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The Role of Glycogen Synthase Kinase-3 (GSK-3) in Alzheimer's Disease

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1. Introduction

Despite its initial discovery as one of five protein kinases activities found to phosphorylate glycogen synthase (GS) in fractionated extracts of rabbit skeletal muscle (Embi et al., 1980; Hemmings et al., 1981), Glycogen Synthase Kinase 3 (GSK-3) is by no means restricted to a role in glycogen metabolism. Indeed, the enzyme targets a wide variety of proteins involved in signalling, metabolism, structural proteins and a remarkable number of transcription factors and plays a far more pleiotropic role than first imagined (Woodgett, 2006). Genetic analyses and the use of selective inhibitors have shown that GSK-3 plays critical roles in development, metabolic homeostasis, neuronal growth and differentiation (Hur & Zhon, 2010), cell polarity, cell fate and apoptosis. Its unique position in modulating the function of a diverse series of proteins in combination with its association with a wide variety of human disorders, from neurodegenerative diseases, stroke, bipolar disorder to diabetes and cancer, has attracted significant attention to the protein both as a therapeutic target and as a means to understand the molecular basis of these disorders.

In particular, the involvement of GSK-3 in several key pathophysiological pathways leading to Alzheimer's disease (AD) and neurodegenerative diseases has placed this enzyme in a central position in this disorder. Thus, GSK-3 has recently been proposed as a link between the two major pathological pathways in AD, amyloid and tau (Hernández et al., 2010; Muyllaert et al., 2008) and even a "GSK-3 hypothesis of AD", suggesting that GSK-3 might be a casual mediator of the disease, has been put forward (Hooper et al., 2008). This review will focus on describing the key role that GSK-3 plays in AD pathobiology and the use of GSK-3 inhibition as a potential therapeutic approach to treat this disease.

2. GSK-3 structure and regulation

GSK-3 is a highly conserved protein kinase belonging to the CMGC family of serine/threonine protein kinases, as genes encoding the enzyme have been identified in every eukaryotic genome that has been investigated, such as *Dictyostelium discoideum* (Kim et al., 1999), *Xenopus laevis* (Itoh et al., 1995), *Drosophila melanogaster* (Ruel et al., 1993) or parasites such like *Plasmodium falciparum*, *Trypanosoma brucei* or *Leishmania donovani* (Osolodkin et al., 2011). Mammalian GSK-3 is encoded by two genes, *gsk-3a* and *gsk-3β*

(Frame & Cohen, 2001; Grimes & Jope, 2001), that encode proteins of 51 and 47 kDa, respectively and which display 84% overall identity (98% within their catalytic domains), with the main difference being an extra Gly-rich stretch in the N-terminal domain of GSK-3 α (Woodgett, 1990). Mammalian GSK-3 α and β are each widely expressed although some tissues show preferential levels of some of the two proteins. Furthermore, an alternatively splicing event between exons 8 and 9 of GSK-3 β gives rise in neurons to a splice variant (GSK-3 β 2) containing a 13 amino acids insertion within the kinase domain near to the substrate binding pocket (Mukai et al., 2002). How this insertion affects kinase activity or regulation remains unclear, although some differences between GSK-3 β 1 and GSK-3 β 2 isoforms have already been described (see below).

Crystallographic studies have revealed the three-dimensional structure of GSK-3 β (Dajani et al., 2001; ter Haar et al., 2001), having the overall shape common to most kinases, with a small N-terminal lobe mostly consisting of β -sheets and a large C-terminal lobe essentially formed of α -helices (Noble et al., 2005). The ATP binding pocket is located between the two lobes and it is well conserved among kinases (Bain et al., 2007). Very recently, a comparison of the human and parasite GSK-3 ATP binding sites has opened the possibility of developing selective drugs specifically affecting parasite GSK-3 (Osolodkin et al., 2011).

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 amino acids 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-3 β has also provided a model for the binding of pre-phosphorylated 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. GSK-3 β uses the phosphorylated serine or threonine at position +4 of the substrate to align the two domains for optimal catalytic activity (Dajani et al., 2001; ter Haar et al., 2001).

Furthermore, crystal structures of GSK-3 β complexes with interacting proteins FRAT/GBP and axin have allowed defining the molecular basis for those interactions, which play critical roles 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 (Ferkey & Kimelman, 2002; Fraser et al., 2002) but reveal significant differences in the detailed interactions, and identify key residues mediating the differential interaction with both proteins. This ability of GSK-3 β 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 (Dajani et al., 2003).

GSK-3 is ubiquitously expressed and, unlike most kinases, has a relatively high activity in resting, unstimulated cells while it is normally reduced in response to a variety of extracellular stimuli (Frame & Cohen, 2001). In mammals, GSK-3 α 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 as for instance, both isoforms are highly expressed in neural tissue. Neither gene appears to be acutely regulated at the transcriptional level whereas the proteins are controlled post-translationally, largely through protein-protein interactions or by post-translational regulation.

Given that chemical inhibitors of GSK-3 are unable to discriminate between the various GSK-3 isoforms, evaluation of isoform-specific functions it is not possible by using these compounds. However, evidence for isoform-specific roles has now emerged from mouse models (see below). For instance, some recent findings suggest that there are tissue- and isoform-specific roles in regulation of glucose metabolism (Patel et al., 2008; Mol Cell Biol), as GSK-3 α seems to be the predominant regulator of GS and glycogen synthesis in liver whereas GSK-3 β has a more prevalent role within skeletal muscle and pancreas. Also, although the effect of the inserted sequence on kinase activity, substrate specificity or requirement for priming of substrates remains unclear, the neuron-specific alternatively spliced GSK-3 β 2 isoform appears to phosphorylate unprimed residues on tau and MAP1B to a lesser extent than GSK-3 β 1 (Mukai et al. 2002, Wood-Kaczmar *et al.* 2009).

