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GSK3 as a sensor determining cell fate in the brain

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Glycogen synthase kinase 3 (GSK3) is an unusual serine/threonine kinase that controls many neuronal functions, including neurite outgrowth, synapse formation, neurotransmission, and neurogenesis. It mediates these functions by phosphorylating a wide range of substrates involved in gene transcription, metabolism, apoptosis, cytoskeletal dynamics, signal transduction, lipid membrane dynamics, and trafficking, amongst others. This complicated list of diverse substrates generally follow a more simple pattern: substrates negatively regulated by GSK3-mediated phosphorylation favor a proliferative/survival state, while substrates positively regulated by GSK3 favor a more differentiated/functional state. Accordingly, GSK3 activity is higher in differentiated cells than undifferentiated cells and physiological (Wnt, growth factors) and pharmacological inhibitors of GSK3 promote the proliferative capacity of embryonic stem cells. In the brain, the level of GSK3 activity influences neural progenitor cell proliferation/differentiation in neuroplasticity and repair, as well as efficient neurotransmission in differentiated adult neurons. While defects in GSK3 activity are unlikely to be the primary cause of neurodegenerative diseases, therapeutic regulation of its activity to promote a proliferative/survival versus differentiated/mature functional environment in the brain could be a powerful strategy for treatment of neurodegenerative and other mental disorders.

Keywords: GSK3, phosphorylation, kinase, substrate, proliferation, differentiation, neural progenitor, Alzheimer's disease

GLYCOGEN SYNTHASE KINASE 3

Glycogen synthase kinase 3 (GSK3) is a Ser/Thr protein kinase that is expressed in all mammalian tissues and subcellular organelles, but particularly highly in the brain. There are two isoforms encoded by separate genes (GSK3α and GSK3β), although their functions are often indistinguishable. GSK3 is critical for normal function of the central nervous system, where it regulates a variety of neuronal functions, including neurotransmission, neurite outgrowth, growth-cone dynamics, cytoskeletal dynamics, synaptic plasticity, endocytosis, apoptosis, and neurogenesis. Interestingly, it is one of the most unusual kinases in the human genome for three main reasons; (1) Most (if not all) substrates require prior phosphorylation by another kinase before they can be efficiently phosphorylated at Ser/Thr residues by GSK3. This process is known as "priming" and occurs four or five residues C-terminal to the GSK3 target site. (2) GSK3 is highly active in cells under basal conditions. This is partly due to constitutive phosphorylation of a conserved tyrosine residue on the activation loop of the kinase domain (Tyr279 in GSK3α, Tyr216 in GSK3β) that is absolutely required for kinase activity (Cole et al., 2004a). (3) Phosphorylation of GSK3 at an N-terminal serine residue inhibits its kinase activity (Ser21 in GSK3α, Ser9 in GSK3β). This phosphoserine acts as a pseudo-substrate and binds to the phosphate-binding pocket on GSK3, preventing interaction with primed substrates (Frame et al., 2001). Phosphorylation at this site is mediated by members of the AGC family of kinases (e.g., Akt) and commonly occurs downstream of insulin, growth factor, and PI3K signaling. 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 (Thomas et al., 1999; Ding et al., 2000).

GSK3 AND ALZHEIMER'S DISEASE

Glycogen synthase kinase 3 is implicated in the development of Alzheimer's disease (AD), principally because it phosphorylates Tau and increases its propensity to aggregate into neurofibrillary tangles, which together with β-amyloid plaques are a characteristic lesion of the disease. Transgenic mice displaying increased GSK3 activity develop hyperphosphorylated Tau and other neurological defects (Lucas et al., 2001), while treatment of transgenic mice with a GSK3 inhibitor (lithium) reduces the number of tangles in their brains (Noble et al., 2005). A similar microtubule-binding protein called CRMP2 is also hyperphosphorylated by GSK3 in the brains of AD patients (Cole et al., 2007; Williamson et al., 2011). The amyloid precursor protein has been reported to be a GSK3 substrate (Aplin et al., 1996), while Aβ peptide production is reported to be regulated by GSK3 (Sun et al., 2002; Li et al., 2003; Phiel et al., 2003; Ryder et al., 2003; Su et al., 2004; Sereno et al., 2009), although these observations have been recently disputed (Jaworski et al., 2011). Thus, GSK3 has been implicated in many pathologic processes leading to AD. However, it is unlikely that defects in GSK3 *per se* are a direct cause of AD, since no mutations, polymorphisms, or dramatic biochemical changes have been consistently detected in

Abbreviations: AD, Alzheimer's disease; ES, embryonic stem (cell); GSK3, glycogen synthase kinase 3; NPC, neural progenitor cell.

