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Regulation of Cell Fate in the Brain by GSK3

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1. Introduction

The human brain is the most complex object in the known universe. It contains ~100 billion neurons, each forming between 1,000 and 10,000 connections with other neurons to form interconnecting circuits containing up to 1,000 trillion connections. This extreme complexity arises from a single cell at conception. Therefore, brain development needs to be tightly controlled to ensure proper patterning and circuit formation. Defects in this process lead to debilitating neurodevelopmental disorders, including autism and microcephaly. Until recently, it was assumed that once an adult brain had matured, it was 'fixed' or 'hard-wired' and no new neurons were generated. However, research over the last 15-20 years has dem-onstrated the existence of neural precursor cells (NPC's) that produce and incorporate new neurons into existing circuits of the adult brain, a process known as neurogenesis [1]. Adult neurogenesis is similar in organisation and mechanism to early brain development [2]. The ability to control neurogenesis could enable the brain to repair itself following injury (e.g. stroke, spinal chord injury, head trauma) and to enhance mental functioning (e.g. delay or prevent age-related cognitive decline and neurodegenerative diseases). Therefore, there is a clear and urgent need to understand the mechanisms controlling neurogenesis in the devel-oping and adult brain.

NPC's give rise to all cell types in the brain by undergoing asymmetric cell division, generating one daughter cell that retains pluripotency and another daughter cell that is committed to a neuronal or glial fate. In adults, this predominantly occurs in the dentate gyrus of the hippo-campus and the subventricular zone/olfactory system. Thousands of new cells are generated every day in the hippocampus, although less than half survive beyond a few weeks to permanently integrate into adult brain circuits [3]. This process is an important component of neuroplasticity in the hippocampus, facilitating learning and memory. Other brain regions are thought to have limited neurogenic potential that might be induced following injury. The fate of NPC's is controlled by extracellular stimuli (e.g. growth factors, Wnt, Notch, Hedgehog)

that trigger intracellular signalling pathways and changes in gene transcription, although the transcriptional regulators targeted by these signalling pathways and their target genes are yet to be fully clarified.

2. GSK3 and neurogenesis

In 1980, Hammond and Dale noticed that lithium treatment of grey collie dogs increased their blood cell counts, which they suggested was due to increased proliferation of haematopoetic stem cells [4]. This was confirmed shortly afterwards [5], although the mechanism for lithium's action was not understood. In 1996, GSK3 was identified as a key target of lithium in cells [6], although it was another 8 years before a key role for GSK3 in regulating stem cell pluripotency was elucidated [7, 8]. Pharmacological inhibition of GSK3 activity was shown to maintain the undifferentiated phenotype in mouse and human embryonic stem (ES) cells, while its withdrawal promoted differentiation into multiple cell lineages [7]. More recently, it was demonstrated that the complex mixture of cytokines, growth factors, hormones, serum and feeder cells traditionally used to maintain self-renewal of ES cells can be replaced with two pharmacological inhibitors; a MAPK inhibitor and a GSK3 inhibitor [8], thus emphasizing the importance of GSK3 for regulating pluripotency. The GSK3 substrates c-myc [9] and Klf5 [10] are among several transcription factors that have been used to induce pluripotency (iPS system). Thus, GSK3 is a key regulator of neurogenesis, although the precise molecular mechanisms are not yet fully understood. This review provides an overview of the extracellular stimuli and intracellular signalling pathways controlling GSK3 activity, as well as the downstream targets of GSK3 directly linking it to cellular proliferation and differentiation in the brain. GSK3 inhibitors are currently in clinical trials for several neurological disorders associated with impaired neurogenesis, therefore it is timely that cell fate pathways involving GSK3 are delineated.

3. GSK3

GSK3 is a Ser/Thr kinase of the CMGC family of proline-directed kinases that is highly conserved in all eukaryotes. In mammals, it is ubiquitously expressed in all tissues and subcellular organelles, most highly in the brain [11]. There are 2 isoforms encoded by separate genes (chromosome 19q13.2 for GSK3α and chromosome 3q13.3 for GSK3β) [11]. Their kinase domains are 98% homologous and their substrate specificities are similar, but not identical [12]. A splice variant of GSK3β containing a 13 amino acid insert in the catalytic domain is specifically expressed in the brain [13], although its function is only just beginning to be investigated [12]. Interestingly, GSK3 is one of the most unusual kinases in the human genome for 3 main reasons; 1) Most (if not all) substrates require 'priming' phosphorylation 4 or 5 residues C-terminal to the GSK3 target site by another kinase *before* they can be efficiently phosphorylated by GSK3 [14]. 2) GSK3 is highly active in cells under basal conditions, opposite to most other kinases. 3) Phosphorylation of GSK3 at an N-terminal serine residue *inhibits* its kinase activity