Three decades after its discovery as a protein kinase involved in glycogen metabolism, GSK-3 has 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 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. Control of GSK-3 activity occurs by complex mechanisms that are each dependent upon specific signalling pathways (for a recent review see Medina & Wandosell, 2011).

Early on, GSK-3 was proved to be a dual specificity kinase differentially regulated by tyrosine and serine/threonine phosphorylation (Wang et al., 1994). 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 auto-phosphorylation (Frame & Cohen, 2001; Harwood, 2001). Thus, four different regions and residues have been described in the GSK-3 molecule. First, it has been clearly established that phosphorylation of serine residue at positions 21 in GSK-3 α and 9 in GSK-3 β , correlates with the inhibition of its kinase activity (Frame et al., 2001; Stambolic et al., 1994; Sutherland et al., 1993). Many protein kinases are capable of phosphorylating GSK-3 at this residue, such as Akt, ILK, PKA, p90Rsk (Delcommenne et al., 1998; Fang et al., 2000), 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 but also in other cell types (Cardona-Gomez et al., 2004). In addition, phosphorylation at threonine 43, present only in the isoform GSK-3 β , by ERK also correlates with GSK-3 inhibition (Ding et al., 2005), whereas residues serine 389 and threonine 390 present in GSK-3 β have been shown to be phosphorylated by p38 MAPK (Thornton et al., 2008), increasing the capacity of Ser-9 to be phosphorylated rather than promoting a direct inhibition.

In contrast, tyrosine phosphorylation present in positions 279 in GSK-3 α or 216 in GSK-3 β , correlates with an increase of its kinase activity (Hughes et al., 1993). Different candidate kinases such as Pyk-2 and Fyn have been reported to be able to phosphorylate GSK-3 on this residue *in vitro*, as MEK1/2 has been shown to do it in mammalian fibroblasts (Hartigan et al., 2001; Lesort et al., 1999) or ZAK1 in *Dictyostelium discoideum* (Kim et al., 1999; 2002), although no homologue of this latter kinase has been found in mammals. More recently, it has been suggested that phosphotyrosine in GSK-3 in mammalian systems might arise from a chaperone-dependent intra-molecular autophosphorylation event perhaps regulated by Hsp90 (Cole et al., 2004 Biochem J; Lockhead et al., 2006; Wang et al., 1994). Molecular

dynamics and crystallographic studies clearly suggest that pTyr216 renders the kinase active through interactions with Arg220 and Arg223, stabilizing the activation loop and allowing full substrate accessibility (Buch et al., 2010; Cohen & Goedert, 2004). Very recently, it has been also shown that the extent of phosphorylation at both Ser9 and Tyr216 residues is very similar in both GSK-3 β splice variants, β 1 and β 2 (Soutar et al., 2010).

On the other hand, tyrosine phosphorylation of residue 216 or 279 increased in neuronal cells following exposure to LPA (Sayas et al., 1999) and also upon exposure of neurons to β -amyloid or PrP (Muñoz-Montañó et al., 1997; Perez et al., 2003; Takashima et al., 1998) in a clear correlation with an increase on 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 (Simon et al., 2008). Thus, taken all together, in addition to the reported tyrosine 216/279 autophosphorylation mechanism proposed, some as-yet-unidentified tyrosine kinases and/or phosphatases may also regulate GSK-3 activity by phosphorylation of this particular residue.

Another mechanism of GSK-3 regulation through post-translational modification 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 (Goñi-Oliver et al., 2007). 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 post-mortem brain tissue (Goñi-Oliver et al., 2009a). 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 induces the cleavage of β -catenin at the N-terminus, generating stable and truncated forms which maintain its transcriptional capacity (Abe & Takeichi, 2007). Likewise, GSK-3 truncation is mediated by extracellular calcium and can be inhibited by memantine (Goñi-Oliver et al., 2009b), a NMDA antagonist used for the treatment of Alzheimer's disease. Interestingly, GSK-3 β has also been recently shown to be cleaved at the N-terminus (and subsequently activated) by matrix metallo-proteinase 2 (MMP-2) in cardiomyoblasts (Kanadasamy & Schulz, 2009).

Besides post-translational modifications, GSK-3 activity can also be regulated by protein complex association, for instance through its interaction with structural (scaffold) proteins. It is well known that GSK-3 contributes to Wnt signalling by participating in a multiprotein complex formed by axin, β -catenin and adenomatous polyposis coli (APC), among others (for review see, i.e. Moon et al., 2004). Indeed, in the absence of the Wnt ligand, GSK-3 is able to phosphorylate β -catenin and targeting it for proteasome degradation (Aberle et al., 1997) whereas in its presence GSK-3 is unable to do that, increasing β -catenin cytosolic levels and eventually mediating TCF/LEF-mediated transcription at the nucleus. Recent data suggest that this complex may be specific for the GSK-3 β 2 splice isoform (Castaño et al., 2010). Recent evidence also supports a neuroprotective role for Wnt signaling in neurodegenerative disorders such as AD (Inestrosa & Toledo, 2008).

