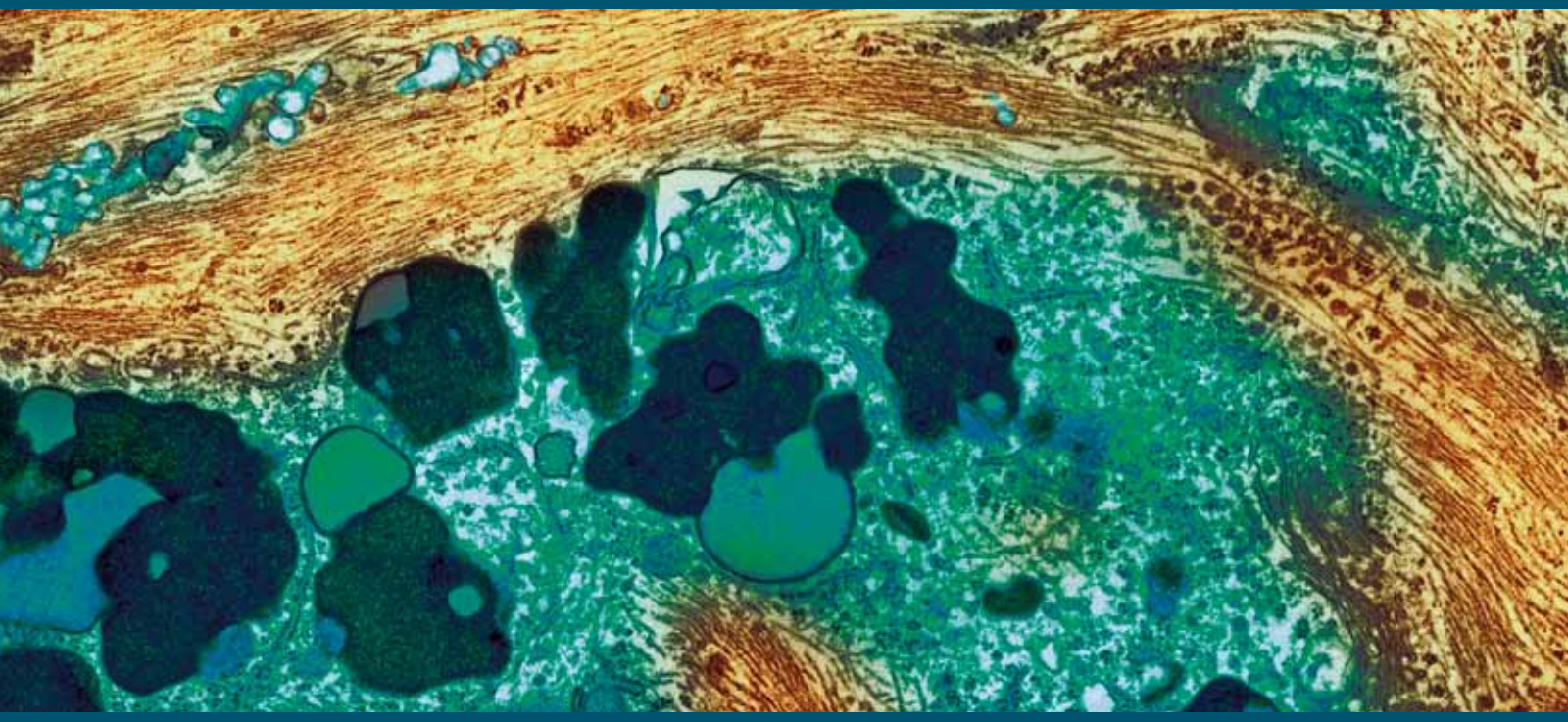
International Journal of Alzheimer's Disease

Glycogen Synthase Kinase-3

Guest Editors: Peter Crouch, Adam Cole, Michael Cousin, Ana Martinez, and Katja Kanninen



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Editorial Board

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International Journal of Alzheimer's Disease
Volume 2011, Article ID 279234, 1 page
doi:10.4061/2011/279234

Editorial

Glycogen Synthase Kinase-3

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Received 2 November 2011; Accepted 2 November 2011

Glycogen synthase kinase-3 (GSK3) is a ubiquitous and promiscuous kinase that has been studied extensively for over four decades. Initial reports beginning in the 1970s described its role in cellular metabolic pathways fundamental to glucose metabolism, but in more recent years the number of reports describing aberrant GSK3 activity in pathological conditions has risen dramatically.

Interest in GSK3 in the field of Alzheimer's disease was first sparked in the early 1990s by papers that described the ability of GSK3 to phosphorylate tau. Excessive tau phosphorylation is present in Alzheimer's-disease-affected brain. These early papers provided new insight to the mechanisms that may contribute to tau pathology of Alzheimer's, with GSK3 as a potential central figure. Since then, the research effort invested into GSK3 in Alzheimer's disease has expanded, and mechanistic studies now demonstrate a functional relationship between not only GSK3 and tau, but also GSK3 and amyloid-β. Through weight of numbers, strong evidence now indicates that GSK3 is associated with the two key pathological features of Alzheimer's-disease-affected brain: neurofibrillary tangles and amyloid plaques.

The scope of this special issue is to provide an overview of the data that implicate GSK3 in Alzheimer's disease. As an introduction, the special issue begins with a review of the regulation of GSK3 activity (M. Medina and F. Wandosell). This is followed by a report that provides caution by articulating the need to demonstrate the bona fide substrates of GSK3 (C. Sutherland). The role of GSK3 in the brain and neuronal function is then introduced by two reports. The first is on presynaptic function of GSK3 (K. J. Smillie and M. A. Cousin) and the second on GSK3 in brain development and neuronal plasticity (P. Salcedo-Tello et al.). After this the contribution of GSK3 to neurodegenerative diseases is described in four reviews. The first describes GSK3 in neurodegenerative diseases in general (P. Lei et al.) while the following three discuss more specific aspects of Alzheimer's disease, including cell survival mechanisms (M. A. Mines et al.), inflammation (j. Koistinaho et al.), and tau phosphorylation (D. P. Hanger and W. Nobel). Finally, the special issue concludes with two reviews on therapeutic strategies for Alzheimer's disease that focus on GSK3. The potential of targeting GSK3 for therapeutic benefit against oxidative stress is presented (K. Kanninen et al.), followed by an appraisal of GSK3 inhibitors in the next horizon (A. Martinez et al.).

Since several GSK3 inhibitors are currently in clinical trials for treatment of neurological and other disorders, we feel this special issue is a timely "snapshot" of our current knowledge of GSK3 function in healthy and diseased brain, and highlights outstanding issues for future research on this important brain kinase.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 479249, 12 pages
doi:10.4061/2011/479249

Review Article

Deconstructing GSK-3: The Fine Regulation of Its Activity

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Received 21 February 2011; Accepted 28 February 2011

Academic Editor: Peter Crouch

Glycogen synthase kinase-3 (GSK-3) unique position in modulating the function of a diverse series of proteins in combination with its association with a wide variety of human disorders has attracted significant attention to the protein both as a therapeutic target and as a means to understand the molecular basis of these disorders. GSK-3 is ubiquitously expressed and, unusually, constitutively active in resting, unstimulated cells. In mammals, GSK-3α and β are each expressed widely at both the RNA and protein levels although some tissues show preferential levels of some of the two proteins. Neither gene appears to be acutely regulated at the transcriptional level, whereas the proteins are controlled posttranslationally, largely through protein-protein interactions or by posttranslational regulation. Control of GSK-3 activity thus occurs by complex mechanisms that are each dependent upon specific signalling pathways. Furthermore, GSK-3 appears to be a cellular nexus, integrating several signalling systems, including several second messengers and a wide selection of cellular stimulants. This paper will focus on the different ways to control GSK-3 activity (phosphorylation, protein complex formation, truncation, subcellular localization, etc.), the main signalling pathways involved in its control, and its pathological deregulation.

1. Introduction

Glycogen synthase kinase-3 (GSK-3) is a CMGC serine/threonine protein kinase initially described as one of the kinases that phosphorylates and inhibits glycogen synthase [1]. It is now widely accepted though that GSK-3 plays an important role in various essential physiological processes, such as development, cell cycle, or apoptosis [2]. Apart from glycogen synthase, a plethora of different substrates has been identified in all cellular compartments, that is, metabolic proteins [3], cytoskeletal proteins [4], and transduction [5] and transcription factors [6] (see Table 1).

In neuronal development, GSK-3 has been reported to control morphogenesis and axonal polarity [7], synaptogenesis [8], and survival [9, 10]. In addition, GSK-3 dysfunction has been associated with brain pathological conditions, such as Alzheimer's disease (AD) [11, 12] or prion neurotoxicity [13]. Thus, the deep knowledge of the role of both GSK-3 isoforms in brain metabolism will allow us to understand their contribution to neurodegenerative processes.

GSK-3 unique position in modulating the function of a diverse series of proteins in combination with its association with a wide variety of human disorders has attracted significant attention to the protein both as a therapeutic target and as a means to understand the molecular bases of these disorders. Furthermore, GSK-3 appears to be a cellular nexus, integrating several signalling systems, including numerous second messengers and a wide selection of cellular stimulants.

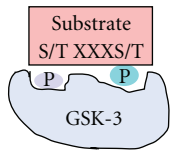
2. GSK-3 Structure

GSK-3 has been highly conserved during evolution, and homolog genes have been identified in virtually every eukaryotic genome investigated, including species, such as *Dictyostelium discoideum*, *Xenopus laevis*, or *Drosophila melanogaster* [14–16]. In mammals, GSK-3 is encoded by two genes known as *gsk-3*α and *gsk-3*β [17, 18] encoding GSK-3α (483 *aa* in humans) and GSK-3β (433 *aa*) proteins with apparent molecular masses of 51 and 47 kDa, respectively. Both isoforms are almost identical (98%) within their ATP binding pocket but differ at their N- and C-terminal domains [19]. A neuron-specific splicing isoform (β2) having an

Table 1: GSK-3 substrates.

Metabolic proteins	Structural proteins	Transcription factors
Glycogen synthase	Tau	AP-1
ATP cytrate lyase	MAP1B	β-catenin
PKA	MAP2	CREB
PDH	NCAM	C/EBP
Acetyl-CoA carboxylase	Neurofilaments	Myc
PP1	CRMP2	NFκB
PP2A inhibitor	Dynein	NFAT
PP2A	Dynamin-like protein	GR
Cyclin D1	MBP	HSF-1
eIF2B	APC	Notch
NGF receptor	Kinesin light chain	p53
Axin		HIF-1
APP		
Bax		
VDAC		
Hexokinase		
Presenilin		
LRP5/6		

GSK3 Selectivity



insertion of 13 *aa* within the substrate-binding domain has also been described [20]. Mammalian GSK-3α and β are each widely expressed although some tissues show preferential levels of some of the two proteins. Neither gene appears to be acutely regulated at the transcriptional level.

Crystallographic studies have revealed the three-dimensional structure of GSK-3β [21, 22]. Its overall shape is shared by all kinases, with a small N-terminal lobe mostly consisting of β-sheets and a large C-terminal lobe essentially formed of α-helices [23]. The ATP binding pocket is located between the two lobes and although, is well conserved among kinases [24], it is possible to obtain selective inhibitors by taking advantage of the small differences that exist between the different kinases. Current availability of crystal structures of complexes of GSK-3β with a variety of ligands, together with molecular modelling approaches, provides the necessary clues for enhancing selectivity towards GSK-3 [22].

Some GSK-3 substrates do not require a very specific sequence, but rather a previous (*primed*) phosphorylation by a *priming* kinase on a Ser or Thr residue located four aminoacids, C-terminal to the Ser or Thr residue to be modified by GSK-3 (see below for regulation through primed phosphorylation). The crystal structure of human GSK-3β has provided a model for the binding of prephosphorylated substrates to the kinase. According to it, *primed* Ser/Thr is recognized by a positively charged binding pocket formed by residues Arg96, Arg180, and Lys205 that facilitates the binding of the phosphate group of primed substrates. GSK3β uses the phosphorylated serine or threonine at position +4 of the substrate to align of the two domains for optimal catalytic activity [21, 22].

Furthermore, crystal structures of GSK-3β complexes with interacting proteins FRAT/GBP and axin have allowed defining the molecular basis for those interactions, which play critical role in some signalling pathways (see below for regulation through protein complex formation). These studies confirm the partial overlap of the binding sites of axin and FRAT1/GBP predicted from genetic and biochemical studies [2, 25] but reveal significant differences in the detailed interactions and identify key residues mediating the differential interaction with both proteins. This ability of GSK-3β to bind two different proteins with high specificity *via* the same binding site is mediated by the conformational plasticity of the 285–299 loop, while some residues in this versatile binding site are involved in interactions with both axin and FRAT; others are involved uniquely with one or the other [26].

3. How Is GSK-3 Activity Controlled?

As already mentioned above, one of the main characteristics of GSK-3 is that its activity is high in resting, unstimulated cells while regulated by extracellular signals that typically induce a rapid and reversible decrease in enzymatic activity. Glycogen synthase kinase-3 is a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation [27]. And for many years, it was believed to be a constitutively active kinase; however, it has become apparent that the activity of GSK-3 may be regulated by a variety of means. In fact, control of GSK-3 activity occurs by complex mechanisms that are each dependent upon specific signalling pathways. Thus, the regulatory mechanisms can be classified as follow.

3.1. Regulation by Phosphorylation. The first regulatory mechanism described of GSK-3 activity involved the phosphorylation of specific residues of GSK-3 by other kinases, and more recently through autophosphorylation [17, 28].

Four different regions and residues have been described in the GSK-3 molecule. The first one corresponds with a serine residue at positions 21 in GSK-3α and 9 in GSK-3β. It has been clearly established that phosphorylation of serine 21 or 9 correlates with the inhibition of its kinase activity [29–31]. Many protein kinases are capable of phosphorylating GSK-3 at this residue, such as Akt, ILK, PKA, and p90Rsk [32, 33], and many physiological situations of inhibition of GSK-3 correlate with serine phosphorylation, such as Insulin/IGF1, NGF, or estradiol treatments, not only in neurons [34].

Additionally, two other regulatory sites have been described. One is the threonine 43, present only in the isoform GSK-3β, which may be phosphorylated by Erk [35].

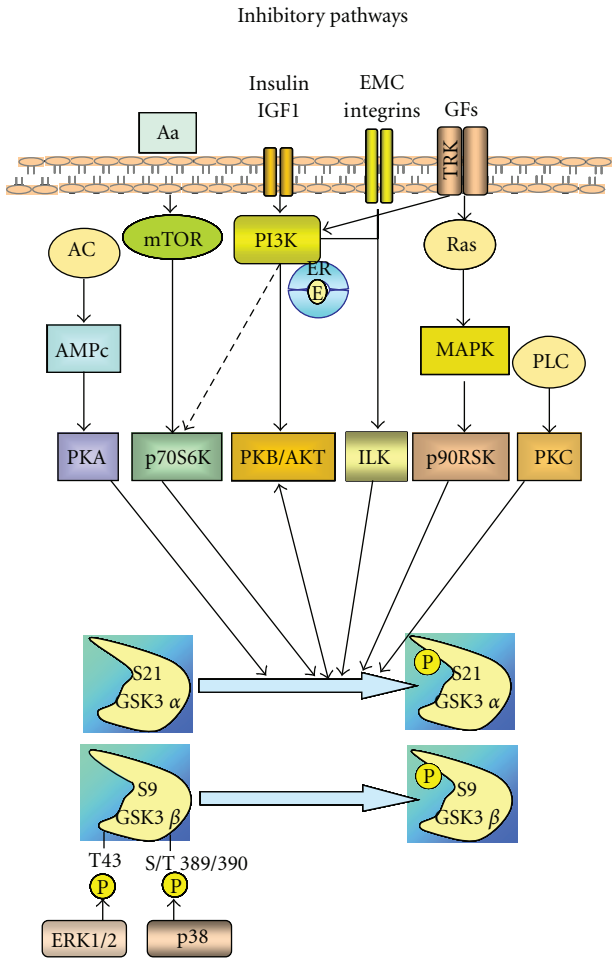


Figure 1

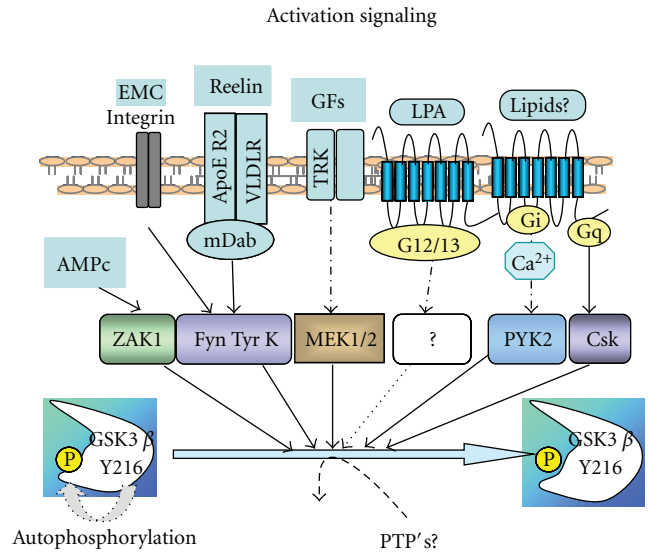


Figure 2

This phosphorylation correlated with GSK-3 inhibition. Second, serine 389 and threonine 390 present in GSK-3β have been shown to be phosphorylated by p38 MAPK [36]. In both cases, the data suggested that this phosphorylation may increase the capacity of Ser-9 to be phosphorylated rather than promote a direct inhibition (see Figure 1).

In contrast, tyrosine phosphorylation present in positions 279 in GSK-3α or 216 in GSK-3β, appears to correlate with an increase of its kinase activity [37]. Different candidates such as Pyk-2 and Fyn kinases have been reported to be able to phosphorylate GSK-3 *in vitro* on tyrosine. In addition, MEK1/2 has been showed to have this capacity only in fibroblasts [38, 39]. This data contrast with those reported in *Dictyostelium discoideum* where there is compelling evidence indicating that ZAK 1 is responsible for generating tyrosine phosphorylation in GSK-3 [14, 40]. However, no homologue of such kinase has been found in mammals.

More recently, an alternative hypothesis has been proposed for the regulation of GSK-3 tyrosine phosphorylation. This hypothesis suggests that in mammalian systems phosphotyrosine in GSK-3 corresponds to an intramolecular autophosphorylation event and may be regulated by Hsp90 [41]. Molecular dynamics and crystallographic studies clearly suggest that Tyr216 renders the kinase active through interactions with Arg220 and Arg223, stabilizing the activation loop and allowing full substrate accessibility [42, 43]. However, this hypothesis still lacks a cellular demonstration.

However, our data indicated that not all pharmacological inhibitors of GSK-3 decrease the level of phosphotyrosine. Therefore, lithium chloride inhibits GSK-3 activity, but this inhibition does not alter its pTyr content [44]. Moreover, in neuronal cells, tyrosine phosphorylation of residue 216 or 279 increased following exposure to LPA [37] and even upon exposure of neurons to β-amyloid or PrP [13, 45, 46] in a clear correlation with an increase in GSK-3 activity. In addition, in many neuronal cells, the pharmacological inhibition of tyrosine phosphatases with *ortho*-vanadate increases the basal level of GSK-3-pTyr [44]. Thus, considering all these data, in addition to this tantalizing autoregulatory system proposed, we hypothesized that some as-yet-unidentified tyrosine kinases and phosphatases may also regulate this kinase (see Figure 2).

3.2. Regulation by Protein Complex Association. One regulatory mechanism that is still not fully understood involves the interaction of the GSK-3 with structural proteins (scaffold proteins). It is well known that GSK-3 contributes to a multiprotein complex formed by axin and adenomatous polyposis coli (APC), among others (for review see, i.e., [47]). This protein complex is the core of canonical Wnt signalling (see below). Indeed, in the absence of ligand, GSK-3 is able to phosphorylate β-catenin for targeting it for proteasome degradation [48]. More recently, some data suggests that this complex may be specific for GSK-3β2 isoform [49], which opens the possibility of a deeper analysis of "specific functions of GSK-3 isoforms."

Another system of protein-kinase interaction was denoted as GSK-3-binding protein (GBP or FRAT) [25, 50].

Three different FRATs have been cloned and characterized; however, their mechanism of action is not well understood. FRAT1 appears to act as an inhibitory system [51], whereas FRAT2 appears to preferentially increase GSK-3-mediated phosphorylation in some residues [52]. Surprisingly, recent data demonstrated that FRAT is dispensable because the triple FRAT-knockout mouse lacks any major defect in brain development [53]. All these data indicated that the precise role of FRAT in GSK-3 regulation is still to be defined.

Using the binding site on GSK-3 for FRAT/GBP, a GSK-3-interacting protein symbolized by GSKIP has been cloned and characterized. GSKIP can block phosphorylation of different substrates and functions as a negative regulator of GSK-3 beta [54].

3.3. Regulation by Priming/Substrate Specificity. As previously mentioned, the specificity of many kinases is governed by a consensus sequence of aminoacids sequence. However, as almost general rule, GSK-3 substrates do not require a very specific sequence, but a previous (*primed*) phosphorylation residue modified by a *priming* kinase located four aminoacids, C-terminal, to the Ser or Thr residue to be modified by GSK-3. The crystal structure of human GSK-3β provides a model for the binding of prephosphorylated substrates to the kinase (PDB ID are 1I09 [22] and 1H8F [21]). According to it, *primed* Ser/Thr is recognized by a positively charged binding pocket formed by residues Arg96, Arg180, and Lys205 that facilitates the binding of the phosphate group of primed substrates.

Some "priming kinases" have been identified, such as cdk-5 [55–57], PAR-1 [58], casein kinase I [59], PK-C [60], or PK-A [57]. However, it is not clear so far whether a second set of "*nonprimed*" substrates may define a different group of functions [61]. In addition, different glycogen synthase kinase-3 isoforms appear to exhibit distinct substrate preference in the brain [62].

3.4. Regulation by Subcellular Localization. In developmental brain, the presence of GSK-3 was high at E18 and peaked on P8, decreasing after that period [63]. In addition, this report showed that the developmental profile of GSK-3α and GSK-3β is different, having β downregulated after birth which suggested a differential role in neuronal development. However, the putative differential role of each isoform has been explored in few reports, that is, [7]. It is important to indicate that a portion of GSK-3, mostly β, has been reported to be associated in the growth cone. This GSK-3 pool appears to respond rapidly, being modified by phosphorylation and/or relocated in the growth cone by external signals such as Semaphorins [64] or NGF [65].

GSK-3 activity is also dependent on its subcellular localization; some data illustrated the presence of GSK-3α and β in many neuronal compartments and in primary neurons, either in axon, dendrite, or in nucleus [66, 67]. In addition, GSK-3 has been found in the cytoplasm, nucleus and the mitochondria [18]. Considering the list of GSK-3 substrates reported, it is evident that most of its activity should occur in the cytoplasm and in the nucleus, while we have less information about GSK-3 potential targets in the mitochondria. Recent data suggested that proteins such as Mcl-1 [68] and hexokinase [69] may be regulated by GSK-3 activity. It has been suggested that nuclear GSK-3 may be involved in phosphorylation of many transcription factors such as cyclin D1, β-catenin, HSF-1, NFAT, and cAMP-response element-binding protein, among others (Table 1), for review see [28, 70, 71]. Also, it has been proposed that GSK-3 in the nucleus may have a role in alternative splicing [72]. In addition, proapoptotic stimuli induce nuclear accumulation of GSK-3β [73]; however, this hypothesis has been not established in other neuronal death paradigms (D. Simon, unpublished observations).

Further insight into GSK-3 regulation has been gained very recently by revealing an essential role of multivesicular endosomes in the Wnt signalling pathway. A combination of protease protection assays, detergent permeabilization, and cryoimmunoelectron microscopy demonstrated that Wnt activation of the Frizzled and LRP6 receptors triggers sequestration of GSK-3 into these membrane-bounded organelles, leading to decreased GSK-3 levels in the cytosol [74]. This process seems to require β-catenin, forming a feed-forward loop by facilitating GSK-3 sequestration.

3.5. Regulation by Proteolytic Cleavage. A new mechanism of GSK-3 regulation has been recently proposed. This regulation involves the removal by calpain of a fragment from the N-terminal region of GSK-3, including the regulatory serines 9/21. After removal of that fragment, GSK-3 becomes activated [75]. The same study showed that both isoforms α and β are cleaved by calpain, although with different susceptibility. Moreover, GSK-3 truncation has been observed in human and mouse postmortem brain tissue [76]. It is noteworthy to consider that a similar mechanism has been described for β-catenin in hippocampal neurons, where after NMDA-receptor-dependent activation, calpain induced the cleavage of β-catenin at the N terminus, generating stable and truncated forms which maintain its transcriptional capacity [77]. Likewise, GSK-3 truncation is mediated by extracellular calcium and can be inhibited by memantine [76], an NMDA antagonist used for the treatment of Alzheimer's disease. Interestingly, GSK-3β has also been recently shown to be cleaved at the N-terminus (and subsequently activated) by matrix metalloproteinase-2 (MMP-2) in cardiomyoblasts [78].

4. Pathways Controlling GSK-3 Activity in Neurons

The regulation of GSK-3, as previously mentioned, is an essential regulatory key controlling many physiological processes in neurons. Many external signals may trigger pathways that finally may activate or inhibit GSK-3 activity, either transiently or in more sustained way. These "physiological pathways" could be subdivided in two major clusters, those that essentially have to inhibit GSK-3 activity, and second, those that may, at least transiently, trigger GSK-3 activity.

4.1. Inhibitory Pathways. Among these pathways, the signalling triggered by Insulin or IGF-1 [19, 79, 80] and NGF/BDNF/NT3 [81, 82] has similar features. These tyrosine kinase receptors initiated cytoplasm signals in which the inhibition of GSK-3 activity is a common feature. It is generally accepted that the kinase implicated in this inhibition is PKB/Akt [29–31], even though kinases such as PKA or ILK have also been implicated [32, 33]. In all cases, phosphorylation on serine 21 and 9 (α and β, resp.) represents the inhibition of the GSK3 kinase activity, as previously mentioned.

The second well-documented pathway is Wnt/Wingless signalling [83, 84]. This signalling has been widely studied, and it has been shown to be essential in early embryonic patterning, cell fate, cellular polarity, and cell movement in both vertebrates and invertebrates [47, 85]. In many if not all cell systems, the canonical Wnt pathway is formed by a set of phylogenetically conserved proteins including the Wnt receptor *frizzled* (*fz*), and a coreceptor LRP5/6; *Dishevelled* (*Dsh*), and a scaffolding protein that activates a complex formed by Axin/APC/GSK3-β/β-catenin [47, 85–88].

In this pathway, in the absence of ligand, GSK-3β phosphorylates β-catenin, among other proteins, this phosphorylation constituting part of a degradation signal for β-catenin. However, in the presence of Wnt, the receptor complex triggers a signal in which *Dsh* inhibits the activity of GSK-3β by a mechanism not completely understood, so far. This system appears to be specific for GSK-3β as no counterpart has been described for GSK-3α to date; however, some GSK-3 activity appears to be necessary for Wnt signalling [89, 90]. More recently, a bioinformatics-based screen for proteins whose stability may be controlled by GSK-3 [91] has led to the identification of a number of multiple Wnt signalling target proteins, suggesting that this pathway controls a broad range of cellular activities apart form β-catenin-mediated transcriptional activation. Furthermore, GSK-3-mediated Wnt signalling seems to regulate the turnover of many cellular proteins [74, 91], indicating that GSK-3 phosphorylation-dependent protein degradation may be a widespread cellular mechanism to regulate a variety of cellular processes in response to extracellular signals [71].

Functional segregation of the insulin/growth factor and Wnt roles requires either that there be no exchange between the subsets of the cellular GSK-3β pool committed to each role, or that the recruitment of GSK-3β to the axin-APC complex can reverse or override inhibitory Ser9 phosphorylation present in a recruited GSK-3β molecule. Phosphatases capable of removing extant Ser9 phosphorylation are certainly known to be associated with the axin-APC complex [92, 93]. Alternatively, the very substantial enhancement in activity towards β-catenin afforded by the axin "scaffolding" may simply allow a primed β-catenin substrate to outcompete a pSer9-GSK-3β N-terminal peptide for access to the substrate-binding site [26].

Estrogens regulate many physiological processes and fulfil a wide range of functions during development and differentiation in mammals of both sexes. The actions of estrogens are mediated by estrogen receptors and have been classified as either "genomic actions" or "nongenomic, rapid actions." The genomic actions are based on the capacity of the estrogen receptors (ERs) to modulate transcriptional activity either directly or through coactivators or corepressors, that is, [94, 95].

More recently, it has been shown that in addition to its direct transcriptional activity, estrogen receptors activate a set of cytoplasm signals in a similar manner to some growth factors. Hence, it has been reported that estradiol acts synergistically with IGF-1 in the brain or in neurons, activating the PI3K/Akt pathway [34, 96, 97]. We described that the addition of estradiol increases the serine phosphorylation of GSK-3. This inhibitory phosphorylation is time- and concentration dependent, and an antagonist of estradiol prevents this event. The kinase responsible is sensitive to the inhibition of the PI3K pathway, and for this reason, it seems that the best candidate would be Akt [98, 99]. A more detailed analysis of these new signals will give us clear evidence whether this pathway is completely convergent with those using PI3K/Akt/GSK3, as previously mentioned.

4.2. Activation Pathways. In neurons, LPA has been shown to induce neurite retraction and the rounding up of neuroblastoma cell lines [100]. In some primary neurons, it also promotes growth cone collapse and neurite retraction [37, 101]. This bioactive lipid acts as a growth factor through specific seven transmembrane domain receptors, denoted as *lpa* 1–4 [102, 103]. We described that GSK3 activity was increased after LPA treatment in diverse neuronal cells of different species in correlation with the neurite retraction process [104, 105]. This activation correlated with an increase in GSK3-PTyr and may be downstream $G\alpha_{12}$ or $G\alpha_{13}$ [101, 105, 106]. The previous inhibition of GSK3 activity prevents, at least in part, the growth cone collapse response. Similarly, it has been reported that three different GSK-3 antagonists (LiCl, SB-216763, and SB-415286) can inhibit the growth cone collapse response induced by Sema 3A [64].

However, the exact mechanism of how this activation of GSK3 occurs is not known, so far. Many reports indicate that in *Dictyostelium discoideum* GSK-3 activity may increase in response to cAMP binding to a heptahelical G-protein-coupled receptor. In this system, a tyrosine kinase and a tyrosine phosphatase have been described as regulators of GSK-3 activity [14, 40], but similar kinase and phosphatase have not been found in mammals.

Furthermore, it has been reported that Reelin and Netrin increased GSK-3 activity, similar to what LPA did. This Netrin or Reelin-dependent GSK-3 activation seems not to be a particular characteristic of the cell line or neuron used but rather a more general physiological process [107–109]. Indeed, even in situations where the final balance is an inhibition of GSK-3 kinase activity, such as following the addition of IGF1/Insulin or after estradiol addition, a transient activation of GSK-3 could be observed [34, 38]. All these data suggest that the upregulation and downregulation of this kinase is more complex than might initially have been considered.

5. Pathological Activation of GSK-3

Deregulation of GSK-3 has been linked to a wide range of human pathological conditions including type II diabetes, muscle wasting, cancer and neurological disorders such as bipolar disorder, schizophrenia, depression, stroke, sleep disorders, and Alzheimer's disease (AD), among others, for a review see [110].

Lithium and valproic acid are mood stabilizers widely used in the chronic treatment of bipolar disorders. Lithium ions directly inhibit GSK-3 [111], most likely by competing with magnesium [112], while valproic acid is able to inhibit GSK-3 activity in relevant therapeutic concentrations in human neuroblastoma cells [113] although *in vivo* direct inhibition of GSK-3 by valproic acid remains a matter of debate [114]. The precise mechanism of action by which lithium exerts its therapeutic effects is not known, but it is conceivable that the acute effects on GSK-3 result in changes in gene regulation and cellular changes which could affect the neuronal plasticity over time [115]. Actually, lithium is also an inhibitor of several phosphomonoesterases [116] and phosphoglucomutases [117], but the fact that GSK-3 has been shown to be significantly inhibited at therapeutic lithium concentrations [118–120] suggests that at least a significant proportion of lithium's therapeutic actions in bipolar disorder results from the inhibition of GSK-3, underlying its importance as a therapeutic target for this disorder [121, 122].

Lymphocytes of patients with schizophrenia show impaired GSK-3 protein levels and activity [123], whereas GSK-3 has been reported to be reduced in the frontal cortex of postmortem schizophrenic brains [124]. Since the Wnt family of genes plays a central role in normal brain development, it is possible that GSK-3 impairment may lead to abnormal neuronal development. More recently, a direct association has been shown between GSK-3 and the N-terminal region of disrupted-in-schizophrenia-1 (DISC1), a strong genetic risk factor associated with schizophrenia [125]. Moreover, mounting evidence suggests that GSK-3 is a crucial node that mediates various cellular processes that are controlled by multiple signalling molecules such as DISC-1, PAR3, PAR6, and Wnt proteins that regulate neurodevelopment [88].

Interestingly, increased levels of GSK-3 have also been reported in postmortem analysis of brains from AD patients compared to age-matched control samples [126], whereas a spatial and temporal pattern of increased active GSK-3β expression correlates with the progression of NFT and neurodegeneration [127]. Apart from being the major kinase to phosphorylate tau both *in vitro* and *in vivo* [128], GSK-3 has been recently proposed as the link between the two major histopathological hallmarks of AD, the extracellular amyloid plaques and the intracellular NFT [129, 130].

Exposure of primary neuronal cultures to Aβ induces activation of GSK-3 [131], tau phosphorylation [132], and cell death [133, 134], whereas blockade of GSK-3 expression by antisense oligonucleotides or its activity by lithium inhibits Aβ-induced toxicity [135, 136]. GSK-3 inhibition *per se* decreases Aβ production in cells and in an animal model of amyloidosis [61, 120], most likely through a mechanism involving inhibition of γ-secretase [137]. Furthermore, amyloid precursor protein (APP) itself is a substrate for GSK-3 *in vitro* and *in vivo* [138]. Finally, modulation of the GSK-3 signalling pathway by chronic lithium treatment of transgenic animals might also have neuroprotective effects by regulating APP maturation and processing [138].

In tauopathies such as frontotemporal dementia with Parkinsonism (FTDP) linked to chromosome 17, the presence of some mutations in tau protein correlates with the onset of the disease [139, 140]. Treatment of transgenic mice overexpressing mutant human tau (P301L, 4RON), with the GSK-3 inhibitor lithium, has been shown to significantly decrease the levels of tau phosphorylation and significantly reduce the levels of aggregated, insoluble tau. Administration in this model of a second GSK-3 inhibitor, AR-A014418, also correlated with reduced insoluble tau levels, supporting the notion that lithium exerts its effect through GSK-3 inhibition [23]. More recently, chronic lithium administration has also been shown to reduce tau phosphorylation in the 3xTg-AD mice [141], but did not significantly alter the amyloid load.

An increase in GSK-3 activity has also been shown to coincide with cell death following middle cerebral artery occlusion in mice which results in cortical infarcts [142], and a reduction in infarct volume with the GSK-3 inhibitor lithium was demonstrated [143], indicating that GSK-3 inhibition may be beneficial in stroke. In fact, pharmacological inhibition of GSK-3 reduced infarct volume and improved behaviour in a focal cerebral ischemia model [144].

6. GSK-3 as a Therapeutic Target

Besides deregulation of its activity in neurodegenerative processes, mounting evidence further suggests a potential role for GSK-3 as a therapeutic target in a range or other pathologies, including pancreatic cancer [145], parenchymal renal diseases [146], and HIV-1-associated dementia [147], among others.

The recent discovery that glycogen synthase kinase-3 (GSK-3) promotes inflammation through nuclear factor *kappa* B (NFκB) has revealed new functions on regulating inflammatory processes [148]. Furthermore, GSK-3 inhibition provides protection from inflammatory conditions in different animal model [149], suggesting that GSK-3 inhibitors may have multiple effects influencing these conditions.

Finally, recent developments suggest an active role of GSK-3β in various human cancers, although its role in tumourigenesis and cancer progression remains controversial. It may function as a "tumour suppressor" for certain types of tumours, whereas it seems to promote growth and development for some others. Deregulation of GSK-3β has been shown to promote gastrointestinal, pancreatic, and liver cancers and glioblastomas. Furthermore, GSK-3β inhibition attenuates cell survival and proliferation, induces cell senescence and apoptosis, and sensitizes tumour cells to chemotherapeutic agents [150] and ionizing radiation [151]. Nevertheless, an attractive target for a variety of human

diseases, its therapeutic potential on tumourigenesis, and cancer chemotherapy still needs to be carefully evaluated [152].

The close involvement of GSK-3 activity in different human pathologies has sparked intense efforts in developing inhibitors as therapeutic agents. Thus, the discovery of small molecule GSK-3 inhibitors in the last few years has not only attracted significant attention to the protein as a therapeutic target but also has provided a means to further understand the physiological functions of GSK-3 and to gain further insight into the molecular basis of those disorders.

In fact, at least one small molecule GSK-3 inhibitor program has made it to the clinic [153]. Tideglusib (NP-12) is a synthetic small molecule form the TDZD chemical class [110] which is currently in phase II development for two CNS indications: Alzheimer's disease and progressive supranuclear palsy (PSP), a tauopathy [154].

7. Conclusion

Three decades after its discovery as a protein kinase involved in glycogen metabolism, GSK-3 was revealed as a key enzyme in regulating many critical cellular processes, providing a link between many different substrates and various signalling pathways as well as gene expression. Modulation of its activity has also turned out to be much more complex than originally thought, as evident from what has been reviewed here. Furthermore, its role in a variety of highly relevant human pathological conditions has drawn significant attention to this enzyme as a potential therapeutic target, and the recent development of specific inhibitors has granted us new tools to dissect out its molecular and physiological functions while providing novel therapeutic agents. Taken all together, the next few years will certainly bring us further insights into the cellular functions of this fascinating enzyme.

Acknowledgments

The authors are grateful to Dr. J. Diaz-Nido, Dr. JJ Garrido, and Dr[a]. I Anton, for corrections to the paper and thoughtful discussions; and Dr[a]. D. Simon for providing some GSK3 schemes. This work was supported by grants from CDTI's program "*CENIT-INGENIO 2010*": "*Consorcio MELIUS*" (2006–2010), and "*Consorcio MIND*" (2006–2010), Ref-050106090004. In addition, FW was supported by Grants from CIBERNED (which was an initiative of ISCIII), "Plan Nacional," DGCYT, SAF2009-12249-C02-01, EU-FP7-2009-CT222887 and by an Institutional grant from the "Fundación Areces." M. Medina acknowledges grant support from EU-FP7 (NeuroGSK3, Project no. 223276) and from CDTI's CENIT program (DENDRIA, Project no. CEN-20101023).

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 505607, 23 pages
doi:10.4061/2011/505607

Review Article

What Are the *bona fide* GSK3 Substrates?

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Received 9 February 2011; Accepted 9 March 2011

Academic Editor: Adam Cole

Nearly 100 proteins are proposed to be substrates for GSK3, suggesting that this enzyme is a fundamental regulator of almost every process in the cell, in every tissue in the body. However, it is not certain how many of these proposed substrates are regulated by GSK3 *in vivo*. Clearly, the identification of the physiological functions of GSK3 will be greatly aided by the identification of its *bona fide* substrates, and the development of GSK3 as a therapeutic target will be highly influenced by this range of actions, hence the need to accurately establish true GSK3 substrates in cells. In this paper the evidence that proposed GSK3 substrates are likely to be physiological targets is assessed, highlighting the key cellular processes that could be modulated by GSK3 activity and inhibition.

1. Introduction

1.1. Why Identify Substrates?

Glycogen synthase kinase-3 (GSK3) was first reported as a glycogen synthase phosphorylating activity in rabbit skeletal muscle (the third to be found, hence GSK3) [1]. GSK3 was later identified as a major tau protein kinase [2]. These substrates immediately focused attention on the importance of GSK3 in glucose metabolism and neurodegeneration, and these remain major areas of GSK3 research. Indeed GSK3 inhibitors, which were initially developed for the treatment of diabetes, are now being investigated for the treatment of Alzheimer's disease, as well as many other conditions [3–5]. These therapeutic programmes have arisen directly from substrate identification; however, more recently the multitude of GSK3 substrates proposed in the literature has lessened therapeutic interest in this enzyme. It is therefore of great importance to establish beyond doubt what the physiological targets of this enzyme are, not only to focus therapeutic potential but also establish actual side effects of manipulating GSK3 activity.

1.2. Problems with False Positives. It is reasonably straightforward to implicate a protein as a substrate for a kinase, with evidence ranging from the existence of a consensus phosphorylation sequence in the primary structure of a protein through to regulation of phosphorylation by manipulation of the protein kinase *in vivo*. Unfortunately, the existence of a consensus sequence is rarely a good predictor of whether a protein will be a substrate of that kinase. Indeed GSK3 target consensus sequences occur in more than half of all known human proteins, most of which are clearly not regulated by GSK3. In addition, phosphorylation *in vitro* does not always correlate with phosphorylation *in vivo*, and great care has to be taken to characterise specificity of reagents, initial rate kinetics, and stiochiometry of phosphorylation *in vitro* and *in vivo* (see below).