(Ser21 in GSK3α, Ser9 in GSK3β) [15, 16]. This phosphoserine acts as a pseudo-substrate and binds to the phosphate-binding pocket on GSK3, preventing interaction with primed substrates [17]. Phosphorylation is mediated by members of the AGC family of kinases (e.g. Akt) and commonly occurs downstream of growth factor and PI3K signaling [15]. Activation of the canonical Wnt signaling pathway also inhibits GSK3 activity, preventing phosphorylation of β-catenin, although this is not mediated by N-terminal phosphorylation, but by protein-protein interactions [18, 19]. GSK3 is also modified by phosphorylation at a tyrosine residue on the activation loop of the kinase domain (Tyr279 for GSK3α and Tyr216 for GSK3β). Phosphorylation at these sites is absolutely required for kinase activity and is most likely constitutively modified (i.e. not regulatable) [20, 21].

4. Neurogenesis in GSK3 mutant mice

Valuable information on the role of GSK3 in cell fate determination has been obtained from mice genetically modified to either increase or decrease expression of GSK3α and β. While GSK3β-knockout mice die in late development due to defects in heart development and/or hepatic apoptosis [22, 23], GSK3β-heterozygous mice and GSK3α-knockout (homozygous) mice are viable and display several behavioural defects, including increased anxiety, decreased aggression and memory defects [24-28]. Also, GSK3α-null mice exhibit decreased numbers and size of Purkinje cells in the cerebellum [24]. Conditional overexpression of GSK3β in the forebrain using the doxycycline/Tet system impaired memory and spatial learning in mice [29]. At the cellular level, GSK3β overexpression increased neuronal cell death, astrocytosis, gliosis and reduced LTP induction. These effects could be restored by reducing GSK3 activity to normal levels by silencing the transgene or by treatment with lithium [30, 31]. In another report, overexpression of GSK3β-S9A in post-natal neurons (Thy-1 promoter) reduced brain size in adult mice, especially in the cerebral cortex, predominantly caused by reduced size of neuronal cell bodies and the somatodendritic compartment [32]. Together, these observations clearly demonstrate that GSK3 is important for healthy development and function of the brain.

In addition to conventional under/over-expression mouse models, GSK3-knockin mice were developed that are insensitive to growth factor inhibition (Ser21/9 mutated to Ala in GSK3α and β, respectively), but remain sensitive to Wnt-induced inhibition [33]. These mice are viable and display no overt developmental or growth defects, but do exhibit increased susceptibility to hyperactivity, stress-induced depression and mild anxiety, as well as abnormal LTP and memory functions [34, 35]. NPC's isolated from GSK3-knockin mice exhibit reduced neurogenesis, despite normal proliferation [36], suggesting defective differentiation/maturation or survival of NPC's. In contrast, mice with double homozygous deletion of GSK3α and β isoforms (i.e. all GSK3 isoforms deleted) display a dramatic increase in proliferation of NPC's and decreased differentiation into post-mitotic neurons [37]. This is accompanied by deregulation of Wnt, Notch, Hedgehog and FGF signalling pathways. In another mouse model, mice expressing a mutant form of the scaffolding protein Disrupted in Schizophrenia (DISC1), which is mutated in schizophrenia and mood disorder patients, display increased GSK3 activity, causing inhibition of the Wnt signaling pathway and decreased NPC proliferation

[38]. Together, these observations suggest that inhibition of GSK3 by the Wnt signalling pathway promotes NPC proliferation, while inhibition of GSK3 by growth factor signalling promotes differentiation of NPC's into post-mitotic neurons.

5. GSK3 and Wnt signaling

Wnt signaling is amongst the most important signaling pathways controlling neurogenesis in the developing and adult brain. Several studies have shown that attenuation of this pathway reduces the number of granule neurons and size of the hippocampus, as well as a reduction in the number of radial glial cells due to proliferation and patterning defects [39-41]. It is well accepted that Wnt's help to maintain the proliferative capacity of ES cells and keep them in an undifferentiated state [7], however there is also evidence for Wnt's being required to promote neural differentiation [42, 43]. These seemingly contradictory views are united in a model whereby Wnt signaling promotes proliferation and inhibits differentiation of pluripotent cells. Inhibition of this pathway promotes initial stages of differentiation into neural progenitors, but reactivation of Wnt signaling is required at later stages of the differentiation program to generate mature neurons [44, 45]. Indeed, in adult neurogenesis in the dentate gyrus of the hippocampus, Wnt's are secreted by local astrocytes where they predominantly regulate differentiation into mature neurons (mostly inhibitory GABAergic interneurons), rather than proliferation of NPC's [46-48].