AD patients, nor any other types of neurodegenerative, developmental, or psychiatric disorders. Instead, a key function of GSK3 is to act as an "environmental sensor," by relaying signals from extracellular stimuli (e.g., growth factors, insulin, Wnt) to signaling and transcriptional machinery inside the cell to influence cell fate. This implies that pharmacological manipulation of GSK3 in the brain could be used to selectively promote survival, proliferation, differentiation, neurogenesis, or neuroplasticity in diseased brains. This type of therapy could be used to artificially create an environment in the brain that delays/prevents disease development, or promotes neurogenesis and neuroplasticity to compensate for specific insults. Indeed, encouraging data is now emerging showing chronic lithium treatment improves cognitive function in human patients and mouse models of neurodegeneration and ischemic stroke (for a review, see Chiu and Chuang, 2010). Although GSK3 is not the only *in vivo* target of lithium (e.g., phosphoinositol phosphatases), these effects are consistent with the known actions of GSK3. It remains to be seen what benefits more selective and potent GSK3 inhibitors might provide.

GSK3 SUBSTRATES

In order to fully understand the function of GSK3 in the brain, it is essential to characterize its substrates, since this is the primary function of a kinase and it is the substrates that mediate the functional effects directed by GSK3. Ultimately, all physiological substrates of GSK3 should be cataloged and assigned to particular functions regulated by GSK3 (e.g., neurogenesis, neurite outgrowth, neurotransmission, cytoskeletal regulation). This exercise would delineate the mechanisms by which GSK3 maintains healthy brain function. Importantly, it could identify new therapeutic targets downstream of GSK3 that could be exploited for the treatment of mental and neurodegenerative diseases. Theoretically, these could be more specific with less side effects than targeting GSK3, which is a pleiotropic kinase with many different substrates involved in diverse cellular functions.

So far, over 70 substrates have been identified for GSK3, although caution should be taken since many substrates have been reported with various levels of confidence/evidence (for a full review, see Sutherland, 2011). Reported substrates include a number of cytoskeletal, signaling, and DNA-binding proteins. Interestingly, a pattern emerges whereby many substrates that are negatively regulated by GSK3 are involved in proliferation/survival of cells, whereas substrates that are positively regulated by GSK3 are predominantly expressed and function in mature, differentiated cells. Key substrates that contribute to cellular proliferation, differentiation, and survival are listed in **Tables 1** and **2** and discussed below.

GSK3 AND PROLIFERATION

For some time, it has been known that pharmacological inhibition of GSK3 activity maintains the proliferative state of embryonic stem (ES) cells (Sato et al., 2004; Ying et al., 2008). The GSK3 substrates c-myc (Hall et al., 2009) and Klf5 (Jiang et al., 2008) are among several transcription factors that have been used to induce pluripotency (iPS system). GSK3 has also been implicated as a key regulator of adult neurogenesis (generation and incorporation of new neurons into existing circuits of adult brains). Genetic (Eom and Jope, 2009; Kim et al., 2009; Mao et al., 2009) and pharmacological (Sato et al., 2004; Ying et al., 2008; Bone et al., 2009) inhibition of GSK3 activity increases proliferation of neural progenitor cells (NPC's), but decreases differentiation and incorporation of newborn neurons into the adult brain. Together, these observations demonstrate that low levels of GSK3 activity promote proliferation in ES cells and NPC's. This correlates with signaling pathways upstream of GSK3 that inhibit GSK3 activity and promote proliferation (e.g., Wnt, growth factors).