Moreover, another GSK-3-binding protein (GBP or FRAT) has been reported to regulate GSK-3 enzymatic activity (Itoh et al., 1995; Li et al., 1999). From the three different FRATs that have been cloned and characterized, FRAT1 appears to act as an inhibitory system (Yost et al., 1998) whereas FRAT2 appears to preferentially increase GSK-3-mediated phosphorylation in some residues (Stoothoff et al., 2005). Surprisingly, the triple FRAT-knockout mouse lacks any major defect in brain development (van Amerongen et al., 2005),

which underlines the need to better define the precise role of FRAT in GSK-3 regulation and brain physiology. Furthermore, using the binding site on GSK-3 for FRAT/GBP, a GSK-3-interacting protein (GSKIP) has been identified that can block phosphorylation of different substrates and functions as a negative regulator of GSK-3 β (Chou et al., 2006). Other proteins have also been proposed to further contribute to GSK-3 regulation through physical interaction with it. Thus, DISC-1 (Disrupted In Schizophrenia-1) regulates neural progenitor proliferation via the β -catenin/GSK-3 β pathway, whereby DISC-1 stabilizes β -catenin by inhibiting GSK-3 β activity through a direct binding (Mao et al., 2009). Finally, the dimeric scaffold protein 14-3-3 has been shown to co-elute from brain microtubules together with tau and GSK-3 β and this interaction has been proposed to facilitate the interaction of the kinase with some of its substrates (Agarwal-Mawal et al., 2003).

As already mentioned, an unusual property of GSK3 is that most of its substrates require prior phosphorylation (priming) at a residue 4 or 5 amino acids C-terminal to the target residue (Frame & Cohen 2001), thus providing another mechanism of regulation of the GSK-3 activity. Some priming kinases have been identified, such as cdk5 (Alonso et al., 2006; Noble et al., 2003; Sengupta et al., 1997), PAR-1 (Nishimura et al., 2004), casein kinase I (Amit et al. 2002), PKC (Liu et al., 2003) or PKA (Sengupta et al., 1997). That said, there are examples of unprimed substrates reported, although is not entirely clear as yet whether this second set of unprimed substrates may define a different group of functions (Twomey & McCarthy, 2006).

3. GSK-3 activity in AD brain

Compared to age-matched control samples, increased levels of GSK-3 have been found in post-mortem analysis of brains from AD patients (Pei et al., 1997) while a spatial and temporal pattern of increased active GSK-3 β expression correlating with the progression of neurofibrillary tangles (NFT) and neurodegeneration has also been shown (Leroy et al., 2002). Thus, GSK-3 has been shown to localize to pre-tangle neurons, dystrophic neurites and NFTs in AD brain (Pei et al., 1997). Neurons actively undergoing granulovacuolar degeneration are also immunopositive for active GSK3 β (Leroy et al., 2002). Taken all together, although direct evidence might be lacking, all these studies strongly suggest that GSK-3 β activity is increased in the brains of patients suffering from AD.

GSK-3 β is the major kinase to phosphorylate tau protein both *in vitro* and *in vivo*. Furthermore, GSK-3 β has been proposed as the link between the two major histopathological hallmarks of AD, the extracellular amyloid plaques and the intracellular NFT (Hernández et al., 2010; Ittner and Götz, 2011; Muyllaert et al., 2008). Exposure of primary neuronal cultures to A β induces activation of GSK-3 β , tau phosphorylation and cell death, whereas blockade of GSK-3 expression by antisense oligonucleotides or its activity by lithium inhibits A β -induced toxicity (Alvarez et al., 1999; Hoshi et al., 2003; Wei et al., 2000). GSK-3 β -deficient mice die during embryonic development (Hoeflich *et al.* 2000, Liu *et al.* 2007) whereas GSK-3 β heterozygous (+/-) mice are viable, although they show some neurological abnormalities, including reduced aggression, increased anxiety, reduced exploratory activity, poor memory consolidation and reduced responsiveness to amphetamine (O'Brien *et al.* 2004, Kimura *et al.* 2008, Beaulieu *et al.* 2008). Conversely, transgenic mice over-expressing GSK-3 β result in behavioural changes that appear to recapitulate hyperactivity observed in the manic phase of bipolar disorder (Prickaerts *et al.* 2006).

On the other hand, mice lacking GSK-3 α are viable and develop normally (MacAulay *et al.* 2007), but display enhanced glucose tolerance and insulin sensitivity accompanied by reduced fat mass. Interestingly, GSK-3 α knock-out mice show reduced exploratory activity and aggression, similar to the GSK-3 β heterozygotes, but in addition have decreased locomotion, impaired co-ordination and a deficit in fear conditioning (Kaidanovich-Beilin *et al.* 2009). These different phenotypes in mice lacking one isoform or the other suggest non-redundant functions of the GSK-3 genes in the brain, while the overlapping behavioural problems between GSK-3 α knockout (KO) and GSK-3 β heterozygous (+/-) mice suggest some common substrates.

Furthermore, loss of both GSK-3 isoforms specifically in the brain results in increased self-renewal of neuronal progenitor cells, but reduced neurogenesis (Kim *et al.* 2009), while double GSK3 α/β knock-in mice in which endogenous isoforms are replaced by mutant proteins where Ser21/9 have been mutated to Ala21/9 respectively, thus preventing repression by growth factor signalling, exhibit impaired neuronal precursor cell proliferation (Eom & Jope 2009). These data underscore the critical role that proper regulation of expression and activity of GSK-3 play in the maturation of these cells during mammalian brain development.

However, we must be careful when interpreting data from transgenic or KO animals since some strain specificity has been recently observed, at least in the case of improved insulin sensitivity and hepatic glucose homeostasis phenotype observed upon global inactivation of GSK-3 α (Patel *et al.*, 2011).