GSK3 is a key component of the Wnt signaling pathway. In unstimulated cells, GSK3 is part of a multi-protein complex with APC, Axin and other proteins that facilitates phosphorylation of β-catenin by CK1 at Ser45, followed by phosphorylation of Ser33/37/41 by GSK3. This creates a recognition site for the E3 ubiquitin ligase βTrCP, which ubiquitinates β-catenin and targets it for degradation via the proteasome. In Wnt-stimulated cells, this multi-protein complex is disrupted, preventing phosphorylation and subsequent ubiquination of β-catenin, thus stabilizing the protein and increasing its cellular abundance. This leads to translocation to the nucleus, where β-catenin binds to several transcription factors facilitating transcription of target genes involved in cell fate regulation, including c-myc [49], NeuroD1 [48, 50, 51], Prox1 [52] and LINE-1 [50]. In adult neurogenesis, Wnt-induced expression of NeuroD1 is required for survival and maturation of adult-born neurons [48, 50, 51]. The prototypcial binding partners of β-catenin in the nucleus are members of the TCF/LEF family, which have been shown to be central mediators of tumourigenesis in the colon, breast and other tissues. In NPC's, activation of the Wnt pathway and elevated transcriptional activity of β-catenin has been shown to promote proliferation and inhibit neuronal differentiation [53, 54], while inhibition of Wnt signaling promotes neuronal differentiation [55, 56]. Accordingly, deletion of GSK3 isoforms promotes proliferation and inhibits differentiation of stem cells [57], consistent with pharmacological GSK3 inhibitors and Wnt-induced inhibition of GSK3 activity [7, 8]. This is dependent on elevated levels of transcriptionally active β-catenin [58], but surprisingly not by members of the TCF/LEF family. Instead, β-catenin was shown to bind to another transcription factor called Oct4, which increased expression of the pluripotency regulator and stem cell marker Nanog [58, 59].

Some transcriptional targets of the Wnt pathway are also targeted by GSK3 post-translation-ally. For example, *c-myc* is an established target of the Wnt pathway that promotes cell cycle progression and proliferation [49]. Meanwhile, its protein product is directly phosphorylated by GSK3 at Thr58, which targets it for ubiquitination by the E3 ligase Fbw7, followed by proteasome-mediated degradation [60, 61]. Thus, Wnt-induced inhibition of GSK3 activity could promote high c-myc activity both transcriptionally and post-translationally. However, it has not yet been proven that Wnt-mediated inhibition of GSK3 reduces phosphorylation of c-myc, or any protein other than β-catenin for that matter. Alternatively, simultaneous stimulation of cells by Wnt and growth factors would activate c-myc transcription and reduce its phosphorylation and degradation, respectively, thus combining to increase c-myc abun-dance. This would promote proliferation and inhibit differentiation of NPC's. Interestingly, a viral oncogenic form of c-myc is mutated at the GSK3 target site (Thr58) [60]. This mutation prevents phosphorylation of c-myc by GSK3 and subsequent ubiquitination, thus stabilizing the protein and driving uncontrolled proliferation in tumourigenesis. Thus, emphasizing the importance of phosphorylation of c-myc by GSK3 in the regulation of cell fate.

Other isoforms of c-myc are also phosphorylated and targeted for degradation by GSK3 (i.e. L-myc, N-myc). In NPC's, deletion of c- and L-myc does not affect proliferation/differentiation, while deletion of N-myc significantly decreases NPC proliferation and impairs differentiation into mature neurons [62], suggesting that N-myc is the critical member of this family regulating neurogenesis and brain development [63, 64]. Like c-myc, GSK3 phosphorylates N-myc at Thr58 to promote ubiquitination by Fbw7 and degradation by the lysosome [65]. This is antagonized by growth factor-mediated inhibition of GSK3 activity (e.g. IGF1). Phosphoryla-tion of N-myc by GSK3 requires prior 'priming' phosphorylation at Ser62 by Cdk1, which is increased during mitosis, causing increased N-myc degradation [65]. This was shown to be important for exiting the cell cycle – the first step along the differentiation pathway. Cdk1 activity is dependent on binding to its co-factors cyclin A and B1 [66], whose transcription is controlled by the Hedgehog pathway, as is the transcription of N-myc [67, 68]. Therefore, N-myc appears to be a point at which multiple signaling pathways involving GSK3 intersect in NPC's to control cell fate.

Some substrates of GSK3 are upstream of the Wnt pathway and can regulate its activity. For example, hypoxia-inducible factor 1 α (HIF1α) is a basic helix-loop-helix (bHLH)-structured transcription factor that is induced by low oxygen conditions to activate transcription of genes that provide protection and adaption of cells to oxidative stress and hypoxic conditions. HIF1α is phosphorylated by GSK3, promoting its degradation by the proteasome [69, 70]. Recently, it was shown that HIF1α promotes Wnt activation and transcription of TCF/LEF members in undifferentiated, but not differentiated cells [71]. Low GSK3 activity in undiffer-entiated cells would reduce GSK3-mediated phosphorylation and degradation of HIF1α, thus stabilizing the protein and leading to activation of the Wnt pathway. Simultaneously, low GSK3 activity (downstream of Wnt) would prevent β-catenin phosphorylation/degradation, increasing its transcriptional activity with TCF/LEF. Interestingly, the authors show that the subgranular zone of the dentate gyrus containing NPC's is hypoxic due to fewer blood vessels in the region and contains relatively high levels of HIF1α and transcriptionally active β-catenin.