Several transcription factors are directly phosphorylated by GSK3 within an [ST]PPx[ST]P or [ST]PxL[ST]P motif. Following priming by another kinase (often a Cdk or MAPK), phosphorylation by GSK3 creates a binding site for E3 ubiquitin ligases that ubiquitinate the protein and target it for proteasome-mediated degradation. Many of these transcription factors have short half-lives, largely due to the actions of GSK3, which is highly active under basal conditions in differentiated cells, including post-mitotic neurons. However, GSK3 activity levels are comparatively lower in ES cells and NPC's, induced by persistent growth factor and Wnt signaling to maintain the proliferative capacity of these cells (Cartwright et al., 2005). 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. Such GSK3 targets include well-known proliferative factors, such as c-myc, c-jun, β-catenin, cyclin E1, and Klf5 (**Tables 1** and **2**; **Figure 1**). Recent studies suggest that attenuating GSK3-mediated degradation of β-catenin, a key effector of the Wnt signaling pathway, is vital for maintaining ES proliferation and pluripotency (Mao et al., 2009; Kelly et al., 2011; Wray et al., 2011). Interestingly, a viral oncogenic form of c-jun (v-jun) is mutated at the GSK3 target site (Ser239). This prevents phosphorylation by GSK3 and subsequent ubiquitination, thus stabilizing the protein and driving uncontrolled proliferation in tumourigenesis (Wei et al., 2005). Similarly, the GSK3 phosphosite (Thr58) is mutated in the viral oncogenic form of c-myc (v-myc; Pulverer et al., 1994). While it is established that low GSK3 activity levels are required for maintaining the proliferative capacity of ES cells and NPC's, there are many DNA-binding substrates of GSK3 implicated in this process and their precise roles and relative importance are only beginning to be clarified.

GSK3 AND DIFFERENTIATION

Not only does low GSK3 activity promote proliferation, it also prevents differentiation. GSK3α/β double knockout ES cells are severely compromised in their ability to differentiate, largely due to hyperactivation of the Wnt signaling pathway (Doble et al., 2007), while conditional deletion of both isoforms in NPC's in mice suppressed the generation of post-mitotic neurons (Kim et al., 2009). Also, expression of mutant GSK3 and RNAi-mediated knockdown impairs neuronal polarization in cultured primary neurons (Jiang et al., 2005; Yoshimura et al., 2005; Kim et al., 2009). GSK3 knockin mice expressing GSK3α/β (Ser21/9Ala) that are insensitive to growth factor-induced inhibition exhibited reduced neurogenesis and behavioral defects, despite normal NPC proliferation (Eom and Jope, 2009; Ackermann et al., 2010), suggesting defective differentiation/maturation of NPC's. In contrast, mice expressing mutant DISC1 (mutated in schizophrenia patients)

Table 1 | Substrates involved in proliferation/survival that are negatively regulated by GSK3.

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
c-myc	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Moberg et al. (2004), Welcker et al. (2004), Yada et al. (2004)
c-jun	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Wei et al. (2005)
β-Catenin	Transcription factor and oncogene – promotes proliferation	Promotes degradation of the protein	Ikeda et al. (1998)
HIF1α	Transcription factor induced by hypoxia. Activates transcription of genes promoting adaptation/survival	Promotes degradation of the protein	Mottet et al. (2003)
HSF1	Transcription factor that promotes expression heat shock factors to protect cells from environmental stress	Reduces DNA-binding and transcriptional activity	Chu et al. (1998)
Klf5	Transcription factor that promotes cell proliferation	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Liu et al. (2010), Zhao et al. (2010)
CyclinE1	Activating cofactor for Cdk2, promoting cell cycle progression	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Welcker et al. (2003)
Mef2D	Transcription factor that promotes survival and activity-dependent synapse formation	Inhibits its transcriptional activity, antagonizing neuronal survival but antagonizing neuronal differentiation.	Wang et al. (2009)
Gli3 (Ci155)	Target of the hedgehog signaling pathway that is important for patterning during development. Full-length Gli3 (Ci155) is a transcriptional activator, while the truncated form is a transcriptional repressor.	Promotes β-TrCP-mediated ubiquitination and proteolytic processing	Jia et al. (2002), Price and Kalderon (2002), Pan et al. (2006), Tempe et al. (2006), Wang and Li (2006)
Snail	Transcription factor that regulates E-cadherin expression during epithelial–mesenchymal transitions (development)	Promotes β-TrCP-mediated ubiquitination and degradation, also promotes translocation from the nucleus to the cytoplasm	Zhou et al. (2004)
NDRG1	Regulated by the cell cycle and cell differentiation, although cellular function is not yet clear	Unknown	Murray et al. (2004)
BCL3	Transcription factor and oncoprotein that regulates NFkB signaling	Promotes ubiquitin and proteasome-mediated degradation	Viatour et al. (2004)
MCL1	Pro-survival member of the Bcl2 family of proteins controlling apoptosis. Overexpressed in some cancer types.	Promotes degradation of the protein via Fbw7-mediated ubiquitination and the proteosome	Maurer et al. (2006)
RBL2	Involved in heterochromatin formation and structure. A key regulator of entry into the cell cycle	Not yet clear	Litovchick et al. (2004)
Smad1	Transcription factor and key mediator of BMP signaling in embryogenesis and tissue homeostasis	Promotes ubiquitination by Smurf1 and proteasome-mediated degradation	Fuentealba et al. (2007), Sapkota et al. (2007)
eIF-2B	Activates initiation of protein translation from mRNA transcripts	Phosphorylation inhibits eIF-2B activity, reducing protein translation	Welsh and Proud (1993)
Myocardin	Muscle-specific transcription factor and SRF-dependent cofactor that promotes expression of contraction-related genes	Inhibits its transcriptional activity and promotes CHIP or UBR5-mediated ubiquitination and degradation by the proteasome	Badorff et al. (2005), Xie et al. (2009), Hu et al. (2010)
VDAC1	Voltage-dependent anion channel in the mitochondrial outer membrane. Mediates cytochrome *c* release from mitochondria during apoptosis	Reduces binding to hexokinase 1, which is overexpressed in many transformed cells, thereby reducing aerobic glycolysis and ATP production in tumor cells	Pastorino et al. (2005)
IRS1	Adaptor protein that mediates signaling downstream of insulin and growth factor receptors	Reduces tyrosine phosphorylation of IRS1, attenuating insulin, and growth factor signaling	Eldar-Finkelman and Krebs (1997), Liberman and Eldar-Finkelman (2005)