1.3. Criteria for Confidence. Establishing whether a proposed substrate is a true physiological substrate of GSK3 is not straightforward; however, three major criteria, if met, can improve confidence. *Firstly*, highly purified GSK3 (keeping in mind that many commercial preparations are contaminated with copurifying kinases) should phosphorylate the proposed substrate at a significant rate *in vitro* (ideally in comparison to other well-characterized substrates), and at residues on the substrate that are phosphorylated *in vivo*. *Secondly*, manipulation of GSK3 activity in cells and *in vivo* (by genetic, pharmacological, *and* physiological means) should change the phosphorylation of the specific residue targeted by GSK3 *in vitro* (i.e., GSK3 inhibition should specifically reduce phosphorylation of this site in cells). *Finally*, a function of the substrate should change in parallel

to alteration of phosphorylation and cellular GSK3 activity, while mutation of the GSK3 target residue to alanine should render this function insensitive to GSK3 manipulation.

1.4. Specific Issues Relating to Addressing These Criteria for GSK3 Substrates. Substrate phosphorylation by GSK3 *in vitro* is complicated by the requirement for prephosphorylation (priming) of most characterised substrates [3, 6]. Purified, bacterially expressed recombinant proteins will contain little phosphate, and thus, if a substrate requires priming, the bacterially expressed protein will not be phosphorylated at an appreciable rate by GSK3 *in vitro*. Therefore, prephosphorylation with an appropriate priming kinase is often required in order to permit subsequent phosphorylation by GSK3. In contrast the existence of this priming mechanism provides the opportunity for additional validation of the protein as a GSK3 substrate. Mutation of the priming residue to alanine, or inhibition of the priming kinase in cells, should prevent subsequent phosphorylation by GSK3.

2. GSK3 Biology

2.1. Gene Structure and Splicing. There are two GSK3 genes (GSK3α and GSK3β) that account for all GSK3 activity in mammals [7]. In addition, the GSK3β mRNA undergoes alternative splicing that produces at least two different protein products GSK3β1 and GSK3β2. The catalytic domain is highly conserved between all GSK3 isoforms, although GSK3β2 has a 13 amino acid insert in this domain [8–11]. GSK3α has an N-terminal glycine rich extension that results in a larger relative molecular weight (51 kDa for GSK3α, and 47 kDa for GSK3β1, GSK3β2 exhibits intermediate mobility upon SDS-PAGE of around 49kDa). GSK3α and GSK3β1 are ubiquitously expressed [7], although relative expression does vary from tissue to tissue (e.g., GSK3β is the predominant isoform in brain [11]). In particular, the GSK3β2 isoform is enriched in neurons although the role of this variant remains unclear [8, 10].

2.2. Unusual Aspects of GSK3 Regulation and Substrate Identification. GSK3 is one of the few protein kinases to be inhibited (as opposed to activated) following stimulation of growth factor receptors. The basal activity of GSK3 in resting cells is relatively high while exposure of cells to growth factors, serum, or insulin reduces the specific activity of GSK3 by between 30 and 70% (dependent on cell type and stimuli) within 10 mins. This appears the case for all GSK3 isoforms. Inhibition is predominantly achieved through phosphorylation at a conserved N-terminal serine (Ser-21 in GSK3α and Ser-9 in GSK3β) [12, 13], and growth factors, promote GSK3 phosphorylation by activation of PKB or p90RSK [14, 15] while insulin inhibits GSK3 mainly through PKB [14]. This indicates that phosphorylation of many *bona fide* GSK3 substrates should be reduced upon stimulation of cells with serum, growth factors or insulin (Figure 1).

GSK3 is one of only a handful of the 500 mammalian protein kinases that have a strong preference for substrates that are already phosphorylated. Most of the best described GSK3 substrates require pre-phosphorylation at a residue 4 or 5 amino acids C-terminal to the GSK3 target residue (Table 1(a)), a phenomenon referred to as PRIMING. Hence the general GSK3 substrate consensus sequence is Ser/ThrXXX(PhosphoSer/Thr), where X is any residue. However, proposed substrates of GSK3 exist that do not conform to this sequence, having either a priming site much further from the target site, or no apparent requirement for priming at all (Table 1(a)). It is not yet clear how GSK3 recognises unprimed substrates; however, in almost every example of primed substrate the lack of priming reduces phosphorylation by GSK3 by >90%, demonstrating the importance of the phosphorylated residue C-terminal to the target site. Priming also allows the regulation of the GSK3-substrate reaction by N-terminal phosphorylation of GSK3. GSK3 has a phosphate binding pocket which interacts with the substrate at the primed Ser/Thr and positions it for phosphorylation by GSK3. Phosphorylation of Ser-21/9 of GSK3α/β results in the N-terminal domain of GSK3 interacting with its phosphate binding pocket, preventing recognition of primed substrates [6]. This inhibition can be overcome by increasing substrate concentration (at least *in vitro*), and it suggests that modulation of this aspect of regulation (e.g., by growth factors) would not inhibit phosphorylation of unprimed substrates (Figure 1) [6].

The semaphorin family of axonal guidance molecules induces GSK3 activity at the leading edge of migrating cells through the dephosphorylation of this N-terminal serine [16, 17]. The mechanism is not fully elucidated but involves activation of R-RAS(GAP) and the subsequent suppression of R-Ras [17]. Again this mechanism of regulation suggests that semaphorins regulate only primed substrates of GSK3.

There are reports that GSK3 activity can be *induced* by specific extracellular stimuli, and this regulation appears to be particularly apparent in the brain [18]. In theory, induction of phosphorylation at Tyr216 (GSK3β1 numbering) is a mechanism for regulating GSK3 activity [18]. Phosphorylation of this tyrosine is crucial for proper folding of the catalytic domain, and it occurs through autophosphorylation during synthesis of the GSK3 polypeptide [19]. As such Tyr216 is likely to be constitutively phosphorylated to high stoichiometry [20], yet an increase in Tyr216 phosphorylation was observed in PC12 cells following removal of NGF (and other apoptotic stimuli), correlating with increased GSK3 activity [18]. However, this observation has subsequently been challenged [20].

Interestingly, regulation of GSK3 by the canonical Wnt signaling pathway does not involve N-terminal or tyrosine phosphorylation [21]. Wnt regulation of GSK3 is most likely achieved through disruption of a specific complex including Axin/APC/β-catenin and GSK3 (Figure 1). This mechanism has been well characterized for the phosphorylation and degradation of β-catenin, and could in theory be as effective at inhibiting phosphorylation of unprimed substrates. In addition, this pathway demonstrates the existence of separate intracellular pools of GSK3, since insulin will not regulate β-catenin activity and Wnts will not regulate glycogen synthesis [22–24]. Hence compartmentalization of GSK3 allows

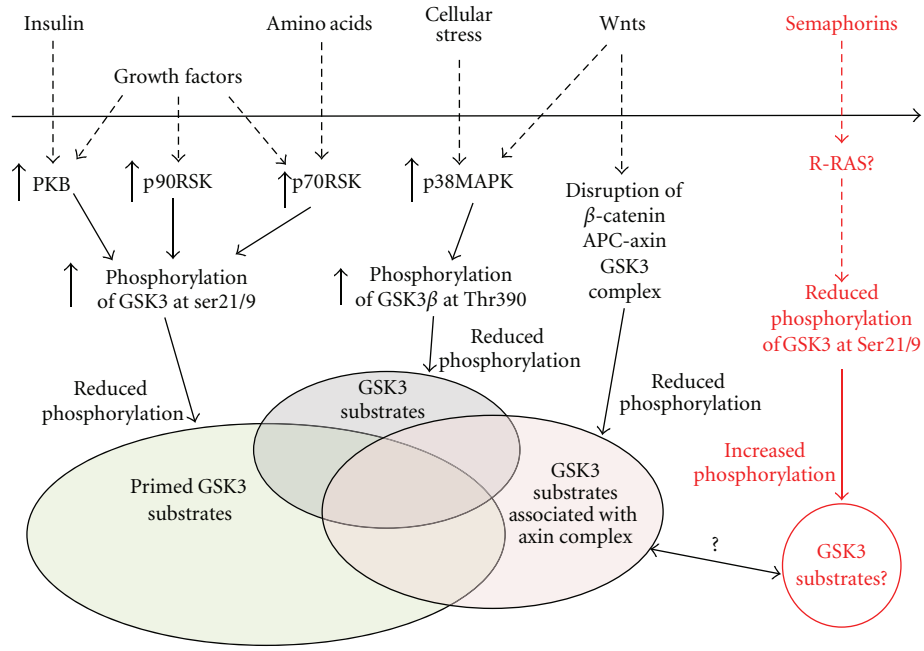

Figure 1: Different signaling pathways regulate GSK3 activity by different mechanisms, and this could permit differential regulation of GSK3 substrate phosphorylation.

differential upstream regulation but also enables differential downstream substrate phosphorylation (Figure 1).

Finally, the stress-induced p38MAPK family can phosphorylate Thr390 of GSK3β reducing its activity, and this also contributes to canonical Wnt signaling and regulation of substrates such as β-catenin [25, 26]. This is of particular interest as the residue is not conserved in GSK3α and thus provides a potential GSK3 isoform specific regulation.

3. GSK3 Substrates: Physiological Function and Therapeutic Potential

3.1. Genetic Studies to Elucidate GSK3 Function. Deletion of the GSK3β gene in mice is lethal [144, 145], while GSK3β heterozygous (+/−) mice exhibit reduced aggression, increased anxiety, reduced exploratory activity, poor memory consolidation, and reduced responsiveness to amphetamine [146–148]. Conversely, overexpression of GSK3β in brain results in hyperactivity and mania [149].

Mice lacking GSK3α are viable and relatively normal [150], exhibiting a small improvement in insulin sensitivity and glucose tolerance. Mice lacking GSK3α specifically in neurons display reduced aggression and exploratory activity, decreased locomotion, impaired co-ordination, and a deficit in fear conditioning [151]. The differential phenotypes between isoform deletions suggest nonredundant functions of the GSK3 genes in the brain, while the overlapping behavioural problems between GSK3α neuronal knockout (KO) and GSK3β (+/−) mice suggest some common substrates.

Deleting both GSK3 isoforms in the brain induces self-renewal of neuronal progenitor cells, but reduced neurogenesis [152]. Mutation of the N-terminal regulatory serine to alanine renders GSK3 insensitive to growth factor regulation. GSK3α/β double knockin mice (where both isoforms are replaced by mutant proteins with Ser to Ala alterations at Ser21 and Ser9, resp., [22]) show impairment of neuronal precursor cell proliferation [153]. Taken together, these data indicate that proper regulation of expression and activity of GSK3 is required for maturation of these cells during mammalian brain development. However, the substrates that mediate this function are unknown. Conversely, overexpression of GSK3β in the brain (using the Thy1 promoter) induces microcephaly [154, 155].

Alternative splicing of GSK3β between exon 8 and 9 gives rise to two main variants of this isoform [8]. GSK3β1 is the most widely expressed; however GSK3β2 (including a 13 amino acid insert due to use of exon 8A) is highly enriched within the brain [8]. The inserted sequence lies within the kinase domain, and there is preliminary evidence that these variants exhibit differential substrate specificity [8, 9, 11]. However, how this impinges on GSK3 function remains unclear.

Therefore, although genetic ablation of one or both genes for GSK3 has provided clues as to the cellular processes that require GSK3 activity, it has not yet established the molecular connections responsible for these phenotypes. Table 1(a) lists more than 100 sequences within 77 proteins that are proposed as substrates of GSK3, virtually none of these have been examined in tissue from GSK3 null animals. Table 1(b) lists the substrates from Table 1(a) where at least 2 of the 3 criteria for confidence (as detailed in Section 1.3) have been met. This represents around half of the sites and proteins listed in Table 1a (all three criteria have been met for very few substrates) and covers a variety of cellular processes, as detailed below.

Table 1: (a) A list of proteins reported to be substrates for GSK3. Where the phosphorylation site, priming mechanism and functional outcome of phosphorylation have been reported, this information is included. ND : not determined. (b) A list of those substrates from (a) that meet at least two out of the three criteria for confidence detailed in Section 1.3 of text, including cellular process likely to be regulated. These are the substrates discussed in more detail in the review.

(a)

Proposed substrate	Target residue(s)	Priming residue (kinase)	Effect of phosphate	Ref
Amyloid precursor protein	Thr743 (APP770) Thr668 (APP695)	ND	Regulates trafficking	[27–29]
APC	1501 1503	1505 (CK1) 1507 (CK1)	Regulates degradation	[30, 31]
ATP-citrate lyase	Thr446, Ser450	Ser454 (unknown)	May regulate activity	[32, 33]
Axin	Ser322/Ser326 (putative)	Ser330	Regulates stability	[34, 35]
Axil	Not Determined		Not reported	[36]
BCL-3	Ser394, Ser398 (putative)	Ser398 (ERK putative)	Regulates degradation	[37]
β-catenin	Ser33, 37, Thr41	Ser45 (CK1)	Regulates degradation	[34]
δ-catenin	Thr1078 (putative)	ND	Regulates degradation	[38]
C/EBPalpha	Thr222, Thr226 (Questioned)	NONE	NONE	[39, 40]
C/EBPbeta	Ser184, Thr179	Thr188 (MAPK)	Induces DNA binding	[41]
	Thr189, Ser185, Ser181, Ser177	NONE	Reduces DNA binding	[42]
Ci-155	Ser852, Ser884, 888	Ser856 (PKA) Ser892 (PKA)	Regulates Degradation	[43, 44]
CLASP	Residues between 594 and 614		Alters affinity for microtubules	[45, 46]
CLASP2	Ser533 and Ser537 (others)	Ser541 (CDK5)	Affects protein-protein interaction	[47]
CRMP2	Thr509, Thr514, Ser518	Ser522 (CDK5)	Regulates axon growth, growth cone collapse, and neuronal polarity	[48, 49]
CRMP4	Thr509, Thr514, Ser518	Ser522	Regulates axon outgrowth and chromosomal alignment	[48, 50]
CREB	Ser129	Ser133 (PKA)	Kinase activation	[51, 52]
CRY2	Ser553	Ser557	Promotes nuclear localisation and degradation	[53]
CTP: phosphocholine cytidylyltransferase	Multiple, within C-term 52 residues	?	No effect on activity	[54]
Cytidine triphosphate synthetase (CTPS)	Ser571	Ser575	Phosphorylation may reduce activity	[55]
Cyclin D1	Thr286	NONE	Nuclear export and degradation	[56]
			Questioned	[57]
Dynamin I	Thr774	Thr778 (CDK5)	Required for activity dependent bulk endocytosis	[58]
Dystrophin	ND	CKII ?	Not reported	[59]
eIF2B	Ser535	Ser539 (DYRK)	Inhibits activity	[60–62]
FAK	Ser722	Ser726	Inhibits activity	[63]
Gephyrin	Ser270	ND	Modulates GABAergic transmission	[64]
Glycogen Synthase	Ser640, 644, 648, 652	Ser656 (CKII)	Reduces activity	[65, 66]
Glucocorticoid receptor	Thr171 (Not conserved in human protein)	NONE	Inhibits GR activity towards some genes	[67]

(a) Continued.

Proposed substrate	Target residue(s)	Priming residue (kinase)	Effect of phosphate	Ref
Heat shock factor 1	Ser303	Ser307 (MAPK)	Reduces DNA binding	[68]
HIF1alpha	Ser551, Thr555, Ser589	ND	Induces proteosomal degradation	[69]
Histone H1.5	Thr10	NONE	Coincides with chromosome condensation	[70]
hnRNP D	Ser83	Ser87	Inhibits transactivation	[71]
IRS1	Ser332	Ser336	Promotes degradation	[72]
c-jun, Jun B, Jun D	Thr239	Thr243	Reduces DNA binding	[73–75]
K-casein	ND	ND	Not reported	[76]
KRP (telokin)	Ser15	Unknown site but ERK2 proposed	Not reported	[77]
LRP6	C-terminal PPPT/SP motifs (Ser1490, Thr1572, Ser1590)	NONE	Not clear	[78, 79]
MafA	Multiple sites, not identified	ND	Phosphorylation induces MafA degradation and prevents insulin gene expression	[80]
MAP1B	Ser1260, Thr1265 Ser1388	NONE Ser1392 (DYRK)	Regulates stability (lithium promotes degradation)	[81–83]
MAP2C	Thr1620, Thr1623	ND	Reduces microtubule binding	[84]
MARK2/PAR1	Ser212	NONE	Regulates activity	[85, 86]
Mcl1	Ser140	Thr144 (JNK)	Permits degradation of Mcl1 in response to UV stress	[87–89]
Mdm2	Ser240, Ser254	Ser244, Ser258 (CK1)	Promotes activity towards p53, reducing p53 levels	[90]
MITF	Ser298	NONE	Increases transactivation	[91]
MLK3	Ser789, Ser793	ND	Activates MLK3 Induces apoptosis in PC2 cells	[92]
MUC1/DF3	Ser40 (possible)	Ser44 (possible)	Inhibits formation of b-catenin-E-cadherin complex	[93]
c-myb	Thr572	ND	Not clear	[94, 95]
c-myc, L-myc	Thr58, Thr62 (c-myc)	Ser62 (ERK1/2)	Promotes degradation	[96–98]
Myocardin	8 serines in two blocks, Ser455—467 and Ser624—636	Yes but kinase not reported	Phosphorylation inhibits myocardin induced transcription	[99]
αNAC (nascent polypeptide associated complex)	Thr159	NONE	Induces transactivation, maybe stability	[100]
NDRG1	Thr342, Ser352, Thr362	Thr346, Thr356, Thr366 (SGK)	Not reported	[101]
neurofilament L	Ser502, 506, 603, 666 (M)	ND	Not reported	[102]
neurofilament M	Ser493 (H)			[103] (M)
neurofilament H				[104] (H)
NFAT	SRR domain SP-2 domain SP-3 domain	ND PKA or DYRK PKA	Induces nuclear exclusion, inhibits DNA binding	[105–107]
Ngn2	231 and 234	ND	Facilitates interaction with LIM TFs, involved in motor neuron determination	[108]

(a) Continued.

Proposed substrate	Target residue(s)	Priming residue (kinase)	Effect of phosphate	Ref
Notch 1C	ND		Stabilises protein	[109]
Nrf2	ND		Inhibits activity by nuclear exclusion	[110, 111]
OMA1	Thr339	Thr239 (MBK-2)	Induces degradation	[112]
p130Rb	Ser948 Ser962 Ser982	Ser952 Ser966 Thr986 all CDK putative	Regulates stability	[113]
p21 CIP1	Thr57	ND	Induces degradation	[114]
p27Kip1	Not fully established	ND	Regulates stability	[115]
p53	Ser33 (GSK3beta only)	Ser37 (DNA-PK)	Increases transcriptional activity	[116]
	Ser315, Ser376	ND	Increases cytoplasmic localisation, degradation, inhibits apoptosis	[117]
p65 RelA	Multiple, including Ser468	ND	Negatively regulates basal activity	[118, 119]
PITK	Ser1013	Ser1017 (CAMKII)	Induces nuclear localization and possibly interaction with PP1C	[120]
Polycystin-2	Ser76	Ser80	Regulates localisation, enhanced in polycystic kidney disease	[121]
PSF- Polypyrimidine tract-binding protein-associated-splicing factor	Thr687	NONE	Regulates interaction with TRAP-150, and CD45 alternative splicing in T cells	[122]
Presenilin-1	Ser397, Ser401 Ser353, Ser357	NONE	Reduces interaction with β-catenin	[123] [124]
Protein phosphatase1 G-subunit	Ser38, 42 (human)	Ser46 (PKA or p90RSK)	Not clear	[125]
Protein phosphatase inhibitor 2	Thr72	Ser86 (CKII)	Inhibits inhibitor, thereby activating PP1	[11, 126, 127]
PTEN	Ser362, Thr366	Ser370 (CK2)	Possibly inhibits activity	[128]
Pyruvate Dehydrogenase	ND		Inhibits activity	[129]
RCN1 (yeast calcineurn regulatory protein-calcipressin)	Ser113	Ser117	Regulates cacineurin signaling	[130]
SC35	ND	Probably	Redistributes this splicing factor	[131]
SKN-1	Ser393, (maybe Ser389 and Thr385)	Ser397	Inhibits activity	[132]
SMAD3	Thr66	ND	Regulates stability	[133]
Snail	Ser97, 101, 108, 112, 116, 120	ND	Regulates degradation and nuclear exclusion (antitumourogenic)	[134]
SREBP1c (processed fragment)	Thr426, Ser430	ND	Promotes degradation	[135]
Stathmin	Ser31	ND	Slight induction of depolymerisation of microtubules	[136]

(a) Continued.

Proposed substrate	Target residue(s)	Priming residue (kinase)	Effect of phosphate	Ref
Tau	Multiple including Ser208, Thr231, 235	Thr212 (DYRK)	Some phosphorylation sites regulate microtubule binding	[62, 137, 138]
	Ser396			
	Ser404, others?			[139]
TSC2	Ser1341, Ser1337	Ser1345 (AMPK)	Activates TSC2 to inhibit mTOR	[140]
VDAC	Thr51	Thr55	Modulates interaction with HKII in mitochondrial membrane	[141]
von Hippel-Lindau (VHL)	Ser68	Ser72 (CKI)	Regulation of MT stabilization	[142]
Zcchc8	Thr492	ND	ND	[143]

(b)

Proposed substrate	Effect of phosphate	Cellular process
Amyloid precursor protein	Regulates Trafficking	Neurobiology
BCL-3	Degradation	Growth and Survival
β-catenin	Degradation	Development
C/EBPbeta	Regulates DNA binding	Endocrine control
		Growth and survival
Ci-155	Regulates degradation	Development
CLASP2	Affects protein-protein interaction	Neurobiology/cell migration
CRMP2	Regulates axon growth, growth cone collapse and neuronal polarity.	Neurobiology
CRMP4	Regulates axon outgrowth	Neurobiology
CREB	Activation	Neurobiology
		Endocrine control
Cytidine triphosphate synthetase (CTPS)	Reduces activity	Cell growth
Dynamin I	Required for activity dependent bulk endocytosis	Neurobiology
eIF2B	Inhibits activity	Cell Growth
FAK	Inhibits activity	Growth and Survival
Glycogen Synthase	Reduces activity	Endocrine control
heat shock factor 1	Reduces DNA binding	Growth and Survival
HIF1alpha	Induces proteosomal degradation	Growth and Survival
Histone H1.5	Coincides with chromosome condensation	Cell division
IRS1	Promotes degradation	Endocrine control
c-jun, Jun B, Jun D	Reduces DNA binding	Growth and survival
MAP1B	Regulates stability	Neurobiology
MAP 2C	Reduces microtubule binding	Neurobiology
MARK2/PAR1	Regulates activity	Neurobiology
Mcl1	Permits degradation of Mcl1 in response to UV stress	Growth and survival
Mdm2	Promotes activity towards p53, reduces p53 levels	Growth and survival
c-myc, L-myc	Promotes degradation	Growth and survival
Myocardin	Inhibits myocardin induced transcription	Development
NDRG1	Not reported	Ion control
NFAT	Regulates nuclear exclusion, Inhibits DNA binding	Immunology
Ngn2	Facilitates interaction with LIM TFs, for motor neuron determination	Development
p130Rb	Promotes stability.	Growth and survival

(b) Continued.

Proposed substrate	Effect of phosphate	Cellular process
protein phosphatase 1 G-subunit	Not clear	Endocrine control
protein phosphatase inhibitor 2	Inhibits inhibitor, thereby activating PP1	Endocrine control
Polycystin-2	Regulates localisation, induced in polycystic kidney disease	Growth and survival
PTEN	Inhibits activity	Growth and survival
RCN1 (yeast calcineurn regulatory protein)	Stimulates cacineurin signaling	Growth and survival Neurobiology
Snail	Induces degradation and nuclear exclusion (antitumourogenic)	Growth and survival
Tau	Modulates interaction with tubulin Increased in AD	Neurobiology
VDAC	Modulates interaction with HKII in mitochondrial membrane	Growth and survival
von Hippel-Lindau (VHL)	Regulation of MT stabilization	Neurobiology

Interestingly, only four of the proteins listed in Table 1(b) appear to have no requirement for priming (C/EBPbeta, histone H1.5, MARK2, and tau (at some sites)). Thus, by far the majority of the well-characterized substrates require priming.

3.2. GSK3 in Energy Homeostasis. Glucose is a vital nutrient for most mammalian cells. It is obtained by ingestion of food but can be generated endogenously in the liver by glycogenolysis or gluconeogenesis (from amino acids or glycerol) during periods of fasting. These processes ensure there is a constant supply of glucose in the blood (around 5 mM), available to all cells in the body. However, high glucose is relatively toxic to tissues and blood proteins, hence there are complex endocrine mechanisms to prevent hyperglycemia (diabetes). Insulin is released from pancreatic β-cells in response to postprandial rising blood glucose, and this hormone combats hyperglycemia by acting on liver, muscle, and fat tissue, promoting glucose storage in the form of glycogen, turning off hepatic gluconeogenesis and promoting adipogenesis (for review see [156, 157]).

Loss of pancreatic β-cells is the main cause of Type 1 diabetes, as these cells are the only endogenous source of insulin. Treatment with exogenous insulin at appropriate times is relatively effective in restoring glucose control in this condition. In contrast, Type 2 diabetes (accounting for about 90% of diabetes) is less well defined, and includes defects in glucose sensing, insulin secretion, and loss of insulin action (insulin resistance). Hence treatment with exogenous insulin is less effective, and alternative approaches, such as insulin sensitizing agents (e.g., metformin) are used to combat this condition. GSK3, as its name indicates, has long been associated with insulin regulation of glucose homeostasis and as such has been investigated as a therapeutic target in diabetes.

3.2.1. Glycogen Synthase. Phosphorylation of glycogen synthase by GSK3 reduces glycogen synthesis (glucose storage) in muscle. Glycogen synthase is constitutively phosphorylated by CKII at Ser656, providing initial priming for a series of phosphorylation events by GSK3 (652, 648, 644, 640), each additional phosphorylation in turn adding to the inhibition of glycogen synthase activity [1, 66]. This places GSK3 in the pathway from insulin, to glucose disposal. More recently, inhibition of GSK3 in cells [158] and *in vivo* [159] was found to reduce hepatic gluconeogenesis, although the GSK3 substrate responsible for this action remains elusive. Clearly, as GSK3 is inhibited in cells treated with insulin pharmacological inhibition of GSK3 should mirror many of the natural actions of insulin including reducing glucose production and enhancing glucose storage to combat hyperglycemia. Therefore many major pharmaceutical companies have generated potent and selective GSK3 inhibitors as potential antidiabetes therapeutics and initial data in animal models suggests efficacy in glucose lowering [159, 160].

3.2.2. CREB and C/EBP. GSK3 also regulates a number of transcription factors with links to endocrine action, in particular the transcription factors C/EBPβ and CREB, which are responsive to hormones that stimulate the generation of the second messenger cAMP [161, 162] or to the fasting signal glucocorticoids [163]. The regulation of C/EBPβ by GSK3 appears complex, with priming of Thr188 by ERK allowing GSK3 to phosphorylate C/EBPβ and induce its DNA binding [41]. Conversely, unprimed phosphorylation of distinct residues (albeit in the same domain of the protein) is reported to reduce DNA binding [42], so it remains unclear which mechanism is invoked upon regulation of GSK3 *in vivo*.

Meanwhile, phosphorylation of CREB at Ser129 by GSK3, following priming by PKA at Ser133, is reported to induce CREB transcriptional activity [51, 52] however the regulation of key CREB-dependent genes by GSK3 in cells or animals remains poorly studied.

In summary, there is little direct evidence that GSK3 regulates these transcription factors as part of physiological responses to the hormones of glucose homeostasis.

3.2.3. Insulin Signaling. GSK3 can regulate cellular phosphorylation indirectly by targeting protein phosphatase-1 (PP1),

a key regulator of insulin signaling. GSK3 phosphorylation of Inhibitor-2, a regulator of PP1, antagonizes Inhibitor-2 function thereby inducing PP1 activity [11, 126, 127]. In this way, inhibition of GSK3 would be predicted to reduce PP1 activity and indirectly induce phosphorylation of cellular proteins (and conversely overexpression of GSK3 may reduce phosphorylation of some proteins). In addition GSK3 phosphorylates the glycogen binding subunit of protein phosphatase-1 (PP1G) following priming by PKA at Ser46, although the effect of phosphorylation on PP1G function is unclear [125]. These were two of the first substrates identified for GSK3 following closely behind glycogen synthase, and hence added to the evidence that GSK3 played a key role in regulation of glycogen metabolism in muscle. More recently GSK3 has been implicated in a negative feedback regulation of insulin signaling, and hence potentially contributing to the insulin resistance found in diabetes as well as other diseases such as polycystic ovarian syndrome and nonalcoholic fatty liver disease. GSK3 phosphorylates insulin receptor substrate (IRS)-1 at Ser332, following priming at Ser336, thereby promoting its degradation [72]. IRS-1 is a key target for the insulin receptor tyrosine kinase [164], hence loss of IRS1 due to aberrant activation of GSK3 would reduce the insulin signaling capacity of the cell.

In summary, GSK3 inhibition has the potential to enhance cellular insulin sensitivity (stabilize IRS1), reduce hepatic glucose production (target unknown) and promote glucose disposal (glycogen synthase), all beneficial to the diabetic patient. Consistent with the molecular predictions, inhibition of GSK3 in an animal model of diabetes reduces the associated hyperglycemia and insulin resistance [159]. However, the ever-growing list of potential GSK3 substrates involved in cell growth (see next section) has dampened the enthusiasm for using GSK3 inhibition as a treatment for a chronic condition such as diabetes (where patients may require treatment for 10–50 years).

3.3. GSK3 in Growth and Survival. The numerous proposed substrates of GSK3 with a role in the control of cell growth and survival suggests that pharmacological manipulation of GSK3 activity may increase the risk of abnormal cell growth or differentiation; however many of these proposed substrates remain poorly characterised.

3.3.1. Bcl3. Bcl3 is a member of the I$\varkappa$B family of NF$\varkappa$B inhibitors that induces transcription of many genes including cyclin D1 [165]. Constitutive Bcl3 phosphorylation at Ser394 by GSK3 (possibly following priming at Ser398) promotes its degradation [37]. Bcl3 expression is upregulated in many tumours, thus GSK3 is proposed to keep the oncogenic potential of Bcl3 in check.

3.3.2. c-Jun. The proto-oncogene c-Jun is one of the components of AP-1, a transcription factor complex believed to play key roles in cell proliferation, survival and death (reviewed in [166, 167]). It is regulated by multisite phosphorylation including N-terminal phosphorylation which induces its transcriptional activity, and C-terminal phosphorylation which inhibits its binding to DNA. JNK and ERK phosphorylate the N-terminal sites on c-jun to activate it, while GSK3 phosphorylates Thr-239 to inhibit c-jun activity (following priming of Ser243 by an unknown kinase) [73–75]. Hence GSK3 inhibition would potentially enhance the action of this oncogene.

3.3.3. Mcl-1. Mcl-1 is an antiapoptotic member of the Bcl2 family that is essential for embryonic development and for the survival of hematopoietic cells. Mcl-1 plays an important role in the sensitization of cells to apoptotic signals. Exposure to UV radiation causes the rapid degradation of Mcl-1 and the release of proapoptotic partner proteins (e.g., Bim). In response to UV (and other cell stress) JNK phosphorylates Mcl-1 at Thr144 priming it for subsequent phosphorylation by GSK3 at Ser140. JNK and GSK3 activities are required for degradation of Mcl-1 in response to stress [87–89], so GSK3 inhibition would antagonize this apoptotic mechanism.

3.3.4. Mdm2. The Mdm2 oncoprotein regulates abundance and activity of the p53 tumor suppressor protein. Phosphorylation of Mdm2 at several contiguous residues within the central conserved domain is key to this function. GSK3 phosphorylates Mdm2 in the central domain both *in vitro* and *in vivo* [90]. Inhibition of GSK3 prevents p53 degradation in an Mdm2-dependent manner, and expression of a S9A GSK3 mutant reduces the accumulation of p53 and induction of its target p21(WAF-1). Therefore inhibition of GSK3 could promote hypophosphorylation of Mdm2 resulting in stabilization of the tumour suppressor p53 [90].

3.3.5. c-Myc. c-Myc is an immediate early gene controlling cell proliferation, differentiation, and apoptosis. It can be primed at Ser62 by ERK2 (extracellular signal-related protein kinase 2), allowing phosphorylation at Thr58 by GSK3, which targets c-Myc for ubiquitin-mediated proteolysis [96]. Thus mitogen-induced dephosphorylation of c-Myc at Thr58 may increase its half-life, similar to the situation proposed for (but less well characterized for) cyclin D1 [168]. Thr58 is mutated in all v-Myc proteins, and restoration of the wild-type threonine severely inhibits transforming potential [169]. Moreover, the T62A mutant c-Myc (GSK3 resistant) potentiates focus formation [170]. There is good evidence that GSK3 is responsible for Thr58 phosphorylation *in vivo*. Firstly, CT99021, a selective GSK3 inhibitor, reduces c-Myc phosphorylation at Thr58 in cells, secondly, c-Myc protein is elevated in GSK3β KO brain, and finally, overexpression of GSK3β in HEK293 cells increases Thr58 phosphorylation [11]. This suggests that GSK3 inhibition *in vivo* would enhance the stability of this oncogenic factor.

Paradoxically, mutation of Ser62 to Ala is reported to destabilize c-Myc, suggesting that the phosphorylation of this residue may stabilize c-Myc and as such play an opposing role to the phosphorylation of Thr58. Thus the regulation of c-Myc by serum appears to run a fine line between stabilization and degradation. Activation of PI 3-kinase and ERK by serum will induce Ser62 phosphorylation but inhibit GSK3 and decrease phosphorylation of Thr58. This

is consistent with the observation that the half-life of c-Myc increases in response to serum [171]. One presumes that upon serum withdrawal, GSK3 activity increases and phosphorylation of the already primed c-Myc enhances the rate of its degradation [96–98].

3.3.6. p130 Retinoblastoma Protein (Rb). The interaction of p130Rb with E2F transcription factors results in active repression of E2F-dependent genes (key for DNA synthesis and cell cycle progression as well as differentiation and DNA damage checkpoints) [172, 173]. The p130Rb protein level is elevated in quiescent cells and decreased in proliferating cells. GSK3 phosphorylates p130Rb during G0, enhancing stability of p130Rb, but does not affect its ability to interact with E2F4 or cyclins [113]. It is conceivable that GSK3 inhibition would thus swing the balance towards p130Rb degradation and cell proliferation.

3.3.7. PTEN. The PTEN tumor suppressor is a phosphatidylinositol D3-phosphatase that antagonizes the action of phosphatidylinositol 3-kinase (PI 3K) and negatively regulates cell growth and survival. CK2 phosphorylates PTEN at Ser370 and Ser385 enabling GSK3 to phosphorylate Ser362 and Thr366 [128]. Expression of T366A mutant PTEN reduces downstream PI 3K signaling to a higher extent than wild-type PTEN suggesting that GSK3 inhibits PTEN activity. In neuronal cell lines-leptin regulates PTEN phosphorylation at Ser362 and Thr366 rather than inducing PI 3K activity in order to control downstream PI 3K signaling [174]. Pharmacological inhibition of GSK3 would therefore be predicted to antagonise the PI 3K signaling pathway and reduce cell growth potential, although this remains to be conclusively proven [128]. How this action of GSK3 interacts with that proposed for regulation of IRS1 described above (where PI 3K signaling would be enhanced by GSK3 inhibition) is also unclear.

3.3.8. Heat Shock Factor (HSF)-1. Mammalian heat shock genes are regulated at the transcriptional level by heat shock factor-1 (HSF-1), a sequence-specific transcription factor. HSF-1 exists as a latent cytoplasmic phosphoprotein but is transformed by dephosphorylation to a nuclear protein that controls the transcription of heat shock genes [175]. HSF-1 is phosphorylated by GSK3 at Ser303, following priming of Ser307 by ERK [68]. GSK3 thus represses the activity of HSF-1, and in a manner reminiscent of c-Myc regulation (Section 3.3.5 above) serum induction of ERK will prime HSF-1 at a time where GSK3 activity is low, presumably to permit a rapid relocalisation of HSF-1 following reduction in ERK signaling. Inhibition of GSK3 would thus be expected to enhance the production of heat shock proteins.

3.3.9. Hypoxia-Inducible Transcription Factor (HIF) 1α. HIF-1α is a transcription factor that is vital for the cellular response to hypoxia; however it also responds to growth factors and hormones following activation of PI 3K signaling [176]. The inhibition or depletion of GSK3 induces HIF-1α expression whereas the overexpression of GSK3 results in the opposite. These effects are mediated through phosphorylation of three serines in the oxygen-dependent degradation domain of HIF-1α, and degradation occurs in a VHL-independent manner [69]. Thus, phosphorylation of HIF-1α by GSK3 is proposed to reduce HIF-1α stability, and GSK3 inhibition (physiologically or pharmacologically) would then promote the action of this transcription factor and alter oxygen sensing and cell growth.

3.3.10. Eukaryotic Initiation Factor 2B (eIF2B). eIF2B is a small G-protein that catalyses the exchange of guanine nucleotides on eIF2, an important regulatory step in the initiation of mRNA translation. GSK3 phosphorylates Ser535 of eIF2B after a priming phosphorylation by DYRK at Ser539 [60–62]. Phosphorylation of these residues inhibits eIF2B thereby reducing translation, and as such GSK3 inhibition could enhance protein synthesis and cell growth.

3.3.11. Polycistin 2 (PC2). Polycystin-2 (PC2) is mutated in 15% of patients with autosomal dominant polycystic kidney disease (ADPKD). It is a nonselective Ca^{2+}-permeable cation channel thought to function at both the cell surface and ER. GSK3 phosphorylates Ser76 of PC2 *in vitro* and the consensus recognition sequence for GSK3 (Ser76/Ser80) is evolutionarily conserved down to lower vertebrates [121]. Inhibition of GSK3 redistributes PC2 from the lateral plasma membrane pool into an intracellular compartment in MDCK cells without a change in primary cilia localization [121]. Hence, it appears that the surface localization of PC2 is regulated by phosphorylation by GSK3, and this contributes to the maintenance of normal glomerular and tubular morphology.

3.3.12. Voltage-Dependent Anion Channel (VDAC). Transformed cells are highly glycolytic and overexpress hexokinase II (HXK II). HXK II binds to the mitochondria through an interaction with VDAC, an abundant outer mitochondrial membrane protein. The binding of HXK II to mitochondria contributes to maintenance of cell viability. Phosphorylation of VDAC by GSK3 prevents binding of HXK II promoting dissociation of HXK II from the mitochondria [141]. Inhibition of PKB potentiates chemotherapy-induced cytotoxicity, an effect that is dependent on GSK3 activation (downstream of PKB) as well as the reduced binding of HXK II to the mitochondria [141]. Hence, enhancing GSK3 activity toward VDAC may potentiate the efficacy of conventional chemotherapeutic agents.

3.3.13. Cytidine Triphosphate Synthetase (CTPS). CTPS catalyzes the rate-limiting step in the *de novo* synthesis of CTP. Phosphorylation of CTPS1 at Ser571 reduces its activity *in vitro,* while phosphorylation of Ser571 in cells is antagonised by the presence of serum [55]. Phosphorylation of Ser571 is reduced (with subsequent induction of CTPS1 activity) in cells following incubation with either the GSK3 inhibitor indirubin-3′-monoxime or GSK3β short interfering RNAs [55]. Hence GSK3 directly regulates CTP

production through the phosphorylation and inhibition of CTPS.

3.3.14. Focal Adhesion Kinase (FAK). GSK3 can phosphorylate FAK at Ser722 [63]. Meanwhile, S722A mutation or dephosphorylation of Ser722 by PP1 increases FAK kinase activity, and cells expressing the S722A mutant FAK display improved cell spreading and faster migration in wound-healing and trans-well assays. The data proposes that GSK3 is a key regulator of FAK activity during cell spreading and migration (and potentially metastasis).

3.4. GSK3 in Neurobiology

3.4.1. Microtubule Function. Neuronal connections are formed during development by a precise and complex pattern of axonal growth, guidance, and synaptogenesis. To achieve this the cells must continually remodel the cytoskeleton in response to external guidance cues that include the Semaphorins, Wnts, and growth factors (for review see [177–179]). Cytoskeletal reorganisation can be accomplished by control of microtubule dynamics and/or the actin cytoskeleton. Microtubule assembly and stability are regulated in large part by the presence and the phosphorylation status of the microtubule-associated proteins (MAPs) [180, 181]. Many of the MAPs are substrates for GSK3.