Thus, decreased oxygen levels could be important for maintaining the NPC niche in the hippocampus via GSK3, HIF1α and the Wnt pathway. It be should be noted that other groups have found that prolonged hypoxia increases the activity of GSK3 in cultured cells and *in vivo* [69, 72]. This might reflect differences between GSK3-mediated phosphorylation and degradation of HIF1α in undifferentiated versus differentiated cells.

6. GSK3, DISC1 and mood disorders

GSK3's involvement in the Wnt pathway is also regulated by the scaffolding protein DISC1. This protein directly binds to GSK3 to inhibit phosphorylation of β-catenin, preventing its degradation and activating its transcriptional activity [38]. This promotes NPC proliferation during embryonic development and in the adult brain. Depletion of DISC1 or expression of DISC1 mutants associated with mood disorders and schizophrenia reduced NPC proliferation and induced schizophrenia and depression-like symptoms in mice [38, 73]. These defects were normalized by administration of pharmacological inhibitors of GSK3. These studies demonstrate that DISC1 is a negative regulator of the Wnt pathway and NPC proliferation by directly inhibiting GSK3-mediated phosphorylation of β-catenin. Interestingly, one schizophrenia-associated mutant of DISC1 (S704C) did not affect GSK3 activity, Wnt signaling or NPC proliferation, but instead impaired neuronal migration in the developing cortex via reduced binding to cytoskeletal proteins (Dixdc1) [73]. Elsewhere, it was shown that DISC1 acts as a molecular switch between proliferation and migration in NPC's, whereby DISC1 inhibits GSK3-mediated phosphorylation of β-catenin and activates its transcriptional activity to drive proliferation of NPC's, while in committed, post-mitotic neurons, DISC1 regulates neuronal migration via another protein called BBS1 and the centrosome [74]. Together, these studies demonstrate that DISC1 regulates NPC proliferation and neuronal migration through GSK3-dependent and independent pathways, respectively.

As well as DISC1, other upstream regulators of GSK3 are genetically linked to mood disorders and schizophrenia, including Akt [75], Neuregulin [76] and the dopamine/β-arrestin signaling complex [77, 78]. GSK3 kinase activity is also inhibited by several mood-stabilizers, anti-depressants and anti-psychotic drugs [6, 79, 80], while genetic manipulation of GSK3 activity in mice produces behaviours correlating with mood disorders [24-28]. A single nucleotide polymorphism in the promoter region of GSK3β has also been correlated with onset of Bipolar disorder [81]. Together, these observations strongly implicate elevated GSK3 activity in the etiology of mood disorders and schizophrenia. Neurogenesis is decreased in these disorders [82, 83], but is promoted by mood stabilizing drugs, such as lithium [84, 85]. Therefore, mood-stabilizing drugs may act (at least in part) by promoting neurogenesis via inhibition of GSK3. If so, promoting neurogenesis using GSK3 inhibitors could also be beneficial to other mental disorders, including neurodegenerative diseases such as Alzheimer's disease and age-related cognitive decline, as well repair following brain injury, such as stroke and spinal chord injuries.

7. GSK3 and Notch signaling

A role for the Notch signaling pathway in regulating development was discovered almost a century ago by pioneering work in fruit flies by John Dexter and Thomas Hunt Morgan [86]. It was later found that absence of Notch in flies caused neuronal hyperplasia and a decrease in glial cells in the brain [87]. Generally, Notch signaling promotes proliferation of NPCs and inhibits their differentiation, but has also been linked with glial cell specification [88, 89], neurite outgrowth [90] and learning and memory [91]. Notch is a family of 4 single-pass transmembrane proteins (Notch1-4) that are expressed at the cell surface of ES cells and NPCs (especially Notch1). Ligand-mediated activation of Notch leads to its proteolytic cleavage by Presenillin-g, releasing the Notch intracellular domain (NICD) for translocation to the nucleus where it binds with the transcription factor RBP-Jk to activate transcription of target genes including the bHLH transcriptional repressors *Hes1* and *Hes5*. These proteins repress transcription of pro-neural genes, thus inhibiting differentiation into neurons and maintaining proliferation of NPC's. Ligands that activate Notch, such as Delta-like 1 (Dll1) and Jagged1 (Jag1) are themselves transmembrane proteins. These are typically located at the cell surface of differentiated neurons that bind and stimulate activation of Notch on neighbouring NPC's. Thus, Notch signaling functions in a localized fashion, whereby a differentiating neuron expressing the ligands Dll1 or Jag1 binds Notch on a neighbouring cell to activate downstream signaling that inhibits differentiation and maintains proliferation of NPC's, thus establishing a stem cell niche in the brain. In other words, Notch signaling prevents equipotent cells from acquiring the same fate.