(Continued)

Table 1 | Continued

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
Bax	Pro-apoptotic member of the Bcl2 family that oligomerizes at the mitochondrial outer membrane, forming a pore to release cytochrome *c*	Promotes translocation to the mitochondria to induce apoptosis	Linseman et al. (2004)
Sufu (exception to the pattern)	Negative regulator of sonic hedgehog pathway, which regulates animal development and cell fate determination. In adults, it maintains the proliferative state of stem cells	Stabilizes Sufu by preventing its degradation and promotes localization in the primary cilium	Chen et al. (2011)
PTEN (exception to the pattern)	Lipid phosphatase and commonly mutated tumor suppressor in human cancers	Phosphorylation stabilizes the protein by reducing degradation	Al-Khouri et al. (2005), Maccario et al. (2007)

Table 2 | Substrates predominantly expressed and functional in mature differentiated cells that are positively regulated by GSK3.

Substrate	Function	Effect of GSK3-mediated phosphorylation	Reference
Polycystin-2 (PKD2)	Non-selective calcium permeable cation channel and part of the TRP channel family, which are broad cellular sensors for multiple stimuli	Promotes translocation to the cell membrane	Streets et al. (2006)
CRMP2	Binds to tubulin heterodimers to promote polymerization of microtubules. Also involved in kinesin-mediated transport and receptor trafficking	Regulates neurite outgrowth and neuronal polarity	Brown et al. (2004), Cole et al. (2004b), Uchida et al. (2005), Yoshimura et al. (2005)
MAP1B	Cytoskeletal component of the developing nervous system with important functions in migrating and differentiating neurons	Unclear, but may destabilize microtubules, making them more dynamic	Goold et al. (1999)
MAP2C	Abundant cytoskeletal components predominantly expressed in neurons	Promotes dissociation from the cytoskeleton, destabilizing microtubules	Sanchez et al. (2000)
Tau	Tubulin-binding protein that stabilizes microtubule structures. Primary constituent of neurofibrillary tangles generated in brains of Alzheimer's Disease and other dementia patients	Reduces binding to tubulin, destabilizing microtubules, making them more dynamic. Promotes aggregation of tau, forming neurofibrillary tangles	Hanger et al. (1992)
β-Adducin	Cytoskeletal-associated protein that links the actin and spectrin networks	Promotes neurite outgrowth	Farghaian et al. (2011)
Dynamin1	GTPase protein that regulates vesicular trafficking processes. Contributes to efficient neurotransmitter release at the pre-synapse	Promotes activity-dependent bulk endocytosis at the pre-synapse, facilitating efficient neurotransmission	Clayton et al. (2010)
CLASP2	Microtubule plus-end tracking protein that promotes the stabilization of dynamic microtubules	Causes dissociation from the plus end of microtubules and other MT-associated proteins	Wittmann and Waterman-Storer (2005), Watanabe et al. (2009)
CaMKKβ	Calcium/CaM dependent protein kinase that regulates learning, memory, migration, neurite outgrowth, and synaptogenesis	Stabilizes newly synthesized protein, decreases calcium/CaM autonomous activity	Green et al. (2011)
Glycogen synthase (exception to the pattern)	Enzyme involved in converting glucose to glycogen for storage	Reduces its enzymatic activity, thus reducing glycogen synthesis and storage	Rylatt et al. (1980)
FAK (exception to the pattern)	Plasma membrane protein and tyrosine kinase involved in cell–cell adhesion	Reduces FAK kinase activity, reducing cell migration	Bianchi et al. (2005)
pVHL (exception to the pattern)	Tumor suppressor that binds and stabilizes microtubules. Important in primary cilium. Component of an E3 ubiquitin ligase complex. Antagonizes cell cycle progression.	Phosphorylation negatively regulates stability (but not binding) of microtubules	Hergovich et al. (2006)