(1) Tau. Tau is a microtubule-associated protein encoded by a single gene on chromosome 17 (MAPT). It is found predominantly in cells of neuronal origin and regulates microtubule assembly, a function that is influenced by gene splicing (there are six possible isoforms of tau), as well as by phosphorylation (phospho-tau has lower affinity for microtubules). Hyperphosphorylated tau is the major protein constituent of neurofibrillary tangles, one of the hallmarks of Alzheimer's disease. Around 50 phosphorylation sites have been identified on tau protein, many specifically associated with neurodegenerative disease, and many of these are known to influence its regulation of microtubule assembly. Regulation of Tau phosphorylation in health and disease has been covered extensively in recent reviews [182–185] and is discussed in detail in a separate review within this issue.

(2) Collapsin Response Mediator Proteins (CRMP). CRMPs are a family of five structurally homologous tubulin-binding proteins implicated in multiple aspects of neuron development and polarisation [49, 50, 186–192]. CRMP1, 2 and 4 are all substrates for GSK3 *in vitro* and *in vivo* [48, 49, 193], being phosphorylated at 3 residues (Ser518, Thr514, and Thr509) by GSK3 subsequent to priming by phosphorylation at Ser522. Priming of CRMP1 and CRMP2 is performed by CDK5 as neither of these phosphorylation events occurs in tissue lacking CDK5, while priming of CRMP4 does not require CDK5 [193]. Phosphorylation of CRMP2 by GSK3 regulates axon growth as well as the number of axons [48, 49, 193], while phosphorylated CRMP2 binds less efficiently to tubulin heterodimers. CRMP2 and CRMP4 are not phosphorylated at 518/514/509 in neurons lacking GSK3β (either genetic or pharmacological ablation) [11, 48], while GSK3β phosphorylates these substrates more avidly than GSK3α *in vitro* [11]. CRMP2 is more heavily phosphorylated in human cortex from Alzheimer's brain compared to age-matched controls [194], and phosphorylated CRMP2 is found in tangles [195]. Phosphorylation of CRMP4 by GSK3 mediates dendrite development in response to inhibitory ligands such as myelin [50]. The CRMPs are excellent substrates for GSK3 *in vitro* being phosphorylated at a relatively high rate and stoichiometry compared to other GSK3 substrates, and are completely dependent on priming.

(3) Microtubule-Associated Protein (MAP) 1B. MAP-1B, a major component of the neuronal cytoskeleton, regulates axonal growth potentially through its ability to bind to and increase the stability of microtubules [196–198]. In contrast to tau, phosphorylated MAP-1B binds to microtubules more avidly than unphosphorylated MAP-1B. Phosphorylated MAP-1B is present mainly in axons while unphosphorylated MAP-1B is present in the cell body and dendrites suggesting localization is regulated by this modification (for review see [199]). Moreover, the level of phosphorylated MAP-1B increases during axonal extension declining to low levels at the end of axonogenesis and a phosphorylated form of MAP-1B is distributed across the axon in a gradient fashion with the highest level at the growth cone [200–202]. GSK3 phosphorylates MAP-1B at Ser1260, Thr1265, and Ser1388, the latter requiring priming at Ser1392 by DYRK. Phosphorylation of these residues stabilizes the MAP-1B as well as contributing to its higher affinity for microtubules [81–83]. GSK3 regulation of MAP1B is a vital link between Wnt-7a signaling and axonal remodeling [81–83].

(4) MAP2C. The microtubule-associated protein 2 (MAP2) proteins, like MAP-1B and tau, are abundant cytoskeletal components predominantly expressed in neurons. MAP2 is phosphorylated *in vitro* and *in situ* by GSK3 at Thr1620 and Thr1623, located in the proline-rich region of MAP2 [84]. Cotransfection of GSK3 and MAP2C in cells promotes phosphorylation of MAP2C, a modification that is sensitive to the presence of lithium chloride (a nonselective inhibitor of GSK3). Additionally, the formation of microtubule bundles, which is observed after transfection with MAP2C, is decreased when GSK3 is co-transfected [84]. Highly phosphorylated MAP2C species are found predominantly unbound to microtubules. These data suggests that GSK3-mediated phosphorylation of MAP2C reduces its binding to microtubules and co-ordinated phosphorylation of MAP2C, tau, and MAP-1B by GSK3 is a major mechanism for regulation of microtubule stability in neurons.

(5) Von Hippel-Lindau (VHL) Tumor Suppressor Gene. Inactivation of the von Hippel-Lindau (VHL) tumor suppressor gene is linked to the development of tumors of the eyes, kidneys, and central nervous system. VHL encodes two gene products, pVHL30 and pVHL19, of which one, pVHL30, associates with microtubules (MTs) to regulate their stability. Phosphorylation of pVHL on Ser68 by GSK3 subsequent to

a priming phosphorylation event at Ser72 (mediated *in vitro* by CKI) regulates the ability of pVHL's to stabilize (but not bind) microtubules [142]. Hence pVHL can be added to the list of GSK3 substrates involved in control of microtubule dynamics.

(6) CLIP-Associating Protein (CLASP) 2. Actin and microtubules are coupled structurally and distributed asymmetrically along the front-rear axis of migrating cells. CLIP-associating proteins (CLASPs) accumulate near the ends of microtubules, particularly at the front of migrating cells, to control microtubule dynamics and cytoskeletal coupling. Regional regulation of GSK3 is proposed to regulate the distribution of CLASPs [45–47]. IQGAP1 is an actin-binding protein, as well as a CLASP-binding protein. GSK3β directly phosphorylates CLASP2 at Ser533 and Ser537 within the IQGAP1 binding domain. Phosphorylation of these residues dissociates CLASP2 from IQGAP1 and microtubules [47]. Overexpression of active GSK3β alters the distribution of wild-type CLASP2 on microtubules, but not that of a non-phosphorylatable CLASP2 mutant. CLASP2 phosphorylated by GSK3 does not accumulate near the ends of microtubules. Thus, phosphorylation of CLASP2 by GSK3 controls the regional linkage of microtubules to actin filaments and hence influences cell movement and axonal guidance [47].

3.4.2. Presynaptic Function of GSK3—Dynamin I. GSK3 will phosphorylate the large GTPase dynamin I at Thr774 following priming at Thr778 by CDK5 [58]. The activity of GSK3 is specifically required for activity-dependent bulk endocytosis (ADBE), but not clathrin-mediated endocytosis. Moreover the specific phosphorylation of Ser774 on dynamin I by GSK3 is both necessary and sufficient for ADBE. This demonstrates a role for GSK3 preparing synaptic vesicles for retrieval during elevated neuronal activity [58].

3.4.3. Neurogenesis—Ngn2. The differentiation of neural progenitors (neurogenesis) involves two coordinated steps: the commitment to neuronal fate and the establishment of cell-type identity [203]. As mentioned earlier, loss of both GSK3 genes in the brain induces self-renewal of neuronal progenitor cells, but reduces neurogenesis [152]. Two conserved serine residues on the bHLH factor neurogenin-2 (Ngn2), namely Ser231 and Ser234, are phosphorylated during motor neuron differentiation [108]. This phosphorylation can be carried out by GSK3 *in vitro*, although it is not clear whether priming is required (either at Ser234 or elsewhere), and phosphorylation facilitates the interaction of Ngn2 with LIM homeodomain transcription factors [108]. In Ngn2 knock-in mice in which these two residues are mutated to alanines (insensitive to GSK3 regulation), motor neuron specification is impaired. Hence, this phosphorylation-dependent cooperativity between Ngn2 and homeodomain transcription factors downstream of GSK3 may contribute to neurogenesis and cell fate decisions in the CNS [108], and could explain at least in part the phenotype of the brain-specific GSK3 KO mouse [152].

3.4.4. GSK3 in Alzheimer's Disease (Tau, APP, CRMP, MARK, and DSCR1). Phosphorylation of the MAPs, tau and CRMP2, at residues targeted by GSK3, is higher in the brains of patients with Alzheimer's disease than age-matched controls. Indeed the phosphorylated forms of these proteins are the main protein constituents of neurofibrillary tangles, implicating excessive GSK3-mediated phosphorylation of MAPs in tangle pathology. Interestingly abnormal GSK3 activity has also been linked to amyloid pathology.

The major component of senile plaques (an early and important hallmark of AD) is the beta amyloid peptide, Ab, a 39–43 amino acid fragment derived from proteolysis of amyloid precursor protein (APP) by β- and γ-secretases [204, 205]. Inhibition of cellular GSK3 by lithium or GSK3β antisense oligonucleotides reduces Ab production in cells without significantly affecting cellular APP levels or APP maturation [28]. In addition, Ab production in the brain of a mouse model of AD is reduced by dosing the animal with lithium [28]. More specifically, GSK3 phosphorylates recombinant APP at Thr743 (numbering for APP 770), although whether this alters processing is not clear [27]. In neurons APP is highly phosphorylated at this site, but Thr743 can be phosphorylated by a number of kinases, including JNK, GSK3, and CDK5. In addition, JNK activity, modulated by GSK3, enhances the traffic of phosphorylated APP to nerve terminals and inhibition of GSK3 and JNK restores calcium oscillations in a hAPP expressing neuronal network [29]. In contrast to the wild-type hAPP, expression of the hAPPT743A mutant in cells does not inhibit calcium oscillations, and the proportion of this mutant APP at the plasma membrane is significantly less than wild-type hAPP. Thus GSK3/JNK phosphorylation controls APP trafficking at the plasma membrane and inhibits neuronal calcium oscillations.

Among the many phosphorylation sites identified in tau, Ser262 is a major site of abnormal phosphorylation in AD brain. One kinase known to phosphorylate this site is MARK2. GSK3 phosphorylates MARK2 *in vitro* at Ser212, one of two reported phosphorylation sites (Thr208 and Ser212) found in the activation loop of MARK2. Downregulation of either GSK3 or MARK2 by siRNAs suppresses the level of phosphorylation of tau on Ser262 suggesting that GSK3 regulates Ser262 of tau indirectly through phosphorylation and activation of MARK2 [85]; however, this has recently been disputed [86].

Calcineurin is a calcium/calmodulin-activated serine/threonine phosphatase (also known as PP2B). Down syndrome candidate region 1 (DSCR1) is the mammalian homologue of the yeast RCN family (more recently referred to as calsipressins) that directly regulates calcineurin [206]. Calcineurin function is well characterized in yeast, where its expression promotes growth in high calcium environments by dephosphorylation of the Tcn1p transcription factor. It also regulates many facets of apoptosis, memory processes,

and skeletal and cardiac muscle growth and differentiation. Hence, by regulating calcineurin, DSCR1 has the potential to influence all of these processes. The DSCR1 gene was isolated from the "Down syndrome candidate region", and in the brain, DSCR1 is predominantly expressed in neurons within the cerebral cortex, hippocampus, substantia nigra, thalamus, and medulla oblongata. DSCR1 mRNA levels are three times higher in patients with extensive neurofibrillary tangles (hallmark of AD) compared to controls [207]. Similarly, postmortem brain samples from Down syndrome patients (who develop AD pathology) also have DSCR1 mRNA levels higher than controls. In addition, exposure of cultured cells to the Ab(1–42) peptide increases expression of DSCR1 [207]. Paradoxically, while increasing Rcn1 expression can inhibit calcineurin signaling in fungal and animal cells, endogenous levels can actually stimulate calcineurin signaling in yeast [130]. The stimulatory effect of yeast Rcn1 requires phosphorylation of a serine residue (conserved in mammals) by a yeast homologue of GSK3 (Mck1). Mutation of this serine in yeast Rcn1, and the human homologue DSCR1, abolishes the stimulatory effects of Rcn1/DSCR1 on calcineurin signaling. Therefore, in healthy cells, GSK3 may switch Rcn1/DSCR1 between stimulatory and inhibitory forms [130]. Whether abnormal GSK3 regulation of DSCR1 contributes to the pathophysiology of AD or Downs syndrome remains unknown.

3.5. GSK3 in Development

3.5.1. Wnt Signaling-Beta Catenin. One of the best-described cellular functions of GSK3 is the regulation of canonical Wnt signaling. This topic is reviewed in excellent detail elsewhere [21, 208–210] and so will not be covered in depth in this review. In short, GSK3β associates with a large protein complex that includes adenomatous polyposis coli (APC), axin, and β-catenin. All three of these proteins are proposed as GSK3 substrates and phosphorylation of each is proposed to regulate the stability of the complex [21, 211, 212]. The phosphorylation of β-catenin by GSK3 is greatly enhanced by the presence of Axin, which acts as a scaffold for the other components [34]. Axin and APC are also substrates of GSK3. Axin phosphorylation by GSK3 stabilizes the protein [35], while APC phosphorylation by GSK3 enhances the interaction of β-catenin and APC [31]. GSK3 phosphorylates Ser33, Ser37, and Thr41 of β-catenin following priming at Ser45 by CKI [213, 214]. These residues lie in a trCP motif and phosphorylation recruits SKP1-cullin1-F-box (SCFβ-TrCP) E3 ligase complex followed by degradation of β-catenin via the 26S proteasome [210]. Exposure of cells to Wnts reduces GSK3 activity in the Axin complex (by disruption of protein:protein interactions or phosphorylation of Thr380) resulting in dephosphorylation and stabilization of β-catenin, which translocates to the nucleus to induce transcription in cooperation with TCF transcription factors. Interestingly the pool of GSK3 associated with Wnt signaling appears distinct to that associated with growth factor signaling [23], probably since the mechanism of inhibition is distinct in each case and the Wnt sensitive GSK3 is sequestered within microvesicles [24]. This targeting of SCFβ-TrCP to substrates of GSK3 may be more widespread than currently appreciated and may allow Wnts to alter the stability of a wide range of cellular proteins [24].

3.5.2. Hedgehog (Hh) Signaling-Ci155. The Hh family of secreted proteins controls cell growth and patterning in development, while mutations in components of the Hh signaling pathway are associated with increased human disease [215]. Cubitus interruptus (Ci155) is a transcriptional inducer first identified as a mediator of Hh signaling in *Drosophila*. Exposure of *Drosophila* cells to Hh blocks production of a transcriptional repressor normally generated by proteolytic cleavage of Ci155 [216]. Deletion of GSK3 (sgg in *drosophila*) results in accumulation of the full length Ci155 and the ectopic expression of Hh responsive genes including decapentaplegic (dpp) and wingless (wg), suggesting GSK3 inhibition is part of Hh regulation of Ci155 processing. Ci155 is phosphorylated by GSK3 at three sites (852 and 884/888) after priming (at 856 and 892, resp.) by protein kinase A (PKA) [43, 44]. Mutation of these GSK3 target sites in Ci155 blocks processing and prevents the production of the repressor [43, 44]. Hence GSK3 acts in conjunction with PKA to promote proteolytic processing of Ci155, switching it from a transcriptional inducer to repressor. Hh may reduce Ci155 proteolysis by inhibiting GSK3 and promoting Ci155 dephosphorylation.

3.5.3. Cardiomyocyte Development-Myocardin. Myocardin is a muscle-specific transcription factor whose overexpression induces hypertrophy in neonatal rat cardiomyocytes [99], with increased cell size, total protein amount, and induction of generation of atrial natriuretic factor (ANF). Myocardin is phosphorylated by GSK3 at multiple sites in two regions of the protein between Ser455 to Ser467 and Ser624 to Ser636 [99]. Myocardin-induced ANF transcription and increase in total protein amount are enhanced by LiCl treatment of cells, consistent with GSK3 inhibiting myocardin activity. A phosphorylation-resistant myocardin mutant (8xAla) activated ANF transcription twice as potently as wild-type myocardin [99]. Conversely, a phosphomimetic myocardin mutant (8xAsp) was relatively transcriptionally inactive compared to wild type, in the presence of GSK3 inhibitors. Therefore, the GSK3-myocardin interaction regulates cardiomyocyte hypertrophy.

3.5.4. Epithelial-Mesenchymal Transition-Snail. The epithelial-mesenchymal transition (EMT) occurs during embryonic development and is triggered by Snail, a zinc-finger transcription factor, which acts by repressing E-cadherin transcription. Snail is highly unstable with a half-life of about 25 min. GSK3β binds to and phosphorylates Snail at two consensus motifs including residues 97/101 and 108/112/116/120 [134]. Phosphorylation of the first motif regulates β-Trcp-mediated ubiquitination, whereas phosphorylation of the second motif controls subcellular

Table 2: -GSK3 inhibition *in vivo*. Potential functional outcomes of pharmacological inhibition of GSK3.

Substrate group 1—Metabolic: *Overall Effect is anti-diabetic*	
GS	Increase glycogen synthesis and glucose disposal (anti-diabetic)
Unknown	Turn off hepatic glucose output (anti-diabetic)
CREB	Reduce glucagon action (anti-diabetic)
IRS1	Stabilise IRS1 protein and enhance insulin action (anti-diabetic)
Inhibitor2	Inhibit PP1 (not clear if beneficial)
Substrate group 2—Growth: *Predicted effect would be oncogenic, except for effect on mdm2/p53 and PTEN*	
BCL3	Stabilise BCL3 (increased oncogenic potential)
c-jun	Induce c-jun activity (increased oncogenic potential)
c-myc	Stabilize c-myc protein (increased oncogenic potential)
Mcl-1	Stabilise Mcl-1 (antiapoptotic)
p130Rb	Increase p130Rb degradation (cell cycle progression)
PTEN	Decrease PI3K signaling (decrease growth factor signaling)
IRS1	Stabilise IRS1 and enhance PI3K signaling (increase growth)
HIF1a	Stabilize HIF1a (could induce cell growth)
eIF2B	Enhance protein translation (aid cell growth)
VDAC	Enhance VDAC interaction with mitochondria (antiapoptotic)
CTPS	Enhance CTP production (aid cell growth)
FAK	Increase FAK activity (enhance cell spreading and migration)
Mdm2	Stabilise p53 (tumour suppression)
Substrate group 3—Alzheimer's disease: *Conducive to reducing AD pathology*	
Tau	Reduce tangle formation (anti-AD?)
APP	Reduce abeta production (anti-AD?)
CRMP2	Regulate axon outgrowth, reduce CRMP2 found in AD (anti-AD?)
MARK2	Reduce tau phosphorylation (anti-AD?)
Calcipressin	Regulate calcineurin action (anti-AD?)
Substrate group 4—Wnt and Hh signaling: *Enhanced effect on Wnt and Hh signaling*	
b-catenin	Induce b-catenin levels (induce wnt signaling)
Axin	Reduce axin levels (induce wnt signaling)
APC	Reduce APC b-catenin interaction (induce wnt signaling)
Ci155	Reduce proteolysis of Ci155 (enhanced Hh signaling)
Substrate group 5—Other possible detrimental effects:	
MAP1B	Reduce MAP1B interaction with microtubules (Wnt7a resistance)
MAP2C	Increase MAP2C interaction with microtubules (effect not clear)
CLASP2	Alteration of actin-microtubule interaction (effect not clear)
Dynamin I	Reduced presynaptic ADBE (effect not clear)
Ngn2	Impaired motor neuron designation (developmental?)
PC2	Relocalise PC2 (enhance polycystic kidney disease)
Myocardin	Enhance mycardin action (cardiac hypertrophy?)
NFAT	Nuclear localization (compromise immune system?)
Unknown	Suppress IL-1β, IL-6, TNF, IL-12 (compromise immune system?)
Unknown	Induction of IL-10 (compromise immune system?)

localization [134]. A variant of Snail (Snail-6SerAla), which cannot be phosphorylated at these motifs, is much more stable and resides exclusively in the nucleus to induce EMT. Importantly, inhibition of GSK3 results in the upregulation of Snail and downregulation of E-cadherin *in vivo*. Thus, Snail and GSK3 function as a molecular switch leading to EMT.

3.6. GSK3 in Immunology. There are many studies suggesting that GSK3 plays a key role in both the innate and adaptive immune systems (for recent paper see [217, 218]). There are two main strands of evidence supporting this function for GSK3: firstly that many cytokines and immune stimuli regulate GSK3 activity, and secondly that inhibition of GSK3 alters many aspects of the immune response. Specifically, Toll-like receptors [219, 220], T cell receptor [221], CD28 [221], and interleukin receptors [222] have all been shown to induce inhibitory phosphorylation of GSK3 in cells. In contrast, IFN-γ reduces phosphorylation and activates GSK3 in TLR2-stimulated macrophages [223] or

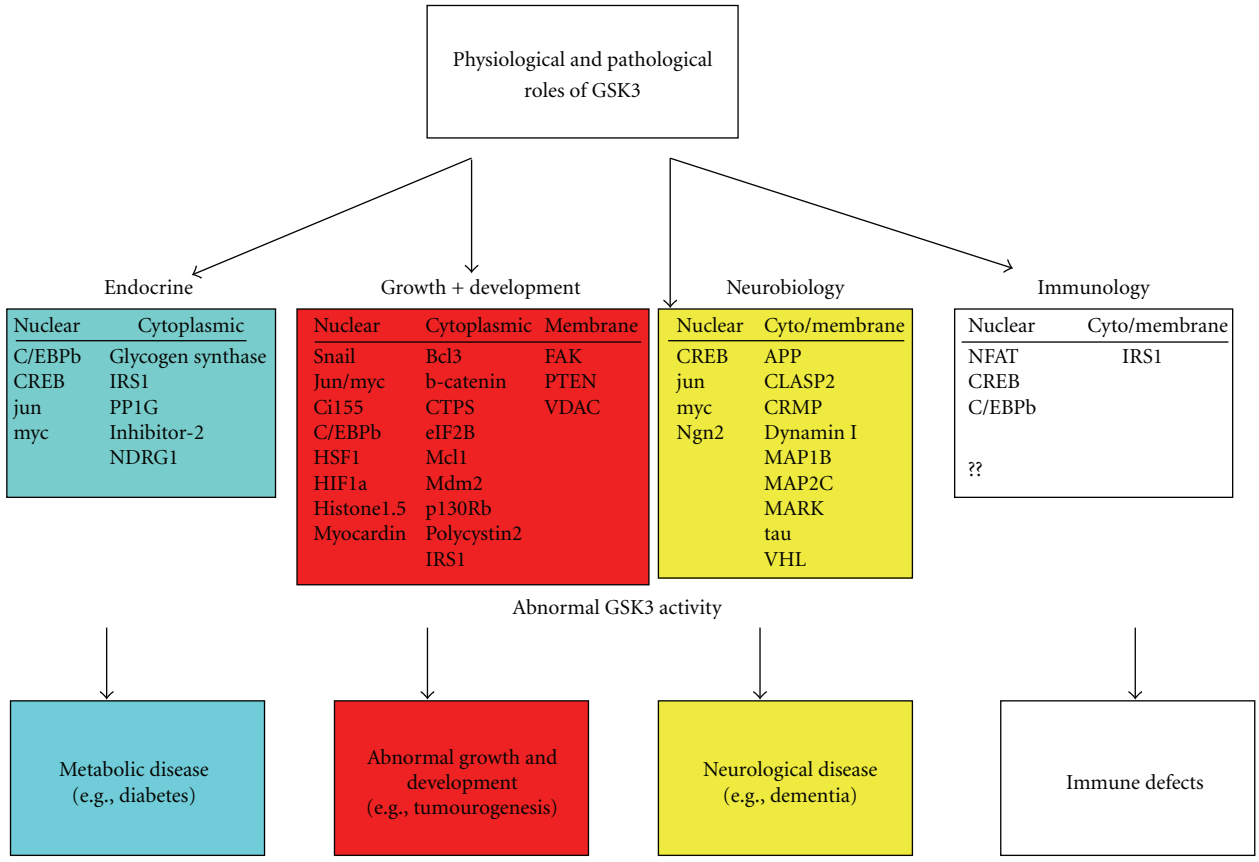

Figure 2: Potential physiological and pathological effects of phosphorylation of proposed GSK3 substrates.

RAW264.7 cells [224]. These studies place GSK3 upstream of STAT3 and STAT1 in the IFN-γ signaling pathway, but have not demonstrated that the STATs are direct targets for GSK3. Inhibition of GSK3, using selective inhibitors or shRNAi, decrease IFN-γ-induced inflammation and this action requires the Src homology-2 domain containing phosphatase 2 (SHP2) [224]. Inhibition of GSK3 activates SHP2, preventing STAT1 activation in late stage IFN-γ stimulation; however, like STATs, SHP2 is not a direct target for GSK3 [224].

Interestingly, pharmacological reduction of GSK3 (admittedly in some cases not with very selective inhibitors) suppresses the production of IL-1β, IL-6, TNF, and IL-12, whilst enhancing production of IL-10 by TLRs [219]. Despite these major effects of GSK3 on cytokine production the specific substrates of GSK3 responsible remain elusive. NFAT, C/EBPβ, and CREB are transcription factors known to regulate many of these genes and both are proposed substrates of GSK3 (Table 1(a)); however these proteins have not been studied in the context of immunological regulation by GSK3. GSK3 inhibition increases the nuclear translocation of several mediators of the immune response, including these transcription factors [217]. GSK-3 phosphorylates a series of conserved serines on NFAT, at least *in vitro*, and phosphorylation of these sites inhibits DNA binding and promotes nuclear exit of NFAT (thereby opposing calcineurin signaling) [105–107]. It remains to be seen how many of the immune effects of GSK3 inhibition are mediated through direct regulation of these transcription factors.

4. Physiological Outcome of GSK3 Inhibition

The literature indicates that inhibition of GSK3 *in vivo* would reduce the phosphorylation of dozens of proteins (Table 1) and influence a wide range of cellular processes (Figure 2), including cell growth, differentiation, survival, and communication. GSK3 inhibition would then be predicted to have numerous unwanted side-effects (Table 2). In particular, the potential oncogenicity of GSK3 inhibition is a major worry, and detrimental effects on the immune system, heart, and development are all possible (Table 2). Taken together with the lethality of genetic ablation of just one isoform of GSK3 (GSK3β), and the important role of GSK3 in Wnt and growth factor signaling, it is perhaps not surprising that despite the huge effort to develop potent inhibitors of GSK3, none have actually made it into Phase 2 clinical trials.

However, it seems rather premature at this time to discount inhibition of GSK3 as a beneficial therapeutic avenue. There is currently a lack of published evidence that truly specific GSK3 inhibitors, at concentrations that produce GSK3 inhibition, are harmful to healthy organisms, yet there is a study demonstrating efficacy at glucose lowering in a model of T2DM with no reported toxic side effects [159].

There are perhaps three major issues that should be addressed to establish whether GSK3 inhibitors should be pursued further for therapeutic potential.

Firstly, how harmful is conditional ablation of GSK3 in adult animals? GSK3 inhibitors would not be used from birth, and very few pharmaceuticals achieve 100% inhibition of their targets, hence it seems unlikely that the pathways responsible for the lethality of the GSK3β knockout or those involved in embryonic development would be of relevance in the treatment of adults for diabetes or Alzheimer's disease.

Secondly, how many of these reported substrates are actually affected by specific GSK3 inhibitors *in vivo*? As discussed in this paper very few of the substrates in Table 1(a) have convincing evidence establishing them as *bona fide* GSK3 substrates, while it has not yet been proven that the phosphorylation (never mind proposed function) of most of the substrates listed in Table 1(b) is significantly affected by specific GSK3 inhibition *in vivo*. A comprehensive analysis of animals receiving efficacious doses of specific GSK3 inhibitors may show that very few of these proteins are functionally affected by such pharmaceuticals.

Finally, most studies that aimed at identifying GSK3 substrates have chronically deleted GSK3 (genetically, siRNAi or high-dose inhibitor). Animals lacking one allele of each GSK3 isoform have a much less severe phenotype, suggesting partial loss of GSK3 even chronically from birth is not oncogenic. In addition, physiological regulation of GSK3 is normally both partial and transient. For example insulin treatment of cells rarely inhibits GSK3 more than 50% and activity returns to normal in a few hours, while Wnt signaling only regulates a very specific pool of GSK3. In addition many of the key processes regulated by GSK3 have feedback mechanisms to overcome abnormal regulation so proposed side effects may not materialize as predicted. It is quite likely that only transient inhibition of GSK3 (back to normal levels of GSK3 activity rather than complete ablation) would be required for many of the beneficial effects of GSK3 inhibition. Therefore there is scope for more elegant intervention of GSK3 function to achieve beneficial responses, without producing complete and chronic inhibition.

One would hope that improved knowledge of GSK3 biology would aid in the development of beneficial interventions. Others have suggested that drugs aimed at the phosphate binding pocket of GSK3 would preferentially inhibit phosphorylation of primed substrates, and therefore reduce potential side effects. However, Table 1(b) would suggest that most substrates of GSK3 do require a priming event. Other possibilities include isoform specific intervention, but to date there is only tantalizing evidence for substrate preference and little evidence for substrates that are completely specific to one GSK3 isoform. However changing the ratio of GSK3 isoform expression in specific tissues may still alter substrate phosphorylation patterns. Possibly the most promising area is the concept of substrate selective inhibition, where a specific pool of GSK3 could be targeted (or avoided). It has been known for some time that GSK3 exists in the Axin-APC complex, and this "GSK3 pool" is distinct from that targeted by growth factors [23, 24]. If other GSK3 complexes exist then the substrates listed in Table 1(b) could be subdivided by the GSK3 complex that regulates them. Inhibition of a GSK3 containing complex would then have a more specific outcome than global GSK3 inhibition.

Acknowledgment

The research ongoing in Dr.Sutherlands lab is supported by the Alzheimer's Research Trust, Diabetes UK, Tenovus Scotland, and the MRC.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 263673, 8 pages
doi:10.4061/2011/263673

Review Article

The Role of GSK3 in Presynaptic Function

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Received 30 November 2010; Accepted 20 January 2011

Academic Editor: Peter Crouch

The past ten years of research have identified a number of key roles for glycogen synthase kinase 3 (GSK3) at the synapse. In terms of presynaptic physiology, critical roles for GSK3 have been revealed in the growth and maturation of the nerve terminal and more recently a key role in the control of activity-dependent bulk endocytosis of synaptic vesicles. This paper will summarise the major roles assigned to GSK3 in both immature and mature nerve terminals, the substrates GSK3 phosphorylates to exert its action, and how GSK3 activity is regulated by different presynaptic signalling cascades. The number of essential roles for GSK3, coupled with the numerous signalling cascades all converging to regulate its activity, suggests that GSK3 is a key integrator of multiple inputs to modulate the strength of neurotransmission. Modulation of these pathways may point to potential mechanisms to overcome synaptic failure in neurodegenerative disorders such as Alzheimer's disease.

1. Introduction

Glycogen synthase kinase 3 (GSK3) is a serine/threonine kinase that was originally identified as a regulator of cell metabolism but has a variety of roles in cellular function including cell survival, proliferation, neural development, and neurotransmission [1, 2]. GSK3 exists as two isoforms encoded by separate genes: GSK3α (51 kDa) and GSK3β (47 kDa) [3]. Both isoforms are ubiquitously expressed and, although structurally similar, perform overlapping but nonidentical functions [3]. GSK3 is constitutively active in most cells and is negatively regulated by phosphorylation at its N-terminus (Ser-21 for GSK3α and Ser-9 for GSK3β) by a variety of upstream signalling cascades [4]. GSK3 is an unusual kinase in that it generally only phosphorylates a substrate after a previous phosphorylation of the substrate by another protein kinase, an event called "priming" [1, 3]. GSK3 has been implicated in several neuronal disorders such as schizophrenia, bipolar disorder, and Alzheimer's disease [4, 5].

A number of the disorders mentioned above are part of a growing list of diseases called synaptopathies that have at their core a defect in synaptic communication [6]. GSK3 is expressed at the synapse and is found in both immature and mature nerve terminals (presynapses) [7–10]. This localisation suggested a role for GSK3 in presynaptic function and over the past 10 years this has been revealed, ranging from axonal growth and synaptogenesis to regulation of synaptic vesicle (SV) recycling in the mature synapse. This paper will summarise these studies and place the function of GSK3 in the context of nerve terminal physiology.

2. GSK3 Function in Immature Nerve Terminals

2.1. GSK3 Role in Axonal Growth and Polarity. GSK3 signalling is essential for multiple aspects of synaptogenesis: the formation of a functional synapse from nascent neurites. For the purposes of this paper, only the presynaptic contribution of GSK3 in synaptogenesis will be considered; however GSK3 also has multiple postsynaptic roles (for reviews see [11, 12]).

One of the first elements of synaptogenesis is axonal growth. There is an essential requirement for GSK3 activity in this process, with numerous studies demonstrating that an inhibition of GSK3 function greatly reduces axon elongation [13–15]. In addition to controlling growth, GSK3 has a key role in establishing neuronal polarity, specifically the differentiation of immature neurites into nascent axons. Inactive GSK3β (phosphorylated at Ser9) is localised at the

tip of all immature neurites before polarization; however, once polarization is triggered, it is restricted to only the single axonal tip [7, 13, 16]. Thus the localised inactivation of GSK3 seems to be critical for axonal polarity to occur. In agreement, inhibition of GSK3 function using pharmacological antagonists, peptide inhibitors or siRNA all induced the formation of multiple axons [7, 15, 16], whereas overexpression of a constitutively active GSK3β mutant (Ser9Ala) inhibited axon formation in primary neuronal culture [7, 16].

The control of localised microtubule dynamics is critical for neuronal polarization [17], and a number of downstream GSK3 substrates have microtubule organising activity. Collapsin response mediator protein 2 (CRMP2) promotes microtubule polymerization and when overexpressed in neurons produced multiple axons [18, 19]. Also, adenomatous polyposis coli (APC) protein stabilises microtubules in its nonphosphorylated form and is enriched in the nascent axon [20, 21]. GSK3 phosphorylates both CRMP2 and APC *in vitro* [16, 20, 22] and inhibition of GSK3 blocks CRMP2 phosphorylation in polarized axonal tips in culture [16]. Furthermore a non-phosphorylatable mutant of CRMP2 does not promote axon elongation but induces multiple axons when overexpressed in neurons [16, 22]. Thus phosphorylation of either CRMP2 or APC by GSK3 inhibits their binding to microtubules [16, 20]. This promotes microtubule polymerization, thus promoting growth and preventing axonal polarization.

The control of GSK3 activity in both axonal growth and polarization is regulated by an array of different growth factors. Classically, GSK3 is negatively regulated via downstream signalling cascades, involving action of phosphatidylinositol 3-kinase (PI3K) and Akt (also known as protein kinase B) [7, 13, 16, 23]. This is supported by studies showing that overexpression of constitutively active Akt or siRNA knockdown of PTEN (phosphatase and tensin homolog; which both inhibit GSK3) resulted in formation of multiple axons in culture [7]. In agreement these effects were prevented by the coexpression of constitutively active GSK3β (Ser9Ala) indicating that the same signalling pathway was involved [7]. Thus inactivation of GSK3 by growth factors is a critical event in axon formation via the prevention of phosphorylation of a number of key microtubule organising substrates (Figure 1).

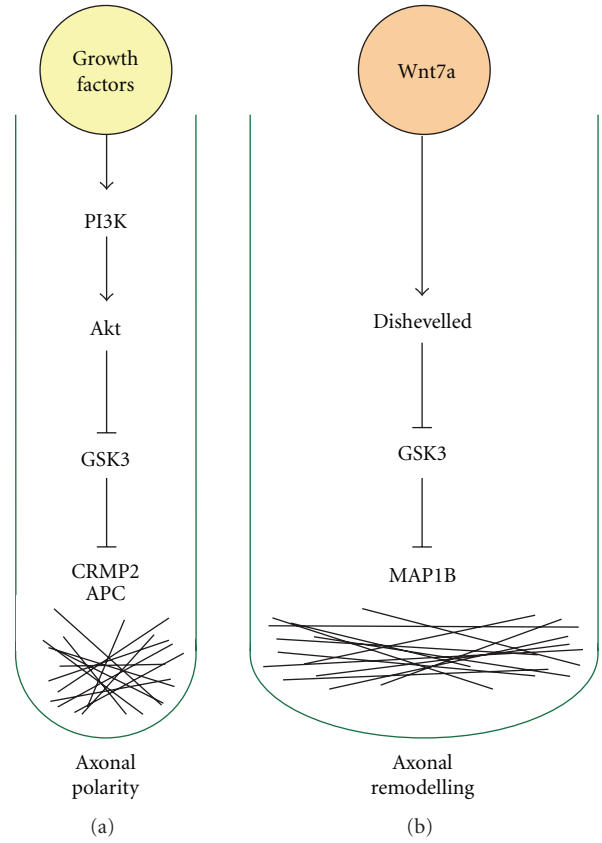

Figure 1: *GSK3 roles, substrates and signalling in immature nerve terminals.* (a) Inhibition of GSK3 activity is essential for the establishment of axonal polarity. GSK3 activity is inhibited by growth factors that signal through the PI3K/Akt signal transduction cascade. When GSK3 is active, it phosphorylates both CRMP-2 and APC, which prevents their interaction with microtubules, arresting microtubule polymerization. When GSK3 is inhibited by growth factors, it cannot phosphorylate CRMP-2 or APC; therefore microtubule polymerization is stimulated. (b) Axonal remodelling describes decreased axonal growth, increased axon diameter, and increased growth cone size when a nascent axon meets a postsynaptic target. Inhibition of GSK3 increases microtubule stability to allow axonal remodelling. GSK3 activity is inhibited by a divergent Wnt signalling cascade. When GSK3 is active, it phosphorylates MAP-1B; which results in increased microtubule instability. When GSK3 is inhibited by Wnts, it cannot phosphorylate MAP-1B therefore microtubules are stabilised.

2.2. GSK3 Role in Axonal Remodelling. GSK3 activity is also critical for the process of axonal remodelling. Axonal remodelling occurs when an axon meets its postsynaptic target and describes the process of decreased axonal growth, increased axon diameter and increased growth cone size. Axonal remodelling in mammalian neurons is controlled by the negative regulation of GSK3 via Wingless Integration (Wnt) proteins.

Wnts are secreted factors that initiate a downstream signalling cascade by binding to Frizzled receptors on the plasma membrane. The canonical Wnt signalling pathway prevents a protein complex of axin, APC, and GSK3 from targeting β-catenin for destruction in the proteasome. β-catenin phosphorylation by GSK3 is a key step in this targeting process. When Wnt binds to Frizzled receptors, this activates the Dishevelled protein to inhibit the function of the "destruction complex" resulting in an inability of GSK3 to phosphorylate β-catenin. Non-phosphorylated β-catenin then translocates to the nucleus to trigger the expression of a number of "Wnt" genes [24].

Axonal remodelling has been studied extensively at the mossy fibre-granule cell synapse in the cerebellum [11], where Wnt7a and Dishevelled both play a key role in this event [8, 25, 26]. Inhibition of GSK3 activity using the antagonist lithium mimics the effect of either Wnt7a or Dishevelled on axonal remodelling [27–29], suggesting

a key downstream role for GSK3. However a divergent pathway to the canonical Wnt route operates in axonal remodelling, where modulation of GSK3 activity results in dynamic changes to the microtubule cytoskeleton rather than alterations in gene expression [11, 12].

The link between the inhibition of GSK3 activity and changes in microtubule dynamics is the microtubule binding protein microtubule-associated protein-1B (MAP-1B). GSK3 phosphorylates MAP-1B both *in vitro* and *in vivo*, which results in altered microtubule organisation and increased microtubule instability within the axon [27, 30–32]. In growth cones the great majority of microtubules are dynamic, whereas on activation of the Wnt7a pathway both stable and dynamic microtubules are present, which form large loop-like structures [26]. This effect of Wnt7a on microtubule dynamics is mimicked by either overexpression of Dishevelled or inhibition of GSK3 [27, 29, 31]. Thus inhibition of GSK3 by a divergent Wnt signalling pathway increases microtubule stability by decreasing MAP-1B phosphorylation and thus allowing axonal remodelling (Figure 1).

2.3. GSK3 Role in Synaptogenesis. After axonal remodelling the growth cone has to differentiate into a nerve terminal. GSK3 again has key roles in this process, via the same divergent Wnt signalling pathway that controls axonal remodelling. For example, Wnt7a is a key retrograde messenger that acts to induce presynaptic maturation, as evidenced by the clustering of vesicle proteins at nascent synaptic sites [26]. Dishevelled is also essential for this process [8]. GSK3 is part of this maturation response too, since its inhibition mimics the effect of Wnt7a and Dishevelled on the clustering of synaptic proteins [26, 28]. The mechanism of GSK3 action is independent of transcription, since Wnt application caused an increase in the size and number of synaptic sites but not the expression of presynaptic proteins [8]. Similarly the Wnt7a knockout mouse displays defective localisation of presynaptic proteins but no decrease in their overall level [8]. β-catenin is also required for SV clustering, suggesting that its phosphorylation by GSK3 may control this event [33, 34]. This is unlikely however, since β-catenin operates independent of Wnt signalling in this process.

A very similar control of synaptogenesis is also observed in *Drosophila,* where the fly homologue of GSK3, *shaggy (Sgg)*, is localised presynaptically. A hypomorphic mutation in *Sgg* resulted in an 80% increase in the number of boutons (nerve terminals), an effect rescued by expression of wild type *Sgg* [35]. Conversely increased expression of *Sgg* reduced the number of synaptic boutons below that seen with wild type [35, 36]. Finally a large increase in bouton number was observed when a dominant negative version of *Sgg* was expressed using a presynaptic driver [35].

Sgg is a likely downstream target of the *Wingless* pathway, since most components of the canonical pathway (*Wingless, Arrow*, and *Dishevelled*) are all expressed presynaptically [36]. In agreement, overexpression of dominant negative *Dishevelled* mimicked the reduction in bouton number observed with *Sgg* overexpression. Conversely *Wingless* overexpression mimicked the effects of presynaptically expressed dominant negative *Sgg* [36].