All these observations and the ones described below strongly suggest a central role of GSK-3 in AD pathogenesis and have also led to several efforts trying to identify sequence variations in the *gsk-3* gene and its promoter. Despite early reports of a lack of genetic association between the *gsk-3* coding sequence or its promoter with AD (Russ *et al.*, 2001), several groups have now reported this association. Thus, a polymorphism in the promoter region (-50) of the *gsk-3 β* gene appears associated with a two-fold increased risk for sporadic AD when analysing 333 sporadic AD patients and 307 control subjects from Spain (Mateo *et al.*, 2006). More recently, a case-control study has found a rare intronic polymorphism in *gsk-3 β* that occurred twice more frequently in AD patients than in aged healthy controls (Schaffer *et al.*, 2010), strongly supporting the notion of a genetic association of the *gsk-3 β* gene with AD. Furthermore, two additional independent studies have reported synergistic effects (epistasis) between the *gsk-3 β* and either the *MAPT* (tau) genes (Kwok *et al.*, 2008) or the p35 subunit of *cdk5* (Mateo *et al.*, 2009) in late-onset AD, further supporting a genetic association between *gsk-3 β* and AD. Interestingly, a genetic polymorphism that increases the ratio of GSK-3 β 1 to GSK-3 β 2 interacts with tau haplotypes and modifies risk in Parkinson's and Alzheimer's disease (Kwok *et al.* 2005, 2008).

4. The role of GSK-3 in tau phosphorylation

Tau protein is a microtubule-associated protein (MAP) that in normal physiological conditions binds to microtubules (MT), regulating their assembly, dynamic behaviour, and spatial organization (Drechsel *et al.*, 1992; LoPresti *et al.*, 1995). Later on, tau has also been shown to regulate the axonal transport of organelles, including mitochondria (Ebner *et al.*, 1998). Tau is primarily, though not exclusively, a neuronal protein encoded by a single gene but with six major isoforms derived by alternative splicing (Goedert *et al.*, 1989; Himmler *et al.*

al., 1989). The interaction between tau and tubulin is mediated by four imperfect repeat domains (31-32 residues) encoded by exons 9-12 (Lee et al., 1989). Alternative splicing of exon 10 gives rise to isoforms with 3 or 4 binding domains (3R and 4R tau) (Goedert et al., 1989). Adult human brain shows a 1:1 ratio of 3R and 4R isoforms whereas foetal brain, however, only expresses 3R tau, demonstrating developmental regulation of exon 10 splicing. Different brain regions also differ in the relative levels of 3R and 4R isoforms with granule cells in the hippocampal formation reported to have only 3R tau (Goedert et al., 1989). Disturbances in this ratio are a common feature in most neurodegenerative tauopathies, including AD.

Within neurons, tau is predominantly found in axons as a highly soluble phosphoprotein. As mentioned in the case of alternative splicing, phosphorylation is also developmentally regulated, with a high tau phosphorylation level during embryogenesis and early development, when only the shortest of the isoforms is being expressed. By contrast, adult brain expresses all six isoforms with relatively reduced phosphorylation levels compared with the foetal one (see [Hanger et al., 2009] for a review).

Upon abnormal phosphorylation, the microtubule-associated protein tau reduces its affinity for and dissociates from microtubules. In AD brains tau accumulates in the neuronal perikarya and processes as paired helical filaments (PHF). It has been suggested that at the single-cell level the defects start with a modification of tau by phosphorylation, resulting in a destabilization of microtubules giving rise to a "pre-tangle" stage. After this stage, the destabilization of microtubules leads to loss of dendritic microtubules and synapses, plasma membrane degeneration, and eventually cell death (Iqbal et al., 2009).

The knowledge accumulated in the last years strongly suggest that tau-induced neurodegeneration is most likely a consequence of a combination of loss of (tau) function as well as gain of (toxic) function. On one hand, tau detachment from microtubules after hyperphosphorylation (or mutations) causes impaired microtubule function and axonal transport and eventually synaptic dysfunction and neurodegeneration (Jaworski et al., 2010). On the other hand, hyperphosphorylated tau molecules tend to self-assemble into filaments such as PHF or straight filaments (SF) that form the NFT. But hyperphosphorylated tau seems to also have the capacity of sequestering normal tau molecules (and perhaps other microtubule-associated proteins) into the aggregates, which will also have a negative impact on the normal microtubule function. At some point after detaching from microtubules and getting into the aggregation process, tau molecules also suffer other post-translational modifications such as truncation (Delobel et al., 2008; Gamblin et al., 2003; Nvak et al., 1993), glycosylation (Wang et al., 1996), O-GlcNAcylation (Arnold et al., 1996; Hart et al., 1996), and ubiquitination (Baner et al., 1991; Mori et al., 1987), which could also contribute to the pathology. For a recent review on the molecular mechanisms by which tau induces neurodegeneration please refer to (Brunden et al., 2009; Iqbal et al., 2009).

Interestingly, recent data strongly indicates that some soluble, oligomeric (pre-filament, immature filaments) tau species, rather than the tangles, are indeed the pathogenic ones (Bretteville & Planel, 2008, Congdon & Duff, 2008), reminiscent of what has happened in recent years in the amyloid field regarding plaques and intermediate A β oligomers (Haass & Selkoe, 2007; Walsh & Selkoe, 2007). For a very long time, tangles or fibrils have been considered to be the pathological species, but it has become clear now that, much like amyloid plaques, NFT are the final stages of a pathological process, but the real damage might actually be done by some

intermediate hyperphosphorylated, most likely soluble tau species (Brunden et al., 2009; Iqbal et al., 2009; Jaworski et al., 2010). In fact, there is some evidence suggesting that NFT might be protective indeed, as tangle-bearing neurons seem to survive for long periods of time (Andorfer et al., 2005; de Calignon et al., 2009; Morsch et al., 1999). More recently, some novel mechanisms of propagation of tau protein misfolding from the extracellular to the intracellular space, both *in vitro* (Frost et al., 2009) and *in vivo* (Clavaguera et al., 2010) have been described. The demonstration of a link between tau oligomers and brain pathology in animal models has lately sparked the interest of tau immunotherapies (Boutajangout et al., 2010; Kaye & Jackson, 2009; Medina, 2011; Sigurdsson, 2008).