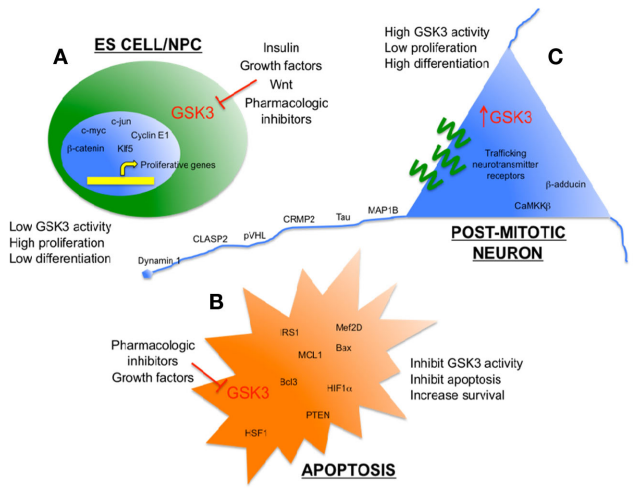

FIGURE 1 | Glycogen synthase kinase 3 as an enzymatic sensor for determining cell fate in the brain. (A) Low levels of GSK3 activity in ES cells/NPC's are maintained by persistent growth factor/Wnt signaling to promote proliferation. Some pro-proliferation transcription factors that are direct targets of GSK3 are shown. **(B)** Low levels of GSK3 activity inhibit apoptosis and promote survival when cells are exposed to toxic stimuli, such as hypoxia and amyloid peptides. Some pro-survival substrates of GSK3 are shown. **(C)** Relatively high levels of GSK3 activity help to promote differentiation and efficient function of mature, post-mitotic neurons, including several cytoskeleton-associated proteins that maintain neuronal morphology and neurotransmission.

caused increased GSK3 activity, inhibition of the Wnt signaling pathway, and decreased NPC proliferation (Mao et al., 2009). This suggests that inhibition of GSK3 by the Wnt signaling pathway promotes NPC proliferation, while inhibition of GSK3 by growth factor signaling promotes differentiation of NPC's into post-mitotic neurons.

Candidate substrates for promoting differentiation include the zinc-finger transcriptional regulator Gli3 (mammalian homolog of Ci155 in the fly), an effector of the hedgehog pathway that is critical for efficient embryo patterning and neural tube formation. In the absence of hedgehog, Gli3 is phosphorylated by GSK3 and CK1 (following priming by PKA), which targets it for ubiquitination and proteolysis, generating a truncated repressor form lacking the C-terminal activation domains (Jia et al., 2002; Price and Kalderon, 2002; Pan et al., 2006; Tempe et al., 2006; Wang and Li, 2006). But in the presence of hedgehog, phosphorylation and processing of Gli3 is inhibited, leading to transactivation by the full-length protein. It has recently been shown that the truncated repressor form of Gli3 is important for differentiation of cortical neurons from neural progenitors, while the full-length active form of Gli3 helps to maintain progenitors in the cell cycle (Wang et al., 2011). This correlates with GSK3 activity, with low GSK3 activity in undifferentiated ES cells increasing the amount of active, full-length Gli3 for maintaining proliferation in ES cells, and high GSK3 activity promoting proteolysis of Gli3 and formation of the truncated repressor form, facilitating differentiation into mature neurons.