Interestingly an increase in microtubule loops was observed in flies with the hypomorphic mutation for *Sgg* [35]. This phenotype is very similar to that observed in mammalian axonal remodelling. In a further parallel, the MAP-1B homolog *Futsch* is required for this microtubule looping effect [35]. *Sgg* also phosphorylates *Futsch in vitro* on at least one conserved MAP-1B phosphorylation site [37]. This suggests that inhibition of *Sgg* by the *Wingless* signalling cascade stabilises the microtubule cytoskeleton by preventing the phosphorylation of *Futsch*. Since there are close parallels in the divergent Wnt/*Wingless* pathways in both mammals and *Drosophila*, it suggests that inhibition of GSK3 phosphorylation of MAP-1B may also be important for synaptogenesis in mammals.

In summary, in the immature nerve terminal GSK3 has key roles in axonal growth, polarity, remodelling, and differentiation. These effects are controlled by a range of different signalling cascades and in general are mediated by the phosphorylation-dependent control of the microtubule cytoskeleton.

3. GSK3 Function in Mature Nerve Terminals

Until recently GSK3 was thought to have a purely developmental role in presynaptic physiology; however, recent studies have suggested that it also performs key functions in adult brain, specifically in neurotransmission. In agreement, GSK3 is enriched in adult nerve terminals [8–10].

3.1. GSK3 Role in Neurotransmitter Release. The primary role of the nerve terminal is to release neurotransmitter, via the fusion of SVs by exocytosis. Initial studies that examined the role of GSK3 in neurotransmitter release suggested that it had no role in the process, since modulation of its activity had no effect on hippocampal neurotransmission [9, 10, 38]. Since these studies mainly investigated the postsynaptic response it could be argued that presynaptic effects were overlooked. However, when paired pulse facilitation (PPF) was examined (which is an indicator of the modulation of neurotransmitter release), a lack of role for GSK3 was again apparent. For example, there was no difference in PPF between wild type hippocampal slices and slices obtained from transgenic mice overexpressing GSK3β [9]. Furthermore PPF was unaltered in hippocampal slices exposed to bath application of GSK3 inhibitors [39]. One study has demonstrated an indirect effect of GSK3 inhibition on PPF, but this was only revealed after inhibition of upstream PI3K [40]. In the same study GSK3 inhibitors partially reversed an inhibition of KCl-evoked glutamate release from synaptosomes produced by PI3K antagonists [40]. Thus there is little evidence to date that GSK3 plays a direct role in neurotransmitter release from central nerve terminals.

Other studies have monitored the role of GSK3 in SV exocytosis rather than neurotransmitter release itself. In agreement with its lack of effect on neurotransmitter release, GSK3 antagonists had no effect on SV exocytosis in

cultured neurons monitored using action potential-evoked unloading of the fluorescent dye FM1-43 [39]. In contrast, overexpression of wild type GSK3β in primary hippocampal cultures retarded FM dye unloading evoked by elevated KCl [41]. This effect was proposed to be due to GSK3-dependent phosphorylation of an intracellular loop of a P/Q-type voltage-dependent calcium channel (VDCC). In support, GSK3β phosphorylated a recombinant GST-fusion protein containing this region *in vitro* and GSK3 antagonists reversed a phosphorylation of P/Q-type VDCCs in synaptosomes that was evoked by inhibition of PI3K [41]. It was proposed that GSK3 phosphorylation inhibits P/Q-type channel activity, since overexpression of wild type GSK3β reduced calcium influx in hippocampal neurons. The lack of an identified phosphorylation site did not allow this hypothesis to be tested directly; however, if true it would provide a mechanism by which the strength of neurotransmission could be regulated by GSK3 phosphorylation, since P/Q-type VDCCs are tightly coupled to neurotransmitter release [42]. Thus no direct role for GSK3 in neurotransmitter release has yet been shown; however, it may indirectly regulate this event via phosphorylation of VDCCs.

3.2. GSK3 Role in Activity-Dependent Bulk Endocytosis. In contrast to its, as yet undetermined, role in neurotransmitter release, GSK3 activity has a key role in SV endocytosis. Two major endocytosis modes exist in nerve terminals to retrieve SVs after their fusion by exocytosis. Clathrin-mediated endocytosis (CME) retrieves single SVs with a fixed rate and is dominant during low-intensity stimulation [43, 44]. In contrast activity-dependent bulk endocytosis (ADBE) is dominant during high-intensity stimulation [45] and generates large endosomes direct from the plasma membrane from which SVs can then bud [46]. Recent studies have shown that GSK3 activity is essential for ADBE but not CME during high-intensity stimulation and have also identified at least one GSK3 substrate whose phosphorylation is essential for the process.

The specific role for GSK3 in ADBE was discovered using a battery of different SV recycling assays in primary neuronal culture [39]. First, uptake of large fluorescent dextran molecules (too large to be accumulated inside single SVs) was inhibited with either GSK3 antagonists or by silencing GSK3β expression using shRNA. Second, pharmacological inhibition of GSK3 reduced the uptake of small fluid phase markers into bulk endosomes but not single SVs. Finally, GSK3 antagonists arrested uptake of FM1-43 (which labels both ADBE and CME modes [47]) but not FM2-10 (which only labels CME). There was an activity-dependent requirement for GSK3 in these events, with no role of GSK3 observed using identical assays during low-intensity stimulation. This agrees with a selective role for the enzyme in ADBE. One further key observation was that acute application of GSK3 antagonists had no effect on ADBE, whereas their continual application resulted in inhibition [39]. This indicated that GSK3 had no role during stimulation itself but was required to phosphorylate a substrate(s) after stimulation had terminated.

A number of endocytosis proteins are rephosphorylated after stimulation of the nerve terminal. They are called the dephosphins since they are co-ordinately dephosphorylated by the calcium-dependent protein phosphatase calcineurin on nerve terminal stimulation [48]. One of these proteins, the large GTPase dynamin I, is phosphorylated on two major sites on its C-terminus in resting nerve terminals (Ser-774 and Ser-778) [49]. The dephosphorylation of these sites by calcineurin recruits the endocytosis protein syndapin I to dynamin I [50]. The activity-dependent dephosphorylation of dynamin I and its interaction with syndapin I and syndapin I itself are all essential requirements for the triggering of ADBE [51].

Since dynamin I dephosphorylation is a critical event in the triggering of ADBE, its subsequent rephosphorylation after stimulation is also essential for the process. Dynamin I was originally thought to be rephosphorylated on both major sites by cyclin-dependent kinase 5 (cdk5, see [49]). In agreement with a key role for this rephosphorylation event, cdk5 activity is essential for ADBE but not CME [52]. However it was recently demonstrated that cdk5 is in fact a priming kinase for GSK3 on dynamin I, with cdk5 phosphorylating Ser-778, allowing GSK3-dependent phosphorylation of Ser-774 [39]. This was shown in both *in vitro* phosphorylation assays and also intact neuronal cultures using selective cdk5 and GSK3 antagonists [39]. A direct functional link between GSK3-dependent phosphorylation of dynamin I to ADBE was shown by the inhibition of dextran uptake by overexpression of dominant negative mutants encompassing mutations at Ser-774 [39]. Thus the rephosphorylation of Ser-774 on dynamin I by GSK3 is an essential requirement for the maintenance of ADBE during high-intensity stimulation. This is the first, and to date the only, demonstration that GSK3 activity controls SV recycling via direct phosphorylation of the SV trafficking machinery.

There are eight different dephosphin proteins, of which at least two are also rephosphorylated by cdk5. The fact that GSK3 acts in concert with cdk5 to phosphorylate dynamin I suggests that these proteins may also be essential for ADBE. These proteins are the lipid phosphatase synaptojanin and the lipid kinase phosphatidylinositol phosphate kinase type Iγ (PIPKIγ) which either break down or synthesise phosphatidylinositol (4,5) bisphosphate (PI(4,5)P_2), respectively [53, 54]. The activity of both of these enzymes is essential for SV endocytosis [55, 56], and importantly both enzymes are activated by their dephosphorylation by calcineurin and are inhibited by their rephosphorylation by cdk5 [57, 58]. Scans of the protein sequences of both synaptojanin and PIPKIγ using bioinformatic prediction software highlight multiple consensus sequences for GSK3 phosphorylation (S/T XXX S/TP, where GSK3 phosphorylates the first S/T and cdk5 the second). Thus it is eminently possible that a cdk5 rephosphorylation event also primes these enzymes for GSK3 phosphorylation after high-intensity stimulation. The determination of whether GSK3 both phosphorylates and/or regulates these enzymes' activity is imperative, since a tight temporal control of lipid metabolism is essential for ADBE [59].

3.3. Regulation of GSK3 in Mature Nerve Terminals. GSK3 is a highly regulated kinase, with its high basal activity negatively regulated by numerous signal transduction cascades [1, 3]. Since GSK3 activity is a key requirement for ADBE, it suggests that activation of a number of these signal transduction cascades may also regulate ADBE. GSK3 regulation may occur in two different instances, either acute control driven by action potential firing or longer-term control driven by activation of signalling cascades by growth factors/hormones.

Depolarization of neurons by prolonged stimulation results in GSK3 phosphorylation and thus its inactivation [60, 61]. This suggests that GSK3 activity could be acutely regulated during transient depolarization by action potential stimulation. This is indeed the case, with phosphorylation of GSK3 being triggered during intense, but not mild action potential stimulation in primary neuronal cultures (Smillie unpublished). Interestingly dynamin I is only dephosphorylated by calcineurin during high-, but not low-intensity stimulation [51]. Thus when dynamin I and the dephosphins are being dephosphorylated by calcineurin, GSK3 is simultaneously inactivated allowing maximal dephosphorylation to occur.

There are a number of possible candidates for the protein kinase that mediates the acute activity-dependent phosphorylation of GSK3. For example, the calcium-dependent protein kinase CaMKII phosphorylates GSK3 during chronic depolarization [62]. Also the activity of Akt is increased by prolonged membrane depolarization [63–65], suggesting that it may also phosphorylate GSK3 under these conditions. Preliminary findings from our laboratory suggest that Akt may be the activity-dependent GSK3 kinase, since Akt activation only occurred during intense, but not mild, neuronal stimulation (Smillie, unpublished). Thus an activity-dependent signal transduction cascade may activate Akt to inhibit GSK3 in nerve terminals, aiding the triggering of ADBE (Figure 2). The identity of the upstream components of this cascade will be of great interest and is currently under investigation.

GSK3 activity in mature nerve terminals can also be controlled in the longer term by classical signalling cascades. To date there are three major pathways identified which converge on GSK3 and all result in its phosphorylation and inhibition. Amino acids signal through the mTOR/S6K pathway, insulin and growth factors signal via a PI3K/Akt pathway, and growth factors also act through activation of MAPK and MAPKAP-K1 [1]. Considering the key role for GSK3 in ADBE, longer-term inhibition by any of these cascades should greatly reduce the functionality of this SV endocytosis mode (Figure 2). In agreement, overexpression of a constitutively active form of Akt results in the arrest of ADBE but not CME in primary neuronal cultures (Smille unpublished). It will be of great interest to identify the ligands that trigger these signal transduction cascades to determine how they control both GSK3 activity and modulate ADBE.

The converse of regulated inhibition of GSK3 via its phosphorylation is its activation by its dephosphorylation [1]. This will be critical for the activity-dependent inhibition of GSK3, since it must then be rapidly dephosphorylated after stimulation to rephosphorylate dynamin I. GSK3 can be dephosphorylated by either PP1 or PP2A protein phosphatases [66]. In agreement, the PP1 and PP2A antagonist okadaic acid increases the basal phosphorylation of GSK3 in both hippocampal slices and primary neuronal cultures [10, 67]. Another possible level of complexity has recently been reported with possible differential dephosphorylation of GSK3α and GSK3β by PP2A and PP1, respectively [68].

In ADBE, GSK3 acts in concert with the calcium-dependent protein phosphatase calcineurin. Interestingly these two enzymes are related on a number of different functional levels. For example, calcineurin activity can be arrested by an endogenous inhibitor called regulator of calcineurin 1 (RCAN1) [69]. Both RCAN1 activity and its degradation are regulated by GSK3-dependent phosphorylation [70–72]. In addition, RCAN1 activity also increases expression of GSK3 [73]. Thus the function and regulation of both calcineurin and GSK3 are tightly controlled by a number of intricate feedback pathways, highlighting the key roles their activities play in nerve terminal function. Thus control of GSK3

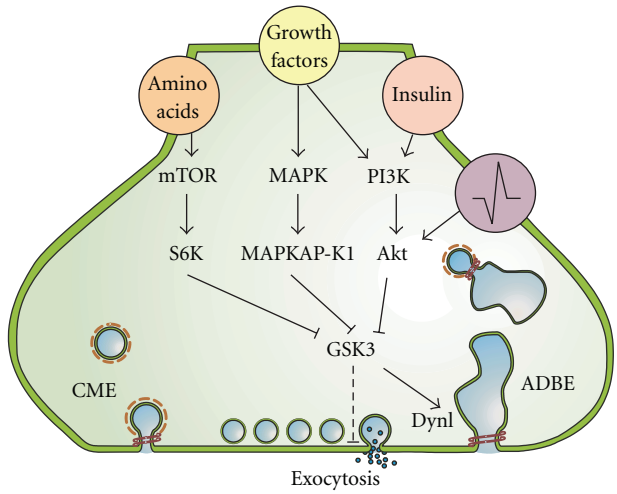

Figure 2: *GSK3-dependent control of SV recycling in mature nerve terminals.* When GSK3 is active, it phosphorylates Ser774 on dynamin I, which inhibits the interaction between dynamin I and syndapin I. This interaction is essential for the triggering of activity-dependent bulk endocytosis (ADBE) and thus during high-intensity stimulation GSK3 is inhibited in an activity-dependent manner by Akt (action potential symbol). After stimulation, GSK3 is active and can rephosphorylate dynamin I. If the rephosphorylation of dynamin I by GSK3 is inhibited, the subsequent cycle of ADBE is arrested. Signal transduction cascades that may regulate GSK3 activity in the longer term are: a mammalian target of rapamycin (mTOR)/S6 kinase pathway (activated by amino acids), a PI3K/Akt pathway (activated by either insulin or growth factors), or a mitogen-activated protein kinase (MAPK)/MAPKAP-K1 pathway (activated by growth factors). GSK3 has no role in clathrin-mediated endocytosis (CME). GSK3 may perform an additional role in SV recycling by negatively regulating neurotransmitter release (dotted line). This is because GSK3 is proposed to phosphorylate P/Q-type calcium channels and inhibit calcium influx into the nerve terminal.

dephosphorylation, and indeed the control of calcineurin by GSK3, may be critical for the rephosphorylation of dynamin I and control ADBE in nerve terminals.

In summary GSK3 has one recently identified key role in the function of the mature nerve terminal, an essential requirement in ADBE. It remains to be seen whether this is its only role in the SV life cycle or whether this is the first of many to be revealed.

4. Presynaptic Function of GSK3 in Alzheimer's Disease

As discussed above, GSK3 activity plays key roles in shaping nerve terminal development and function. Thus regulation of its activity may provide a mechanism to increase synaptic function, especially in neurodegenerative disorders such as Alzheimer's disease. In Alzheimer's disease, synapse loss often precedes neuronal death [74], therefore modulation of the key identified roles of GSK3 in synaptogenesis offers the possibility of either generating new synaptic contacts or at least stabilising existing synapses before irreversible neuronal loss occurs.

Similarly, synaptic failure is one of the first events observed in patients with mild cognitive impairment, a prodromal state of Alzheimer's disease [74]; The essential role for GSK3 in ADBE suggests that its modulation may impact on neurotransmission during periods of intense neuronal activity such as during learning and memory generation. In agreement, inhibition of GSK3 results in increased neurotransmission during high-intensity stimulation [39]. Thus modulation of presynaptic GSK3 activity may either increase synaptic function or at least slow synaptic failure. This is especially interesting since GSK3 activity can be regulated by a wide variety of signal transduction cascades, providing possible entry points for pharmacological intervention in the disease.

In summary, the past 10 years of research have revealed that GSK3 is a key integrator of presynaptic signalling whose output determines the strength of neurotransmission via synaptic remodelling, synaptic strength, or synaptic capacity. The next 10 years will determine whether modulation of any of these key functional roles will facilitate the treatment of neurodegenerative disorders such as Alzheimer's disease.

Acknowledgment

This work was supported by a grant from the Wellcome Trust (Ref: 084277).

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 189728, 12 pages
doi:10.4061/2011/189728

Review Article

GSK3 Function in the Brain during Development, Neuronal Plasticity, and Neurodegeneration

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Received 1 February 2011; Accepted 7 March 2011

Academic Editor: Peter Crouch

GSK3 has diverse functions, including an important role in brain pathology. In this paper, we address the primary functions of GSK3 in development and neuroplasticity, which appear to be interrelated and to mediate age-associated neurological diseases. Specifically, GSK3 plays a pivotal role in controlling neuronal progenitor proliferation and establishment of neuronal polarity during development, and the upstream and downstream signals modulating neuronal GSK3 function affect cytoskeletal reorganization and neuroplasticity throughout the lifespan. Modulation of GSK3 in brain areas subserving cognitive function has become a major focus for treating neuropsychiatric and neurodegenerative diseases. As a crucial node that mediates a variety of neuronal processes, GSK3 is proposed to be a therapeutic target for restoration of synaptic functioning and cognition, particularly in Alzheimer's disease.

1. GSK3 Signaling Pathway

Many diseases of the central nervous system are characterized by changes in the structural organization of neuronal networks, developmental abnormalities, or dysregulation of signaling pathways, leading to altered brain plasticity and, ultimately, neurodegeneration. The proline-directed serine/threonine kinase, glycogen synthase kinase 3 (GSK3), has been suspected to be a contributing factor in psychiatric illness and age-associated neurodegenerative diseases for some time [1]. The involvement of GSK3 misregulation in a variety of brain abnormalities strongly supports its pivotal role as a metabolic crossroads for controlling basic mechanisms of neuronal function from brain bioenergetics to establishment of neuronal circuits, modulation of neuronal polarity, migration, neuronal proliferation, and survival [2]. In particular, the role of GSK3 in phosphorylation of cytoskeletal proteins impacts neuronal plasticity, as cytoskeletal constituents are involved in the development and maintenance of neurites, and changes in the rate of stabilization/destabilization of microtubules (MT) could influence major cellular compartments of neurons, such as dendrites, spines, axons, and synapses.

The metabolic function of GSK3 was first described in glycogen metabolism, as GSK3 phosphorylates glycogen synthase in response to insulin [3]. Since then, research has identified a multitude of substrates and functions for this enzyme. GSK3 exists in cells as two distinct gene products, α and β, which exhibit high homology in the catalytic domain but differ in the N- and C-terminal sequences [4]. GSK3 is ubiquitous throughout the animal kingdom [5] and is widely expressed in all tissues with particularly abundant levels in the brain [4], where the neuron-specific isoform GSK3β2 is found [6].

GSK3 is unique because it is constitutively active, and upstream signals downregulate its activity by phosphorylation at specific residues. The most important phosphoresidues are serine (Ser) 21 for GSK3α and Ser9 for GSK3β, which inhibit its kinase activity [2, 7–10], while phosphorylation on tyrosine (Tyr) residues (Tyr 216/279 for GSK3β and GSK3α, resp.), is required for its activation [11–13]. The latter kind of phosphorylation is mediated by

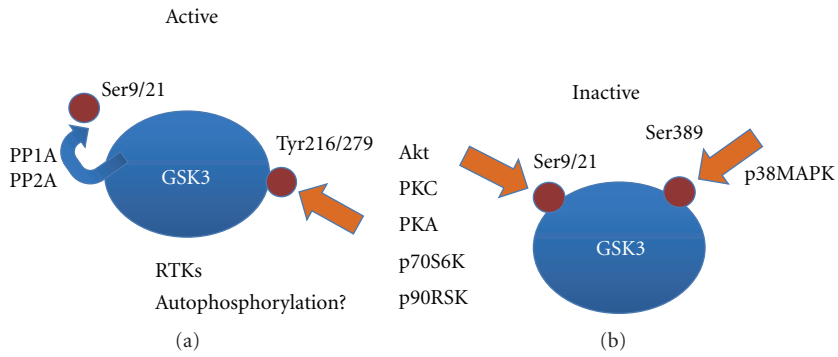

Figure 1: Modulation of GSK3 activity by phosphorylation. Protein phosphatases 1 and 2A activate GSK3 by removing Ser9/21 phosphorylation. It has also been reported that phosphorylation in tyrosine residues by members of the receptor tyrosine kinase family of cell surface receptors (RTKs) or by autophosphorylation may activate GSK3. On the other hand, signaling networks activate several protein kinases, which may bring about phosphorylation of different residues and inhibition of GSK3.

diverse tyrosine kinases [14] or by autophosphorylation [15] (Figure 1).

Multiple kinases can phosphorylate Ser21/9, including Akt, protein kinases A and C, p70S6K, and p90RSK [16]. In contrast, protein phosphatases 1 (PP1) and 2A (PP2A) dephosphorylate the inhibitory site of GSK3, resulting in activation of the enzyme. In addition to the inhibitory phosphorylation of GSK3β described above, an additional inhibitory site at Ser389 has been detected in the brain, which is phosphorylated by p38 mitogen-activated protein kinase (MAPK) [17].

In addition to its phosphorylation state, GSK3 activity may be regulated by proteolysis through disruption of the axin-β-catenin complex [18] or N-terminal cleavage by the calcium-dependent protease calpain [19]. GSK3 activity also depends on its cellular localization. Although GSK3 is predominantly located in the cytosol, it is also present in nuclei and mitochondria, where it is highly activated compared with the cytosolic pool [20]. Nuclear GSK3 regulates the expression of diverse genes via various transcription factors, such as Ap-1, β-catenin, c-myc, and p53 [16]. Subtle control of GSK3-mediated activation and inhibition is required to ensure a proper balance among cell morphoregulation, proliferation, and growth. Thus, prolonged inhibition of GSK3 is associated with hypertrophic cell growth [21], while sustained activation is associated with neurodegeneration [22]. Unlike other kinases, the majority of GSK3 substrates require a "priming" phosphorylation on Ser/Thr residues, which is catalyzed by a protein kinase other than GSK3 [2, 10, 16].

2. Implications of GSK3 Activity in Brain

In adulthood, both GSK3α and GSK3β are expressed in mice adult brain and are particularly enriched in hippocampus, neocortex, and cerebellum [23]. In rodent adult hippocampus GSK3β is more abundant than GSK3α [24], and in aged hippocampus GSK3β is elevated, but not GSK3α [25]. Two splice variants of the GSK3β gene are found in neurons from mouse, rat, and human: GSK3β1 and GSK3β2, the latter being highly expressed during brain development and specific to neurons [6, 26–28]. The two isoforms are differentially involved in phosphorylation of different substrates [29] and establishment of neuronal polarity and axon guidance [2, 30–32].

The importance of GSK3 in brain function has been established by several studies in transgenic mice, which have shown different neurological defects depending of the specific GSK3 isoform involved. While deletion of GSK3β is lethal, heterozygote mice survive and present increased anxiety and reduced exploration [33–35]. Conversely, knockout GSK3α mice are quite normal [36], although neuron-specific knockout of GSK3α results in reduced anxiety, locomotor activity, and aggression [37]. Overexpression of an inhibitory phosphorylation-resistant form of GSK3 results in increased locomotor activity and has been proposed as a model of manic illness [38]. Moreover, overexpressed GSK3β in dentate gyrus results in tau-dependent neurodegeneration of this region [39]. In the brain, GSK3 regulates developmental processes, including neurogenesis, migration, axon growth and guidance, and synaptic plasticity [40], and its activity is controlled through several signaling pathways activated by growth factors, wingless (Wnt) proteins, G-protein-coupled receptors (GPCR), β-arrestin, among other proteins [41].

Abnormal activation of GSK3 has been associated with several neurological and psychiatric disorders that share developmental abnormalities and altered neurocircuitry maintenance, such as schizophrenia, bipolar disorder, autism, and Alzheimer's disease (AD) [42–46]. GSK3 is indeed a common therapeutic target for neuropsychiatric drugs [41, 47].

3. Signaling Pathways Involved in GSK3 Activity in Brain

GSK3 is a downstream component of several signaling pathways in the brain. One of the most studied is the phosphoinositide-3-OH kinase (PI3K)/Akt pathway, which plays a crucial role in differentiation and survival of neuronal and glial cells [48]. Growth signals, Ras proteins [49],

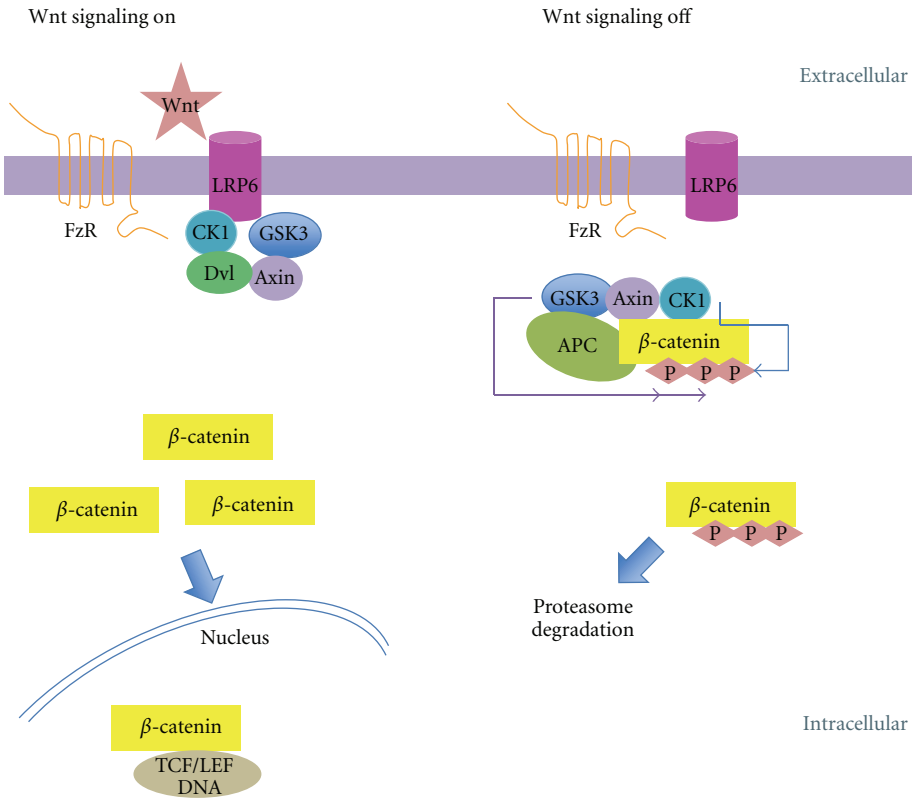

Figure 2: Canonical Wnt signaling and GSK3 regulation. Wnt activation trough Frizzled receptor (FzR) induces destabilization of the protein complex composed of axin, adenomatous polyposis coli (APC) protein, β-catenin, casein kinase (Ck1), and GSK3, which results in GSK3 inhibition leading to the induction of β-catenin/TCF target gene expression. When Wnt signalling is off the GSK3/axin complex is not inhibited and β-catenin phosphorylated and is degraded by the proteasome machinery.

or diminished phosphatase and tensin homolog (PTEN) all activate the catalytic subunit of PI3K, which phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) and activates phosphoinositide-dependent protein kinase-1 (PDK-1). Meanwhile, signaling proteins with pleckstrin homology (PH) domains accumulate at sites of PI3K activation on the inner surface of the plasma membrane through interactions between their PH domains and the phospholipid products of PI3K. Next, the serine-threonine kinase Akt/protein kinase B is recruited and phosphorylated by PDK-1, which stimulates the catalytic activity of Akt, in turn phosphorylating GSK3 to downregulate its activity.

The canonical Wnt pathway is also classically involved in negative regulation of GSK3. Although the role of Wnt proteins in mature neurons remains largely unexplored, recent data indicate that Wnts are important mediators of neuronal function, neuronal morphology, neurogenesis, and synaptic plasticity [50–52]. Interestingly, Wnt signaling has also been implicated in neurological disorders associated with developmental abnormalities, such as schizophrenia [53], as well as in chronic neurodegenerative diseases, such as AD [54]. Extracellular secreted Wnt proteins activate Frizzled receptor and/or the low-density lipoprotein-related protein 5 and 6 (LRP5/6) receptors, leading to the characteristic activation of the Wnt canonical pathway [55]. Due to Frizzled activation, the Dishevelled mammalian homolog Dvl1 is recruited, inducing destabilization of the protein complex composed of axin, adenomatous polyposis coli (APC) protein, β-catenin, and GSK3β, which results in GSK3β inactivation [56]. Inhibition of GSK3β favors an increase in unphosphorylated β-catenin levels, allowing interaction with members of the lymphoid enhancer factor/T-cell factor (LEF/TCF) family of transcription factors and, as a consequence, promoting the expression of cell survival genes [57]. Although the molecular mechanism of GSK3 inhibition is not completely understood, Wnt signaling has recently been reported to trigger the sequestration of GSK3 from the cytosol to multivesicular organelles, preventing its interaction with cytoplasmic substrates [58] (Figure 2).

The outcome is different in the absence of the Wnt stimulation, which may occur due to lack of Wnt ligands or the presence of Wnt negative modulators, such as the extracellular protein Dickkopf-1 (DKK1), which regulates the canonical Wnt signaling, or the secreted Frizzled-related protein, which modulates both canonical and noncanonical Wnt signaling [59]. Under these circumstances, GSK3β is activated and able to phosphorylate its target proteins. Several regulators also target β-catenin/GSK3β signaling. For example, the product of disrupted in schizophrenia 1 (DISC1) gene inhibits GSK3β activity through a direct physical interaction, causing stabilization of β-catenins.

DISC1 loss-of-function in the dentate gyrus has been shown to result in reduced neural progenitor proliferation and to elicit hyperactive and depressive behaviors in mice [60], suggesting the involvement of GSK3β overactivation in mental illnesses, such as depression and schizophrenia. Moreover, DISC1 function seems to be essential for neural progenitor proliferation in embryonic brains and in the dentate gyrus of adult brains through its ability to control GSK3 activity and to maintain β-catenin levels, which ultimately impacts the neural circuitry [60].

GSK3β is also a downstream mediator of dopamine signaling via the dopamine D2 receptor/β-arrestin 2/PP2A complex. In this signaling pathway, Akt activates neuregulin-1 signaling leading to inhibition of GSK3β activity [61]. Interestingly, neuregulin-1 has been also implicated as schizophrenia risk factor [62].

In addition to the described role of GSK3β in neurodevelopment, it has been recently found the potentiation of Notch signalling by PI3K through GSK3β inhibition [63]. The Notch pathway has been implicated in controlling cell fate, differentiation, development as well as synaptic plasticity, learning and memory [64].

4. GSK3: A Switch for Cytoskeletal Reorganization and Synaptic Plasticity

Changes in neuronal morphology and plasticity are affected by GSK3-induced phosphorylation of proteins involved in the modulation of MT and neurofilament stabilization, which affect the cytoskeleton [65]. Among these proteins are tau, microtubule-associated protein 2 (MAP2), microtubule-associated protein 1B (MAP1B), collapsin response mediator protein 2, APC, axin, neurofilaments, kinesin light chain, and cytoplasmic linker protein [9, 16, 30, 31, 40, 53, 66–70].

The induction of polarity during neuronal development is essential for the establishment of circuits that support complex functioning [71, 72]. Subcellular location of the inactive form of GSK3β varies depending on the state of neuronal polarization, as it moves from nonpolarized neurites to the neurite tip that will form the axon at the beginning of the differentiation process. Local inactivation of GSK3 is important to allow axonal growth concurrent with its activation in dendrites [73–76]. These mechanisms support the establishment of neuronal polarity, which is dependent on the stability and dynamism of the MT in each neuronal compartment [40, 53]. The relationship between GSK3β and the microtubule stabilizing protein complex APC-mPar3, which are both present at the tip of the actively growing nascent axon, is important for the establishment of neuronal polarity. Shi and colleagues [74] have demonstrated that spatially regulated GSK3 activity in hippocampal neurons during development leads to axonal generation [74]. The inactivation of GSK3 at the nascent axon is required for mPar3 targeting through APC and kinesin-mediated transport at the plus end of the axonal MT [74].

Two further studies showed that GSK3β inhibition in hippocampal neurons induces formation of multiple axons [75, 76]. However, the role of GSK3 in neurodevelopment remains only partially understood due to contradictory data; other studies have found that GSK3 inhibition induces axonal spreading, reduces axonal elongation, and increases growth cone size, but it does not induce the formation of multiple axons [66, 68, 77–79].

One mechanism related to both synaptic reorganization and MT dynamics is Wnt signaling [80–82], which directs the growing axon towards the synaptic terminal. This process involves the reduction of axonal growth speed and the extension of axonal distal portions at the growth cone [83] until arborization forms functional synaptic endings where the presynaptic apparatus can be assembled. Transmembrane proteins, such as neuroligin/neurexin and cadherins, are also involved in this process and serve to regulate assembly on both sides of the synapse [52, 84]. Wnt proteins have a fundamental role in synapse formation, acting as retrograde signals that regulate assembly of the presynaptic apparatus [84]. Specifically, Wnt7a has a dual function in synaptic differentiation, promoting axon remodeling and increasing incorporation of synaptic proteins [66, 84]. These effects are linked to changes in the reorganization and dynamics (stabilization-destabilization) of MT, which are achieved through the canonical Wnt signaling, independent of the transcription pathway, in which GSK3β activity is inhibited, and, consequently, the phosphorylation state of the axonal MAP1B is reduced [84–86]. The addition of Wnt7a to neuronal cultures reduces MAP1B phosphorylation and induces MT depolymerization from growing areas of the axon, promoting axonal growth cone enlargement and axonal spread [51, 66, 87]. The classical inhibition of GSK3β by lithium chloride (LiCl) reproduces the effects of Wnt7a, inducing axonal arborization and widening and enlargement of the growth cone through remodeling of axonal MT during postnatal development of cerebellar cells [52, 87, 88]. On the other hand, it has been shown that Wnt7a increases the level of Synapsin I (SynI), which is known to be involved in synapse formation, as well as in the maturation and transport of synaptic vesicles in areas of growth [87, 89, 90]. Accumulation of SynI promotes both axonal remodeling and synaptogenesis during cerebellar development [87] and is mimicked by LiCl treatment [66, 88, 91].

GSK3 is also present in mature synapses [92], where its activity, along with that of cyclin-dependent kinase (Cdk5), participates in the recovery of synaptic vesicles during high neuronal activity. During this process, Cdk5 phosphorylates the GTPase dynamin I, and then GSK3β phosphorylates the same dynamin I [93]. Both phosphorylation events are necessary and sufficient to trigger and maintain activity-dependent bulk endocytosis of vesicles [94].

As a result of controlling different morphofunctional aspects of adult brain plasticity, GSK3 also plays a role in long-term potentiation (LTP) [95, 96] and long-term depression (LTD). LTP might be considered the electrophysiological correlate of learning based on its synaptic mechanisms and long-lasting experience-dependent cortical circuits [97–99]. On the other hand, LTD has been suggested as a mechanism to enhance the signal-to-noise ratio of sensory input from stored memories [97]. Some studies have shown that GSK3β inhibition upregulates and maintains LTP [24, 50, 91, 100–102], while GSK3β remains active during

LTD [101]. In rat hippocampus, GSK3β overactivation has been shown to impede LTP and affect synapses by decreasing both synaptic transmission and release of the presynaptic neurotransmitter glutamate [91]. This is regulated by proteins associated with synaptic vesicles, such as SynI [103–108], which is considered to be a synaptic plasticity marker [109, 110]. GSK3β activation inhibits SynI expression after LTP induction and simultaneously disrupts SynI clustering, which results from elevated neuronal activity [91].

An other evidence that underscores the importance of GSK3 in brain plasticity is derived from experiments conducted in rat hippocampus by Gómez de Barreda and colleagues. The authors found that inhibitory phosphorylation of GSK3 at Ser9 increased at the time of LTP induction was maintained for up to one hour *in vivo* and was significantly higher in the hippocampal CA1 and dentate gyrus subregions, which are involved in learning and memory acquisition [39]. Transgenic mice overexpressing GSK3 showed reduced LTP induction [100]. These data confirm the significant participation of GSK3 in LTP regulation by enabling LTP when its catalytic activity is inhibited and preventing LTP when it is overactive. The inhibition of the two main signaling pathways (insulin/PI3K and Wnt) which induced an activation of GSK3 also prevents the induction of LTP [50, 64, 111–113].

GSK3 has been shown to induce LTD through presynaptic and postsynaptic mechanisms. In the presynaptic neuron, upregulation of GSK3 decreases the expression of SynI [91], which has been linked to a decrease in glutamate release [103]. In the postsynaptic neuron, GSK3 activation causes changes in levels of synapse-associated proteins [114, 115], evident as downregulation of the NR2A/B subunits of NMDA receptors and of the scaffolding protein postsynaptic density 93 (PSD93) [24, 91]. In addition, a transient activation of NMDA receptors and endocytosis of AMPA receptors occurs [116, 117], leading to the loss of GSK3 inhibition due to insufficient Ca^{2+} entry. This GSK3 inhibition is mediated by NMDA-PI3K-Akt signaling [112, 118]. Over-activity of GSK3 may also induce MT destabilization in dendrites and axons [80, 86, 119] (Figure 3).

Overexpression of GSK3β in mice prevents the induction of LTP [100] and causes spatial memory deficits [120]. These data suggest that GSK3β plays an essential role in memory formation through three general processes: (i) phosphorylation of substrates involved in synaptic remodeling, necessary for the establishment of new connections, (ii) turnover of cytoskeletal proteins such as MAPs, actin, and tubulin, promoting disassembly, a condition required for a proper synaptic reorganization, and (iii) involvement in the two major forms of synaptic plasticity in the brain, LTP, and LTD [121].

In summary, the functional consequence of GSK3 overactivation in mature neurons is inhibition of LTP and induction of LTD [101, 121], which could be linked to deficiencies of memory and learning characteristic of some neurological diseases, such as AD.

5. GSK3 and Alzheimer's Disease

AD represents a serious epidemiological problem, as it is now recognized as the most common age-related neurodegenerative disease. Evidence supports a role for GSK3 in producing some of the characteristic hallmarks of AD: extracellular accumulation of amyloid-β protein (Aβ) and intraneuronal neurofibrillary tangles (NFTs) composed of hyperphosphorylated forms of tau and inflammatory markers [122]. All of these effects contribute to synaptic and neuronal loss and memory decline [123, 124].

It has been proposed that overactivation of GSK3 in AD leads to inhibition of LTP and may partially explain the learning and memory deficits present early in this neurodegenerative disorder. On the other hand, changes in GSK3 activity may be a molecular link between the two main histopathological markers: Aβ overproduction and tau hyperphosphorylation [39, 46, 125, 126].

The NFTs that accumulate in AD are anomalous filamentous structures composed mainly of abnormal, hyperphosphorylated forms of tau protein [127]. Hence, numerous studies have focused on identification of the protein kinases and phosphatases regulating tau phosphorylation *in vivo*. GSK3β was recognized as a primary kinase involved in tau phosphorylation, as was apparent from the first studies that termed it tau protein kinase-I [128]. Thus, GSK3β has been identified as one of the major enzymes mediating tau hyperphosphorylation at the residues implicated in neurodegenerative tauopathies, including AD [129].