GSK3 was originally found to be associated with the Notch signaling pathway in *Drosophila* development [92]. Since then, it has been shown to bind and phosphorylate the intracellular domain of Notch, stabilizing the protein by reducing its degradation by the proteasome [93]. However, other studies report that phosphorylation *de*stabilizes Notch and promotes its degradation [94, 95]. The reason for these contradictory observations are not clear, although considering that low GSK3 activity and high Notch signaling correlate with the highly proliferative, undifferentiated nature of ES cells/NPC's, it is likely that GSK3-mediated phosphorylation targets Notch for degradation by the proteasome. That is, low GSK3-mediated phosphorylation and degradation of NICD increases its abundance and transcriptional activity to promote proliferation/suppress differentiation. This is consistent with many other proteins that are destabilized by GSK3 phosphorylation, such as c-myc, HIF1α, β-catenin, etc. It is possible that prior phosphorylation by the cyclin C:Cdk8 complex could prime NICD for subsequent phosphorylation by GSK3 [96], although this remains to be proven.

Cross-talk between the Notch and Wnt signaling pathways has been suggested by several studies, although the reports are contradictory. One study shows that Notch binds to the unphosphorylated, transcriptionally-active form of β-catenin, targeting it for degradation by the lysosome, thus suppressing expression of Wnt target genes [97]. At first glance, this is surprising, since both pathways are pro-proliferation/anti-differentiation. However, it should be noted that Notch-mediated degradation of β-catenin does not require ligand activation. Therefore, this mechanism might be more relevant to differentiated cells, such that in the

absence of ligand stimulation, Notch reduces β-catenin levels to antagonize Wnt signaling. Thus, both pathways are suppressed, consistent with a differentiated phenotype. Another report has shown that FGF2-mediated inhibition of GSK3 activity leads to an increase in transcriptionally-active β-catenin in the cell nucleus, which simultaneously activates TCF/LEF and Notch/RBP-Jk transcriptional complexes, promoting proliferation and inhibiting differentiation, respectively [98]. Although both pathways promote maintenance of stem cells as expected, FGF2-mediated inhibition of GSK3 is unexpected, since it is commonly assumed that growth factors do not influence β-catenin levels in the Wnt pathway [19], although this observation has been confirmed by several other groups [99, 100]. It is possible that FGF signaling is an exception to the growth factor rule. All together, it seems clear that there are interactions between Notch and other cell fate pathways involving GSK3, although the precise mechanisms are yet to be fully clarified.

8. GSK3 and Hedgehog signalling

The Hedgehog signaling pathway is a critical regulator of cell fate during development and for maintaining proliferation of cell stem niches in adults, including NPC's in the brain. In mammals, there are 3 Hedgehog proteins, with Sonic hedgehog being the most widely studied. These are secreted proteins that bind to a receptor at the surface of target cells called patched. When Hedgehog binds to patched, patched is released from another receptor protein at the cell surface called smoothened, allowing smoothened levels to increase. Smoothened inhibits the proteolytic cleavage of the zinc-finger proteins Gli (*cubitus interruptus* in flies), which are critical effectors of the Hedgehog pathway. Gli1 and Gli2 are transcriptional activators, while Gli3 is a repressor. These proteins regulate the transcription of cell cycle-related genes, such as cyclins D and E, N-myc, Bcl2, POU3F1, Runx2 and Tbx2, as well as patched in a negative feedback loop. In the absence of Hedgehog ligands, Gli associates with a scaffolding complex containing Cos2 and Fused that facilitates phosphorylation by GSK3 and CK1 (following priming by PKA). This phosphorylation targets Gli for ubiquitination and proteolysis, generating a truncated repressor form lacking the C-terminal activation domains [101-105]. But in the presence of Hedgehog, this signaling complex is disrupted, inhibiting phosphorylation and processing of Gli, leading to accumulation of transcriptionally active full-length protein in the nucleus. Thus, GSK3 antagonises Hedgehog signaling by mediating the degradation of Gli proteins. The scaffolding protein Sufu, a negative regulator of Hedgehog signaling, is also phosphorylated by GSK3, which stabilizes the protein and increases degradation of Gli proteins [106]. Thus, GSK3 promotes Gli degradation directly and via stabilization of Sufu.