Another zinc-finger transcriptional repressor called snail regulates transition of epithelial cells into fibroblast-like mesenchymal cells during development and tumor metastasis, essentially a form of "de-differentiation." Snail suppresses the expression of E-cadherin, a cell–cell adhesion molecule that is critical for maintaining epithelial cell identity. Relatively high activity of GSK3 in epithelial cells promotes phosphorylation and ubiquitin/proteasome-mediated degradation of snail (Zhou et al., 2004). However, in fibroblast/mesenchymal-like cells of human breast tumors where GSK3 activity is lower, snail is stabilized and suppresses E-cadherin expression (Zhou et al., 2004; Yook et al., 2006). Pharmacological inhibition of GSK3 activity in epithelial cells reduces E-cadherin expression and induces a more-mesenchymal-like morphology via increased snail activity (Bachelder et al., 2005). These observations demonstrate that Snail is an example of a GSK3 substrate controlling cellular differentiation. It does not appear to regulate neuronal differentiation (Murray and Gridley, 2006), although it has been shown to regulate cell fate in glioblastoma cells (Han et al., 2011).

Myocardin is a transcription factor that is required for cardiac and skeletal muscle cell differentiation during development. Again, it is not expressed in neurons or glial cells, but interestingly, it is upregulated in vascular smooth muscle cells in the brains of AD patients, where it enhances accumulation of Aβ in blood vessel walls (Bell et al., 2009). Myocardin is phosphorylated by GSK3, targeting it for ubiquitin, and proteasome-mediated degradation (Badorff et al., 2005; Xie et al., 2009; Hu et al., 2010), however it is not yet clear if upregulation of myocardin levels is due to reduced GSK3-mediated phosphorylation and degradation. In summary, several substrates of GSK3 regulating cell differentiation have been identified, although mostly in non-neural cell types and neuron-specific differentiation factors await identification.

GSK3 AND SURVIVAL

Glycogen synthase kinase 3 promotes intrinsic apoptotic signaling in neurons, and overexpression of GSK3 is sufficient to induce apoptosis in cultured cells (Pap and Cooper, 1998; Bijur et al., 2000) and in mouse brain (Lucas et al., 2001). Deletion of the GSK3β isoform in mice causes severe liver degeneration during mid-gestation due to excessive tumor necrosis factor-induced apoptosis (Hoeflich et al., 2000). In contrast, numerous studies have demonstrated that genetic or pharmacologic inhibition of GSK3 protects neurons from a wide range of environmental stresses, including hypoxia and amyloid toxicity, which may be relevant for treatment of stroke and AD patients, respectively (for a review, see Mines et al., 2011).

Several GSK3 substrates have been implicated in regulation of apoptosis. Bax is a pro-apoptotic member of the Bcl2 family that oligomerizes at the mitochondrial outer membrane, forming a pore to release cytochrome *c*, inducing cell death. Phosphorylation of Bax at Ser163 by GSK3 promotes translocation to the mitochondria, whereas inhibition of phosphorylation using lithium reduced Bax translocation and cytochrome *c* release, thus antagonizing apoptosis (Somervaille et al., 2001; Linseman et al., 2004). VDAC1 is a voltage-dependent anion channel in the mitochondrial outer membrane that also mediates cytochrome *c* release during apoptosis and is a direct substrate of GSK3 (Pastorino et al., 2005), although the effect of phosphorylation on cytochrome *c* release from the mitochondria and apoptosis is not yet clear. In

contrast, MCL1 is an anti-apoptotic, pro-survival member of the Bcl2 family, and phosphorylation by GSK3 targets it for degradation by the ubiquitin–proteasome-mediated pathway (Maurer et al., 2006). Thus, low GSK3 activity would reduce phosphorylation and degradation of MCL1, favoring cell survival. Several transcription factor substrates of GSK3 have also been implicated in the balance between apoptosis and cell survival by regulating transcription of pro-apoptotic or pro-survival genes, including the pro-survival factors HIF1α, HSF1, Mef2D, and BCL3. GSK3 phosphorylation of each of these substrates targets them for ubiquitin and proteasome-mediated degradation. In summary, many apoptosis-related GSK3 substrates identified so far are pro-survival, and when GSK3 activity is low (e.g., undifferentiated or pharmacologically treated cells), reduced phosphorylation of substrates protects them against ubiquitin and proteasome-mediated degradation, promoting survival of the cell.