Normally, tau protein contains a total of 85 phosphorylable sites: 45 Ser, 35 Thr, and 5 Tyr. Of these, 40 have been identified as phosphorylated in insoluble tau in AD brain: 28 Ser, 10 Thr, and 2 Tyr, and GSK3β can phosphorylate 23 of these sites [130]. Although GSK3β commonly needs priming phosphorylation of tau, three sites were recently found that can be phosphorylated by GSK3β alone, without priming: Ser396, Ser400, and Ser404 [131]. Furthermore, initial phosphorylation of the Ser214 by cAMP-dependent protein kinase was shown to lead to the rapid modification of four additional sites by GSK3β [131]. Studies in transgenic mouse models have shown that overexpression of GSK3β results in neurodegeneration and have unequivocally demonstrated that GSK3β phosphorylates tau in AD-related phosphoepitopes *in vivo* [93, 132, 133]. Moreover, co-overexpression of tau and GSK3β synergistically increased tau phosphorylation and induced neuronal death in a transgenic model in *Drosophila* [134] while GSK3 inhibition reduces the phosphorylation and aggregation of tau [135, 136]. Similarly, tau hyperphosphorylation and neurodegeneration after GSK3β overexpression are exacerbated by co-overexpression of tau with mutations characteristic of frontotemporal dementia with parkinsonism, associated with chromosome 17 (FDTP-17). This study also showed that tauopathy progression could be prevented by administration of a GSK3β inhibitor at the first signs of pathology [133]. Tau knockout mice overexpressing GSK3β show reduced hippocampal degeneration, indicating that tau partially contributes to the pathology observed in mouse brain [39]. Finally, GSK3β inhibitors decrease tau phosphorylation and amyloid deposition in

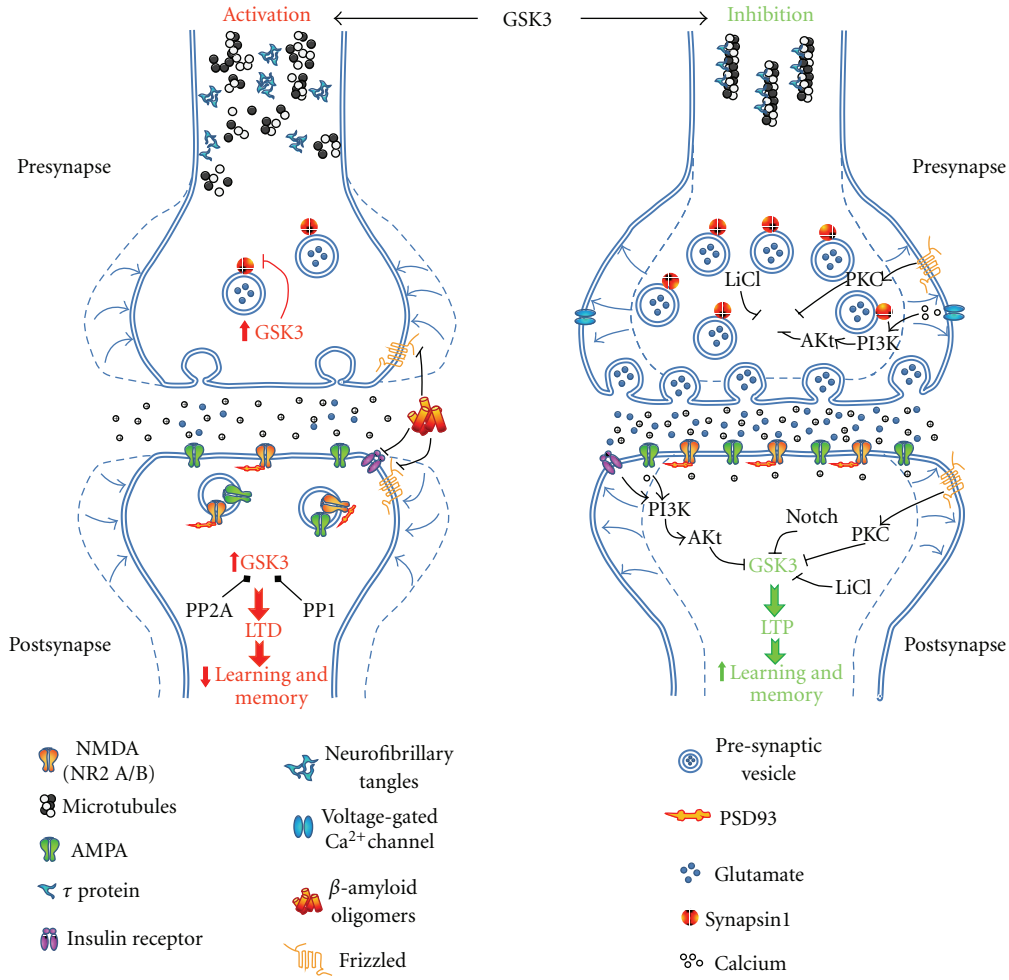

Figure 3: Schematic representation of pre- and postsynaptic mechanisms involved in neuronal plasticity and the role of GSK3. In the presynapse GSK3 activity decreases the expression of SynI reducing the release of glutamate while in postsynapses GSK3 transiently activates NMDA receptors leading to endocytosis of AMPA receptors and reduces the levels of PSD93 protein, favoring LTD. In contrast, Wnt and PI3K signaling pathways or pharmacological inhibition of GSK3 by LiCl supports the induction of LTP, facilitating learning and memory. GSK3 inhibition is also involved in axon and dendritic widening in both pre- and postsynaptic sites. Serine/threonine phosphatases PP1 and PP2A can activate GSK3 regulating phosphor-GSK3 levels through its dephosphorylation. GSK3 is important in the modulation of multiple signaling pathways including Notch pathway that plays an important role in different developmental processes. In AD, amyloid-β oligomers inhibit Wnt and insulin signaling pathways leading to activation of GSK3. In addition, GSK3 overactivation mediates τ hyperphosphorylation and microtubule destabilization.

a double transgenic mouse model coexpressing human mutant amyloid precursor protein (APP) and tau [137]. In brains of AD patients, GSK3β colocalizes with NFT [138], and active GSK3β is present in neuronal cytoplasm of neurons with tangle-like inclusions when abnormal tau hyperphosphorylation begins [139]. In fact, polymorphisms in GSK3 were recently reported to be risk factors for late-onset AD [140, 141].

Evidence suggests that GSK3β regulates APP processing [126, 142], leading to increased production of Aβ. Neuronal exposure to Aβ increases GSK3β activity through PI3K inhibition [143], causing a positive feedback loop. Aβ peptide can regulate GSK3 activity, acting as an insulin receptor antagonist and preventing activation of PI3K and Akt. In turn, the absence of activated Akt prevents the inhibitory phosphorylation of GSK3, increasing its activity [144]. Aβ seems to interfere with the Wnt canonical pathway as well, leading to increased GSK3 activity [145]. Thus, deregulation of GSK3 in AD might be due, in part, to alterations in insulin and Wnt signaling. In the canonical Wnt signaling pathway, the gene for LRP6 coreceptor has been identified as a risk factor for late-onset AD in ApoE4-negative individuals [146]. Interestingly, it has been suggested that the Wnt pathway might be inhibited by ApoE protein, which likely binds to the coreceptor LRP5/6 [147]. Moreover, the ApoE4, implicated in sporadic AD [148], may activate GSK3 [46, 149].

Wnt dysregulation has also been implicated in AD. For example, protein Dickkopf-1 negatively modulates the canonical Wnt signaling pathway and thus activates GSK3. DKK1 colocalizes with NFT and dystrophic neurites in

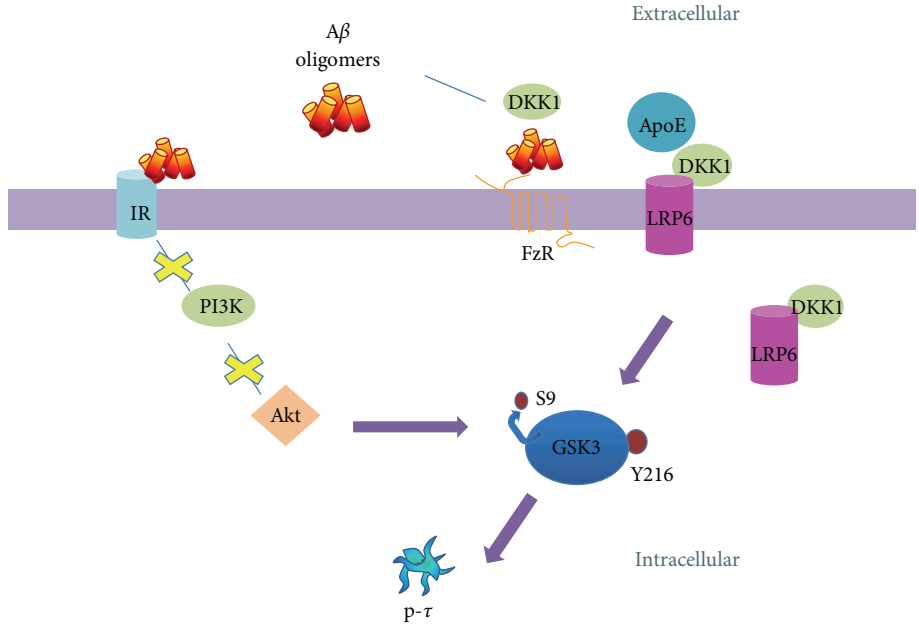

Figure 4: Proposed model of GSK3 activation by amyloid-β protein in AD. Amyloid-β oligomers bind to the insulin receptor and inhibit PI3K/Akt pathway, and Akt is unable to phosphorylate and inactivate GSK3. Aβ also induces the expression of DKK1, which internalizes LRP6 receptor and inhibits Wnt signaling leading to GSK3 activation. Aβ can bind to Frizzled receptor (FzR) and inactivate Wnt signaling as well. ApoE also inhibits this signaling pathway and activates GSK3. Tau hyperphosphorylation and NFT formation may result from GSK3 overactivation.

degenerating neurons of AD brains [150]. Moreover, using Wnt and PI3K signaling inhibitors, cultured cortical neurons have shown increased tau phosphorylation and morphological changes mediated by GSK3β [151]. Taken together, this evidence suggests an important role for GSK3 in AD and supports the notion that GSK3 could be the link between amyloid and tau pathology [46] (Figure 4).

6. Concluding Remarks

GSK3 has attracted a great deal of interest due to the myriad of processes it controls. GSK3 is implicated in many fundamental functions, ranging from bioenergetics to developmental and plasticity events, particularly in the brain. Altered GSK3 activity in the brain negatively influences neuronal structure, which in turn may affect maintenance of neuronal circuits that support cognitive function. The use of therapeutic drugs to control GSK3 activity has been hampered by the variety of substrates targeted by this enzyme and the long-term ramifications of its downstream signaling. Future studies could focus on identifying spatiotemporal expression patterns of specific GSK3 isoforms in the brain with the goal of developing specific inhibitors for clinical use in devastating neurological diseases, such as AD.

Acknowledgments

This work was supported by PAPIIT IN219509-3. P. Salcedo-Tello was supported by CONACYT 220709.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 189246, 9 pages
doi:10.4061/2011/189246

Review Article

GSK-3 in Neurodegenerative Diseases

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Received 27 January 2011; Accepted 7 March 2011

Academic Editor: Peter Crouch

Glycogen synthase kinase-3 (GSK-3) regulates multiple cellular processes, and its dysregulation is implicated in the pathogenesis of diverse diseases. In this paper we will focus on the dysfunction of GSK-3 in Alzheimer's disease and Parkinson's disease. Specifically, GSK-3 is known to interact with tau, β-amyloid (Aβ), and α-synuclein, and as such may be crucially involved in both diseases. Aβ production, for example, is regulated by GSK-3, and its toxicity is mediated by GSK-induced tau phosphorylation and degeneration. α-synuclein is a substrate for GSK-3 and GSK-3 inhibition protects against Parkinsonian toxins. Lithium, a GSK-3 inhibitor, has also been shown to affect tau, Aβ, and α-synuclein in cell culture, and transgenic animal models. Thus, understanding the role of GSK-3 in neurodegenerative diseases will enhance our understanding of the basic mechanisms underlying the pathogenesis of these disorders and also facilitate the identification of new therapeutic avenues.

1. Introduction: GSK-3 Isoforms, Expression, and Neuronal Regulation

Glycogen synthase kinase-3 (GSK-3) is a cellular serine/threonine protein kinase [1, 2], belonging to the glycogen synthase kinase family [1]. It is involved in a number of cellular processes, including the division, proliferation, differentiation, and adhesion of cells [3]. Dysfunction of GSK-3 is implicated in diverse human diseases, including Alzheimer's disease (AD), Parkinson's Disease (PD), type 2 diabetes, bipolar disorder (BPD), and cancer [3, 4]. Two isoforms of GSK-3 have been identified, namely, GSK-3α and GSK-3β, which although encoded by different genes are similarly regulated [5]. GSK-3α (51 kDa) differs to GSK-3β (47 kDa) in that the former has a glycine-rich extension at the amino-terminal end of the protein [5]. Both isoforms are ubiquitously expressed throughout the brain, with high levels of expression seen in the hippocampus, cerebral cortex, and the Purkinje cells of the cerebellum [6]. The expression ratio of these isoforms, however, favors GSK-3β [6, 7].

The crystal structure of GSK-3β reveals a catalytically active dimer [8] conformation that progressively phosphorylates substrates with Ser/Thr pentad repeats [9]. Despite having disparate sequences, the isoforms have a conserved functional domain and share similar substrates, while remaining pharmacologically distinguishable [3]. The independent deletion of GSK-3 isoforms in mice resulted in a distinct profile of substrate phosphorylation [10], suggesting different functions of GSK-3 isoforms in the brain.

The activity of GSK-3 is dependent on phosphorylation at specific sites; phosphorylation of Ser9 of GSK-3β, or Ser21 of GSK-3α, inhibits activity [9], whereas phosphorylation of Tyr216 on GSK-3β and Tyr279 on GSK-3α increases activity [3]. It is thought that deactivation of GSK-3 has more influence on activity rather than activation, as the enzyme is constitutively active and the activation sites can undergo autophosphorylation [11].

The most well-studied GSK-3 regulation pathway is through Akt activation. Insulin stimulation, for example, can activate phosphatidylinositol 3-kinase (PI3K), which phosphorylates Akt (protein kinase B) and in turn inhibits GSK-3 [12–15]. A brief exposure to insulin, however, can also transiently activate GSK-3β by phosphorylating Tyr216 through Fyn, a nonreceptor tyrosine kinase [13]. Other kinases, such as protein kinase C (PKC), inhibit GSK-3 activity by phosphorylating Ser9 [14, 16, 17]. The inhibition

by PKC is additive to the inhibition by PI3K [14]. Additionally, within the brain, p38 mitogen-activated protein kinase (MAPK) inactivates GSK-3β by direct phosphorylation at its C-terminus [18].

Dephosphorylation of GSK-3 at inhibitory sites (thus activating the protein), is coordinated by protein phosphatase 1 (PP1), protein phosphatase 2A (PP2A), and protein phosphatase 2B (PP2B, calcineurin) [19–21]. PP1 preferentially acts as a phosphatase for GSK-3β, while PP2A favors GSK-3α [19]. On the other hand, the overexpression of GSK-3β inhibits PP2A, which may serve as a negative feedback mechanism for GSK-3β activity [22]. GSK-3 and its complex regulatory mechanisms have been extensively studied in a number of neurodegenerative diseases as outlined below.

2. GSK-3 in AD and Tauopathies

Alzheimer's disease is characterized by the accumulation of extracellular senile plaques and intracellular neurofibrillary tangles (NFTs) within the brain (for a review, see [23]). The major component of the plaques, which was first purified and identified from AD brains in the 1980s [24] and later shown to be a product of normal cellular metabolism [25], is β-amyloid (Aβ). Aβ is proteolytically processed from the amyloid precursor protein (APP) [26] via cleavage at the β-secretase site by BACE1 [27], followed by γ-secretase cleavage by presenilin (PS) [28]. The key component of the NFTs on the other hand, is the tau protein [29–31], which was originally identified as an intracellular microtubule stabilizer [32]. Both Aβ and tau are, therefore, fundamentally involved in driving the pathogenesis of AD. With respect to this paper then, it is of note that both these proteins may be modulated by GSK-3. The most well-characterised interactions, however, occur with tau.

2.1. Tau. GSK-3 is one of the main kinases involved in the phosphorylation of tau, a process that is crucial to the function of the protein. The normal phosphorylation of tau determines its affinity for microtubule binding [29, 33–35], with pathological hyperphosphorylation resulting in the dissociation of tau from microtubules and subsequent aggregation to form NFTs (for a review, see [36]). GSK-3β has been found to be associated with normal microtubule-bound tau [37] as well as with the hyperphosphorylated tau deposits in the AD brain [38, 39]. There are several lines of evidence that support a direct functional link between tau phosphorylation and GSK-3. For example, *in vitro* and in cell culture models, both GSK-3α and GSK-3β can phosphorylate tau at various sites that are consistent with the epitopes found to be hyperphosphorylated in AD brains [40–45]. The overexpression of GSK-3β in animal models also promotes the phosphorylation of tau, implicating it as an *in vivo* tau kinase [46–49]. Conversely, the inhibition of GSK-3β activity by either GSK-3 inhibitors or upstream Akt inhibitors reduces tau phosphorylation [50–58]. GSK-3β thus affects tau function through interfering with tau phosphorylation, thereby disrupting microtubule stability [59, 60], self-assembly of microtubules [61, 62], the microtubule-dependent cell processes [63], and regulation of organelle transport and axonal transportation [64–66]. Interestingly, the overexpression of tau also increased GSK-3β activity, which perpetuated the phosphorylation of tau [67].

In addition to effects on phosphorylation, the activation of GSK-3β may also facilitate the aggregation of tau [68–71]. Furthermore, the *in vivo* overexpression of GSK-3β accelerates tau-induced neurodegeneration [47, 49, 71, 72], while the inhibition of its activity reduces tau toxicity [73–75]. Conversely, in the absence of tau, the neurodegenerative and cognitive phenotype observed in GSK3-overexpresing mice is ameliorated, suggesting that tau may mediate GSK-3β toxicity [76]. In addition, GSK-3 may regulate tau-mRNA splicing [77] and expression [78] by disrupting transcription [79].

2.2. β-Amyloid. Accumulating evidence suggests that GSK-3 interferes with the biology of Aβ, which is believed to be upstream of tau in the pathogenesis of AD [23]. Aβ accelerates tau pathology [80, 81] and promotes tau phosphorylation by several mechanisms, including activation of GSK-3β [82–84]. The use of Aβ antibodies both *in vitro* and *in vivo* decreases GSK-3 activity, supporting the interaction between Aβ and GSK-3 [85]. It has also been shown that the activation of GSK by Aβ in primary hippocampal cultures is specific to GSK-3β [86], and that the inhibition of GSK-3β prevents Aβ-induced toxicity to neurons [82, 84, 87, 88]. Likewise, although both isoforms of GSK-3 are hyperactivated in transgenic mice expressing mutant APP (V717I) [89], the data from this model together with that from a model expressing the intracellular domain of APP [90] firmly support the notion that it is the activation of GSK-3β by amyloid that results in downstream pathological effects on tau. The Aβ-induced activation of GSK-3 also only needs to be transient to result in tau hyperphosphorylation and other effects such as mitochondrial trafficking impairments [91]. Finally, tau null mice are protected against Aβ-induced toxicity [92, 93] and GSK-induced toxicity [76], which taken together with the discussed data highlight the complex interaction between GSK, Aβ, and tau. This is further complicated by the fact that GSK-3 is involved in APP processing and subsequent Aβ production.

The amyloid precursor protein and PS1 are substrates of GSK-3 [94–98], and GSK-3α is thought to regulate Aβ production by interfering with APP cleavage at the γ-secretase step [99]. Co-overexpression of GSK-3α and APP in CHO cells increased the level of Aβ in a dose-dependent manner, while selective reduction of GSK-3α protein expression by RNAi decreased Aβ levels [99]. Although Phiel et al. [99] showed an opposite role of GSK-3β in their study, GSK-3β was later shown to decrease Aβ levels by an unknown pathway [100]. Nevertheless, the genetic or pharmacological deactivation of GSK-3 reduces Aβ and its associated toxicity, ameliorates Aβ-induced behavioral deficits, and rescues neuronal loss in APP-overexpressing mouse models [101–103], thus strongly implicating GSK-3 in the pathogenesis of AD.

3. GSK-3 in Parkinson's Disease

Parkinson's disease is characterized by dopaminergic neuron degeneration in the substantia nigra pars compacta (SNpc) with Lewy body (LB) pathology, accompanied by clinically defined parkinsonism [104]. As there is a potential role of tau emerging in PD [105–107], then the function of GSK-3 in PD has also thus been investigated. The examination of postmortem tissue from PD patients has revealed that GSK-3β, phosphorylated at Ser9, is specifically localised within the halo of LBs [108] and that GSK-3β activity is also elevated in the striatum [109]. This latter finding has been recapitulated in mouse models of PD [110]. Increased GSK-3 levels have also been reported in peripheral blood lymphocytes in PD patients [111], and polymorphisms in GSK-3β, which affect its transcription and splicing, are also associated with disease risk in PD when stratifying by tau haplotype [112, 113].

Mechanistically there is evidence to support an interaction between α-synuclein, a 16 kDa natively unstructured protein that is fundamentally involved in the pathogenesis of PD, and GSK. Aggregated α-synuclein species are the main component of LBs and single nucleotide polymorphisms and duplication or triplication of the α-synuclein gene cause familial Parkinsonian degeneration [104]. α-synuclein, which is a substrate for GSK-3β phosphorylation, may also modulate the activation of GSK-3β [114]; GSK-3β phosphorylation at Tyr216 (which activates GSK-3 activity) is also abolished in cells lacking α-synuclein and in α-synuclein knockout mice [110]. The potential role of GSK-3β in PD has been elucidated in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) model of PD, where the inhibition of GSK-3β protects against MPTP toxicity *in vitro* and *in vivo* [56, 110, 115] and decreases α-synuclein protein expression [56]. Taken together, these data strongly implicate GSK-3 in the pathogenesis of PD. The neuroprotective possibilities of GSK-3 inhibition on Aβ, tau and α-synuclein pathology have thus been explored in depth, most extensively with lithium.

4. Lithium: A GSK-3 Inhibitor

Lithium, a monovalent cation, affects multiple cellular processes in model organisms and humans (for a review, see [116]). Importantly, it has been used as a mood stabilizer and primary therapy for BPD since its discovery by Cade in 1949 [117]. Although effective in many cases, lithium exhibits a narrow therapeutic window, and side effects may occur within the therapeutic dose range [118]. Lithium is suggested to have several molecular targets in BPD, one leading mechanism of action is the inhibition of GSK-3 [116].

Haplo-insufficiency of GSK-3β mimics behavioral and molecular effects of lithium [119], while GSK-3β overexpression mimics mania and hyperactivity in a mouse model [120], supporting GSK-3β as a relevant target of lithium action [121]. With an inhibitory Ki of 2 mM [121], lithium inhibits both GSK-3α and GSK-3β directly through competitive inhibition of Mg^{2+} [122], and indirectly through the modulation of post-translational modifications of GSK-3 [123, 124] in a number of species [125]. Lithium is selective for GSK-3α and GSK-3β and does not inhibit other protein kinases tested *in vitro* and *in vivo*, including casein kinase II, protein kinase A and C, MAPK, and CDK5 [121, 126]. When utilized at therapeutic concentrations in various cell culture models, lithium reduces tau phosphorylation [53, 127–129] and the processing of APP to generate Aβ [99, 130, 131], suggesting that lithium may have important implications in both AD and BPD. However, some of these findings have been disputed, with lithium shown to increase β-secretase activity and to subsequently elevate extracellular Aβ levels in CHO cells and rat cortical neurons [132]. In this case, the activity of γ-secretase was unaltered, suggesting that the lithium-induced elevation of Aβ was independent of GSK-3 inhibition [132]. In addition, lithium treatment has been shown to reduce tau protein and mRNA levels in cultured cortical neurons [79].

Nevertheless, lithium has been assessed for its potential efficacy in treating "AD-like" pathology *in vivo*. In wild-type rats, lithium has been shown to reduce tau phosphorylation and inhibit GSK-3 activity [133] and to also enhance spatial memory [134, 135]. Using transgenic animals characterised by progressive Aβ deposition, lithium treatment has been consistently found to decrease Aβ levels and APP phosphorylation, as well as to reduce GSK-3 activity and tau phosphorylation [99, 101, 136–138]. In contrast to previous cell culture studies, however, β-secretase activity has been unaffected [101, 132]. Lithium treatment has also been shown to prevent Aβ toxicity [136], preserve dendritic structure [101], facilitate neurogenesis [138], and rescue Aβ-induced cognitive impairment [101, 137, 138].

Less clear, however, is the efficacy of lithium against tau-mediated degeneration. Mice that overexpress disease-linked tau exhibit reduced tau phosphorylation with lithium treatment [55, 73–75, 139]. In addition, tau transgenic models have attenuated axonal degeneration with lithium treatment [55], but no motor or working memory recovery [139]. Lithium-treated 3XTg mice (harbouring both tau and Aβ pathology) have reduced GSK-3 activity and tau phosphorylation, but no change in Aβ levels or working memory [126]. However, in accordance with cell culture studies [53, 127–129] GSK-3 activity remained the same in a long-term (5 months) lithium trial [74], possibly suggesting that the protection offered by lithium is GSK-3 independent. The authors [74] alternatively suggested that lithium reduced the tau lesion primarily by promoting its ubiquitination and degradation rather than by inhibiting its phosphorylation through GSK-3.

While these *in vitro* and *in vivo* studies reveal a beneficial effect of lithium on tau and Aβ pathology, a number of observational studies and case reports have provided conflicting evidence, with both positive [140–144] and negative outcomes [145, 146] on dementia reported. Despite this, lithium has recently been evaluated as a therapy for AD in a 10-week multicenter, randomized, single-blind, and placebo-controlled trial [147]. GSK-3 activity was monitored in lymphocytes at 1-2 week intervals, total and phosphorylated tau levels were assessed in the CSF, and $A\beta_{(1-42)}$ levels were assessed in the CSF and plasma at the end of

treatment. Cognitive function was assessed using the Mini-Mental State Examination (MMSE), the Alzheimer's disease Assessment Scale-Cognitive subscale (ADAS-Cog) and the Neuropsychiatric Inventory (NPI). The study concluded that lithium was not an effective therapeutic for AD, as there were no significant effects on any of the endpoint measurements. A post-hoc examination on a subset of individuals did, however, reveal an increase in serum BDNF that was inversely correlated with decreased ADAS-Cog sum scores [148]. Further long-term studies are required to determine the safety and efficiency of lithium or other GSK-3 inhibitors for the treatment of AD.

In pursuit of GSK-3 regulation in PD, lithium has also been tested in animal models of this disease. The data, however, are not conclusive, with lithium shown to both protect against the dopamine depletion resulting from MPTP toxicity [149] and to also cause a decrease in brain dopamine (DA) release [150] that leads to deficits in DA levels [151]. Furthermore, lithium treatment does not prevent dopaminergic neuron loss in the related 6-OHDA model of PD [152]. There is, therefore, currently little evidence to support lithium as a treatment strategy for PD. The data on the use of lithium in other human neurodegenerative diseases is also not compelling.

Lithium, for example, has also been investigated as a therapy for one of the motor-neuron diseases, amyotrophic lateral sclerosis (ALS), despite the lack of an established connection with GSK-3. Although lithium was found to delay disease onset and to reduce neurological deficits in both ALS mouse models and a small human trial [153, 154], other mouse and human trials have shown detrimental effects [155, 156]. The potential utility of lithium in ALS, or indeed in any of the neurodegenerative disorders outlined above, remains unclear. It is likely that lithium has other activities, independent of GSK-3, that may mediate its pharmacodynamics.

5. Concluding Remarks

We have summarized the latest knowledge regarding GSK-3 and its involvement in neurodegenerative diseases such as AD and PD. Although extensive research has been undertaken in the last decade, the role of GSK-3 in disease pathogenesis has yet to be fully elucidated. The inhibition of GSK-3 may be a potential target for AD, since it has regulatory effects on both Aβ and tau. Similarly, GSK-3 inhibition could interact with α-synuclein to affect the pathogenesis of PD. The intriguing preclinical data, however, has yet to be translated into an effective pharmacotherapy for neurodegeneration, perhaps in part owing to the complex regulation of GSK and its activity on multiple substrates. Future endeavors should investigate alternative modulators of GSK-3 and annotate more precise mechanisms of how the isoforms of GSK-3 participate in neurodegeneration.

Acknowledgments

This paper funded by the National Health and Medical Research Council of Australia and the Australian Research Council. The authors thank Y. H. Hung for helpful discussions and proofreading.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 861072, 11 pages
doi:10.4061/2011/861072

Review Article

Regulation of Cell Survival Mechanisms in Alzheimer's Disease by Glycogen Synthase Kinase-3

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Received 15 January 2011; Accepted 9 March 2011

Academic Editor: Adam Cole

A pivotal role has emerged for glycogen synthase kinase-3 (GSK3) as an important contributor to Alzheimer's disease pathology. Evidence for the involvement of GSK3 in Alzheimer's disease pathology and neuronal loss comes from studies of GSK3 overexpression, GSK3 localization studies, multiple relationships between GSK3 and amyloid β-peptide (Aβ), interactions between GSK3 and the microtubule-associated tau protein, and GSK3-mediated apoptotic cell death. Apoptotic signaling proceeds by either an intrinsic pathway or an extrinsic pathway. GSK3 is well established to promote intrinsic apoptotic signaling induced by many insults, several of which may contribute to neuronal loss in Alzheimer's disease. Particularly important is evidence that GSK3 promotes intrinsic apoptotic signaling induced by Aβ. GSK3 appears to promote intrinsic apoptotic signaling by modulating proteins in the apoptosis signaling pathway and by modulating transcription factors that regulate the expression of proteins involved in apoptosis. Thus, GSK3 appears to contribute to several neuropathological mechanisms in Alzheimer's disease, including apoptosis-mediated neuronal loss.

1. Introduction

Ten years ago we first noted that glycogen synthase kinase-3 (GSK3) appeared to be linked to all of the major pathological mechanisms that had been identified in Alzheimer's disease [1]. Since then, a remarkable amount of new evidence has solidified the central role of GSK3 in Alzheimer's disease neuropathology, as exemplified by this entire issue being devoted to the subject. Among the early identified links between GSK3 and Alzheimer's disease was the discovery that GSK3 promotes the intrinsic apoptotic signaling pathway that may be partly responsible for neuronal loss in Alzheimer's disease [2]. Here we review the multiple cellular pathways influenced by GSK3 that may contribute to changes in cell viability in Alzheimer's disease.

2. Overview of Cell Death in Alzheimer's Disease

Among the known mechanisms that may contribute to loss of neurons in Alzheimer's disease brain, apoptosis has received the most attention. Apoptotic signaling is generally classified as proceeding by either an intrinsic pathway or an extrinsic pathway. Of these, the intrinsic apoptotic signaling pathway has predominated in studies of Alzheimer's disease. Intrinsic apoptotic signaling is most often induced by intracellular damage that leads to mitochondrial release of cytochrome c and the activation of intracellular cysteine proteases called caspases [3], particularly caspase-9 and caspase-3, with a variety of other pro-apoptotic mediators and caspases contributing to the eventual outcome

of apoptosis [4]. Extrinsic apoptotic signaling is initiated by stimulation of plasma membrane death receptors that initiate apoptosis by activation of caspase-8, and subsequent apoptotic signaling can proceed through the mitochondrial pathway or independently of mitochondria by caspase-8-mediated direct activation of caspase-3 [5]. Of these two apoptotic signaling pathways, the intrinsic system has been the focus of the great majority of studies of apoptotic cell death mechanisms in Alzheimer's disease.

3. GSK3 Promotes Intrinsic Apoptotic Signaling

Much evidence indicates that promotion of the intrinsic apoptotic signaling pathway by GSK3 may be particularly important in the apoptosis and neuronal loss that occurs in Alzheimer's disease. This is because GSK3 has been shown to promote apoptosis following a wide range of insults that activate the intrinsic apoptotic signaling pathway [2]. In order to promote intrinsic apoptotic signaling, GSK3 must be active. The major mechanism regulating GSK3 activity is phosphorylation of an N-terminal serine in each of the two paralogs (commonly called isoforms) of GSK3, serine9-GSK3β or serine21-GSK3α. Phosphorylation of these regulatory serines inhibits GSK3, thus signaling activities that reduce GSK3 serine-phosphorylation activate GSK3. The inhibitory serines in GSK3 can be phosphorylated by several different kinases. The most often studied of these is Akt (also called protein kinase B), which itself is activated by multiple receptor-coupled signaling pathways that signal through phosphatidylinositol 3-kinase (PI3K), such as signaling induced by a variety of neurotrophin receptors. Thus, one mechanism by which GSK3 can be activated is by signals that reduce its serine-phosphorylation mediated by Akt or other kinases. A widely used method to study the actions of GSK3β is to express GSK3β with a serine9-to-alanine9 mutation (S9A-GSK3β) to maintain expressed GSK3β fully active. GSK3 also must be phosphorylated on a tyrosine residue for full activity, tyrosine216-GSK3β or tyrosine279-GSK3α. Although the mechanisms regulating tyrosine-phosphorylation of GSK3 are still not well-understood, a number of reports have indicated that GSK3 activity can be increased by signals that increase tyrosine-phosphorylated GSK3.

3.1. Overexpression of GSK3 Is Sufficient to Activate Apoptosis. Overexpression of GSK3 in cells or rodent brains has been shown to induce apoptosis and neuronal death in many reports. The first study of this type showed that that transient overexpression of wild-type GSK3β was sufficient to induce apoptosis in cultured PC12 cells [6]. Furthermore, this report showed that expression of a dominant-negative kinase-dead mutant of GSK3β was sufficient to reduce apoptosis that was induced by inhibition of PI3K, demonstrating that GSK3 is a major mediator of apoptosis in conditions of reduced PI3K activity [6]. Bijur and colleagues [7] extended those findings to show that although relatively low levels of over-expressed GSK3β did not induce apoptosis in human neuroblastoma SH-SY5Y cells, pro-apoptotic signaling was greatly increased by modestly elevated levels of GSK3β, demonstrating that increased GSK3 activity promotes apoptotic signaling induced by a variety of toxic agents [7]. These and other *in vitro* studies demonstrating that increased GSK3 activity can promote activation of the intrinsic apoptotic signaling pathway and that inhibition of GSK3 provides protection from apoptosis have been previously reviewed in detail [1, 2].

The results of *in vitro* studies that showed promotion of intrinsic apoptotic signaling by GSK3 raised the question of whether abnormal increases in GSK3 *in vivo* may contribute to neuronal death in neurodegenerative diseases, such as Alzheimer's disease. One approach to test this that has been productive is to study transgenic mice over-expressing GSK3. Spittaels and colleagues [8] studied transgenic mice over-expressing constitutively active S9A-GSK3β and found hyperphosphorylation of the microtubule-associated protein tau and altered behaviors in sensorimotor tasks in these mice. Mice postnataly over-expressing S9A-GSK3β driven by the thy-1 promoter in neurons exhibited decreased brain volume and cell size, increased neuronal densities, and learning deficits in the Morris water maze [9]. Lucas and colleagues [10] created transgenic mice over-expressing GSK3β in regions specifically relevant to Alzheimer's disease, the hippocampus and neuronal layers I–VI of the cortex. These mice displayed evidence of apoptosis activation, including increased TUNEL staining and caspase-3 activation in the dentate gyrus [10]. Concomitant with increased markers of apoptosis, the GSK3β-over-expressing mice exhibited activated astrocytes and microglia. These mice also displayed deficits in learning in the Morris water maze, but tau filaments were concluded to not be involved in the learning deficits [11]. Further studies of these mice took advantage of the capability of terminating GSK3β overexpression with doxycyclin treatment, which reduced GSK3β levels, reduced tau phosphorylation, increased microtubule polymerization, reduced reactive astrocytosis, restored spatial memory, and decreased levels of active caspase-3 [12]. When the tetracycline-regulated conditional transgenic mice were crossed with mice over-expressing tau carrying a FTDP-17 mutation, GSK3-mediated hyperphosphorylated tau had an increased propensity to form filaments, leading to neurofibrillary tangles (NFTs), and displayed microencephaly at 18 months of age [13]. Expression of constitutively active S9A-GSKβ in the cortex and hippocampus caused hyperphosphorylated tau, neurofibrillary tangles, and morphological changes in neuronal structure [14]. Mice expressing human P301L tau (JNLP3 mice), expressing mutant amyloid precursor protein (Tg2576 mice), and expressing both P301L tau mutation and mutant APP protein (TAPP mice), all displayed increased tyrosine-phosphorylated GSK3α/β in spinal cord and amygdala neurons characterized by granulovacular degenerative granules and neurofibrillary tangles in the JNPL3 and TAPP mice [15]. Avila and colleagues [16] reported that mice over-expressing GSK3β had a 2-fold increase in tau levels and a decrease in dentate gyrus volume, and suggested that increased GSK3β activity, particularly in the dentate gyrus, hinders neurogenesis, thereby promoting the decreased tissue volume. Collectively, these findings in

transgenic mice indicate that GSK3 promotes pathological process associated with Alzheimer's disease, but whether GSK3 promoted decreases in neuronal viability often was not directly investigated due to the difficulty in capturing transient markers of apoptosis in *in vivo* studies.

3.2. Localization of GSK3 in Alzheimer's Disease Brain. Localization studies in postmortem Alzheimer's disease brain have been used to determine if GSK3 is accumulated or activated in areas with prominent neurodegeneration. Pei and colleagues [17] reported increased GSK3α and GSK3β immunoreactivities in plaques and CA1 hippocampal neurons, and co-staining with Congo red indicated that many cells with increased GSK3β immunoreactivity contained hyperphosphorylated tau and neurofibrillary tangles. Subsequently, Pei and colleagues [18] compared non-diseased brains, deemed Stage 0 cases, to Alzheimer's disease-like brains from middle-aged and senescent patients, classified as stages A–C according to the extent of amyloid deposition, and NF I–VI according to the extent of neurofibrillary tangle pathology. They found only moderate active GSK3β staining in normal brains (Stage 0) in neurons of the entorhinal cortex and CA1 and CA2 regions of the hippocampus [18]. Inactive GSK3β staining was pronounced in entorhinal cortical neurons and the hippocampal CA1 region relative to staining for active GSK3. Stage 0/NF I-II brains also had increased inactive GSK3β, relative to active GSK3, immunoreactivity in the entorhinal cortex and hippocampal CA1 region. Stage III/IV brains showed increased tau phosphorylation immunoreactivity (AT8 antibody) and tangle formation in the entorhinal cortex and hippocampal CA1 region, and tangle-containing neurons also had increased active, as well as inactive, GSK3 immunoreactivity, suggesting increases in both GSK3 levels and activity as disease pathology progressed. Stage V/VI brains exhibited AT8 immunoreactivity and tangle inclusions throughout the entorhinal and temporal cortices and the hippocampus. Most inclusion-positive neurons stained intensely for active GSK3, while little or no inactive GSK3 immunoreactivity was recorded in cortical or hippocampal tissues. Collectively, Pei and colleagues [18] clearly defined an Alzheimer's disease progression profile detailing increased tau phosphorylation and increased GSK3β expression and activity in cortical and hippocampal tissues as disease pathology worsened. Ferrer and colleagues [19] also found increased GSK3 immunoreactivity in degenerating neurons characterized by tangle-like inclusions in Stage III and Stage VI postmortem Alzheimer's disease entorhinal cortex and hippocampus. Furthermore, GSK3 colocalized with 40–80% of neurons with hyperphosphorylated tau (PHF-1 antibody), thereby supporting the notion that GSK3 expression and/or activity increases as Alzheimer's disease progresses.

GSK3 immunoreactivity has been reported to be increased at sites of granulovacular degeneration, a pathological characteristic of Alzheimer's disease [15, 19, 20]. Leroy and colleagues [20] reported increased GSK3β and phospho-tyr216-GSK3β immunoreactivity in neuronal cell bodies and dendrites of postmortem human hippocampal tissues. Increases in GSK3 immunoreactivity co-localized specifically with granulovacular degenerative granules, and there were no detectable changes in GSK3 immunoreactivity within neurofibrillary tangles. Ferrer and colleagues [19] also reported increased GSK3 immunoreactivity in granulovacular degenerative bodies located in neuronal cell bodies, and also found increased GSK3 immunoreactivity in glial cells in postmortem human brain tissues.

3.3. Toxicity Associated with Amyloid-β Peptide (Aβ). Substantial evidence has demonstrated that Aβ activates GSK3 by decreasing its inhibitory serine-phosphorylation, which appears to contribute to Aβ-induced increased tau phosphorylation and to Aβ-induced neurotoxicity [21–30]. These studies showing Aβ-induced activation of GSK3 have used a variety of peptides, including A$\beta_{1–40}$, A$\beta_{1–42}$, and the 25–35 peptide fragment, indicating that accumulation of any of these may activate GSK3, although perhaps by utilizing different signaling mechanisms, which remain to be identified. Takashima and colleagues [21–24, 31] first identified a neuroprotective effect of inhibiting GSK3 (at that time also called tau protein kinase-1) against Aβ-induced toxicity. They found that in cultured rat hippocampal neurons Aβ treatment increased GSK3β activity and pretreatment with GSK3β antisense oligonucleotides prevented Aβ-induced cell death and reduced tau phosphorylation. These studies indicated that GSK3 is involved in Aβ-induced tau phosphorylation and neurotoxicity. Subsequent reports also demonstrated that inhibitors of GSK3, such as lithium or SB216763, reduced Aβ-induced tau phosphorylation and cell death in cultured neurons [27, 29, 32]. Inestrosa and colleagues [33] found that treatment with lithium prevented Aβ_{1-42}-induced morphological changes, specifically shrunken soma and affected dendritic and axonal processes, and reduced Aβ-induced decreases in cell viability of primary rat hippocampal neuronal cultures [33]. After injection of Aβ into rat hippocampus, increased GSK3 immunoreactivity was found near Aβ deposits [33]. Treatment with SB216763 or GSK inhibitor VIII also prevented Aβ-induced caspase-3 activation *in vivo*, decreased TUNEL positive neurons, prevented tau-phosphorylation, reduced microglia activation, decreased cytochrome c release from the mitochondria to the cytosol, and improved deficits in the Morris water maze [27, 28]. GSK3 inhibitor VIII or lithium reduced A$\beta_{1–42}$-induced reduction in cell viability and reduced markers of apoptosis [28]. Lithium treatment decreased cortical tau phosphorylation and aggregates, and reduced axonal degeneration [34]. Administration of the GSK3 inhibitor NP12 decreased tau phosphorylation, decreased Aβ deposition, and improved performance in the Morris water maze in amyloid precursor protein (APP) transgenic mice, and reduced neuronal loss in the CA1 region of the hippocampus and the entorhinal cortex [35]. Rockenstein and colleagues [36] also reported neuroprotective effects of inhibiting GSK3 with lithium using APP transgenic mice, with improvements in the Morris water maze task, decreased Aβ immunoreactivity, decreased phospho-tau immunoreactivity, and an increase in MAP2 staining (indicative of increased neuron

density) after treatment with lithium. The role of GSK3 was further examined by crossing mice conditionally expressing a dominant-negative (DN) GSK3β construct with hAPP transgenic mice. These hAPP x DN-GSK3β mice displayed improved performance in the Morris water maze, increased MAP2 immunoreactivity, decreased Aβ immunoreactivity, decreased phospho-tau immunoreactivity, and normal cell morphologies, when compared to hAPP transgenic littermates, suggesting that inhibition of GSK3 can phenotypically rescue hAPP mice [36]. Ma and colleagues [37] showed that antibodies directed against Aβ increased inhibitory serine-phosphorylation of GSK3, which was associated with a decrease in neurotoxicity. Altogether, these and additional reports have firmly established that Aβ activates GSK3 and that reducing GSK3 activity provides protection from Aβ-induced neurotoxicity.