The Hedgehog pathway is similar to the Wnt pathway, in that it utilizes the constitutive activity of GSK3 to negatively regulate the key transcriptional effector of each pathway (β-catenin for Wnt, Gli for Hedgehog). Ligand-mediated activation of both pathways reduces phosphorylation of these key effectors by disrupting their respective signaling/scaffolding complexes without directly inhibiting GSK3 activity (i.e. they exclusively reduce phosphorylation of a specific substrate). In contrast, growth factor signaling directly inhibits GSK3 kinase activity

via phosphorylation of the N-terminal serine residues. Therefore, it seems that the Wnt and Hedgehog pathways regulate specific substrates within their respective signaling complexes, while growth factor stimulation affects a wider range of GSK3 substrates (Fig.1). It should also be mentioned that signaling complexes containing the scaffolding protein AKAP220 have been shown to promote inhibitory Ser21/9 phosphorylation of GSK3 [107, 108]. Hence, the number of scaffolding/signal complex-associated proteins reported to bind and regulate GSK3 activity is increasing, suggesting that this mechanism might be common, rather than an exception, although this remains to be proven. Nevertheless, it is highly likely that other signaling complexes regulating GSK3 are likely to be discovered.
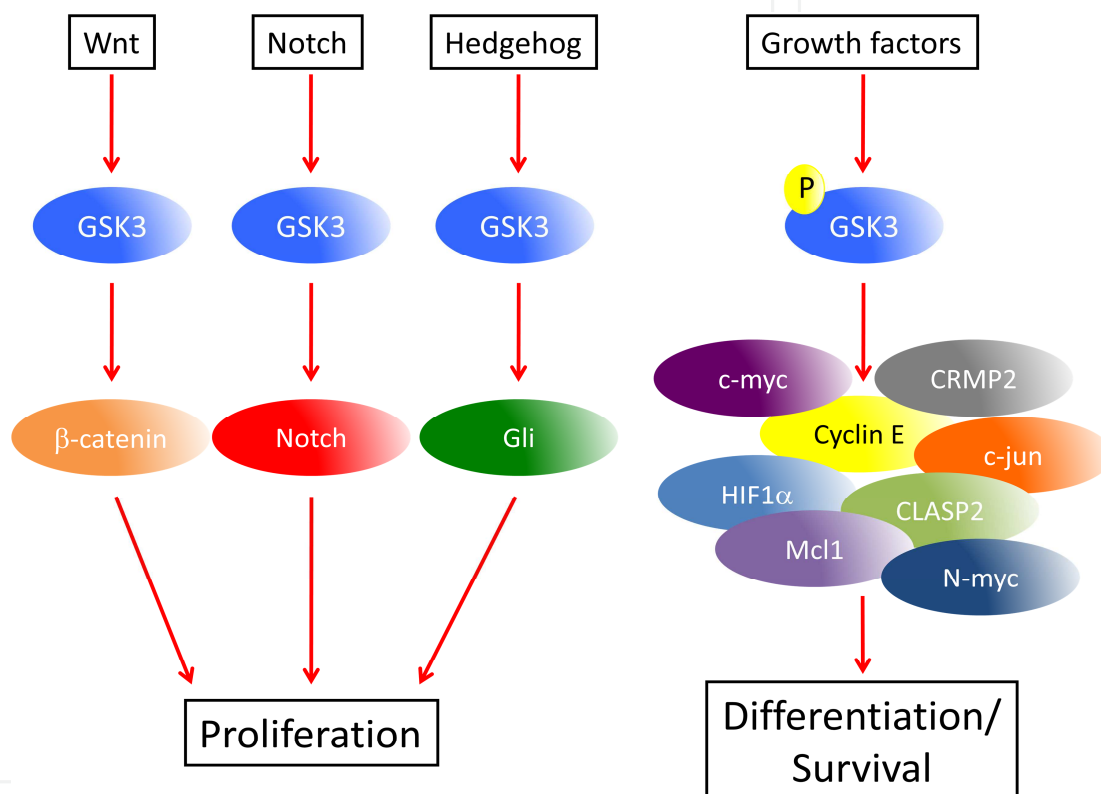

Figure 1. Signalling pathways regulating cell fate target different subsets of GSK3 substrates. Ligand-stimulated activation of the Wnt, Notch and Hedgehog pathways prevents phosphorylation of a single substrate by disrupting multi-subunit signalling complexes required to mediate their phosphorylation, driving proliferation. In contrast, growth factor-mediated inhibition of GSK3 via phosphorylation of N-terminal serine residues reduces phosphorylation of many substrates, promoting survival and differentiation of newly generated neurons.

9. GSK3 and growth factor signaling

In general, growth factors positively influence neurogenesis in the developing and adult brain by supporting differentiation and survival of newly generated neurons. Direct injection of many growth factors into the subventricular zone of mice increases neurogenesis, including

FGF [109], EGF [110], TGF [110], CNTF [111]. Among the most potent growth factors are the neurotrophins (BDNF, NGF, NT-3, NT-4), whereby direct administration [112] or genetic deletion [113] increases and reduces neurogenesis, respectively. Accordingly, their receptor molecules, called Trk receptors, are predominantly expressed by post-mitotic neurons, but not NPC's or neural crest cells [114]. BDNF signaling, in particular, has been shown to promote survival of newly-generated neurons [115], while defects in BDNF signaling have been linked to decreased neurogenesis and neuronal survival in several neurological disorders, including Alzheimer's disease, age-related cognitive decline, Bipolar disorder and Schizophrenia (for reviews, see [116, 117]).

