (EIF2B)[131] and tuberous sclerosis 2 (TSC2)[107].

Concluding remarks and future directions

An algorithm that integrates consensus sequence motifs with contextual information has predicted that GSK3 is a kinase with a large number of substrates[77]. Although further studies are required to validate the physiological relevance of this prediction, the large number of putative substrates suggests that GSK3 could affect a broad range of cellular activities (FIG. 7). Indeed, recent studies implicate GSK3 in many fundamental processes during neural development, and a number of neurological disorders are associated with deficits in GSK3 signalling. It is therefore important to understand GSK3 signalling, and this requires, first, a re-evaluation of the involvement of GSK3 using more reliable methods to detect GSK3 activation or inactivation, and second, the identification of additional *in vivo* GSK3 substrates.

The emerging picture is that GSK3 activity is mainly controlled by protein–protein interactions. Considering its many substrates, regulation of GSK3 by a complex and dynamic interaction network is an attractive model because it allows pathway-specific control of GSK3 activity through signal-dependent formation of protein complexes as well as precise regulation of GSK3 activity towards particular substrates. It remains to be determined whether multi-protein complexes, as exemplified in the Wnt signalling pathway, form a widespread mechanism for controlling the specificity of GSK3 actions. A distinguishable characteristic of GSK3 is the requirement of a priming event that involves activation of another kinase. Thus, in combination with the signalling that directly regulates GSK3 activity, signalling that regulates the phosphorylation status of its substrates allows GSK3 signalling to achieve further specificity.

With respect to GSK3 regulation, studies so far have focused on the inhibitory mechanism. However, it is becoming increasingly apparent that activation of GSK3 is an important aspect of the regulatory scheme, and this suggests that a delicate balance between activation and inactivation is required to shape GSK3 signalling. The data so far support the view that GSK3 activity is subjected to multiple layers of sophisticated regulation to ensure that GSK3 encounters its substrates in the right place at the right time. It will be of great interest to investigate how the complexity of GSK3 signalling is orchestrated *in vivo* to exert exquisite control over a plethora of cellular processes during neural development.

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Acknowledgements
The authors were supported by the Whitehall Foundation (to F.Q.Z), the Basil O'Connor Starter Scholar Research Award from the March of Dimes foundation (to F.Q.Z) and the US National Institutes of Health grant 1R01NS064288. We apologize to authors whose work we were unable to include owing to space constraints.

Competing interests statement
The authors declare no competing financial interests.

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