GSK-3 induces tau phosphorylation in several primed and unprimed PHF phosphoepitopes, both *in vitro* and in cell cultures. Activation of the insulin or Wnt signalling pathways increase tau phosphorylation mediated by GSK-3 (Caricasole et al., 2004; Lesort et al., 1999). Furthermore, some genetic studies show an association of Wnt signalling with AD through the low-density lipoprotein receptor-related protein 6 (LRP6), a co-receptor for Wnt signalling, which has been identified as a genetic risk for a subpopulation of late onset AD (De Ferrari et al., 2007). In addition, epidemiological and genetic studies also associate diabetes and insulin resistance with AD (Biessels & Kappelle, 2005; Hamilton et al., 2007; Reiman et al., 2007).

Persistent tau phosphorylation might result in neuritic dystrophy. Lipophosphatidic acid treated neurons result in GSK-3-dependent persistent tau phosphorylation followed by neurite retraction and growth cone collapse (Sayas et al., 2002). Several animal models, which exhibit persistent tau phosphorylation, also display neuritic dystrophy. For instance mice lacking either Reelin, mammalian disabled (mDab1), or VLDLR2 and ApoER2 exhibit persistent tau phosphorylation and have neuritic dystrophy and cytoskeletal abnormalities associated with them (Hiesberger et al., 1999; Sheldon et al., 1997). It is conceivable that persistent phosphorylation by GSK-3 results in neuritic dystrophy and subsequent cytoskeletal breakdown. In *Drosophila*, tau overexpression in combination with phosphorylation by the *Drosophila* GSK-3 homolog *Shaggy*, exacerbated neurodegeneration induced by tau overexpression alone, leading to neurofibrillary pathology (Jackson et al., 2002).

Recent evidence points out to GSK-3 linking tau and neuronal polarity through a protein called CRMP-2 (collapsing response mediator protein-2) which is essential for regulating axon growth and promotes assembly of microtubules (Cole et al., 2004). GSK-3 not only phosphorylates tau but also several CRMPs (Cole et al., 2006), including CRMP-2 (Yoshimura et al., 2005) at Thr514, a residue crucial for controlling its activity. Low levels of phosphorylated CRMP-2 at that residue are present in the growth cone and are associated with axon growth, which is consistent with previous data demonstrating that inhibition of GSK-3 results in enhanced neurite outgrowth (Muñoz-Montaña et al., 1999). These data were substantially backed up by a different group (Jiang et al., 2005) that also found that GSK-3 is spatially regulated, with the ratio of inactive (phosphorylated at S9) versus active (unphosphorylated) being highest in the axon tips, consistent with the fact that higher levels of unphosphorylated CRMP-2 drive axon development, and hence, neural polarity.

5. The role of GSK-3 in A β formation and neurotoxicity

While not universally accepted, the so-called amyloid hypothesis of AD has provided the main conceptual framework for studying the causes of the diseases and developing new therapeutic interventions during the last quarter of century. According to it, the gradual

cerebral accumulation of soluble and insoluble assemblies of the amyloid A β peptide triggers a cascade of biochemical and cellular alterations that produce the clinical phenotype of AD (Hardy & Higgins, 1992; Hardy & Selkoe, 2002; Selkoe, 1991). The reasons for elevated A β levels in most patients with sporadic, late-onset AD are unknown, but recent evidence suggest that these could turn out to include increased neuronal release of A β during some kind of synaptic activity (Selkoe, 2002; 2008).

GSK-3 inhibition *per se* decreases A β production in cells and in an animal model of amyloidosis, as shown using non-isoform selective pharmacological inhibitors such as lithium, kenpaullone as well as small interfering RNA against the α isoform of GSK-3 (Phiel et al., 2003; Su et al., 2004; Sun et al., 2002). The exact mechanism by which this occurs remains unclear and in fact the isoform specificity of the effect on A β production is still highly controversial. However, the observation that amyloid precursor protein (APP) C-terminal fragments accumulate in the presence of these inhibitors suggests that GSK-3 may influence γ -secretase activity. γ -secretase activity is a multiprotein complex that is necessary for the terminal cleavage of APP to generate the A β fragment. Interestingly, inhibition of GSK-3 failed to demonstrate accumulation of C-terminal fragments of the Notch protein, which is also a substrate for γ -secretase (Phiel et al., 2003). Actually, GSK-3 has been shown to bind and phosphorylate presenilin 1 (PS1), the catalytic component of the γ -secretase complex, acting perhaps as a docking protein and regulating phosphorylation of some GSK-3 substrates such as tau and β -catenin (Palacino et al., 2001; Su et al., 2004; Takashima et al., 1998; Tesco & Tanzi, 2002; Twomey & McCarthy, 2006). PS1 has been shown to inactivate GSK-3 through PI3K/Akt signalling, preventing tau phosphorylation and apoptosis. Interestingly, PS1 FAD mutations inhibit PS1-dependent PI3K/Akt signalling, facilitating GSK-3 and thus tau phosphorylation (Baki et al., 2004). Furthermore, APP has also been shown to be a substrate for GSK-3 *in vitro* (Aplin et al., 1996) and *in vivo* (Rockenstein et al., 2007), suggesting a role of GSK-3 in APP transport and maturation (da Cruz e Silva & da Cruz e Silva, 2003; Lee et al., 2003) from the early secretory pathway through the axon terminals, perhaps controlling APP processing. Finally, modulation of the GSK-3 signalling pathway by chronic lithium treatment of transgenic animals has been shown to have neuroprotective effects by regulating APP maturation and processing (Rockenstein et al., 2007).