GSK3 is an established target of many growth factor signaling pathways. Ligand-binding to their respective receptors at the cell surface induces dimerisation/oligomerisation, activating their intrinsic kinase activities and autophosphorylation of key tyrosine residues. This provides binding sites for SH2 and SH3-domain-containing adaptor proteins, such as IRS and Grb proteins, promoting recruitment and activation of phosphatidylinositol 3 kinase (PI3K). This lipid kinase converts phosphatidylinositol 4,5 bisphosphate (PIP2) to PIP3, recruiting members of the AGC family of kinases (e.g. PKB/Akt), where they are phosphorylated and activated by PDK1. These AGC kinases phosphorylate an N-terminal serine on GSK3 (Ser21 on GSK3α, Ser9 on GSK3β) that binds to the substrate binding site on GSK3, thus acting as a pseudo-substrate to inhibit phosphorylation of primed substrates [17].

Numerous studies have demonstrated that pharmacological inhibitors of GSK3 promote survival of neurons subjected to a range of toxic stimuli (for a review, see [118]), while over-expression of GSK3 promotes neuronal apoptosis *in vitro* and *in vivo* [29, 119, 120]. Importantly, GSK3α/β -knockin mice (S21/9A) that are insensitive to growth factor inhibition exhibit reduced neurogenesis, despite normal proliferation of NPC's [36]. As noted previously, this could suggest defective differentiation patterns in the NPC's, but could equally represent decreased survival of newly generated neurons. In addition, several substrates of GSK3 are associated with cell survival (discussed below). Together, these observations strongly suggest that growth factor-mediated inhibition of GSK3 appears to be a key mechanism by which growth factors promote survival of newly generated neurons during neurogenesis.

A key downstream target of GSK3 promoting survival is Mcl1. This anti-apoptotic, pro-survival member of the Bcl2 family is directly phosphorylated by GSK3 at Ser155 and Ser159, targeting it for ubiquitination by E3 ligases (Fbw7, βTrCP and/or Trim17) and degradation by the proteasome [121, 122]. Upon growth factor stimulation, GSK3 is inhibited, reducing GSK3-mediated phosphorylation of Mcl1, thus stabilizing the protein and promoting neuronal survival. The Notch signaling pathway has also been shown to promote survival of neurons via Mcl-1, although the role of GSK3 was not investigated [123]. NPC's and newly-committed neurons in the subventricular zone and surrounding areas of mice express high levels of Mcl-1, while Mcl-1 deficiency caused widespread apoptosis, especially in newly-committed neurons as they migrate away from this region [124]. This suggests that a key target of growth factor

and GSK3-mediated protection of neurons is via Mcl-1. Indeed, nutrient deprivation correlates with decreased levels of Mcl-1 and induction of apoptosis in neurons, which was dependent on phosphorylation by GSK3 [125, 126]. In contrast, Bax is a pro-apoptotic member of the Bcl2 family. Phosphorylation by GSK3 at Ser163 promotes its translocation to the mitochondrial outer membrane, where it oligomerizes to form a pore promoting cytochrome c release and cell death [127, 128]. Inhibition of GSK3 via growth factor signaling or therapeutics (e.g. lithium) antagonizes Bax translocation and cytochrome c release, promoting cell survival.

Other downstream targets of GSK3 that promote neuronal survival include the transcription factors HIF1α, HSF1 [129], Mef2D [130] and Bcl3 [131] by activating transcription of pro-survival genes. For example, increased activity of GSK3 during potassium withdrawal from cerebellar granule neurons leads to increased phosphorylation and degradation of Mef2D, which could be blocked by a GSK3-resistant form of Mef2D [130]. The neurotrophins NGF and BDNF promote Mef2D stability by reducing GSK3-mediated degradation, leading to increased transcription of its target gene Bcl-w, an anti-apoptotic member of the Bcl-2 family [132].