Studies of the mechanism by which Aβ activates GSK3 have indicated the involvement of the PI3K-Akt pathway, which normally maintains inhibitory serine-phosphorylation of GSK3. Aβ treatment was shown to cause time-dependent decreases in PI3K activity and increases in GSK3 activity [22]. Treatment of cultured cells with $A\beta_{1–42}$ reduced Akt phosphorylation, indicative of decreased Akt activity [38, 39], activated GSK3β [38], and activated caspase-3 [25], suggesting that decreased Akt activity contributes to Aβ-induced activation of GSK3, which promotes apoptosis.

In addition to acting downstream of Aβ in its neurotoxic signaling, GSK3 likely also influences the neurotoxicity of Aβ by regulating APP processing and the production of Aβ. Takashima and colleagues [24] found that GSK3β associated with presenilin-1 in postmortem Alzheimer's disease cortical tissues and in COS-7 cells transiently transfected with wild-type presenilin-1, which raised the possibility that GSK3 may regulate Aβ production. This was found in studies that showed reducing GSK3 activity *in vitro* or *in vivo* diminished the production of Aβ [40–42]. The mechanism by which GSK3 promotes Aβ production remains to be determined, but may be related to its phosphorylation and regulation of presenilin-1 [24, 43] or of APP [44].

β-Catenin destabilization has been suggested to be a contributing factor in Aβ-induced GSK3-mediated neurotoxicity. GSK3 promotes the degradation of β-catenin, and nuclear β-catenin levels were decreased in response to acute Aβ treatments, indicating that Aβ-induced activation of GSK3 led to increased degradation of β-catenin [45, 46]. Lucas and colleagues [10] reported decreased nuclear β-catenin levels in GSK3 over-expressing mice. Presenilin-1 (PS1), a GSK3 substrate, can regulate the turnover of β-catenin [47, 48]. Kang and colleagues [48] found that GSK3 co-immunoprecipitated with PS1 but not with mutant M146L or ΔX9 PS1. Overexpression of PS1 also increased the GSK3β-β-catenin association, thereby facilitating GSK3-mediated phosphorylation and subsequent degradation of β-catenin. PS1 mutants were later linked to increased GSK3 activity via decreased PI3K/Akt signaling, thereby promoting decreased inhibitory serine-phosphorylation of GSK3 in primary neuronal cultures [49]. In cultured $PS1^{-/-}$ neurons the activated GSK3 was associated with increased caspase-3 activation [49]. In HEK293 and SK-N-MC cells, Kwok and colleagues [50] transiently over-expressed GSK3$\beta\Delta$exon9+11, which lacks exons 9 and 11 and is characterized by an increased propensity to phosphorylate tau, and found decreased β-catenin levels and signaling. Transient transfection of tau decreased β-catenin levels by 25%, and co-expression of tau and GSK3$\beta\Delta$exon9+11 reversed the GSK3-mediated decrease in β-catenin signaling. Inestrosa and colleagues have reported in detail that activation of Wnt signaling, which inhibits GSK3-mediated phosphorylation and degradation of β-catenin, is neuroprotective against Aβ toxicity [33, 51–53]. Thus, reduced levels of Wnt signalling-associated β-catenin may contribute to GSK3-mediated neurotoxicity induced by Aβ production and promoted by mutations in PS1 in Alzheimer's disease.

3.4. Toxicity Associated with Tau. The microtubule-associated protein tau is one of the most well characterized substrates of GSK3 [54]. Phosphorylation of tau by GSK3 promotes tau dissociation from microtubules, increasing destabilization of microtubules [55]. Conversely, inhibition of GSK3 promotes tau binding to microtubules and assembly of microtubules [56]. As noted above, several studies have reported that the GSK3-mediated increase in tau phosphorylation in Alzheimer's disease may result in part from Aβ-induced activation of GSK3. GSK3-mediated tau phosphorylation in Alzheimer's disease has been suggested to promote tau oligomerization, which can be toxic [57, 58], and aggregation of tau and eventual neurodegeneration [54, 59]. Sahara and colleagues [60] reported that overexpression of tau in SH-SY5Y cells resulted in increased tau phosphorylation and increased caspase-3 activity, suggesting a role in pro-apoptotic signaling and cell death. It is possible that GSK3β-mediated hyperphosphorylation of tau may promote tau-mediated, as well as Aβ-mediated, neurotoxicity.

Transgenic mice have also been used to study the interactions between tau and GSK3. Using protein preparations from the brains and spinal cords of double transgenic mice over-expressing GSK3β and human tau40-1, an isoform of tau containing an additional 29 and 58 amino acid sequence that promotes Alzheimer's disease-like pathologies [61], Spittaels and colleagues [8] found decreased tau binding to microtubules in double transgenic mice, as compared to transgenic mice littermates expressing human tau40-1 alone. The relationship between GSK3 and tau was found to be more than a mere protein-protein interaction, as Kwok and colleagues [50] found interactions between the GSK3β and tau (MAPT) genes associated with increased risk and incidence of Alzheimer's disease. Using senescence-accelerated mice (SAM), Tajes and colleagues [62] showed that inhibition of GSK3 with lithium decreased calpain activation and decreased caspase-3 activity. Primary neuronal cultures treated with the GSK3 inhibitors lithium or SB415286 exhibited decreased neurite disintegration, neuronal shrinkage, and nuclear condensation, further implicating GSK3 in neurodegenerative disease progression [62]. In transgenic mice expressing mutant tau, chronic lithium

treatment reduced tau aggregation [63]. Evidence of tau-related toxicity has been bolstered by studies of tau-knockout mice [64]. Mice conditionally over-expressing GSK3 and lacking tau performed better in the Morris water maze task, as compared to GSK3 over-expressing littermates [64]. Knockout of tau reduced GSK3-mediated shrinkage of the dentate gyrus and reduced reactive microglia, as GSK3-only over-expressing littermates were characterized by increased brain shrinkage and increased reactive microglia compared to control and tau-knockout mice.

In addition to hyperphosphorylation of tau, GSK3 has also been linked to alternate splicing of tau, thereby possibly promoting pro-apoptotic oligomerization and tau-induced cell death [65]. Inclusion of exon 10 likely promotes increased binding of tau and stabilization of microtubules, thereby combating tau aggregate-mediated neurofibrillary tangle formation and neurodegeneration observed in Alzheimer's disease. Hernández and colleagues [65] examined the relationship between GSK3 and alternative splicing of tau and found that in primary mouse cortical neurons treatment with GSK3 inhibitors lithium or AR-A014418 decreased alternative splicing of tau and promoted the increased presence of exon 10 in tau, which promotes microtubule bundling and stabilization, as compared to exon 10-absent tau [65, 66]. Alternative splicing of tau has also been linked to caspase-mediated cleavage and aggregation of tau in Alzheimer's disease [67]. Alternative forms of tau have been linked to increased tau aggregation in other cells and cell systems [68].

In contrast to reports of tau oligomerization contributing to neurotoxicity, a few reports suggest a neuroprotective role for tau. Mouse neuroblastoma cells stably over-expressing tau were less affected by apoptotic stimuli, including staurosporine, camptothecin, and H_2O_2 treatments, and over-expressed tau blocked GSK3 overexpression-mediated increases in cell death, actions that may have resulted from tau binding to GSK3 to block its induction of β-catenin degradation, allowing up-regulated levels of β-catenin, which supports cell survival [69]. Recently, Wang and colleagues [70] found that overexpression of human tau, *in vivo* in transgenic mice and *in vitro* in N2a cells, decreased p53 levels, decreased mitochondrial cytochrome c release, and decreased caspase-9 and caspase-3 activation. Treatment with lithium exaggerated the decrease in p53 expression and increased pro-apoptotic processes [70]. Thus, the connections between tau and GSK3 in affecting neurodegeneration remain to be further clarified and may be complicated by employing overexpression approaches.

3.5. GSK3 Promotes Insults Associated with Alzheimer's Disease. As previously reviewed [2], GSK3 promotes apoptosis induced by many insults that activate the intrinsic apoptotic signaling pathway, some of which may contribute to neuronal loss in Alzheimer's disease. For example, oxidative stress is increased in Alzheimer's disease, as indicated by increased markers of oxidative stress found in postmortem Alzheimer's disease brain [71–73], and has been associated with the loss of neuronal viability, and GSK3 promotes oxidative stress-induced cell death [2]. For example, Schäfer and colleagues [74] found that resistance to oxidative stress was associated with decreased GSK3 activity. Aβ treatment of cells increases oxidative stress [75, 76], as well as activates GSK3, which may contribute to apoptosis. Several reports showed that GSK3 inhibitors reduce toxicity of oxidative stress [77, 78]. Thus, inhibition of GSK3 may be neuroprotective in Alzheimer's disease in part by reducing oxidative stress-induced neurotoxicity.

Neurotrophic factor deficiency has been linked with neuronal loss in Alzheimer's disease. Studies of insulin-like growth factor-I (IGF-1) are particularly interesting because IGF-1 deficiency has been linked to Alzheimer's disease and IGF-1-induced cellular signaling contributes to maintaining inhibition of GSK3 by activating the PI3K-Akt pathway. Additionally, GSK3 inhibition has been linked to increases in IGF-I in the brain [79]. Bolós and colleagues [79] used megalin, an IGF-I receptor interacting protein that is associated with transport of IGF-1, and found that in MDCK cells transiently transfected with or without mini-megalin, a cDNA encoding the two peri-membrane extracellular cysteine-rich domains, the trans-membrane region, and the cytoplasmic region of the megalin gene, treatment with the GSK3 inhibitor NP12 stimulated internalization of IGF-I and cell-surface megalin expression. Moreover, treatment of APP/PS1 transgenic mice with NP12 significantly increased both brain and CSF IGF-I levels. Collectively this data suggests that inhibition of over-active GSK3β that appears to occur in Alzheimer's disease can promote IGF-I expression and counteract Aβ-induced toxicity. Brain-derived neurotrophic factor (BDNF) activation of TrkB receptors is also responsible for activation of the PI3K/Akt pathway and inhibition of GSK3β via Ser9 phosphorylation [80–82]. Decreases in hippocampal and cortical BDNF levels have been reported in Alzheimer's disease [83–85], which could promote an increase in GSK3 activity. Elliott and colleagues [85] showed that in neuronally differentiated P19 mouse embryonic carcinoma cells, BDNF altered tau phosphorylation, and that inhibition of GSK3 with lithium reduced tau phosphorylation. BDNF has also been linked to promotion of anti-apoptotic signaling via the PI3K/Akt pathway. Hetman and colleagues [86] found that trophic factor withdrawal promoted inhibition of the cell survival mediator PI3K and activated the pro-apoptotic GSK3, which was reversed by PI3K activating treatments, such as BDNF, by treatment with a GSK3 inhibitor, or after transient transfection of a kinase-dead GSK3 mutant. Overexpression of wild-type or mutant β-catenin, in which all GSK3β-targeted serines were mutated to alanines, had no effect on GSK3β-mediated neuronal apoptosis [86]. Thus, neurotrophin deficiency in Alzheimer's disease may contribute to abnormally active GSK3 that can promote neurotoxicity.

3.6. Mechanisms by Which GSK3 May Impede Cell Survival from Insults. GSK3 has been reported to promote apoptosis by regulating the actions of proteins involved in apoptosis signaling and by regulating transcription factors known to

regulate the expression of apoptosis modulators. For example, GSK3 has been reported to regulate Bax, a pro-apoptotic Bcl2 family member that is commonly associated with the release of cytochrome c. Under apoptotic conditions, Bax undergoes a conformational change associated with its translocation from the cytosol to the mitochondria where it facilitates cytochrome c release in apoptotic signaling [87–89]. GSK3 can directly phosphorylate Bax on Ser-163, which results in the activation of Bax [90] and inhibition of GSK3 with lithium prevented Bax activation and subsequent cytochrome c release [91]. Another Bcl2 family member, Mcl-1, an anti-apoptotic protein that can be induced after cellular stress to promote cell survival, is phosphorylated on Ser159 by GSK3 to promote Mcl1 degradation, thereby reducing the protective action of Mcl-1 [92, 93]. By these and other actions on the apoptotic signaling pathway, GSK3 can reduce cellular resilience to stress and promote apoptotic signaling.

Several transcription factors that are inhibited by GSK3 normally promote mechanisms that promote cellular survival responses to stresses that are potentially lethal insults [1]. These include heat shock factor protein 1 (HSF-1), cyclic AMP response element-binding protein (CREB), and others, impairments of which are well-documented to increase the susceptibility of cells to toxic insults. HSF-1, for example, promotes the expression of heat shock proteins, chaperones that combat cellular stress. Chu and colleagues [94] reported that GSK3 reduced HSF-1 activity and increased susceptibility to environmental stressors. Xavier and colleagues [95] showed that overexpression of GSK3β repressed HSF-1 transcriptional activity and DNA-binding. CREB, which can support cell survival and is activated by phosphorylation at Ser133, also can be negatively regulated by GSK3 [1, 96]. Activation of CREB has been reported to be impaired in Alzheimer's disease hippocampal tissues [97, 98]. Since GSK3 inhibits CREB activity [1, 96, 99], increased GSK3 activity may contribute to the Alzheimer's disease-induced decrease of phospho-CREB-mediated neuroprotection. Thus, by regulating these and other transcription factors that influence the expression of proteins that modulate cellular responses to stress [1], GSK3 may contribute to setting the threshold for apoptotic signaling, which may be lowered in Alzheimer's disease.

3.7. GSK3 Promotes Decreased Cell Survival in Other Neurodegenerative Diseases. Many components of neurodegenerative processes are common among various neurodegenerative diseases, including apoptosis and mechanisms regulating apoptosis. Thus, it is not surprising that, similarly with Alzheimer's disease, GSK3 has also been linked to neuronal death in other neurological diseases. For example, prion disease shares with Alzheimer's disease accumulations of protein aggregates and neuronal death [100, 101]. Mouse embryonic cortical neurons (E17) treated with varying concentrations of prion protein (PrP) peptide exhibited increased GSK3 activity and increased tau phosphorylation, which was prevented by pretreatment with lithium. GSK3β activation and hyperphosphorylation of tau has also been identified in Lafora Disease, an autosomal recessive form of progressive myoclonus epilepsy that is characterized by dementia and rapid neurological deterioration [102]. Amyotrophic lateral sclerosis has been linked to mutations in superoxide dismutase type 1 (SOD1), and expression of mutant SOD1 in motor neurons increased GSK3 activity and apoptosis, and GSK3 inhibitors provided protection from apoptosis [77]. Thus, there appear to be a variety of disease-associated conditions that can cause abnormal activation of GSK3 that contributes to the neurodegenerative process.

4. GSK3 Impedes Extrinsic Apoptotic Signaling

In contrast to the many studies of intrinsic apoptotic signaling mechanisms in association with loss of cell viability in Alzheimer's disease, few studies have addressed the possibility that death receptor-mediated extrinsic apoptotic signaling is involved in Alzheimer's disease. Plasma membrane death receptors that can initiate apoptosis are members of the tumor necrosis factor (TNF) receptor family that contain conserved intracellular death domains, which includes Fas (CD95/Apo1), TNF-R1 (p55/CD120a), TNF-related apoptosis-inducing ligand receptor-1 (TRAIL-R1/DR4), and TRAIL-R2 (DR5/Apo2/TRICK2/KILLER). Studies in postmortem Alzheimer's disease brain and particularly in Aβ-treated cells *in vitro* have provided some evidence for increased death receptor-induced apoptotic signaling pathway [103–111]. However, the contribution of death receptor-initiated apoptosis in Alzheimer's disease remains to be firmly established.

Although it remains unclear if death receptors contribute to cell loss in Alzheimer's disease, we can surmise that GSK3 is highly unlikely to contribute to this potential neuropathological mechanism. This is because GSK3 impairs death receptor-induced apoptotic signaling, as opposed to its promotion of intrinsic apoptotic signaling [2]. The concept that GSK3 inhibits death receptor-induced apoptosis followed the discovery that GSK3β knockout mice died during embryonic development due to massive hepatocyte apoptosis [112], which demonstrated that GSK3β is an important inhibitor of TNFα-induced apoptosis. This inhibitory effect of GSK3 on extrinsic apoptotic signaling was extended to all other death receptors, as reviewed [2]. The mechanism for this action was found to be due to the presence of GSK3 in a death receptor-associated anti-apoptotic complex that impedes the initiation of apoptotic signaling [113]. Thus, several studies have clearly established that GSK3 is anti-apoptotic in death receptor-mediated signaling.

If death receptor-induced apoptosis does contribute to cell loss in Alzheimer's disease, the anti-apoptotic action of GSK3 in this process could very likely limit the application of inhibitors of GSK3 as therapeutic agents in Alzheimer's disease because they would be able to promote extrinsic apoptotic signaling. This complication was exquisitely demonstrated in a study of the effects of *in vivo* treatment with the GSK3 inhibitor lithium, which demonstrated increased neuronal apoptosis mediated by lithium's promotion of Fas-mediated apoptotic signaling [114]. Whether or not this detrimental action of GSK3 inhibitors would be deleterious

in Alzheimer's disease depends on whether death receptor-induced apoptotic pathways are activated in Alzheimer's disease, a question that remains unresolved.

5. Conclusions

GSK3 has been shown to be associated with the major neuropathological markers of Alzheimer's disease and to be abnormally activated or expressed in Alzheimer's disease brains, particularly in association with neuropathological or degenerative markers. GSK3 is activated by Aβ and promotes both Aβ production and its neurotoxic actions. GSK3 phosphorylates tau and may promote oligomerization of tau and its aggregation, which can contribute to neurotoxicity. Apoptosis may contribute to neuronal loss in Alzheimer's disease, and GSK3 promotes intrinsic apoptotic signaling induced by many insults, some of which may be involved in neurodegeneration in Alzheimer's disease. GSK3 promotes intrinsic apoptotic signaling both by regulating signaling proteins involved in apoptosis and regulating transcription factors that control the expression of proteins that modulate cellular responses to stress. Altogether, much evidence indicates that GSK3 is an integral component of the neurodegenerative processes in Alzheimer's disease.

Acknowledgment

The authors' research was supported by grants from the NIMH (MH092970, MH090236, and MH038752).

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 129753, 9 pages
doi:10.4061/2011/129753

Review Article

Glycogen Synthase Kinase-3β: A Mediator of Inflammation in Alzheimer's Disease?

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Received 7 February 2011; Accepted 4 March 2011

Academic Editor: Peter Crouch

Proliferation and activation of microglial cells is a neuropathological characteristic of brain injury and neurodegeneration, including Alzheimer's disease. Microglia act as the first and main form of immune defense in the nervous system. While the primary function of microglia is to survey and maintain the cellular environment optimal for neurons in the brain parenchyma by actively scavenging the brain for damaged brain cells and foreign proteins or particles, sustained activation of microglia may result in high production of proinflammatory mediators that disturb normal brain functions and even cause neuronal injury. Glycogen synthase kinase-3β has been recently identified as a major regulator of immune system and mediates inflammatory responses in microglia. Glycogen synthase kinase-3β has been extensively investigated in connection to tau and amyloid β toxicity, whereas reports on the role of this enzyme in neuroinflammation in Alzheimer's disease are negligible. Here we review and discuss the role of glycogen synthase-3β in immune cells in the context of Alzheimer's disease pathology.

1. Inflammation in Alzheimer's Disease

In addition to progressive loss of neurons and accumulation of intra- and extracellular protein deposits, chronic inflammation is a major pathological hallmark of Alzheimer's disease (AD) [1, 2]. Neuroinflammation in AD is characterized by the existence of inflammatory mediator cells surrounding the β-amyloid (Aβ) plaques and sites of neuronal injury [3–5]. Even though microglia, the main immune cells of the brain, have been extensively studied in AD, the exact role of inflammation in the disease pathogenesis remains elusive [3–10]. There is substantial evidence that microglia and the monocytic cells derived from the blood or bone marrow at least initially protect neurons from neurotoxic accumulation of Aβ and even release neurotrophic factors and extracellular proteases which may support neuronal survival and regeneration [5–11]. On the other hand, extensive and long-term release of proinflammatory mediators and reactive oxygen or nitrogen species (ROS and RNS) by the inflammatory cells is thought to accelerate neurodegeneration and disturb cognitive functions [3–10].

The primary inflammatory cells in the central nervous system are microglia, constituting around 10% of all cells in the brain. They represent the innate immune system and form the first line of defense against invading pathogens in the brain [12–14]. Microglia serve as sensors for disturbed brain tissue homeostasis as they accumulate and proliferate locally in response to neuronal injury or penetration of foreign material in the brain [13, 14]. In AD, such activation can result from extracellular deposition of Aβ, neuronal injury caused by Aβ or tau toxicity [5–15], to some extent from ischemic or traumatic brain injury, and may be contributed even by local or systemic infection [16, 17]. In addition to microglia, astrocytes, pericytes, endothelial cells and neurons are thought to play a role in inflammatory responses relevant to AD [18]. However, most of the data on the impact of inflammation in AD originate from studies with microglia.

2. Glycogen Synthase Kinase 3-β in the Nervous System

Glycogen synthase kinase 3 (GSK-3) is a multifunctional serine/threonine kinase present in all eukaryotes. There are two highly homologous isoforms of GSK-3, GSK-3α and GSK-3β, that are usually equivalent in actions. In addition, there is an alternatively spliced GSK-3β variant that encodes GSK-3β2, which has a 13-residue insert in the kinase domain [19–25] and is expressed exclusively in the nervous system [19–25]. GSK-3 shows partial constitutive activity and is known to phosphorylate more than 50 different substrates. The most important mechanism for regulation of GSK-3β activity is inhibitory phosphorylation of Ser9 by protein kinase A (PKA) protein kinase B (PKB)/Akt and protein kinase C (KPC). Other kinases may phosphorylate the regulatory Ser9 as well. Activation of GSK-3β is enhanced when also the regulatory Tyr216 is phosphorylated [19–25].

In the brain, GSK-3β is known to be involved in neurogenesis, neuronal migration, neuronal polarization, and axon growth and guidance. GSK3β2 shows the highest expression in the nervous system during development and seems to have a special role in neuronal morphogenesis [25–32]. GSK-3β affects axon growth by controlling microtubule dynamics through phosphorylation of microtubule-associated proteins (MAPs) such as Tau, MAP-1B and adenomatous polyposis coli [25–32]. Importantly, GSK-3β plays a key role in neuropathology of AD, schizophrenia, autism and Parkinson's disease (PD). Also, the polymorphisms in GSK3β interact with the microtubule-associated protein Tau (MAPT) haplotypes to increase the risk for idiopathic PD and AD [32–35].

There is substantial evidence that activation of GSK-3β contributes to tau pathology, Aβ synthesis, and apoptotic neuronal death and it is thus not surprising that GSK-3β has been recognized as a potential therapeutic target in AD [35–37]. However, GSK-3β is a well-known regulator of innate and adaptive immune responses and plays a key role also in pathways of microglial activation relevant to AD [19, 20, 38–41]. Considering that neuroinflammation is a characteristic of AD brain pathology, the role of GSK-3β in glial cells is of great interest. The therapeutically interesting role of GSK-3β in regulating inflammation in AD is emphasized by the fact that various forms of Aβ promote microglial activation and release of proinflammatory mediators and ROS/RNS. In addition, in vitro studies suggest that microglial activation may in turn induce accumulation of tau in neurites though microglial ROS production [15].

3. GSK-3β and Migration of Microglia

Migration of blood and bone marrow-derived monocytic cells as well as endogenous microglia to the sites of brain injury and abnormal proteins, such as Aβ, is a necessary step before production of proinflammatory mediators or neurotoxins and attempts of phagocytosis [10, 11, 13, 42–48]. GSK-3β has been reported to be a key kinase regulating migration of various cell types, such as different stem cells and other cells related to development, cancer cells, endothelial cells, and blood-derived inflammatory cells [39, 49–52]. Similarly, GSK-3β has been shown to promote microglial migration both in vitro and in situ [39]. When random and directed migration of BV2 cells were studied using transwell migration and scratch assays, respectively, GSK inhibitors were found to inhibit both types of microglial migration by far more than 50% [39]. The same authors also demonstrated that GSK-3β mediates migration of endogenous mouse microglia in response to slice injury of hippocampus [39]. It is possible that GSK-3β promotes migration/mobility of microglia at least partially by triggering upregulation of CD11b, the αMβ2 integrin and complement receptor, which are needed for adhesion and migration of leukocytes, including microglia [23]. Studies on the role of GSK3 in migration of other cell types support the conclusion that GSK-3β controls multiple pathways involved in migration [28, 30, 51, 53].

4. GSK-3β and Microglial Inflammatory Cytokines, Chemokines and Reactive Oxygen/Nitrogen Species

The production of proinflammatory molecules is a crucial feature of cells needed for innate immune response and the most widely investigated function of microglia in neuroinflammation coupled to AD. Microglia are able to secrete a variety of cytokines and chemokines upon activation by Aβ. These include interleukin 1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor alpha (TNF-α), as well as chemokines such as macrophage inflammatory protein-1 (MIP-1) and monocyte chemotactic protein-1 (MCP-1) [54]. These secretory products have been postulated to contribute to neuronal death seen in AD brain. In general, cytokines function by regulating the intensity and duration of the immune response [55, 56]. Thus, IL-1 can induce IL-6 production and stimulate synthesis and release of nitric oxide by triggering inducible nitric oxide synthase (iNOS) [57]. This neuroinflammatory stimulation of microglia is further characterized by activation of the complement cascade and induction of the prostanoid generating enzyme cyclooxygenase-2 (COX-2) [57–60]. In addition to this general proinflammatory role, Aβ-induced release of cytokines may promote further Aβ production in microglia. Certain cytokines, such as IL-1, can interact with the amyloid precursor protein (APP) processing pathway resulting in increased cleavage of Aβ [61]. In turn, fibrillar Aβ has been reported to increase neurotoxic secretory products, proinflammatory cytokines and RNS/ROS [5–7]. Eventually, these interactions between cytokines and APP processing establish a self-propagating cycle of neuronal injury [62, 63]. Indeed, several lines of evidence suggest that continuous cytokine production and inflammation-driven cascades cause further activation and recruitment of microglia and can exacerbate disease progression or even sensitize to AD pathology [8, 9]. This continuous reactive microgliosis has been described as the cycle of neuronal death: as in AD brain the cause (Aβ) of microglial activation is not effectively cleared, microglia

may enhance their secretion of inflammatory mediators and thus promote toxicity to nearby neurons. However, the causal relationship between microglial activation, cytokine production, Aβ accumulation and neuronal death has not been completely resolved [14].

It is important to note that microglia have also a potential beneficial role in neuroinflammation when another general category of cytokines are released. These antiinflammatory cytokines include IL-1 receptor antagonist (IL-1Ra), IL-4, IL-10, and tumor growth factor beta (TGF-β) [64–68]. IL-4 and TGF-β have a potential to reduce the expression and activity of CD40 and class II MHC [69]. TGFβ has also been reported to reduce Aβ burden in the brain parenchyma in a transgenic mouse model of AD [64]. On the other hand, IL-4 can counteract AD pathology by selectively inducing the clearance of oligomeric Aβ by primary microglia [64, 65]. Similarly, IL-10 can reduce proinflammatory state of microglia by inhibiting the synthesis of cytokines TNFα, IL-1, IL-6, IL-12, granulocyte-macrophage colony stimulating factor (GM-CSF) and chemokines MIP-2, MCP-1 and RANTES [66, 67, 70].

5. Signaling of GSK-3β in Inflammation

GSK-3β is a major regulator of the balance between the above-mentioned proinflammatory and antiinflammatory mediators in immune cells, including microglia [38, 39]. This regulation is manifested by multiple pathways and include interactions with nuclear factor κB (NF-κB) and mixed lineage kinase 3 (MLK3)/c-Jun N-terminal kinase (JNK) signaling pathways [38, 39, 49] (Figure 1).

NF-κB is a dimer protein complex that controls the DNA transcription. In resting microglia, the NF-κB dimers are sequestered in the cytoplasm by inhibitors of κB (IκBs) [71]. Activation of the NF-κB is triggered by the signals that result in degradation of IκB, thereby freeing the NF-κB complex to enter the nucleus and interact with the DNA binding sites of NF-κB [72, 73]. Activation of NF-κB mediates expression of several proinflammatory cytokines and iNOS [74]. Once activated, NF-κB transcriptional activity is further regulated by inducible posttranslational modifications, including phosphorylation and acetylation [49, 75–80]. In certain conditions, GSK-3β may regulate NF-κB activation by phosphorylation of p65 subunit of NF-κB upon TNFα treatment, whereas in cultured microglia LPS treatment induces NF-κB activation by increasing acetylation of p65 at lysine 310 through GSK-3β [49, 75–80]. In fact, several studies support the idea that such acetylation of p65 is required for the full transcriptional activity of NF-κB and that GSK-3β increases the p65 binding of the coactivator CREB-binding protein (CBP), which has acetylase activity. CBP is present in limited amounts and also binds and acetylates transcription factor CREB. Thus, these two transcription factors, the p65 subunit of NF-κB and CREB, compete for CBP and activation of GSK-3β pathway shifts the balance in favor of NF-κB [20, 49, 75–80]. The GSK-3β-mediated increase in NF-κB activity results in expression of proinflammatory cytokines and chemokines, such as TNFα, IL-6 and MCP-1. Simultaneously, the expression of IL-10 is reduced, partially because of the reduced DNA binding activity of CREB and also AP1, which are the main transcription factors contributing to IL-10 expression. The eventual proinflammatory effect of GSK-3β signaling is mediated by reduced IL-10 expression, which leads to further increased synthesis of various cytokines and chemokines [20, 49, 75–80].

β-catenin is a transcriptional coactivator of WNT signaling and a direct target of GSK-3β phosphorylation. β-catenin regulates cell proliferation and inhibits NF-κB [81, 82]. Upon GSK-3β phosphorylation, β-catenin enters the proteasomal degradation pathway resulting in reduced inhibition of NF-κB and thereby increased NF-κB-mediated inflammatory responses [81, 82]. β-catenin expression is increased in microglia of transgenic AD mice and Wnt signaling has been reported to play a role in impaired cognitive functions in transgenic AD mouse models [83, 84].

Activation of IL-6 receptors executes proinflammatory response through activation of STAT3 transcription factor, leading to increased expression of proinflammatory molecules, including IL-6 itself. GSK3β selectively promotes STAT3 and STAT5 activation and thereby IL6-induced proinflammatory responses [38, 49, 85].

Finally, certain proinflammatory responses, such as the LPS-induced activation of microglia involve JNK pathway that is regulated by MLK3. GSK-3β phosphorylation may be needed for proper function and dimerization of MLK3, which eventually leads to increased activity of JNK pathway and TNF-α synthesis [49, 86].

Even though there is substantial evidence for proinflammatory role of GSK-3β in several cell types, including microglial cell lines and primary rodent microglia, there are also studies demonstrating an opposite role for GSK-3β. The contradictory results most likely reflect the dependence on the type of cell, stimulus and experimental conditions as the targets of GSK-3β phosphorylation are numerous and of interacting signaling pathways [87–89].

6. GSK-3β and Phagocytosis

Phagocytosis is a main function of microglia. In vitro microglia have the capacity to phagocytose Aβ, but several studies have failed to show actual Aβ-laden vesicles in microglial cells in animal models of AD or in AD [4, 11, 64]. At least the capacity of successful phagocytosis by microglia is very limited in AD brain and not sufficient to prevent the formation of Aβ plaques [4, 11, 64]. However, modulation of microglial activity may enable microglia to effectively phagocytose Aβ as evidenced by activation of microglia for example by Aβ opsonisation [90, 91]. In models of AD, the pathway resulting in Aβ phagocytosis is initiated when Aβ binds a complex of microglial surface receptors consisting of the $\alpha_6\beta_1$ integrin, CD36, CD47, and the class A scavenger receptor (SRA) [10]. In addition, Toll-like receptors (TLRs) which function as dimers and are often coupled to CD14 coreceptors, functionally interact with other partners of the microglial Aβ binding receptor complex [92–96] and

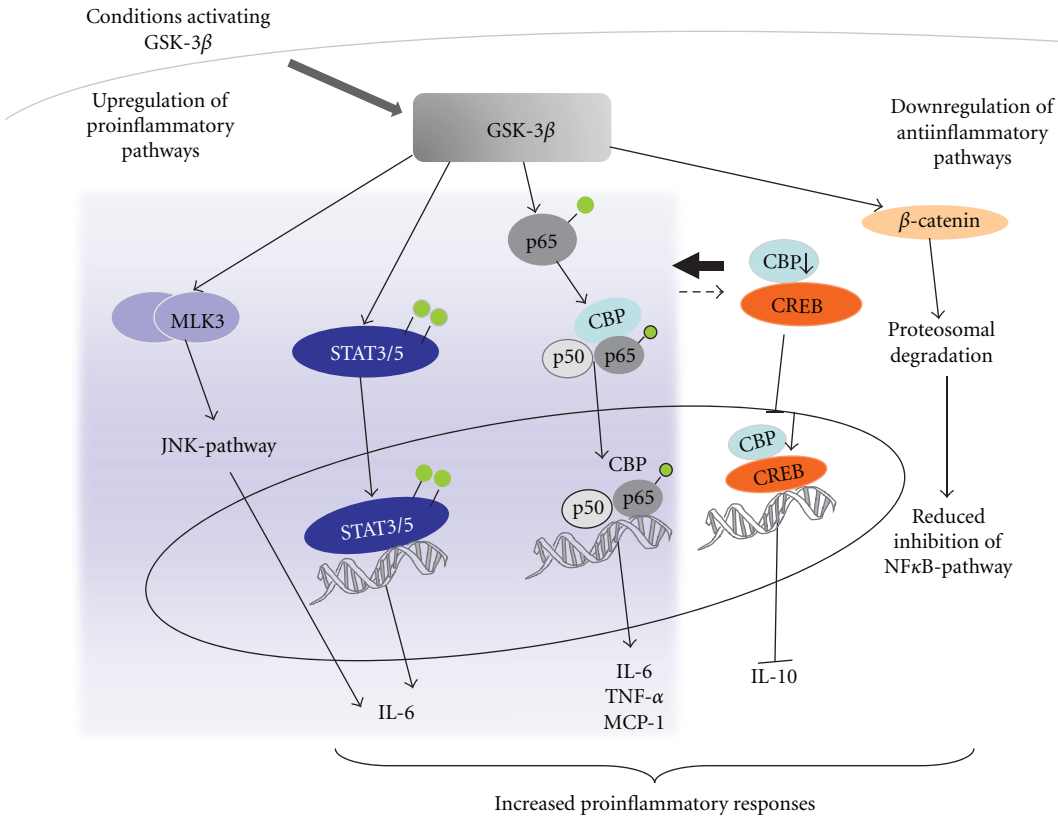

Figure 1: GSK-3β regulates the production of microglial inflammatory molecules. GSK-3β activation has been shown to increase the production of proinflammatory mediators via multiple mechanisms. GSK-3β is able to activate JNK-, STAT3/5- and NF-κB pathways leading to increased cytokine and chemokine production. GSK-3β is able to increase the binding of coactivator CREB-binding protein (CBP) to p65 thus enhancing NF-κB mediated transcription. Since CREB competes for CBP binding, activation of GSK-3β shifts the balance in favor of NF-κB pathway and CREB-mediated induction of IL-10 is reduced. Moreover, GSK-3β activation leads to proteosomal degradation of beta-catenin, thus resulting in reduced inhibition of NF-κB-activation. All these events lead to enhanced production of proinflammatory molecules.

execute phagocytosis associated with increased ROS. In turn, engagement of this receptor complex activates tyrosine kinase-based signaling cascades [10, 97, 98] resulting in beneficial phagocytosis but also in production of reactive oxygen species (ROS) and secretion of cytokines [99, 100].

The role of TLR2 and TLR4 in Aβ phagocytosis and AD is emphasized by numerous studies. The expression of TLR2 and TLR4 receptors are upregulated in both AD brains and in related transgenic mouse models of AD. Also, the microglia associated with Aβ plaques show increased levels of mRNA coding for TLR2, -4, -5, -7, and -9 [101]. In addition, AD mice deficient in TLR4 show increased brain Aβ burden. Stimulation of microglial cells with TLR2 and TLR4 ligands boosts indirect clearance of Aβ *in vitro* [102]. Moreover, induction of monocyte recruitment in response to foreign particles, including Aβ, may require activation of TLR-based signaling pathway. Gene delivery of TLR2-lentivirus into the bone marrow cells can rescue the cognitive decline of TLR2 deficient AD mice [103]. Upon Aβ stimulation, monocytes from normal subjects upregulate TLRs, whereas monocytes from AD patients may fail to do so [104]. Also, the level of TLR4 in monocytic cells of AD patients may be lower compared to levels of TLR4 in the same cell population of healthy controls. Finally, bisdemethoxycurcumin, an anti-inflammatory compound, improves the defective clearance of Aβ and the transcription and translation of TLR2-4 in monocytic cells of AD patients [104]. These studies point to the importance of TLR signaling in the phagocytic activity of blood-derived monocytic cells in AD.

Signaling of several TLRs, including TLR2, TLR4, TLR5, and TLR9 is regulated by GSK-3β in human monocytes and is coupled to production of cytokines [39, 41, 105] (Figure 2). Stimulation of TLR receptors activates phosphatidylinositol 3-OH kinase (PI(3)K) pathway activated Akt leading to phosphorylation and inhibition of GSK-3β. As a result, the cells produce less proinflammatory molecules but upregulates production of antiinflammatory cytokines, such as IL-10 [105].

Another pathway relevant for Aβ clearance is triggered by activation of CD40R, a transmembrane receptor of the TNF gene superfamily that is expressed on a variety of cells, such as monocytes, B-cells, antigen presenting cells, endothelial, smooth muscle cells, fibroblasts, and microglia [50]. CD40L is an immunoregulatory molecule that is expressed by activated T-cells, for example. By preventing the CD40-CD40L interaction in AD transgenic mice [106, 107] the Aβ burden

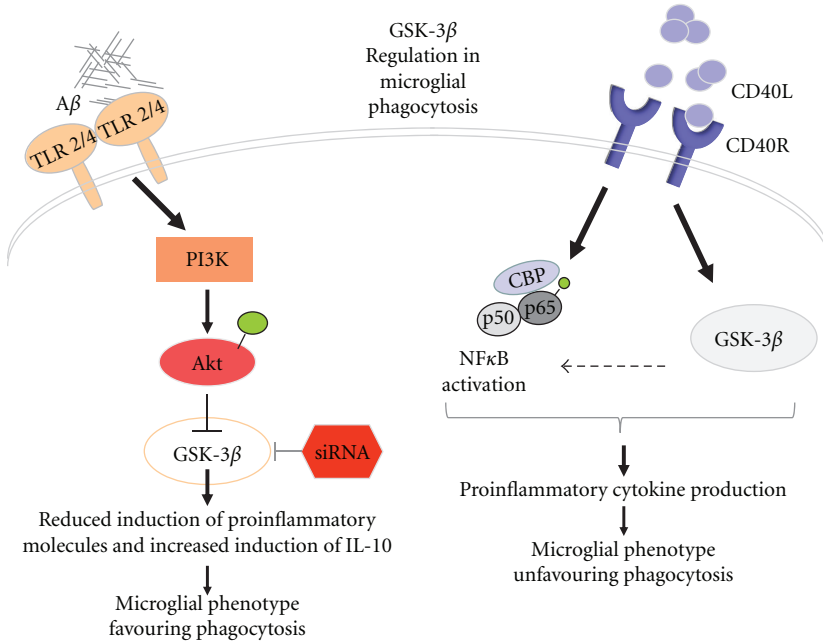

Figure 2: GSK-3β in the regulation of microglial phagocytosis. Microglial phagocytosis has been shown to be enhanced through activation of TLR2/4 pathway. Binding of Aβ to TLR2/4 may result in activation of PI3K signalling eventually leading to inhibition of GSK-3β activation. This in turn shifts the cellular balance towards increase in the production of antiinflammatory cytokines favouring phagocytic microglial phenotype. On the other hand, CD40R-CD40L interaction results in both NF-κB and GSK-3β activation thus increasing proinflammatory cytokine production. This may shift the phenotype of microglial cells being less capable of clearing Aβ.

is reduced. Aβ stimulation in the presence of CD40-CD40L interaction has been demonstrated to cause diminished microglial phagocytosis and a shift in balance towards an adaptive, antigen-presenting state [108]. It is conceivable that CD40R is activated in microglial cells in AD. The interaction between CD40 and CD40L enhances the expression of cytokines, chemokines, matrix metalloproteinases, growth factors, and adhesion molecules, mainly through the stimulation of NF-κB and also by GSK-3β, which has a role in CD40-mediated response and polarization of naïve CD4+ T cells to Th2 cells [50, 51].