A substantial body of evidence has established the toxic properties of extracellular A β peptides on neuronal cells (Selkoe, 2008). Non-neuronal cells however are generally resistant to A β treatment, with some exceptions such as endothelial cells and smooth muscle cells (Suhara et al., 2003). On the other hand, oligomers of the A β peptide have been reported to act as antagonists for insulin (Towsend et al. 2007) or Wnt (Magdesian et al. 2008) receptors, resulting in an increase in GSK-3 activity. Also, a PS1 lack of function by mutations such as those present in some familial AD patients has been suggested to result in an increase of GSK-3 activity (Baki et al., 2004).

As mentioned, the aggregation of A β peptide into soluble oligomers is considered an early event in Alzheimer's disease and the presence of these aggregates seems to lead to neurodegeneration in the context of this disease. However, the mechanisms underlying A β -induced neurotoxicity are not completely understood. Although previous studies in mice have suggested that GSK-3 alters A β levels via modulation of APP processing (Phiel et al., 2003; Rockenstein et al., 2007), the direct effects of the enzyme on A β toxicity, and in the adult nervous system, have not been examined in depth. A recent study has tackled this

particular issue of the specific role of GSK-3 in regulating A β 42 toxicity in adult neurons *in vivo*, by modulating its activity in an adult-onset *Drosophila* model of Alzheimer's disease (Sofola et al., 2010). This study shows that GSK-3 inhibition ameliorates A β 42 toxicity in adult flies, and also highlights a novel mechanism of protection by which GSK-3 directly regulates A β 42 levels in the absence of any effects on APP processing.

6. The role of GSK-3 in synaptic plasticity, learning and memory

GSK-3 has also been shown to phosphorylate and inhibit kinesin-mediated motility. Fast axonal transport misregulation has been hypothesized to play a role in Alzheimer's disease pathogenesis (Morfini et al., 2002). Fibrillar A β binds to and induces the clustering of the integrin receptors, leading to the activation of paxillin and focal adhesion kinases. Interestingly, active GSK-3 associates with focal adhesion proteins suggesting the possibility that GSK-3 might mediate neuritic dystrophy via these interactions (Grace & Busciglio, 2003). Abnormal increase in GSK-3 activity has been shown to cause neurodegeneration and interfere with synaptic plasticity (for review see Bhat & Budd, 2002; Bhat & Froelich-Fabre, 2004).

Another important aspect of GSK-3 function is its role in the assembly and disassembly of synapses determining synaptic plasticity. Regarding memory, some states of synaptic plasticity may be considered as a balance between long-term potentiation (LTP) and long-term depression (LTD), with the former strengthening synaptic connections and the latter weakening them. Interestingly, GSK-3 appears to be a key factor in swaying that balance (Hooper et al., 2007; Peineau et al., 2007) since after LTP induction, GSK-3 becomes temporarily inactivated, support for LTD is lost and LTP comes out on top. This is relevant from the drug discovery point of view, since it implies that inhibition of GSK-3 might boost LTP and depress LTD, in principle a good thing for learning and memory. The precise molecular mechanism by which GSK-3 influences these processes remains to be elucidated, although some preliminary data seems to suggest that installation or maintenance of AMPA receptors might play a role (Peinau et al., 2007). Several GSK-3 downstream substrates such as CRMP-2 or the cAMP responsive element-binding protein (CREB) are also involved in synaptic remodelling, a key process required for memory formation. All this evidence has led to propose that GSK-3 acts as a gate through which LTP and memory are established (Hooper et al., 2008) and that memory failure in AD may be due to the inhibition of LTP by GSK-3 overactivity, with neuronal loss ensuing during disease progression.

It is well established that A β oligomers inhibit LTP and enhance LTD (Shieh et al., 2003; Walsh et al., 2002; Selkoe, 2008), although the precise mechanisms by which A β interferes with long-term plasticity have remained largely unknown. Very recently, GSK-3 has revealed as a key enzyme in mediating A β -induced LTP inhibition (Jo et al., 2011). In this study, treatment of rat hippocampal slices with A β oligomers induced caspase 3-mediated cleavage of Akt-1, resulting in GSK-3 activation. Consistent with it, treatment with a GSK-3 inhibitor completely prevented A β oligomers from inhibiting LTP.

7. Lithium as a GSK-3 inhibitor

The finding that the mood stabilizing drug lithium directly inhibited GSK-3 initially sparked the interest for this enzyme as a potential target for mood disorders. Lithium and valproic

acid are mood stabilizers widely used in the chronic treatment of bipolar disorders. Lithium ions directly inhibit GSK-3 (Klein & Melton, 1996), most likely by competing with magnesium, while valproic acid inhibits GSK-3 activity in relevant therapeutic concentrations in human neuroblastoma cells (Chen et al., 1999), most likely through an indirect mechanism (Rosenberg, 2007).

The mechanism of action by which lithium exerts its therapeutic effects is not known but it is conceivable that the acute effects on GSK-3 results in changes in gene regulation and cellular changes which could affect the neuronal plasticity over time (Gould & Manji, 2002; Jope, 1999; Lennox & Hahn, 2000). Lithium also inhibits at least four phosphomonoesterases (including inositol monophosphatase) (York et al., 1995), and phosphoglucomutase (Ray & Szymanski, 1978; Stambolic & Woodgett, 1994), apart from GSK-3 (Klein & Melton, 1996; Li-Smerin et al., 2001). That said, GSK-3 is significantly inhibited at therapeutic lithium concentrations (Gould & Manji, 2002; Phiel et al., 2003; Shaldubina et al., 2001). Thus, if a significant proportion of lithium's therapeutic actions in bipolar disorder results from the inhibition of GSK-3, then this enzyme would be an important target for bipolar disorder (Li et al., 2002; Rowe et al., 2007).