10. GSK3, ubiquitin ligases and proteasome-mediated degradation

It is noticeable that many substrates of GSK3 are targeted for ubiquitination and proteasome-mediated degradation following phosphorylation by GSK3. Most of these are transcription factors that are phosphorylated by GSK3 within an [ST]PPx[ST]P motif, including c-myc [61, 133, 134], c-jun [135], Klf5 [136, 137], cyclin E1 [138], Gli3 [101, 102] and snail [139]. These transcription factors have short half-lives, largely due to the actions of GSK3, which is highly active under basal conditions in mature, differentiated cells. However, GSK3 activity levels are comparatively lower in undifferentiated cells, induced by persistent Wnt, Notch, Hedge-hog and/or growth factor signaling to maintain the proliferative capacity of these cells [140]. Here, phosphorylation and ubiquitination of transcription factors by GSK3 is reduced, thus stabilizing the proteins (prolonging their half-lives) and contributing to stem/precursor cell proliferation. A common E3 ligase targeting GSK3 substrates is Fbw7. In fact, most of the reported targets of Fbw7 are established GSK3 substrates (see [141]). Genetic studies in mice indicate that Fbw7 is required for differentiation and survival of NPC's. For example, brain-specific deletion of Fbw7 increased apoptosis of NPC's, which could be rescued by concomitant deletion of c-jun [142]. Similar results were observed for cerebellar development in mice [143]. NPC differentiation was also impaired, particularly a decrease in the number of mature neurons and increased glial progenitors (although no change in mature astrocytes). This was alleviated by inhibition of Notch signaling. Supporting this, a separate study showed that brain-specific deletion of Fbw7 skewed the differentiation of NPC's towards astrocytes, rather than neurons, which could be restored with a pharmacological inhibitor of the Notch pathway [144]. Meanwhile, the maintenance/proliferation of the NPC's was not affected. Together, these observations show that GSK3 and Fbw7 share common substrates that regulate the differentiation and survival of neurons.

11. GSK3 and NPC migration and polarization

Correct positioning of newly generated neurons is crucial during development and for healthy function of the adult brain. Neuronal migration is tightly regulated by extracellular cues, including Wnts and growth factors. Accordingly, there is emerging evidence that their common downstream target, GSK3, may be involved in this process, primarily via regulation of cytoskeleton-associated proteins. Migration requires cellular polarization and extension at the leading edge of the cell. This involves dynamic reorganization of microtubules by a variety of microtubule-binding proteins. Several of these are directly phosphorylated and regulated by GSK3, including Tau, CRMP2, MAP1B, MAP2C, CLASP2, pVHL and APC (for reviews, see [145, 146]). Phosphorylation of many of these substrates by GSK3 reduces their ability to bind microtubules, thus making them less stable. Interestingly, GSK3 activity is typically low at the leading edge of migrating cells or at the tips of growing neurites in neuronal polarization [147-149]. This would promote substrate binding and stabilization of microtubules, facilitating forward movement of the cell's leading edge or growing neurite. Similarly, local inhibition of GSK3 activity is essential for polarization of newly generated neurons and growth of the nascent axon [150, 151]. Meanwhile, global inhibition of GSK3 induces formation of multiple axons [150, 152]. Several upstream inhibitors of GSK3 have been implicated in this process, including Cdc42 [149], ILK [153], LKB1 [147] and Akt [150]. Apart from the latter, these signaling proteins are not established regulators of GSK3 activity, so the precise molecular mechanisms by which they inhibit GSK3 activity await clarification.

12. Conclusions

It is clear that GSK3 is an important target of several signaling pathways controlling cell fate in the brain. It is also clear that many of these pathways can be activated simultaneously in the same cells/tissues. One possibility is that GSK3 acts as an integrator of these simultaneous inputs to determine the cellular outcome. That is, GSK3 acts as a node for multiple signaling pathways and the sum of these inhibitory signals dictates cell fate. This is unlikely, since although each of these pathways target GSK3, their downstream targets are different. For example, growth factor signaling inhibits phosphorylation of CRMP2, but not β-catenin, and *vice versa* for Wnt [19, 154, 155]. An alternative explanation is that different subsets of GSK3 substrates are selectively affected by particular stimuli. That is, ligand-stimulated activation of the Wnt, Notch and Hedgehog pathways prevents phosphorylation of a single substrate (β-catenin, Notch and Gli, respectively) by disrupting multi-subunit signaling complexes required to mediate their phosphorylation. This increases the stability of these proteins, translocation to the cell nucleus and regulation of gene transcription programs that promote proliferation of ES cells/NPC's. In contrast, growth factor-mediated inhibition of GSK3 via phosphorylation of N-terminal serine residues reduces phosphorylation of many substrates (although not β-catenin), promoting survival and differentiation of newly generated neurons. It is possible that signaling complexes associated with β-catenin (and perhaps Notch and Gli) are able to surmount the inhibitory N-terminal serine phosphorylation induced by growth

factor signaling. Indeed, the protein phosphatase PP2A is a known component of the β-catenin/APC/Axin signaling complex [156] and PP2A is able to activate GSK3 by dephosphorylating its N-terminal serine residue [157]. If this is true, these signaling complexes could be seen to *activate* GSK3 activity (at least phosphorylation of a particular substrate e.g. β-catenin). It might be more accurate to say that GSK3 is *not* inhibited by these pathways, but rather GSK3 *antagonizes* them. Then upon ligand stimulation, this function of GSK3 is alleviated by disruption of the signaling complex, activating the pathway. It is important to catalogue the pathway-specific targets of GSK3 and their effects on cell fate and survival, since this knowledge could identify novel therapeutic targets for artificially controlling neurogenesis and promoting recovery in diseased or damaged brains.

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