7. Concluding Remarks

Inflammation and especially microglial activation is a contributory factor in neurodegeneration, including AD. Without question, GSK-3β is a central mediator molecule of harmful inflammatory mechanisms relevant to AD. Several studies convincingly link the role of tau and Aβ to increased activity of GSK-3β in the brain [109–114]. Indeed, both human and rodent model studies on AD indicate that inhibition of GSK-3β can be expected to be beneficial in AD [115–120]. Even though some small molecules inhibiting GSK-3β reduce memory/learning deficits and also inflammation in transgenic mouse models of AD [115–120], the link between GSK-3β and harmful inflammation in AD has not been much explored. There are hardly any investigations on the Aβ or tau-related harmful inflammation through mechanisms involving GSK-3β. Based on the overall literature on inflammation, microglia, and AD, we hypothesize that GSK-3β is a potential therapeutic target uniting Aβ deposition, tau aggregation, and inflammation, which represent all the key components of AD pathology.

Acknowledgments

The authors thank The Academy of Finland and The Sigrid Juselius Foundation for their support.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 352805, 11 pages
doi:10.4061/2011/352805

Review Article

Functional Implications of Glycogen Synthase Kinase-3-Mediated Tau Phosphorylation

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Received 15 April 2011; Accepted 6 May 2011

Academic Editor: Adam Cole

Tau is primarily a neuronal microtubule-associated protein that has functions related to the stabilisation of microtubules. Phosphorylation of tau is an important dynamic and regulatory element involved in the binding of tau to tubulin. Thus, highly phosphorylated tau is more likely to be present in the cytosolic compartment of neurons, whereas reduced phosphate burden allows tau to bind to and stabilise the microtubule cytoskeleton. Highly phosphorylated forms of tau are deposited in the brain in a range of neurodegenerative disorders including Alzheimer's disease, progressive supranuclear palsy, and frontotemporal lobar degeneration associated with Pick bodies. A key candidate kinase for both physiological and pathological tau phosphorylation is glycogen synthase kinase-3 (GSK-3). Multiple phosphorylation sites have been identified on tau exposed to GSK-3 *in vitro* and in cells. In this review, we highlight recent data suggesting a role for GSK-3 activity on physiological tau function and on tau dysfunction in neurodegenerative disease.

1. Introduction

The microtubule-associated protein tau is a normally soluble phosphoprotein found predominantly in neurons [1]. The structure of tau comprises three broadly defined regions, an N-terminal projection domain, that is thought to be responsible for its interaction with membranes and other proteins, a central proline-rich domain, and a C-terminal microtubule-binding repeat region (Figure 1). Phosphorylation of tau is usually a very rapid and reversible process, which is mediated by the opposing actions of several protein kinases and phosphatases [2]. Tau phosphorylation is increased during embryonic development, and in neurodegenerative conditions, in which tau deposition is a characteristic feature [3, 4]. Such disorders include Alzheimer's disease, progressive supranuclear palsy (PSP), and frontotemporal lobar degeneration associated with Pick bodies, amongst others, collectively termed the "tauopathies". A common factor to all of these diseases is the presence of aggregated and highly phosphorylated tau in the brain. These aggregates characteristically form intracellular inclusions comprised predominantly of tau with reduced solubility and increased reactivity to phospho-specific tau antibodies. Determining the key kinases that may be involved in the development and progression of disease pathology is an important research goal. In this review, we highlight the links between glycogen synthase kinase-3 (GSK-3) activity and tau function in normal and diseased brain.

2. The Microtubule-Associated Protein Tau

2.1. Tau Function and Localisation. Tau is present in the adult human central nervous system (CNS) as six isoforms that are generated from alternative splicing of a single gene. These isoforms differ from each other by the presence of none, one, or two inserts of 29 amino acids in the N-terminus of the molecule, and the inclusion of either three (3R) or four (4R) repeated stretches of approximately 30 amino acids that comprise the microtubule-binding region of the molecule (Figure 1). Tau in embryonic brain comprises primarily the smallest (0N3R) tau isoform, and this single isoform is gradually replaced with the six adult

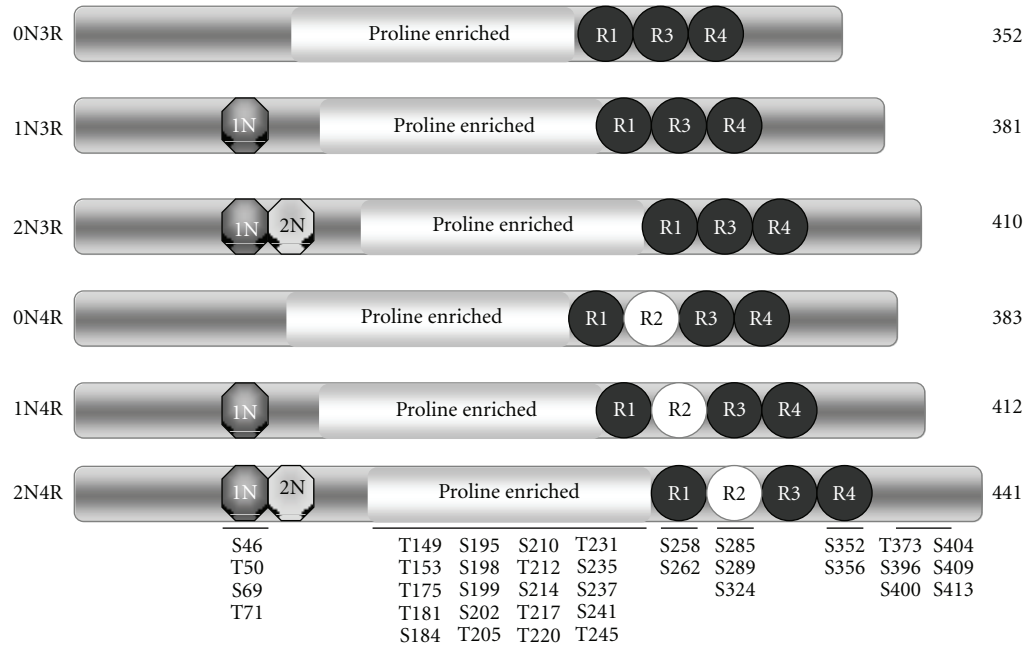

FIGURE 1: Tau isoforms in the human CNS and identified GSK-3 phosphorylation sites. The diagram illustrates the six isoforms of tau present in the human CNS. The longest tau isoform includes alternatively spliced exons 2, 3, and 10. Exons 2 and 3 encode two short amino acid inserts near the N-terminus of the molecule (1N and 2N, respectively). Exclusion of exons 2 and 3 gives rise to 0N tau isoforms, inclusion of exon 2 produces 1N tau isoforms, and inclusion of exons 2 and 3 results in the expression of 2N tau isoforms. Exon 10 encodes an additional microtubule-binding repeat domain (R2) that is present in 4R, but absent from 3R, tau isoforms. The number of amino acids in each tau isoform is indicated on the right. The centre of the molecule comprises a proline-enriched region that harbours the majority of the identified GSK-3 phosphorylation sites in 2N4R tau. Serine (S) and threonine (T) residues that have been identified as being phosphorylated by GSK-3 *in vitro* are indicated below.

isoforms during development [5, 6]. In normal adult human brain, the ratio of the tau isoforms harbouring three or four microtubule-binding repeats is approximately equal. However, this ratio is altered in favour of expression of the 4R tau isoforms in several neurodegenerative tauopathies [7], although increased relative expression of 3R tau isoforms has also been observed in frontotemporal lobar degeneration associated with Pick bodies [8]. Thus, abnormal tau splicing and phosphorylation are both events that are closely associated with neurodegeneration.

In neurons, the primary location of tau is in axons, where it is presumed to act as a stabilising protein for the microtubule cytoskeleton. Tau has an important function in maintaining microtubule dynamic instability, through dual processes that result in the lengthening and shortening of microtubules in response to external signals [9]. Increased tau phosphorylation leads to its detachment from tubulin, thereby, enhancing microtubule disassembly and reducing microtubule stability [10, 11]. In contrast, dephosphorylation of tau leads to an increase in its binding to tubulin, accelerated microtubule growth, and stabilisation of the microtubule cytoskeleton. Altered microtubule stability is particularly important during neurodevelopment when increased phosphorylation of tau reduces its binding to microtubules, allowing the rapid extension and retraction of exploratory neurites. Spatial and temporal changes in tau phosphorylation have been reported during neuronal differentiation [12], and some phosphorylated epitopes on tau (e.g., PHF-1, corresponding to phosphorylated Ser396/Ser404 in tau) have been associated with early stages of axon formation [13], indicating a role for tau in the development of neuronal polarity. In disease, highly phosphorylated forms of tau bind less well to microtubules, resulting in a loss of the microtubule-stabilising properties of tau and ultimately the collapse of the neuronal cytoskeleton. This has the effect of disrupting axonal transport and negatively impacting on the delivery of organelles, neurotransmitters, and other proteins to and from the cell body, with a consequent detrimental effect at synaptic termini [14–16].

Tau phosphorylation also influences the positioning of tau in dendrites, and the association of tau with plasma membranes and nuclei. Elevated phosphorylation results in the relocalisation of tau from axons into the somatodendritic region of neurons. Interactions between tau and the non-receptor tyrosine kinase, Fyn, result in increased Fyn localisation in dendrites [17]. In models of Alzheimer's disease, increased Fyn activity in response to neurotoxic stimuli such as β-amyloid (Aβ), enables Fyn to phosphorylate subunit 2B (NR2B) of the N-methyl-D-aspartate (NMDA) receptor, increasing the stability of this complex with the postsynaptic density (PSD) protein, PSD-95, and ultimately enhancing neurotoxicity [17]. In contrast, dephosphorylation of tau increases its association with plasma membranes [18, 19], which may also influence neurodegenerative processes by

Table 1: Comparison of phosphorylation sites in human Alzheimer and control brain with recombinant tau phosphorylated by GSK-3 *in vitro*.

Tau residue number	Alzheimer tau	Control brain tau	GSK-3
Tyr18	A		
Ser46	A	•	•
Thr50			•
Ser68	•		
Thr69	•		•
Thr71	•		
Ser113	•		
Thr123	A		
Ser131			
Thr149			•
Thr153	A		•
Thr175	•		•
Thr181	•	•	•
Ser184	•		•
Ser185	•		
Ser191	•		
Ser195			•
Tyr197	•		
Ser198	•	•	•
Ser199	•	•	•
Ser202	•	•	•
Thr205	A	•	•
Ser208	•		
Ser210	•		•
Thr212	•	•	•
Ser214	•		•
Thr217	•	•	•
Thr220			•
Thr231	•	•	•
Ser235	•	•	•
Ser237	•		•
Ser238	•		
Ser241			•
Thr245			•
Ser258	•		•
Ser262	•		•
Ser285			•
Ser289	•		•
Ser305			•
Ser324			•
Ser352			•
Ser356	•		•
Thr373			•
Tyr394	•		
Ser396	•	•	•
Ser400	•	•	•
Thr403	•		
Ser404	•	•	•
Ser409	•		•
Ser412	•	∗∗	
Ser413	•	∗∗	•
Thr414	∗	∗∗	
Ser416	A*	•	
Ser422	•		
Thr427	•		
Ser433	•		
Ser435	•		

Numbering of residues refers to 2N4R human tau, the largest isoform present in the human CNS.
• indicates tau phosphorylation sites identified by direct means.
A indicates tau phosphorylation sites identified using phospho-specific tau antibodies.
∗ indicates that one of the two closely spaced phosphorylation sites indicated (Thr414, Ser416) is phosphorylated in tau from AD brain.
∗∗ indicates that two of the three closely spaced phosphorylation sites indicated (Ser412, Ser413, Thr414) are phosphorylated in tau from control human brain.

mediating the interactions of tau with membrane-associated cell-signalling molecules such as Src-family kinases and phospholipase C-γ [20–22].

A pool of tau has also been identified in the nucleus of cultured cells and in brain tissue [23–28]. In human neuroblastoma cells, tau has been identified in the nucleolar organiser region [23], and, thus, tau-DNA binding may be involved in nucleolar organisation [27]. It has been reported that the phosphorylation of tau, particularly in response to heat stress, increases the amount of tau found in neuronal nuclei [29], although this finding is somewhat controversial [30]. The microtubule-binding domain of tau associates with RNA [31] and DNA [32], and the association of nuclear tau with DNA in response to stress is believed to play a role in protecting DNA from damage [29]. Tau has been found to be associated with the, predominantly nuclear protein, mammalian solute transport protein-2 (SUT2). SUT2 is localised to SC35-positive speckles in the nucleus, and it may have a role in mRNA processing [33]. Expression of this protein in C. elegans potentiates tau neurotoxicity [34, 35]. In addition, mammalian SUT2 is reduced in disease-affected regions of Alzheimer brain, under conditions where tau phosphorylation is increased, and SUT2 expression is increased when tau is overexpressed in mammalian cells in culture [34]. RNAi knockdown of SUT2 in cultured human cells overexpressing tau results in a marked decrease in tau aggregation, with no apparent effect on soluble tau species, suggesting a possible role for SUT2 in the pathogenesis of tauopathies [34]. Thus, tau may mediate neurodegeneration by changes in phosphorylation that result in both losses and gains of tau function.

2.2. Tau Phosphorylation. Tau possesses 80 phosphorylatable serine and threonine residues, approximately 20% of which have been identified as being phosphorylated in normal human brain (Table 1). A further five tyrosine residues in

tau are also amenable to phosphorylation by tyrosine kinases, including Fyn, Syk, c-Abl, and Lck [36, 37]. A large number of serine/threonine kinases have been reported to phosphorylate tau *in vitro*, but the identity of the protein kinase(s) responsible for regulating physiological tau phosphorylation remains unknown [7]. Candidate serine/threonine-directed tau kinases include GSK-3, casein kinase 1 (CK1), and cyclin-dependent kinase-5 (cdk5), amongst others [7]. It seems likely that more than one kinase may be involved in the phosphorylation of tau. However, substantial evidence exists to support a major role for GSK-3 in both physiological and pathological tau phosphorylation.

3. Glycogen Synthase Kinase-3

3.1. GSK-3 Isoforms. GSK-3 exists as two isoforms, α and β, which share 85% sequence identity and are encoded by distinct genes located on chromosomes 19 and 3, respectively [38]. GSK-3α and GSK-3β both phosphorylate tau *in vitro* and appear as granules with slightly differing morphologies and densities in pyramidal cells of hippocampal neurons [39]. There are two variants of GSK-3β, with GSK-3β2 differing from GSK-3β1 by the presence of an additional insertion of 13 amino acids. GSK-3β2 is enriched in neurons, where it is present in cell bodies, neuritis, and growth cones [40]. GSK-3α and GSK-3β share many substrates and appear to be able to compensate partially for each other, although they also appear to have distinct functions [41]. A recent report has shown that, whereas GSK-3β is present in birds, GSK-3α is absent, indicating that the GSK-3α isoform is not required either for viability or for normal physiological function in birds [42]. Furthermore, knockout of GSK-3α in mice results in increased insulin sensitivity [43], suggesting a role for GSK-3α in glucose metabolism that cannot be replaced by GSK-3β. In contrast, complete knockout of GSK-3β in mice is embryonic lethal [44].

3.2. Regulation of GSK-3 Activity. The kinase activity of GSK-3 is regulated by its phosphorylation at serine and tyrosine residues. Phosphorylation of GSK-3α/β at Tyr216 and Tyr279 is believed to maintain the constitutive activity of GSK-3 in neurons (reviewed in [45]), while the phosphorylation of Ser21 on GSK-3α or Ser9 on GSK-3β negatively regulates GSK-3 activity [45], and phosphorylation at these residues is believed to the predominant mediator of GSK-3 activity *in vivo* [46]. Both protein phosphatase 1, (PP1) and PP2A are known to target Ser21/9 and thus regulate GSK-3 activity [47]. Indeed, PP activation with okadaic acid leads to increased phosphorylation of S9 and, hence, inhibition of GSK-3β, which results in reduced tau phosphorylation [48]. Other phosphatases could also be involved in GSK-3 regulation since calcineurin (PP2B) also dephosphorylates and hence activates GSK-3 [49]. In addition, one of the many substrates of GSK-3 is the inhibitory subunit (inhibitor-2) of PP1. The phosphorylation of this subunit leads to its activation which results in the inhibition of PP1 [50], and decreased GSK-3 activity, demonstrating one mechanism through which GSK-3 activity may be regulated.

3.3. GSK-3 Regulation of Tau Splicing. In addition to phosphorylating tau, GSK-3 can influence tau splicing. GSK-3 has been shown to phosphorylate nuclear SC35, an enhancer of splicing elements that regulate exon 10 splicing in tau [51, 52]. Aβ application to cultured cells activates GSK-3 [53], leading to the phosphorylation of SC35 in parallel with enhanced splicing of tau exon 10 and decreased expression of 4R tau [54]. These events can be suppressed by the inhibition of GSK-3 activity with lithium or following siRNA knockdown of GSK-3 [54]. Despite these findings, most research investigating GSK-3 regulation of tau function has concentrated on GSK-3-mediated phosphorylation of tau.

3.4. GSK-3 Regulation of Tau Phosphorylation. Tau is a good substrate for GSK-3 *in vitro* [39, 55], in cultured nonneuronal cells [56], and in transgenic mice overexpressing GSK-3 [57]. In brain, tau exists in a complex with GSK-3 and the scaffolding protein 14-3-3 [58]. 14-3-3 recognises GSK-3 phosphorylated at Ser9, and indeed GSK-3 in this complex is phosphorylated at Ser9 in brain [58, 59]. The association of tau with this complex is believed to regulate its phosphorylation by GSK-3, since in human embryonic kidney cells, tau phosphorylation by GSK-3 is suppressed in the absence of 14-3-3, but GSK-3 is active and phosphorylates tau if 14-3-3 is present [59].

4. *In vitro* Phosphorylation of Tau by GSK-3

Tau is phosphorylated by GSK-3 on approximately 40 different serine and threonine residues, at least *in vitro* [60, 61]. The importance of this finding should not be underestimated since few kinases have been shown to target this number of sites in tau. Indeed, CK1 is the only other kinase that has been reported to phosphorylate a similar number of tau residues. Treatment of primary neuronal cortical cultures with specific inhibitors of either GSK-3 or CK1 reduces tau phosphorylation, suggesting that these kinases could have functionally important roles in neurons [7, 14, 60]. The phosphorylation of tau by GSK-3 or CK1 also reduces the ability of tau to promote microtubule assembly *in vitro* and in cells [62, 63]. These results rank GSK-3 and CK1 as targeting the greatest number of phosphorylatable residues on tau and implicate these two protein kinases in physiological tau phosphorylation in neurons. It remains to be seen whether the activities of GSK-3 and/or CK1 are modified in diseases in which increased phosphorylation of tau is a characteristic feature.

4.1. Potential Priming of Tau for GSK-3 Phosphorylation. It is well established that GSK-3 preferentially phosphorylates many of its substrates after they have been prephosphorylated by other kinases, and this seems also to be true for tau phosphorylation by GSK-3. Other examples of GSK-3 substrates that require prephosphorylation by another kinase before recognition by GSK-3 include glycogen synthase, inhibitor-2 of PP1, the regulatory subunit of cyclic AMP-dependent protein kinase (PKA), cAMP response element-binding (CREB), β-catenin, and kinesin light chain [46, 64]. Most priming phosphorylations for GSK-3 occur at

an amino acid located four residues C-terminal to the target residue [65]. However, there are some exceptions to this rule, and priming events have been reported that occur five or six residues from the GSK-3 target site, for example, in collapsin response mediator protein-2 and also in tau [66, 67]. The GSK-3 priming phosphorylation is frequently provided by the activity of PKA, CK1, or CK2 on unphosphorylated substrates, although other kinases, such as members of the mitogen-activated protein kinase family, or cdk5, can also initiate priming on some GSK-3 substrates [68, 69]. As has previously been shown, CK1 primes β-catenin for subsequent phosphorylation by GSK-3 (reviewed in [69]), and this might also occur on tau because the rate of GSK-3 phosphorylation of tau is increased when it is first phosphorylated by CK1 [70]. Substrate priming, therefore, may represent an important regulatory element of GSK-3 signalling since the activity of GSK-3 has been reported to differ for its primed and nonprimed substrates [68]. In the case of tau, this is supported by the observation that targeting GSK-3 phosphorylation of tau to either unprimed or primed sites has a differential impact on the binding of tau to microtubules [71, 72].

There are 24 short sequences of amino acids in tau that conform to the strict consensus sequence Ser/Thr-XXX-Ser/Thr (indicating pairs of serine or threonine residues separated by any three amino acids), that could be implicated in priming for GSK-3. Fourteen of these serine/threonine pairs have been found to be phosphorylated *in vitro* by GSK-3 (Table 1), and six pairs contain a proline residue immediately C-terminal to the target sequence. Five of the 10 remaining paired amino acids are phosphorylated by GSK-3 on only one of the two serine or threonine residues and five pairs have not been shown to be phosphorylated by GSK-3. Relaxation of the consensus sequence to include a separation of five or six amino acids between potential priming sites would allow the inclusion of further sets of amino acids, including Ser214, Ser210, Thr205, and Ser199 (see below). Overall, therefore, tau appears to fulfil several of the requirements for GSK-3 substrates, including multiple Ser/Thr-Pro sequences with nearby N-terminal phosphorylatable amino acids closely opposed, which could allow for kinase priming, either by GSK-3 itself or by other tau kinases.

Priming phosphorylation on tau residues Ser235 and Ser404 by other kinases, including cdk5, has been shown to promote phosphorylation by GSK-3 at Thr231 and Ser400, respectively [71, 72]. Interestingly, the stretch of amino acids in tau that includes the phosphorylatable residues, Ser396, Ser400, and Ser404, can be directly phosphorylated by GSK-3 without the prior activity of other kinases [67]. However, phosphorylation at Ser404 is critical to this process and substitution of this residue by alanine ablates phosphorylation of both Ser396 and Ser400. It appears, therefore, that the primary phosphorylation of Ser404 by GSK-3 can itself serve as a primed residue for the subsequent sequential phosphorylation of tau at Ser400 and Ser396 by GSK-3. However, the functional significance of many of the other potential GSK-3 priming sites in tau has not been widely investigated.

Prephosphorylation of tau by PKA for subsequent GSK-3 phosphorylation in rat brain appears to particularly enhance the overall amount of GSK-3 phosphorylation [73]. Furthermore, the initial phosphorylation of tau by PKA results in a different spectrum of phosphorylation sites generated by the action of GSK-3 compared to those produced when tau is exposed to GSK-3 alone, demonstrating that prior phosphorylation by PKA alters the recognition of tau by GSK-3 [73]. Moreover, initial phosphorylation of tau by PKA, which occurs primarily on Ser214, results in the subsequent phosphorylation by GSK-3 of four closely spaced residues, namely Ser210, Thr205, Ser199, and Ser195, each separated by 4–6 amino acids [67]. Interestingly, this study also revealed an interaction between the low-density lipoprotein receptor-binding domain of apolipoprotein E (ApoE), a major genetic risk factor for Alzheimer's disease, and GSK-3-phosphorylated tau [67]. Such an interaction was not observed with either nonphosphorylated tau or following extensive phosphorylation of tau by PKA. These results suggest that the pattern of phosphorylation sites generated by the action of GSK-3 on tau may be critical for its interaction with ApoE.

4.2. GSK-3α and GSK-3β1/2 Isoforms Differentially Phosphorylate Tau. Suppressing the expression of individual GSK-3α and GSK-3β isoforms results in differing tau phosphorylation patterns [74]. The induction of a phosphorylation-induced shift in electrophoretic mobility of tau, following incubation with GSK-3, also appears to be favoured preferentially by GSK-3β, rather than GSK-3α, as is tau phosphorylation at the antibody epitopes recognised by tau-1 (Ser199–Ser202) and 8D8 (Ser396) [39]. Together, these results support the view that GSK-3α and GSK-3β are likely to have differing preferences for tau that may be related to the distinct, but overlapping, intracellular locations of these kinases in neurons.

There is also a difference in the kinetics and sites of tau phosphorylation induced by the two GSK-3β isoforms, with GSK-3β2 appearing to phosphorylate tau more slowly than GSK-3β1 and on different, with some overlapping, tau residues, even under conditions in which other GSK-3β substrates, such as amyloid precursor protein, are phosphorylated equally [75, 76]. For example, Ser396 in tau is phosphorylated by both splice variants, whereas Ser199 is a significant target of GSK-3β1, but not of GSK-3β2 activity. It has recently been suggested that the interaction of GSK-3β2 with tau is weaker than that of GSK-3β1 [76]. Taken together, these results suggest that, not only are there differential activities of GSK-3α and GSK-3β towards tau, there are also partially overlapping, but distinct, tau phosphorylation sites recognised by each of the two isoforms of GSK-3β [74–77].

5. Tau Phosphorylation in Human Brain

In the tauopathies, tau is present in both normally phosphorylated and highly phosphorylated forms, the latter of which may be potentially pathological since it is most commonly observed as intraneuronal aggregates or glial inclusions.

Tau is normally a highly soluble protein under nondenaturing conditions, but the aggregated tau present in diseased brain displays a significantly reduced solubility and an increased reactivity to phospho-specific tau antibodies [78]. These characteristic features have facilitated purification strategies for tau from diseased brain exhibiting tau pathology which yield relatively enriched preparations of highly phosphorylated tau. Such preparations have resulted in the identification of phosphorylation sites on insoluble tau isolated from human tauopathy brain, thereby providing clues to the identities of protein kinases that are potentially involved in the pathogenesis or development of these neurodegenerative diseases [60, 79–81].

In particular, these approaches have been used to identify phosphorylation sites on purified tau from Alzheimer's disease and PSP brain material, as well as soluble tau from control adult and foetal brain [60, 82, 83]. These studies, combined with results obtained from immuno-labelling with phospho-specific tau antibodies, have led to the identification of approximately 45 phosphorylation sites on tau from Alzheimer brain (Table 1). In comparison, only 15 phosphorylation sites have been detected in insoluble tau from PSP brain and 16 sites on control brain tau, not all of which overlap, and for some of which no kinase has yet been identified [7].

A comparison of tau extracted from human brain with tau phosphorylated using protein kinases *in vitro* shows that GSK-3 remains a principal candidate kinase for both physiological and pathological tau phosphorylation (Table 1). Although GSK-3 has been reported to be associated with neurofibrillary pathology in Alzheimer's disease and related neurodegenerative disorders [84, 85], other studies have not identified such colocalisation [39, 86], and it remains to be seen if GSK-3 remains associated with tau pathology in diseased brain. In the case of tau extracted from neurologically normal human brain, all but two residues have been shown to be phosphorylated by GSK-3. These two sites, Ser412 and Thr414, are located within a group of three closely spaced residues, including Ser413, of which only two residues are phosphorylated in human brain. Since GSK-3 phosphorylates Ser413, it is possible that only a single site, either Ser412 or Thr414, remains unphosphorylated by GSK-3, and, of these, Ser412 is phosphorylated by both CK1 and PKA, and potentially also by CK2. This suggests that, under physiological conditions, tau in human brain may be phosphorylated primarily by GSK-3, with potential contributions from CK1, CK2, or PKA. Under pathological conditions such as Alzheimer's disease, in which tau becomes deposited in the brain, GSK-3 accounts for some 27 of the approximately 45 tau phosphorylation sites identified in insoluble tau. The remaining phosphorylation sites are accounted for the activities of a combination of other kinases, including the tyrosine kinases, c-Abl, and Fyn, although there remain a few sites for which a kinase has not yet been identified. Thus GSK-3 plays a prominent role in both the physiological and pathological phosphorylation of tau in human brain.

6. Cellular Models of Tau Phosphorylation by GSK-3

Further information regarding the role of GSK-3 in tau phosphorylation and function has been gained from cell models. In cells, the involvement of GSK-3 was first revealed when GSK-3 over-expression was shown to disrupt tau binding to microtubules, resulting in a diffuse cytoplasmic localisation of tau [56]. This staining pattern contrasts with the strongly fibrillar pattern displayed when tau is expressed alone. Cellular phosphorylation of tau by GSK-3β over-expressed in mammalian cells decreases tau binding to microtubules and influences microtubule organisation [87, 88]. This process is inhibited by mouse dishevelled-1, thereby potentially implicating the wingless signalling pathway in tau phosphorylation mediated by GSK-3 [89].

Investigations in human neuronal NT2N cells have shown that tau phosphorylation at multiple sites recognised by phospho-specific tau antibodies could be prevented by lithium treatment, which inhibits GSK-3 activity [90]. Lithium also promotes tau binding to microtubules and subsequent microtubule polymerisation, suggesting a role for GSK-3 in this normal physiological process [90]. It is important to note that lithium is not a specific inhibitor of GSK-3 as it also effectively inhibits inositol monophosphatase, as well as a family of structurally related phosphomonoesterases (reviewed in [91]). However, the reported effects of lithium on tau phosphorylation in cells have subsequently been validated in studies using more selective GSK-3 inhibitors [74, 92, 93]. The examination of endogenous tau phosphorylation by GSK-3 in primary rat neurons has revealed a number of lithium-sensitive sites, suggesting that these might represent the physiological targets of GSK-3 on tau [14, 94]. Interestingly, in the latter study, the inhibition of GSK-3, either by lithium or by the more specific GSK-3 inhibitor, SB-415286, resulted not only in reduced tau phosphorylation but also in reduced axonal transport of tau [14]. This led to the idea that tau phosphorylation mediated by GSK-3 could influence tau axonal transport, at least in primary neuronal cultures [14]. In an *ex vivo* study, perfusion of preformed filaments of tau into isolated squid axoplasm resulted in the inhibition of anterograde transport of membrane-bound organelles [95]. Interestingly, inhibiting GSK-3 activity, or perfusion of inhibitor-2 to inhibit PP1 activity, overcame the detrimental effect of tau filaments on axonal transport in this model [95]. These results, thus, support the notion that GSK-3 has an important role in relation to tau function in neurons.

Inhibiting GSK-3 with SB-415286 also protects cultured neurons from cell death induced by reduced phosphatidylinositol 3-kinase activity, and this protection is paralleled by decreased tau phosphorylation [92]. These results suggest that the state of tau phosphorylation by GSK-3 in cells is important for the maintenance of healthy functional neurons, and changes in tau phosphorylation are likely be indicative of reduced neuronal viability.

7. Animal Models of Tau Phosphorylation by GSK-3

7.1. Drosophila. Over-expression of tau together with GSK-3 (the human orthologue of *shaggy/Zw3* in Drosophila) has been demonstrated to exacerbate the neurodegeneration resulting from expression of tau alone in Drosophila melanogaster [96], suggesting that the phosphorylation of tau by GSK-3 is involved in the neurodegenerative process. The presence of partition-defective 1 (PAR-1), the Drosophila orthologue of mammalian microtubule affinity regulating kinase (MARK), has been shown to be required to initiate GSK-3 and cdk5 phosphorylation of tau in flies [97]. However, reports differ as to whether PAR-1 activity enhances [97, 98] or suppresses [15, 99, 100] tau-induced toxicity, although PAR-1 over expression in Drosophila appears to increase neuronal loss [97]. MARK phosphorylates tau on Ser262 and Ser356 *in vitro*, both of which are located within the microtubule-binding domain of tau, and this phosphorylation results in the disruption of microtubules [101]. MARK phosphorylation, therefore, results in the detachment of tau from microtubules, and this may be related to its involvement in microtubule-based axonal transport in neurons [102]. Interestingly, MARK itself is phosphorylated by GSK-3, resulting in MARK inactivation [103], suggesting the possible involvement of multiple tau kinases in this model, however the relevance GSK-3 and MARK interaction to tau phosphorylation has not yet been determined.

More recent studies have found that tau phosphorylation in Drosophila is not dependent on GSK-3 activity. A mutant form of tau, described as GSK-3 resistant, retained toxicity in flies, and, under these conditions, mutant tau exhibited an increased affinity for binding to microtubules [98]. Notably, while GSK-3 phosphorylation of tau was impaired in these flies, toxicity was unaffected, suggesting that the ability of tau to bind to microtubules, or possibly the propensity of GSK-3 to alter the oligomerisation state of tau, may be critical for neurodegeneration.

Drosophila express a form of tau with limited homology to human tau, especially in the microtubule-binding region [104]. There is less than 50% amino acid identity between the presumed microtubule-binding domain of Drosophila and mammalian tau. Coupled with the presence of an additional microtubule-binding repeat in flies, these considerable differences between invertebrate and mammalian tau suggest that the results obtained by overexpressing GSK-3 in flies may differ from those obtained in the presence of human tau. The relationship, therefore, between tau toxicity and tau phosphorylation in Drosophila warrants further investigation if it is to be applied to study the physiological relevance of GSK-3 phosphorylation of tau.

7.2. Mice. The influence of GSK-3-mediated tau phosphorylation in mammals has been studied in various mouse models (reviewed in [105]). The postnatal over-expression of GSK-3 in mice results in GSK-3 activation and the development of several characteristics of human tauopathy including elevated tau phosphorylation, reactive gliosis, spatial learning deficits, and neuronal death [106, 107]. These features can be reversed by suppressing GSK-3β expression [108], indicating that tau is a prime substrate for GSK-3 *in vivo*. Indeed, the axonopathy and motor impairment resulting from over-expression of human 2N4R tau in mice is alleviated when GSK-3β is coexpressed [109], with this beneficial effect believed to occur as a result of GSK-3 phosphorylation of tau removing excess tau from microtubules. Further support for a potential pathological role of GSK-3-phosphorylated tau has come from studies showing that GSK-3 inhibition in transgenic mice expressing disease-associated mutant human tau reduces tau phosphorylation and aggregation [110–114] as well as axonal degeneration [111].

8. Conclusions

Changes in the phosphorylation state of tau have a major impact on its subcellular localisation and function in neurons. Major changes in the phosphorylation state of tau are evident under two apparently unrelated conditions, that is, during early neuronal development and during neurodegenerative processes that lead to the deposition of highly phosphorylated pathological tau in the brain, such as in Alzheimer's disease. Therefore, determining the nature of the kinases involved in this process *in vivo* is an important research goal that will improve our understanding of these processes.

GSK-3 is a key candidate kinase for tau phosphorylation, under both physiological and pathological conditions. Multiple serine and threonine residues on tau are targeted by GSK-3 *in vitro*, with many of these sites being coincident with those phosphorylated *in vivo*. Together with the, possibly coordinated, activity of other kinases, including CK1, cdk5, PKA and/or MARK, GSK-3 is well positioned to act on tau in such a way that will result in significant and rapid tau phosphorylation and, hence, modulation of its function in neurons. Understanding the regulation of GSK-3 activity in neurons, including the possible differential effects of the related GSK-3α, GSK-3β1, and GSK-3β2 isoforms, should lead to further elucidation of the mechanisms underlying tau phosphorylation and may ultimately lead to new therapeutic targets for neurodegenerative disease.

Abbreviations

Aβ:	β-amyloid
ApoE:	Apolipoprotein E
cdk5:	Cyclin-dependent kinase-5
CK:	Casein kinase
CNS:	Central nervous system
CREB:	Cyclic AMP response element binding
GSK-3:	Glycogen synthase kinase-3
MARK:	Microtubule-affinity regulating kinase
NMDAR:	N-methyl-D-aspartate receptor
NR2B:	Subunit 2B of the NMDA receptor
PAR-1:	Partition-defective 1
PKA:	Cyclic AMP-dependent protein kinase
PP:	Protein phosphatase

PP2B: Calcineurin
PSD: Postsynaptic density
PSP: Progressive supranuclear palsy
Ser: Serine
SUT2: Solute transport protein-2
Thr: Threonine
Tyr: Tyrosine.

Acknowledgments

Work in the authors' laboratories is funded by the UK Medical Research Council, The Wellcome Trust, Alzheimer's Research UK, The Henry Smith Charity, and The Royal Society.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 985085, 9 pages
doi:10.4061/2011/985085

Review Article

Targeting Glycogen Synthase Kinase-3β for Therapeutic Benefit against Oxidative Stress in Alzheimer's Disease: Involvement of the Nrf2-ARE Pathway

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Received 29 December 2010; Accepted 1 March 2011

Academic Editor: Peter Crouch

Specific regions of the Alzheimer's disease (AD) brain are burdened with extracellular protein deposits, the accumulation of which is concomitant with a complex cascade of overlapping events. Many of these pathological processes produce oxidative stress. Under normal conditions, oxidative stress leads to the activation of defensive gene expression that promotes cell survival. At the forefront of defence is the nuclear factor erythroid 2-related factor 2 (Nrf2), a transcription factor that regulates a broad spectrum of protective genes. Glycogen synthase kinase-3β (GSK-3β) regulates Nrf2, thus making this kinase a potential target for therapeutic intervention aiming to boost the protective activation of Nrf2. This paper aims to review the neuroprotective role of Nrf2 in AD, with special emphasis on the role of GSK-3β in the regulation of the Nrf2 pathway. We also examine the potential of inducing GSK-3β by small-molecule activators, dithiocarbamates, which potentially exert their beneficial therapeutic effects via the activation of the Nrf2 pathway.

1. Alzheimer's Disease and Oxidative Stress

Alzheimer's disease is a common age-associated dementia characterized by pathological, progressive loss of neurons and synapses, accumulation of intra- and extracellular protein deposits, and gliosis. The amyloid hypothesis of AD postulates that amyloid-beta (Aβ) deposition and neurotoxicity play a causative role in AD [1]. Although the mechanisms through which Aβ exerts its toxicity are numerous [2], it appears that oxidative injury is central in the pathogenesis of AD [3–5].

Oxidative stress results from an imbalance between the production and removal of physiologically important molecules collectively called reactive oxygen species (ROS) [6–8]. Oxidative stress damages all cellular macromolecules and when uncontrolled, leads to irreparable oxidative injury and cell death. The imbalance between the production and removal of ROS occurs when endogenous defence systems are overwhelmed or exhausted, usually due to disease or as part of normal aging. It is thus not surprising that oxidative stress is involved in the pathogenesis of several neurodegenerative disorders, including AD.

Oxidative stress is a central feature of AD, and, in fact, it may even be one of the first pathogenic events during disease progression [5]. Markers of oxidative damage such as protein carbonyls [9, 10] and elevated lipid peroxidation [11, 12] precede pathological changes and are found in the brains of AD patients. Importantly, antioxidant defence is impaired in mouse models of AD; the levels and activities of protective enzymes including superoxide dismutase (SOD) and glutathione peroxidase are altered [13].

2. GSK-3β and AD

GSK-3β is a serine/threonine kinase that regulates diverse cellular functions ranging from glycogen metabolism to gene transcription and cell survival [14, 15].

Several lines of evidence directly link GSK-3β to the neuropathology of AD [14, 16, 17]. In fact, the recently described GSK hypothesis of AD depicts how overactivation of GSK-3β accounts for the pathology of AD [16]. GSK-3β expression is elevated in the brain of AD patients and is implicated with at least three major pathological hallmarks of the disease [18, 19]. First, overactivity of GSK-3β accounts, at least in part, for tau hyperphosphorylation [20]. In human brain, active GSK-3β has been detected in neurons loaded with neurofibrillary tangles [21], and *in vitro* evidence link increased GSK-3β activation to tau hyperphosphorylation. In fact, Aβ can activate GSK-3β, leading eventually to increased tau phosphorylation and loss of microtubule binding. In agreement with the *in vitro* evidence, mice overexpressing GSK-3β display prominent tau hyperphosphorylation [22]. Secondly, acetylcholine synthesis is suppressed by GSK-3β, thus implicating the kinase in the cholinergic deficit characteristic of the disease [23]. This effect has been shown to be mediated by GSK-3β-dependent inactivation of pyruvate dehydrogenase (PDH), leading to the depletion of acetyl coenzyme A, an important precursor in acetylcholine synthesis [23]. Thirdly, overactivity of the kinase causes increased production of toxic Aβ. GSK-3β has been shown to interact with presenilin, thus increasing the production of the amyloid precursor protein (APP) and subsequently toxic Aβ. The increased production of Aβ then leads to synaptic deficits and memory impairment [24–27]. GSK-3β can also directly phosphorylate APP *in vitro* [28], and, interestingly, AD-related mutated presenilin-1 is able to both directly and indirectly activate GSK-3β [29, 30]. Not surprisingly, the inhibition of GSK-3β blocks the accumulation of Aβ and reduces plaque burden in transgenic mice modelling AD [24], while the overexpression of GSK-3β causes memory deficits [31, 32]. Certain antibodies that reduce brain Aβ burden also mediate their protective effect by inhibiting GSK-3β activation [33]. Finally, GSK-3β is an important mediator of apoptosis [34], thus its dysregulation may also directly contribute to AD-associated neuronal loss.