In spite of these attributes, lithium has a narrow therapeutic window (blood serum levels 0.6 to 1.2 mM) above which side effects are intolerable. Overdose can lead to severe neurological dysfunction and in some cases death. Non-CNS side effects of lithium (not uncommonly within therapeutic levels) include tremor, polyuria, polydipsia, nausea, and weight gain. Lithium can have adverse reactions with other drug classes including diuretics, NSAIDs, and other drugs that alter kidney function (see Gould & Manji, 2006 for a review).

There are only a few observational studies that have attempted to address the clinical effect of lithium in patients with AD. A retrospective study with a large sample of patients with dementia resulted in an increased risk of AD in patients who had been treated with lithium within 4 years prior to diagnosis (Ayuso-Mateo et al., 2001), although it is possible that this is partially accounted for by the increased occurrence of depression associated with AD. Moreover, a single case study reported in dementia patient showed that lithium treatment alleviated symptoms of aggression and agitation, while cognition persisted after 1.5 years of treatment (Havens et al., 1982). Furthermore, a significantly increased global cognitive ability as measured by MMSE in non-demented patients appears associated with lithium intake (Terao et al., 2006). The study design and low sample size precludes however to draw any causative conclusion from those studies.

Some pilot studies have been carried out to directly address the effect of lithium treatment in AD patients. An open label feasibility and tolerability study on a small cohort of 22 subjects patients receiving a low dose of lithium was carried out in UK, reported a high discontinuation rate despite few, relatively mild and reversible side effects (MacDonald et al., 2008). A second randomized, single-blind, placebo-controlled, parallel group, multicentre 10-week study was carried out in Germany as a proof-of-principle (Hampel et al., 2009). A total of 71 patients with mild AD (MMSE scores between 21 and 26) were treated with lithium or placebo for 10 weeks after which neuropsychological and neuropsychiatric assessment was performed together with some biomarkers determinations in plasma (A β 1-42), lymphocytes (GSK-3 activity) and CSF (total tau, phospho-tau, and A β 1-42). In spite of the fact that lithium plasma levels were within the therapeutic range, no treatment effect was observed in any the cognition assessment scales used or the selected biomarkers. Given the short time of treatment of this study, the possibility that lithium has long-term effects on cognition or any other biomarker in AD remains to be tested.

8. Development of GSK-3 inhibitors and their therapeutic potential

The unique position of GSK-3 as a pivotal and central player in the pathogenesis of both sporadic and familial forms of AD has attracted significant attention to this enzyme as a therapeutic target and also as a means to understand the molecular basis underlying AD and related disorders. This has led to the synthesis of a high number of GSK-3 inhibitors, some of which are currently being tested in phase II proof-of-concept clinical trials (Mangialasche et al., 2010; Medina & Avila, 2010). Inhibition of GSK-3 with small molecules would be expected to slow down progression of neurodegeneration in AD and perhaps other tauopathies as well.

A number of novel potent and fairly selective small-molecule inhibitors of GSK-3 activity from different chemical families have recently been described, including hymenialdisine, indirubins, paullones, maleimides, amino pyrazoles, thiazoles, and 2,4-disubstituted thiadiazolidinones (TDZD) (reviewed in Medina & Castro, 2008). Most of them are ATP-competitive inhibitors, although more recently new small molecule derivatives that exhibit substrate competitive inhibition activity toward GSK-3 have been reported. Since the different GSK-3 isoforms display a high degree of homology within the ATP binding site, inhibitors are unable to exhibit isoform selectivity, as they all show similar potencies towards purified GSK-3 α and GSK-3 β .

Although the ATP-competitive inhibitors occupy the general area of the highly conserved ATP-binding site, they do explore other available space nearby depending upon their structure and it is possible to obtain selective inhibitors by taking advantage of the small differences that exist between the different kinases. Crystal structures of GSK-3 β complexed with a variety of ligands, together with molecular modelling approaches, provide the necessary clues for enhancing selectivity towards GSK-3 (Patel et al., 2007; ter Haar et al., 2001). All ATP-binding site inhibitors make hydrogen bonds with backbone atoms of the kinase domain hinge (residues Asp 133 to Thr 138). The hydrogen bonds are the same as observed with ATP although different inhibitors make different combinations of hydrogen bonds. For instance, the two indirubin complexes (PDB 1UV5 and 1Q41) have four hydrogen bonds. In contrast, the Alsterpaullone complex (PDB 1Q3W) only has three hydrogen bonds (with the two backbone atoms of Val 135).

Some GSK-3 inhibitors also target other areas of the ATP pocket. For instance the nitro-group of the Alsterpaullone (PDB 1Q3W) and the chlorine of I-5 (3-anilino-4-arylmaleimide) interact with the conserved catalytic lysine, Lys 85. The bromine atom of 6-bromoindirubin (PDB 1UV5) is buried in the hydrophobic pocket of GSK-3 β between residues Leu 132, Leu 130 and Met 101. This is a pocket that is often targeted to increase the selectivity of the inhibitor since it is one of the most diverse areas in the ATP-binding site of kinases and has been successfully used for instance to increase the selectivity in favour of p38 α over ERK2. The GSK-3 β ATP-binding site inhibitors do not cover the γ -phosphate transfer area. Targeting this part of the ATP-binding site does not appear to improve the selectivity of the inhibitor, although it may improve the potency as additional contacts between the inhibitor and the protein are established (ter Haar et al., 2006).