GSK3 AND NEURONAL MORPHOLOGY

GSK3 is an important regulator of neuronal morphology and synapse formation in mature, post-mitotic neurons. Pharmacologic inhibition of GSK3 activity reduces the rate of axon elongation in hippocampal neurons, increases the size of growth cones (Owen and Gordon-Weeks, 2003), and disturbs polarity, leading to the formation of multiple axon-like processes (Gartner et al., 2006; Garrido et al., 2007). Treatment of cerebellar granule cells with a GSK3 inhibitor increased the number of synapses on mossy fibers (Hall et al., 2000), whereas inactivation of the *Drosophila* homolog of GSK3, *shaggy*, promoted synapse formation at neuromuscular junctions by increasing the number of synaptic boutons (Franco et al., 2004). Assuming these interventions were selective, then taken together they demonstrate that GSK3 regulates synapse formation. Accordingly, neurotrophin and growth factor stimuli (e.g., BDNF, NGF, IGF-1) that inhibit GSK3 activity, promote neurite outgrowth, and synapse formation.

Several groups recently demonstrated that the actin-capping protein β-adducin is critical for synapse stability and turnover, underlying learning and memory in flies and mammals (Bednarek and Caroni, 2011; Pielage et al., 2011; Ruediger et al., 2011). Moreover, dynamic disassembly and reassembly of synapses by β-adducin is regulated by phosphorylation at its C-terminal region by PKC. This domain is also targeted by GSK3 following priming by Cdk5 and phosphorylation by these kinases is necessary for β-adducin's ability to promote neurite outgrowth in cultured primary neurons (Farghaian et al., 2011). Therefore, it will be interesting to see if GSK3-mediated phosphorylation of β-adducin also regulates synapse formation and stability. Several other cytoskeleton-associated proteins are phosphorylated by GSK3 in mature, post-mitotic neurons, in particular the tubulin-binding proteins Tau, MAP1B, MAP2, CRMP2, CLASP2, and pVHL. Phosphorylation of these substrates causes their dissociation from tubulin, destabilizing the microtubule structure. In post-mitotic neurons where GSK3 activity levels are relatively high, this would reduce interactions between GSK3 substrates and tubulin, resulting in destabilization of microtubules. Accordingly, non-phospho-mutant forms of substrates (e.g., CRMP2, MAP1B) increase the stability of microtubules, causing impaired neurite outgrowth, and polarity in cultured primary neurons (Cole et al., 2004b; Trivedi et al., 2005; Yoshimura et al., 2005). In theory, high levels of phosphorylation of these substrates would promote their dissociation from microtubules, favoring dynamic remodeling of the cytoskeleton, and enhancing neuroplasticity, although this is yet to be proven *in vivo*.

GSK3 AND NEUROTRANSMISSION

A systematic screen of Ser/Thr kinases using a panel of pharmacological inhibitors revealed that GSK3 was the only kinase among 58 Ser/Thr kinases that was required for induction of NMDA-induced long-term depression (LTD) in hippocampal CA1 pyramidal neurons (Peineau et al., 2009). LTD increases GSK3 activity via decreased phosphorylation of Ser21/9 at its N-terminus, while NMDA-induced long-term potentiation (LTP) reduces GSK3 activity by increasing Ser21/9 phosphorylation (Hooper et al., 2007; Peineau et al., 2007). Meanwhile, GSK3 inhibitors do not affect baseline synaptic transmission (Peineau et al., 2007; Zhu et al., 2007; Li et al., 2009). GSK3 regulates transmission at both the pre- and post-synapse. For example, high GSK3 activity reduces glutamate release from the pre-synapse, inhibiting LTP (Hooper et al., 2007; Zhu et al., 2007, 2010), while retrieval of synaptic vesicles at the pre-synapse by endocytosis requires GSK3 (Clayton et al., 2010). Dynamin 1 is a large GTPase that regulates vesicle endocytosis at the pre-synapse. Phosphorylation by GSK3 at Ser774 is required for re-uptake of neurotransmitters during times of elevated neuronal activity (Clayton et al., 2010). Thus, relatively high GSK3 activity in differentiated neurons would be expected to activate Dynamin 1 and facilitate efficient recycling of neurotransmitters at the synapse. At the post-synapse, pharmacological inhibition of GSK3 decreases surface expression of NMDA and AMPA receptors (Chen et al., 2007; Wei et al., 2010). CRMP2 is a GSK3 substrate that has been implicated in trafficking of transmembrane proteins to the cell surface (Nishimura et al., 2003; Brittain et al., 2011), although the effect that phosphorylation has on this process has not yet been determined. Together, these observations demonstrate a clear requirement for GSK3 at the synapse, although the synaptic substrates that mediate these effects remain to be fully uncovered.