GSK-3β phosphorylates a diverse group of substrates, including over 20 transcription factors [14, 35]. It is hypothesized that GSK-3β-mediated impaired activation of transcription factors compromises the ability of cells to respond adequately to stressful conditions. Indeed, GSK-3β is inhibitory towards the activation of transcription factors such as heat shock factor-1 [36, 37] and cyclic AMP response element-binding protein [14, 38], which are important in cell survival mechanisms after potentially toxic insults [39–41]. However, it is important to note that GSK-3β activation may also affect cell survival by other mechanisms. For example, activated GSK-3β can inactivate an important mediator in the citric acid cycle, PDH, and may thus impair the energy supply in neurons [23].

As the majority of the transcription factors controlled by GSK-3β are involved in cellular survival pathways, the modulation of transcription factors by GSK-3β is likely an important survival mechanism against various stresses, including oxidative stress. It is interesting to note that oxidative stress itself may also be related to GSK-3β activation. For example, oxidative stress induces overactivation of GSK-3β in neuronal cells [42], while the inhibition of GSK-3β is involved in the control of oxidative stress in neuronal hippocampal cell lines [43].

3. Endogenous Defence against Oxidative Stress

Under normal conditions, oxidative stress leads to the activation of a battery of defensive gene expression that leads to detoxification, prevention of free radical generation and cell survival [44]. At the forefront of defence against oxidative stress is the transcription factor named nuclear factor erythroid 2-related factor (Nrf) 2. Human Nrf2 was first isolated in 1994 from a hemin-induced K562 erythroid cell line and showed high sequence specificity to the known p45 subunit of NF-E2 [45, 46]. Nrf2 regulates a broad spectrum of enzymes and proteins involved in the disposition of harmful compounds causing oxidative stress. The classes of genes regulated by Nrf2 affect such diverse functions as detoxification of electrophiles, free radical metabolism, glutathione metabolism, proteasome function, and calcium homeostasis [47]. Microarray analyses have revealed numerous Nrf2-dependent genes that confer protection against oxidative stress *in vitro* [48, 49, 49, 50]. Some of the well-characterized cytoprotective genes controlled by Nrf2 include heme oxygenases (HOs), NAD(P)H : quinone oxidoreductases, superoxide dismutases (SODs) and the rate-limiting enzymes of glutathione synthesis consisting of catalytic (GCLC) and modifier (GCLM) subunits [51–53].

Transcription factors such as Nrf2 are considered key targets of numerous signalling pathways because they have the critical role of transferring information from the extracellular environment to the nucleus to regulate multiple functions. The induction of the Nrf2-controlled protective response requires at least three essential components; antioxidant response elements (AREs), Nrf2, and Kelch ECH-associating protein 1 (Keap1) [47]. AREs are enhancer sequences that control the basal and inducible expression of protective genes in response to oxidative stress, xenobiotics, heavy metals and ultraviolet light [44, 54, 55]. Nrf2 is the principal transcription factor that binds to the ARE and induces the expression of ARE-driven cytoprotective genes. Keap1 is a cytosolic, actin-associated protein that suppresses the activity of Nrf2 by sequestering it in the cytoplasm and by targeting it for proteasomal degradation.

The abundance of Nrf2 inside the nucleus is constantly regulated by positive and negative stimuli that affect nuclear import and export, binding to the ARE, as well as the degradation of Nrf2 [44]. It is well known that Keap1 [56] is an inhibitor of Nrf2 that targets Nrf2 for degradation and thus promotes low, basal expression of cytoprotective genes under normal physiological conditions. Previously, it was thought that oxidative stimuli activate Nrf2 by promoting its dissociation from Keap1; however, as the binding affinity between the two proteins is not affected by oxidative stress

[57–59], it is now understood that the main function of Keap1 is to serve as an adapter for the Cullin3/Ring Box 1 E3 ubiquitin ligase complex [60, 61]. Keap1 binding to Cullin3 and Nrf2 leads to the ubiquitination and degradation of Nrf2 through the 26S proteasome [62, 63]. Keap1 also functions as ROS sensor molecule, the cysteine residues of which are modified upon conditions of oxidative stress [64, 65]. Oxidative stress impairs the ability of Keap1 to target Nrf2 for degradation, most likely by triggering an alteration in Keap1 conformation.

4. Nrf2 in AD

The activation of the Nrf2-ARE pathway is beneficial in animal models of various diseases of the central nervous system, including chronic neurodegenerative diseases such as Parkinson's disease and acute insults such as brain ischemia and brain trauma [66–69]. However, in comparison to other neurodegenerative disorders, the role of Nrf2 in AD has received relatively little attention. Currently, it is known that while Nrf2 is not a susceptibility gene for AD, common variants of the gene encoding Nrf2 may affect disease progression [70]. In the human AD brain, the amount of nuclear Nrf2 is reduced in the hippocampus [71]. Histochemical analyses demonstrate that Nrf2 predominantly localizes to the cytoplasm of AD-affected hippocampal neurons, suggesting that despite oxidative stress, Nrf2-mediated transcription is not induced in AD patients. However, this study utilized brain tissue demonstrating full-blown AD pathology; it is not clear whether the finding is a cause or consequence of the ongoing pathological events and cell death. In fact, it has been suggested that the induction of Nrf2, and levels and activity of its cytoprotective target enzymes may display a time-dependent alteration in AD [66].

We recently showed that attenuation of the Nrf2-ARE pathway coincides with disease progression in the APdE9 transgenic mice modelling AD [72]. The Nrf2-pathway was impaired in transgenic AD mice concomitantly with increased brain Aβ burden. This AD-associated reduction in Nrf2 was recently confirmed by Choudry et al., who described a 50% reduction in Nrf2 levels in transgenic AD mice [73].

A growing body of literature suggests that Nrf2 is neuroprotective in AD. Induction of the Nrf2-ARE pathway by small therapeutic molecules protects against neuronal dysfunction and toxicity mediated by Aβ *in vitro* [72, 74–76]. Interestingly, Nrf2 can also exert its protective effects by suppressing oxidative stress-induced Aβ formation [74, 77] and by inducing the 26S proteasome, thus facilitating the removal of toxic Aβ [78]. Protection against Aβ toxicity by coffee extract has also been shown to occur via the induction of the Nrf2-ARE pathway in *Caenorhabditis elegans* [79].

In addition to assessing the effect of small molecule inducers of the Nrf2-ARE pathway on Aβ toxicity, we recently studied therapeutic and disease-modifying properties of Nrf2-ARE induction by gene transfer in transgenic mice modelling AD [80]. The long-term effect of Nrf2-ARE activation was studied *in vivo* by employing a gene therapy approach where human Nrf2 was directly injected into the hippocampi of transgenic AD mice, an area of the brain important for learning and memory that is directly affected by AD pathology. Evident improvement in cognitive abilities was achieved when transgenic AD mice were treated with the Nrf2 vector at the age of 9 months and assessed in the Morris water maze 6 months later. This effect was associated with the induction of the Nrf2-pathway, suggesting that strategies aimed at boosting the Nrf2-ARE pathway constitute a potential therapeutic approach for AD.

5. GSK-3β in the Regulation of the Nrf2-ARE Pathway

As GSK-3β controls a variety of targets in several cellular pathways, it is not surprising that the kinase is also implicated in the regulation of Nrf2. GSK-3β exerts a negative form of regulation on Nrf2 by controlling it is subcellular distribution [81] (Figure 1). Long-term exposure to hydrogen peroxide causes downregulation of Akt, activation of GSK-3β, and translocation of Nrf2 from the nucleus to the cytosol, thus limiting the antioxidant response of cells [82]. This is particularly important in conditions of prolonged oxidative stress, such as AD, and highlights the importance of this kinase in chronic neurodegenerative disorders. The inhibition of GSK-3β results in nuclear accumulation and the elevation of transcriptional activity of Nrf2 [82], indicating that GSK-3β is a fundamental element of Nrf2-ARE downregulation after oxidative injury. The mechanism of GSK-3β-mediated Nrf2 inhibition appears to involve the tyrosine kinase Fyn, which is phosphorylated by activated GSK-3β and leads to nuclear localization of Fyn. Activated Fyn phosphorylates tyrosine 568 of Nrf2 [83] in the nucleus, leading to Nrf2 export and dampening of protective gene transcription [83, 84]. Once excluded from the nucleus, Nrf2 is degraded. Very recently, it was shown that transfection with a constitutively active genetic variant of GSK-3β completely inhibits nuclear accumulation of Nrf2, providing further support for the role of GSK-3β in controlling Nrf2 activation [85].

Further evidence for the importance of GSK-3β in the regulation of Nrf2 is demonstrated by the finding that activation of the muscarinic M1 receptor induces Nrf2 through a signalling cascade involving protein kinase C-mediated inhibition of GSK-3β [86]. Moreover, GSK-3β is known to regulate oxidative stress protein SKN-1, the functional counterpart of Nrf2, in *Caenorhabditis elegans* [87]. Taken together, these data suggest that increased activation of GSK-3β leads to a dampening of the protective Nrf2-ARE pathway.

6. Dithiocarbamates as Nrf2-Inducing Pharmacological Agents Targeted to GSK-3β Activation

Stimulation of the Nrf2-ARE pathway by small-molecule activators represents an appealing strategy to upregulate the endogenous defence mechanism of cells against oxidative stress. At least nine classes of Nrf2 inducers have been described [88] and several of these are protective in models of neurodegenerative diseases. However, more potent, safe,

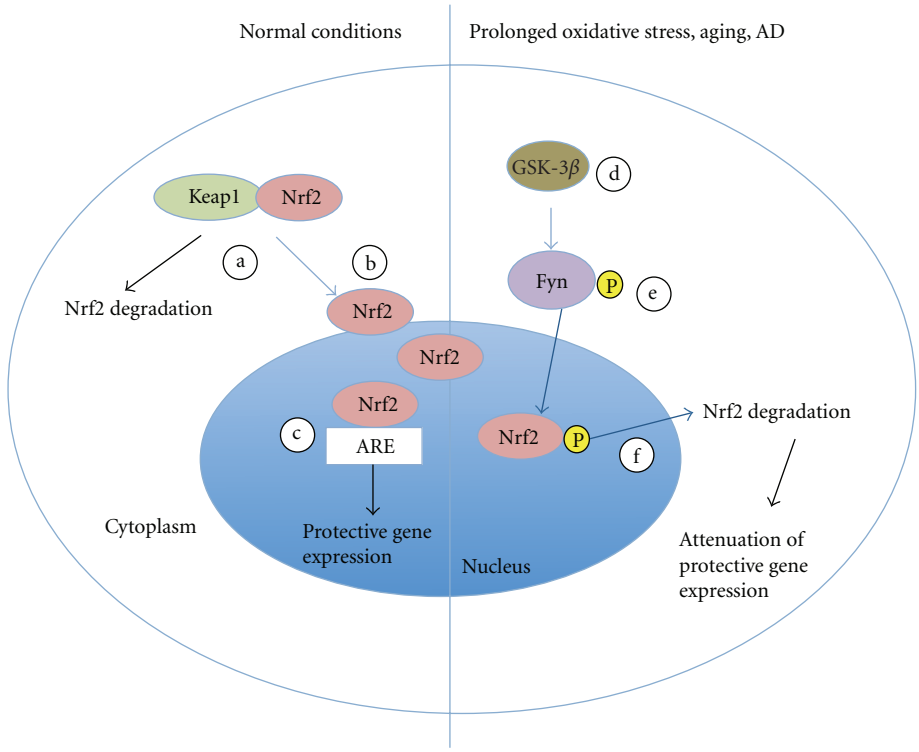

Figure 1: Induction of the Nrf2-ARE pathway under normal and pathological conditions: potential role of GSK-3β as a negative regulator of Nrf2. (a) In normal conditions, actin-associated Keap1 sequesters Nrf2 in the cytosol and targets it for degradation. (b) Endogenously and exogenously generated ROS alter the interaction between Nrf2 and its repressor, resulting in the accumulation of Nrf2 in the cytoplasm. Nrf2 translocates into the nucleus. (c) An abundance of Nrf2 in the nucleus results in the binding of Nrf2 to the ARE and the increased expression of protective, Nrf2-controlled genes. (d) In conditions of prolonged oxidative stress, GSK-3β is activated. (e) Activated GSK-3β phosphorylates Fyn, causing nuclear translocation. (f) Nuclear Fyn phosphorylates Nrf2, leading to nuclear export of Nrf2 and degradation in the cytosol.

and specific activators of the Nrf2-ARE pathway that cross the blood brain barrier (BBB) need to be explored in relevant models of AD.

Dithiocarbamates are attractive drug candidates for many diseases as they are BBB permeant metal chelating compounds that possess antioxidant and anti-inflammatory properties [89] and are clinically approved for treatment of alcohol addiction (Antabus) and heavy metal poisoning. In addition to its well-characterized role in the inhibition of nuclear factor-κB [90, 91], pyrrolidine dithiocarbamate (PDTC) has potent antioxidant properties and is able to scavenge ROS [90–93]. It is becoming increasingly evident that PDTC also has the potential to activate endogenous antioxidant gene expression. *In vitro* studies suggest that PDTC treatment results in the activation and nuclear translocation of Nrf2 [94, 95]. Moreover, the cytoprotective, Nrf2-controlled proteins, HO-1, GCLM, and SOD, are potently induced in response to PDTC *in vitro* [94, 96–98]. Interestingly, it has also been shown that PDTC can act as a pro-oxidant [99]. Indeed, PDTC induces apoptosis in several *in vitro* models [100, 101, 101, 102] and may also be toxic to neurons *in vivo* [103]. Whether the action of PDTC is anti- or pro-oxidant has been reported to depend on the dose of PDTC and the presence of metal ions [104]. It may well be that PDTC can exert its effects on Nrf2-mediated gene transcription by mimicking an oxidative insult (and thus acting as a pro-oxidant) that triggers Nrf2 activation.

PDTC is known to reduce activated GSK-3β signalling in neonatal hypoxia-ischemia [105]. We also showed that while the activity of GSK-3β is increased in the brains of transgenic AD mice [106], a 7-month treatment with PDTC reduces the amount of active GSK-3β with concomitant improvement in the spatial learning of the treated mice. This effect may involve the metal-chelating ability of PDTC. It is known that PDTC can transport extracellular copper into cells [107] and depending on the situation at hand, this may have different effects. For example, copper transport into cells is most likely beneficial in AD animal models [108–110], where pools of intracellular copper are depleted or unevenly or inefficiently distributed within the brain parenchyma. In contrast, under normal conditions, an increase in cellular copper levels may cause an increase in free radical production and apoptosis [111, 112]. As copper can induce the Akt pathway, we hypothesize that the PDTC-mediated increase in intracellular copper could trigger the phosphorylation of Akt, leading to reduced GSK-3β activity.

To analyze the association of Nrf2 with the beneficial effect of PDTC treatment, we assessed the potential of

PDTC in protection against Aβ toxicity in primary cultures prepared from Nrf2 knockout mice. While PDTC protected against Aβ toxicity in wild-type neuronal cultures, the beneficial effect was abolished in cultures prepared from knock-out mice (Kanninen, K et al., unpublished data). Moreover, PDTC treatment of neuronal cultures induced Nrf2 target genes (Kanninen, K et al., unpublished data). Taken together, these data indicate that Nrf2 is required for the beneficial effect of PDTC against Aβ toxicity *in vitro* and suggest that Nrf2-ARE induction may be associated with the beneficial effect of PDTC in AD. However, further studies are required to clarify and specify the involvement of GSK-3β in aberrant regulation of Nrf2 in AD.

7. Targeting GSK-3β for Therapeutic Benefit in AD: Involvement of the Nrf2-ARE Pathway

While the detailed mechanisms behind the impairment of the Nrf2-ARE pathway in transgenic AD mice remain unresolved, it is possible that it involves GSK-3β. Considering that GSK-3β can inactivate Nrf2 [81, 86], it is conceivable that the Nrf2-ARE pathway is dampened in the aged AD transgenic mouse brain through the increased activity of GSK-3β. In fact, long-term oxidative stress causes GSK-3β activation and reduces nuclear Nrf2, suggestive of downregulation of the Nrf2-ARE pathway [82]. This hypothesis is further supported by the finding that pharmacological treatments, which inhibit GSK-3β, have been reported to reduce Aβ pathology and cognitive impairment in AD mice [24]. Moreover, lithium, a GSK-3β inhibitor, has been shown to promote the transcriptional activity of Nrf2 [82].

While modulating the activity of GSK-3β is known to be beneficial in models of AD, studying the mechanism of action of modulators of the kinase is important in understanding the diverse pathways GSK-3β is involved in and the numerous effects modulation may have. For example, treatment with small molecules such as PDTC prevents cognitive impairment in transgenic AD mice [106], not only by the inhibition of GSK-3β, but also potentially via the activation of the Nrf2-ARE pathway.

8. Concluding Remarks

Due to the major social and economical burden caused by the aging of populations and the subsequent increase in the incidences of neurodegenerative diseases, potential novel targets for effective AD therapeutics are urgently needed. Despite extensive research and knowledge that oxidative stress is a central pathological feature of AD, several therapeutic approaches targeted to this aspect of disease have failed, most likely because they have targeted only one aspect such as the decline of a single antioxidant. Considering the complexity of the antioxidant system, it seems reasonable to consider that the induction of endogenous protective pathways, such as the Nrf2-ARE pathway against oxidative stress, is a viable strategy for delaying the progression of injury and cell death.

It is clear that understanding the mechanisms of regulation of the Nrf2-ARE pathway and how these mechanisms are impaired in disease is central in deciphering how we can modulate this protective pathway against oxidative stress associated with AD. While several other regulatory pathways of Nrf2-ARE have been described and GSK-3β modulation in AD is also known to be beneficial in several ways, studying the influence of GSK-3β on Nrf2-ARE is certainly an important path to pursue in order to better understand how to combat the oxidative stress associated with AD.

Acknowledgments

The authors thank the Academy of Finland, the Finnish Cultural Foundation, the Orion-Farmos Research Foundation, and the Sigrid Juselius Foundation for their support.

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SAGE-Hindawi Access to Research
International Journal of Alzheimer's Disease
Volume 2011, Article ID 280502, 7 pages
doi:10.4061/2011/280502

Review Article

Glycogen Synthase Kinase 3 Inhibitors in the Next Horizon for Alzheimer's Disease Treatment

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Received 17 March 2011; Accepted 3 May 2011

Academic Editor: Peter Crouch

Glycogen synthase kinase 3 (GSK-3), a proline/serine protein kinase ubiquitously expressed and involved in many cellular signaling pathways, plays a key role in the pathogenesis of Alzheimer's disease (AD) being probably the link between β-amyloid and tau pathology. A great effort has recently been done in the discovery and development of different new molecules, of synthetic and natural origin, able to inhibit this enzyme, and several kinetics mechanisms of binding have been described. The small molecule called tideglusib belonging to the thiadiazolidindione family is currently on phase IIb clinical trials for AD. The potential risks and benefits of this new kind of disease modifying drugs for the future therapy of AD are discussed in this paper.

1. Introduction

After more than a century from its discovery and several decades of intensive research, the cause of Alzheimer's disease (AD) remains yet unknown [1]. As a consequence, it is very difficult to discover and/or develop effective therapies. For this reason, current therapies are mostly palliative and there exists a significant unmet medical need in the treatment of this devastating condition.

Different etiological hypotheses for AD origin have been considered in the last thirty years focused partially on the different histopathological lesions found in the brain of AD patients. The first one was the cholinergic hypothesis that considers the disease as a consequence of a deficit in the neurotransmitter acetylcholine. It is implicated in the cognitive process and consequently to its decline [2]. Although this strategy has severe limitations, drug research in this field has succeeded and today we have on the market four of the five drugs currently approved for the palliative treatment of AD based on drug research following this hypothesis [3]. The acetylcholinesterase inhibitors produced a slight enhancement in patient's cognitive abilities that partially improve temporarily their quality of life, but, in fact, the neurodegenerative disease does not stop [4].

In the eighties the amyloid cascade hypothesis emerged, and it was the most long considered theory [5]. It is based on the β-amyloid overproduction as responsible for the senile plaque formation and for the neurotoxicity that leads to the progressive neuronal death. However, controversial data about if β-amyloid is the cause of the disease or one of the main risk factors for AD are reported [6]. This dilemma is more pronounced recently because of the phase III clinical trials failure of the compounds discovered and developed based on amyloid hypothesis, such tramiprosate [7], tarenflurbil [8], and semagacestat [9].

The third postulated theory at the end of the past century was the tau-based hypothesis [10]. It is based on aberrant tau protein, a microtubule-associated protein that stabilizes the neuronal cytoskeleton, as the origin of AD pathology. As the time estimated to develop a new drug is around fifteen years, there is no time yet to see what happened with drugs specifically developed based on this hypothesis. Currently there are two phase IIb clinical trials with two different compounds, tideglusib and methylene blue. Both compounds have reported some positive results in the increase of cognitive level of AD patients after the first treatments on phase IIa clinical trials [11, 12]. In the meanwhile, intensive research on the physiology and pathology of tau protein leads to the discovery of two kinases responsible for its posttranslational aberrant modifications. After cloning, these kinases were identified more than ten years ago, as the well-known glycogen synthase kinase 3 (GSK-3) and cyclin

dependent kinase 5 (CDK-5), as the enzymes responsible for aberrant hyperphosphorylation of tau protein [13].

2. The GSK-3 Hypothesis for AD

In the last years a proposed alternative theory postulated that GSK-3 may play a more instigative role in the etiology of AD, being the link between β-amyloid and tau protein [14]. It has been suggested that aberrant wnt or insulin signaling results in increased GSK-3 function, and this could be responsible for the observed hyperphosphorylation of tau and the formation of neurofibrillary tangles. This point is of utmost importance when growing evidence considered AD as diabetes type III, which means that a deficit of brain insulin signaling is the cause of AD [15]. In addition, elevated GSK-3 activity may induce increased β-amyloid formation through its action on γ-secretase and thereby give rise to the primary neuropathological lesion observed in AD, the senile plaques [16]. GSK-3 has also been demonstrated to be involved in the mechanism underlying memory and learning, and dysregulation of the enzyme function may explain some of the early cognitive deficiencies observed in AD [17]. Moreover, overactivity of GSK-3 activates microglia through different cell signaling pathways involving, among others, NFκB and leading to the neuronal death [18]. Today there is no doubt about the GSK-3 upregulation in the brains of AD patients, and it is not clear if an overactivation and/or overexpression is the cause of its exacerbated activity [19]. All these observations point directly to GSK-3 as an excellent target to effectively treat all the clinical symptoms present in this devastating neurodegenerative disease. Currently, GSK-3 inhibitors represent promising disease modifying agents for AD neurodegeneration [20], a disease affecting the brain of the patients and the heart of their caregivers.

3. GSK-3 Inhibitors as Drugs for Alzheimer's Disease: Risk versus Benefits

GSK-3 is a proline-directed serine/threonine kinase. It affects the phosphorylation of a wide range of substrates and is involved in the regulation of many and diverse cellular functions, including metabolism, differentiation, proliferation, and apoptosis. GSK-3 is constitutively active, ubiquitous, and essential for life as it is demonstrated in many studies [21]. Its basal activity is regulated by many different mechanisms of action including phosphorylation at different residues (leading to inactive or superactive enzyme functions), protein-protein interactions, and inhibition by endogenous peptides [22]. Our organism is prepared to restore, through compensative mechanism of action, a deficit in the expression and/or activity of the enzyme [23]. However, it is not able to downregulate GSK-3 with an endogenous alternative mechanism of action when this enzyme is exacerbated in different pathological conditions such as AD or diabetes type II. Thus, a smooth inhibition of GSK-3 able to restore levels of activity to physiological ones would be enough to produce an important therapeutic effect.

Moreover, GSK-3 is also known to play a key role in glucose metabolism and was first identified as the enzyme responsible for affecting the inhibitory phosphorylation of glycogen synthase (GS). This inhibitory effect leads to the reduction of rate conversion of glucose to glycogen, giving rise to elevations in blood glucose levels [24]. This function is controlled by insulin that through its binding to its receptor leads indirectly to activation of protein kinase B and subsequent phosphorylation of a key serine residue in the N-terminal domain of GSK-3 inactivating its function [25]. The major mechanism by which insulin stimulates the activity of GS in skeletal muscle is inactivation of GSK-3. Insulin treatment causes about a 50% inhibition of GSK-3 activity, a level sufficient to activate GS in skeletal muscle. Different experiments using ATP-competitive GSK-3 inhibitors showed insulin sensitization without elevation of β-catenin levels. These data suggested that mild inhibition of GSK-3 (30–40%) may be sufficient for insulin sensitization while a much more degree of inactivation (>75%) of GSK-3 may be required for invoking growth-promoting effects [26].

An unrelated mechanism of GSK-3 inhibition operates in the wnt signaling cascade, a cellular pathway involved in controlling cell fate, differentiation, and proliferation. In this system, GSK-3 is complexed with APC, axin, and β-catenin, as well as other proteins [22]. When the wnt system is in a nonstimulated state, GSK-3 phosphorylates axin and APC, the effect of which is to create a more tightly associated complex. CK1, which is also associated with this protein assembly, performs an initial phosphorylation of β-catenin, and then, as priming substrate for GSK-3, it is hyperphosphorylated by this last enzyme resulting in its dissociation from the complex and its subsequent ubiquitination and destruction by proteasome. However, following binding of wnt ligands to their receptors, axin is displaced from the complex with GSK-3 as a result of binding of the latter to FRAT peptide, frequently rearranged in advanced T-cell lymphomas [27]. This leads to the dissociation of the destruction complex, the consequence of which is that β-catenin is no longer effectively phosphorylated and degraded. Cytosolic accumulation of β-catenin promotes its translocation to the nucleus where it binds to DNA and produces the transcription of certain oncogenes. This fact may raise the primary concern for GSK-3 targeted therapeutics and relate to the potential to induce transformation of nonmalignant cells or exacerbate preexisting malignancies through their actions on β-catenin. However, several *in vivo* experiments do not confirm this risk [28].

Apparently GSK-3 inhibition may be associated with significant mechanism-based toxicities, potentially ranging from hypoglycemia to tumorigenesis. However, it is worth mentioning that lithium has been used as standard therapeutic for the treatment of bipolar disorder since the 1950s. This agent is a weak inhibitor of GSK-3 that exerts its effect *in vivo* through a mixed mechanism of direct inhibition and activation of PKC-α, the latter of which leads to increased GSK-3Ser9/21 phosphorylation, the inactivated enzyme state. At therapeutic doses, lithium is estimated to inhibit approximately a 25% of total GSK-3 activity, and this inhibition degree has not been associated with hypoglycemia,

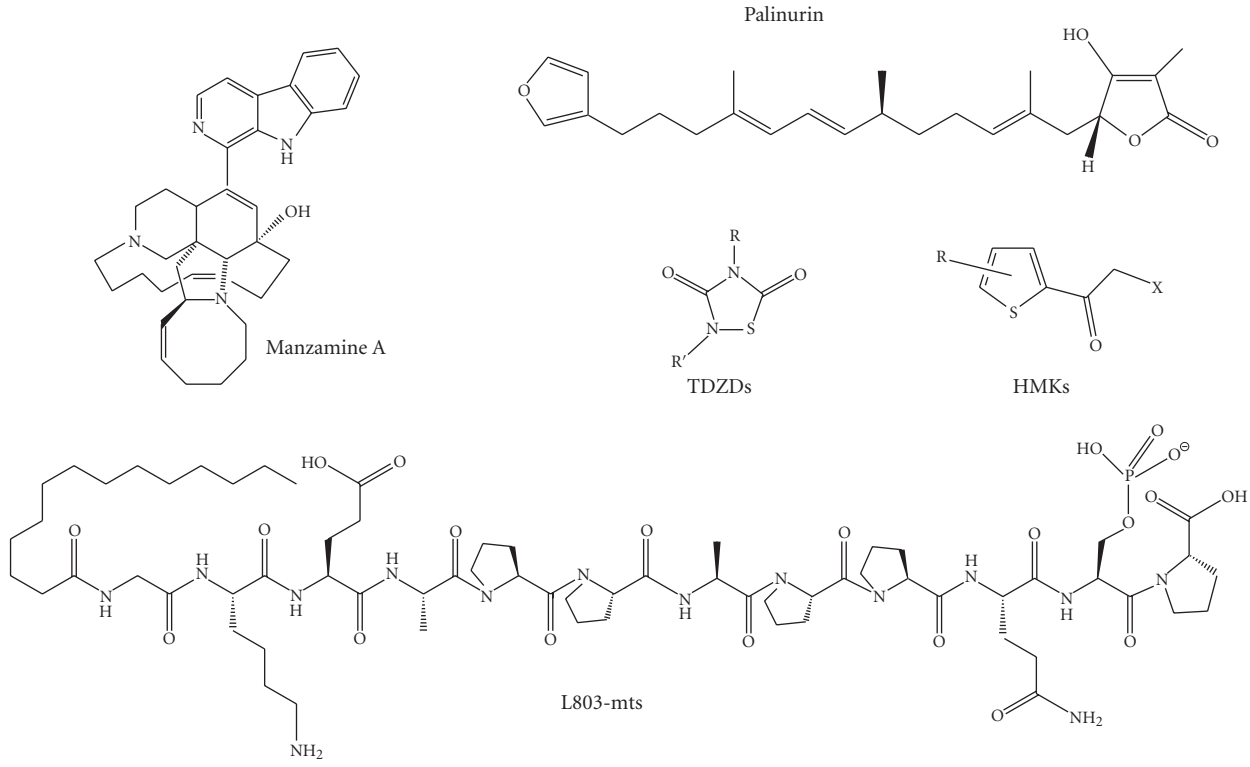

Figure 1: non-ATP competitive GSK-3 inhibitors.

increased levels of tumorigenesis, or deaths from cancer. These epidemiological data are the most compelling argument for the potential safety of GSK-3 inhibitors, even considering that the final resulting *in vivo* GSK-3 inhibition through direct and indirect mechanism of action produced by the GSK-3 inhibitor treatment should be smooth and not greater than 25% of total activity. This means that, in pathological conditions, the GSK-3 inhibitor would be able to decrease the upregulation of the enzyme and, in the case that this treatment would slow down the GSK-3 physiological levels, other compensatory mechanisms of action would play the restorative function.

Another important challenge for a GSK-3 inhibitor as an AD treatment is its specific brain distribution. The drug needs to cross the blood-brain barrier to exert its action in the regulation of exacerbated GSK-3 brain levels. Usually this is not an easy task for any kind of drug, moreover when oral bioavailability is the preferred administration route for chronic AD treatment. It is very difficult to balance the equilibrium between molecular lipophilicity to enter into the brain and molecular hydrophilicity to be orally administrated, and this reason has ruled out several promising GSK-3 inhibitors from the race to the market. In addition, having suitable brain-to-plasma ratios may be determinant in achieving an adequate therapeutic index by limiting the peripheral exposure required to drive CNS efficacy. Once more, a smooth GSK-3 inhibitor is here required because in that case plasma levels of the GSK-3 inhibitor should not decrease more than 25% of physiological enzyme activity to avoid adverse and toxic effects.

4. ATP versus Non-ATP Competitive GSK-3 Inhibitors as Drugs

Over the past ten years a number of chemically diverse families of GSK-3 inhibitors have been discovered. They have been reviewed periodically [29, 30]. The structural chemical diversity of compounds reported in this area has expanded considerably, especially in the patent literature, showing the great interest and expectation that this new kind of powerful drugs has arisen both in pharmaceutical and academic laboratories [31]. However, the great majority of these compounds compete with the ATP in their binding to GSK-3.

Human kinome is formed by more than 500 kinases with a more or less conserved site for binding the ATP, a common phosphate donor molecule used by all of them in their phosphorylating physiological function [32]. Thus, GSK-3 inhibitors that are non-ATP competitive are potentially attractive for several reasons, first of all because they may show better cellular and *in vivo* potency in comparison with competitive inhibitors having comparable absorption-distribution-metabolism-excretion (ADME) properties due to the absence of endogenous ATP competition. In addition, much better kinase selectivity may be expected from inhibitors that bind outside the ATP pocket. Moreover, this kind of kinase inhibitors should have lower values of IC_{50}, which in the case of GSK-3 is not only beneficial but also necessary to avoid toxicity. Thus, non-ATP competitive GSK-3 inhibitors arise as the unique real potential drugs for the treatment of at least chronic diseases as AD.

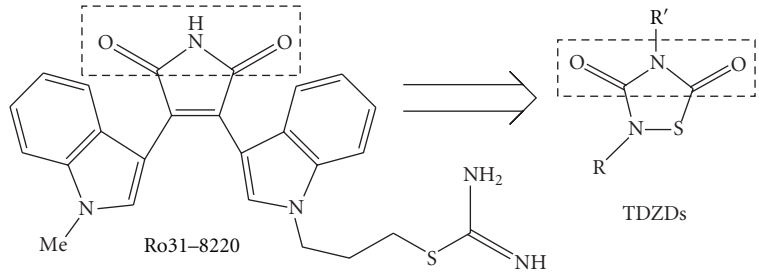

Figure 2: Common features between Ro31–8220 and TDZDs.

There are few GSK-3 inhibitor families reported with non-ATP competitive mechanism of action (Figure 1). The first one was the thiadiazolidindione family (TDZDs) [33]. TDZD-8, commercially available from different sources, has been one of the most useful pharmacological tools in the chemical genetic approach followed by many scientists to explore GSK-3 functions, as it is reflected in the great number of published manuscripts on the field using this small molecule [34, 35]. More important for AD therapy, and probably for society, is tideglusib, a TDZD compound currently on phase IIb clinical trials for AD and the rare tauopathy called progressive supranuclear palsy (PSP).

In addition to TDZDs, two marine natural compounds, the alkaloid manzamine [36] and the sesquiterpene palinurin, have been reported as cell permeable non-ATP competitive GSK-3 inhibitors able to reduce tau phosphorylation in cell cultures [37]. The binding site of manzamine has been recently postulated by molecular modeling studies as an allosteric site at the back of ATP site, just in the junction of C-terminal and N-terminal globes of GSK-3, being the first GSK-3 allosteric modulator described [38]. At the moment, nothing is known about the potential binding site of palinurin although some quantitative structure activity relationship has been reported [39]. These compounds are valuable candidates, with privileged scaffolds provided by the nature, to be considered for further optimization of synthetic, biological, and ADME properties in a drug development process.

Another promising group of GSK-3 inhibitors is those specifically designed to interact with the priming substrate site present on GSK-3 [40]. The ability of GSK-3 for binding to prephosphorylated (priming) substrates and introduce a new phosphate group in the fourth amino acid from the priming site has been considered by Eldar-Finkelman group to provide substrate competitive GSK-3 inhibitors all of which are based on peptide scaffolds [41]. The small peptide L803-mts has proven to be effective in decreasing the early depressive behavior induced by mild traumatic brain injury [42] and in the improvement of glucose homeostasis in obese mice (ob/ob), an animal model of type II diabetes [43].

Finally, irreversible inhibitors of GSK-3 have just been reported representing a good alternative to avoid resistance in a future drug treatment as it is the case in other therapeutic areas as cancer where the therapy with protein kinase inhibitors is more advanced [44]. Halomethylketones (HMKs) are irreversible inhibitors with IC_{50} values in the low micromolar range, able to decrease tau phosphorylation in cell cultures, good kinase and neurotransmitter selectivity, and ability to cross the blood-brain barrier [45, 46]. GSK-3 inhibitor VII, commercially available, belongs to this class of compounds, and it is yet used in chemical genetic approaches for the study of GSK-3. Important is the fact that biophysical methods such mass spectrometry and the use of these last pharmacological tools have proven experimental data to show that Cys199 of GSK-3 is a key amino acid to modulate its activity [47], opening new opportunities for the future design of specific and valuables inhibitors.

5. TDZDs: GSK-3 Inhibitors in Clinical Trials for AD

As mentioned before, the TDZDs were the first non-ATP GSK-3 inhibitors reported in the literature [33]. The inhibitory kinase activity of TDZDs was discovered in a GSK-3-directed program initiated in the late nineties in the Medicinal Chemistry Institute, CSIC. This activity was determined in a radiometric assay incubating together rabbit recombinant GSK-3β enzyme, the peptide derived from the sequence of the human glycogen synthase GS-1 as substrate, and [γ-^{32}P]ATP in the appropriate buffer and temperature [48]. When the GSK-3 inhibition for the known PKC inhibitor Ro31–8220 was reported [49], we decided to include in our screening program some of the side products obtained in our laboratory in the synthesis of biological active compounds such as potassium channel openers [50], muscarinic agonist [51], or acetylcholinesterase inhibitors [52]. The main reason for this decision was the common features present in the chemical structure of TDZDs and Ro31–8220, mainly the 1,3-dicarbonyl moiety in a five-membered ring with a nitrogen atom between both carbonyl groups (Figure 2). The results of the screening showed that three of the four compounds tested had an IC_{50}, the compound concentration that inhibits 50% of the enzyme activity, in the low micromolar range [53]. After the discovery of TDZDs as GSK-3 inhibitors, different compounds with the maleimide core were reported as inhibitors of this versatile enzyme [54, 55], pointing out the relevance of this 1,3-dicarbonyl substitution joined by a nitrogen atom in a pentagonal ring for the interaction with GSK-3. Our extensive SAR studies showed that the thiadiazolidine scaffold is the best one to interact with GSK-3 [56].

All the work done in the hit to lead process and lead selection has been reported recently [57], and currently the clinical development of the most advanced candidate, called tideglusib and previously known as NP031112 or NP-12, is sponsored by a Spanish biotechnological company. In summer 2010, data from the phase II clinical trial of tideglusib were reported showing a trend in the cognition increase of the twenty mild to moderate AD patients treated for 24 weeks [58]. Now, clinical phase IIb trial is ongoing and the orphan drug status has been achieved for the development of tideglusib in the rare tauopathy PSP [59]. The recruitment of patients is closed and the results are expected by the end of this year.

In preclinical studies, the compound has shown an important neuroprotective effect using a kainate excitotoxicity model and i.c.v. and oral administration [60]. Moreover, very relevant for the future benchmark to clinical translation to AD patients are the results obtained in the chronic oral treatment for 3 months performed with tideglusib to the double transgenic APP×tau rodent model [61]. An increase in cognition using the Morris water maze test was observed after the treatment while all the histopathological features present on the mice brain related to AD pathology were reduced, such as the β-amyloid plaque load, the hyperphosphorylation of tau protein, the gliosis, and, what it is more important, the number of neuron deaths both in cortex and hippocampus [62]. Very interesting is the fact recently reported regarding the increase of insulin growth factor 1 (IGF-1) in mice brains, both in wild-type and APP×PS1 mice, after oral treatment with NP-12 for five days [63]. IGF-1 is a potent neurotrophic peptide with therapeutic value for many neurodegenerative diseases including AD pathology [64]. These increased IGF-1 levels in mice would mean that there is not only a direct action of GSK-3 inhibitors in the brain and there is also an effect on some peripheral signalling pathways (as activation of the IGF-1 transporter megalin) that could be modified with this innovative treatment improving the AD pathology.

6. Conclusions

Clear evidence, both in cell and animal models and in AD brain patients, points to GSK-3 as one of the key players with a unique pivotal role in AD pathology. As the upregulation of the enzyme is the fatal cause for neurodegeneration, its inhibitors arise without any doubt as promising drugs for a future disease modifying treatment [65]. Some data suggested that smooth inhibition of the enzyme, not more than 25%, is enough to restore the overactivated levels in the brain to the normal function and it is not too much to promote alternative physiological mechanism to compensate the 25% downactivity in other tissues with normal GSK-3 function.

Although two of the GSK-3 inhibitors which entered the clinical trials in the last five years failed in the first phases of development, it is clear that it was caused by the intrinsic toxicity of these two compounds related to their chemical structure, the potent inhibition on the enzyme, and the ATP competitiveness in their binding to GSK-3. In the meanwhile, NP-12 (tideglusib), the unique ATP noncompetitive GSK-3 inhibitor that entered the clinical trials in May 2006, advances adequately in its long way to become an effective and accessible drug. Moreover, the great interest arising in GSK-3 inhibitors has led to the search for GSK-3 as a potential biomarker not only to follow the treatment progression but also to stratify the patients for better clinical trials design, and it could be possible to diagnose correctly and on time AD neuropathology [66]. All together locate GSK-3 inhibitors in the next horizon for AD treatment.

Acknowledgments

The authors gratefully acknowledge the financial support of Ministry of Science and Innovation MICINN (Project SAF2009-13015-C02-01) and Instituto de Salud Carlos III ISCiii Project no. RD07/0060/0015 (RETICS program). D. I. Perez acknowledges a postdoctoral fellowship from the CSIC (JAE program). There is no commercial conflict to declare.

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