Some physiological peptides act as GSK-3 inhibitors, including GBP, a maternal *Xenopus* GSK-3 binding protein homologous to a mammalian T cell proto-oncogene (Yost et al., 1998) and p24, a heat resistant GSK-3 binding protein (Martín et al., 2002). That finding led to a synthetic strategy to develop new inhibitors, such as L803-mts, a peptidic inhibitor that binds to the substrate site (Plotkin et al., 2003). L803-mts has been more recently used to

examine the impact of long-term *in vivo* inhibition of GSK-3 and its effects in specific tissues (Kaidanovich-Beilin & Eldar-Finkelman, 2006).

One classical approach for identifying GSK-3 inhibitors has exploited screening programs specifically aimed at finding new hits among compounds that exhibit other pharmacological profiles. However, the availability of X-ray crystallographic data of GSK-3 β and several of its complexes with different inhibitors (ter Haar, 2006) in recent years has enabled the application of rational drug optimisation programs to discover new lead compounds. Molecular docking studies on the inhibitors of GSK-3 kinase in the enzyme binding sites of the X-ray complexes studies provide valuable insights into computational strategies useful for the identification of potential GSK-3 inhibitors (Gadakar et al., 2007). As a result of the great amount of information concerning current GSK-3 inhibitors, there are a huge number of reported empirical structure-activity relationships (SAR) that may guide a rational design of more potent and selective inhibitors. However, only a few studies based on Quantitative Structure-Activity Relationships (QSAR) are available for predicting the inhibitor potency against this specific kinase, and they involve mainly molecular modelling and 3D-QSAR (Medina & Castro, 2008).

The last few years have seen the synthesis of quite a number of fairly selective, potent GSK-3 inhibitors which have started to show *in vivo* efficacy in a diverse array of animal models of human diseases, including Alzheimer's disease. Despite the challenges faced by this approach with respect to safety and specificity, a number of efforts are underway to develop kinase inhibitors and in fact, Noscira's tideglusib (NP12), is already in phase II clinical trials for the treatment of both Alzheimer's disease and progressive supranuclear palsy (PSP), a tauopathy (Medina & Castro, 2008; Medina & Avila, 2010).

9. Conclusion

Three decades after its discovery as a protein kinase involved in glycogen metabolism, GSK-3 has revealed as a cellular nexus, integrating several signalling systems, including several second messengers and a wide selection of cellular stimulants. Modulation of its activity has also turned out to be much more complex than originally thought as control of GSK-3 activity occurs by complex mechanisms that are each dependent upon specific signalling pathways, including post-translational modifications, protein complex formation and subcellular localization. Although there seems to be a good degree of functional overlapping between the different isoforms, some tissue- and isoform-specific functions and substrates are starting to emerge and more will most likely be discovered within the next few years and will open the possibility to design better, more specific inhibitors.

Deregulation or abnormal GSK-3 activity appears to be associated with various relevant pathologies, including Alzheimer's disease, as the enzyme is uniquely positioned as a key, central player in AD pathogenesis, having a critical role in key events such as tau phosphorylation, A β formation and neurotoxicity, microtubule dynamics, synaptic plasticity, neuritic dystrophy, cognition, neuronal survival, and neurodegeneration. Furthermore, recent reports point out to a genetic association of the *gsk-3* gene with the risk of AD either by itself or synergistically with tau or cdk5 genes.

Drug discovery and development efforts for AD in the last two decades have primarily focused on targets defined by the amyloid cascade hypothesis, so far with disappointing results, underscoring the need of novel therapeutic approaches and targets. A significant effort has being made in the last few years to synthesize a high number of fairly selective,

potent GSK-3 inhibitors, while some of them have shown *in vivo* efficacy in various animal models of AD. Some of the known drug discovery and development challenges will be faced: lack of good predictive animal models, lack of good validated biomarkers of disease progression, clinical trial design, early diagnosis and treatment, definition of target population, difficulties in demonstrating disease modifying effects, etc. Despite the challenges faced by this approach with respect to safety and specificity, a number of efforts are underway to develop GSK-3 inhibitors as useful drugs for the treatment of AD as some compounds have already reached phase II clinical trials and some proof-of-concept studies are currently ongoing or planned.

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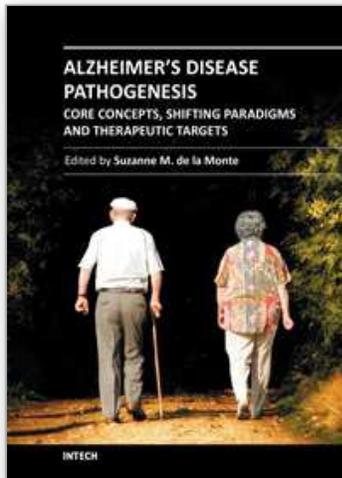
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Alzheimer's Disease Pathogenesis: Core Concepts, Shifting Paradigms, and Therapeutic Targets, delivers the concepts embodied within its title. This exciting book presents the full array of theories about the causes of Alzheimer's, including fresh concepts that have gained ground among both professionals and the lay public. Acknowledged experts provide highly informative yet critical reviews of the factors that most likely contribute to Alzheimer's, including genetics, metabolic deficiencies, oxidative stress, and possibly environmental exposures. Evidence that Alzheimer's resembles a brain form of diabetes is discussed from different perspectives, ranging from disease mechanisms to therapeutics. This book is further energized by discussions of how neurotransmitter deficits, neuro-inflammation, and oxidative stress impair neuronal plasticity and contribute to Alzheimer's neurodegeneration. The diversity of topics presented in just the right depth will interest clinicians and researchers alike. This book inspires confidence that effective treatments could be developed based upon the expanding list of potential therapeutic targets.

How to reference

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