CONCLUSION

When analyzing the substrates of GSK3, a pattern emerges whereby those that are negatively regulated by GSK3 are commonly involved in promoting proliferation and/or survival, while substrates that are positively regulated by phosphorylation are predominantly expressed in differentiated post-mitotic neurons and are required for efficient function of mature neurons. The former substrates include pro-proliferation transcription factors or pro-survival proteins targeted for ubiquitin-mediated degradation by GSK3, while the latter are often cytoskeleton-associated proteins. Thus, low GSK3 activity levels are conducive to proliferative ES cells and NPC's, while higher GSK3 activity is required for efficient function of differentiated neurons. This pattern implies that pharmacologic manipulation of GSK3 activity can be used to influence cell fate between proliferative/undifferentiated and mature/differentiated states, as has already been successfully demonstrated for ES cells. In the brain, inhibition of GSK3 would promote proliferation of NPC's, while high levels of GSK3 would

promote neuronal differentiation and efficient function of post-mitotic neurons. Also, it is possible that high GSK3 activity in post-mitotic neurons could promote neuroplasticity, learning, and memory via increased dynamics of the cytoskeleton. Manipulation of GSK3 activity may be of great therapeutic benefit for neurodegenerative and other mental disorders. In AD, the use of pharmacologic inhibitors of GSK3 has been proposed to decrease phosphorylation of Tau, reducing its aggregation and formation of neurofibrillary tangles. This strategy has shown some success in mouse models of AD (Perez et al., 2003; Nakashima et al., 2005; Noble et al., 2005; Leroy et al., 2010). In elderly humans and AD patients, chronic (but not acute) treatment with GSK3 inhibitors reduced decline in cognitive and memory function (Nunes et al., 2007; Chiu and Chuang, 2010; Kessing et al., 2010; Forlenza et al., 2011). These studies have been performed using lithium, a relatively weak and non-specific inhibitor of GSK3, so it is necessary to advance these studies using more potent and specific inhibitors of GSK3.

Another exciting potential therapeutic use of GSK3 inhibitors in the clinic is to maintain neuron survival under stressful conditions, including neurodegenerative diseases and acute injuries, such as stroke. Since GSK3 inhibitors are such effective inhibitors of neuronal apoptosis (at least *in vitro*), rapid administration of these drugs could help to prevent neuronal loss during the immediate period following injury. By keeping these neurons alive, one might expect an improved prognosis for functional recovery. It might also promote proliferation of NPC's that could later be induced to differentiate into functional post-mitotic neurons to compensate for damages incurred at the site of injury. So far, several groups have elegantly demonstrated that lithium treatment effectively protects neurons and even promotes migration of stem cells to affected regions (Chiu and Chuang, 2010; Tsai et al., 2011).

Of course, there is the danger that inhibition of GSK3 activity could impede the basic function of post-mitotic neurons. However, it should be remembered that very few drugs inhibit kinases 100%, therefore any treatments are likely to reduce GSK3 activity, not completely inhibit it. Also, GSK3 substrates that are relatively resistant to phosphatases are beginning to be discovered [e.g., β-adducin (Farghaian et al., 2011), CRMP2 (Cole et al., 2008)] and moderate reduction of GSK3 activity is unlikely to affect the stoichiometry of phosphorylation of these substrates. This provides another good reason for identifying and characterizing each individual substrate of GSK3 in the brain. Importantly, downstream targets of GSK3 that are specifically involved in a particular neuronal process (e.g., neurogenesis, neurotransmission) may prove to be better therapeutic targets than GSK3, being more potent and selective with fewer side effects. Therefore, the full catalog of GSK3 substrates and their physiological functions needs to be completed.

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