

GSK-3 α/β kinases and amyloid production *in vivo*ARISING FROM C. J. Phiel, C. A. Wilson, V. M.-Y. Lee & P. S. Klein *Nature* **423**, 435–439 (2003)

A major unresolved issue in Alzheimer's disease is identifying the mechanisms that regulate proteolytic processing of amyloid precursor protein (APP)—glycogen synthase kinase-3 (GSK-3) isozymes are thought to be important in this regulation. Phiel *et al.*¹ proposed that GSK-3 α , but not GSK-3 β , controls production of amyloid¹. We analysed the proteolytic processing of mouse and human APP in mouse brain *in vivo* in five different genetic and viral models. Our data do not yield evidence for either GSK-3 α -mediated or GSK-3 β -mediated control of APP processing in brain *in vivo*.

GSK-3 β is believed to be central to the pathogenesis of Alzheimer's disease, linking amyloid and tau pathology^{2–5}. Unlike GSK-3 β , neither physiological functions nor pathological roles of GSK-3 α are well explored⁶. To analyse the function of both GSK-3 kinases in brain, we generated mice that were completely deficient in GSK-3 α (denoted as *Gsk3a*^{KO}) as well as mice with neuron-specific GSK-3 α or GSK-3 β deficiency using *Cre/loxP* as for presenilin 1 (ref. 7).

The contribution of GSK-3 α to the processing of APP was analysed by Phiel *et al.*¹ by silencing with short interfering RNA in cells and by pharmaceutical inhibition with Li⁺ ions (an inhibitor of both GSK-3 isozymes). We approached this problem genetically by generating two strains of GSK-3 α -deficient mice, denoted as *Gsk3a*^{KO} and *Gsk3a*^{n-/-} (complete and neuron-specific GSK-3 α deficiency, respectively). In *Gsk3a*^{KO} mice, the gene was inactivated completely in all tissues, demonstrated by genotyping, immunohistochemistry and western

blotting (data not shown). In the brains of *Gsk3a*^{n-/-} mice, some residual activity was expected because GSK-3 enzymes are expressed in non-neuronal cell types in the CNS^{5,8} (Fig. 1). GSK-3 α deficiency had no evident impact on GSK-3 β levels, demonstrating their independent regulation (Fig. 1).

First, we analysed biochemically the proteolytic processing of endogenous mouse APP in brains of adult *Gsk3a*^{n-/-} mice by measuring the APP metabolites in total brain extracts by validated methods^{5,7,9–11}. Neuronal deficiency of GSK-3 α did not affect levels of immature and mature APP, APP phosphorylated at T668 (pT668), secreted ectodomain APP, nor of carboxy-terminal fragments (Fig. 1a). Levels of mouse amyloid peptides in the brain, measured by specific enzyme-linked immunosorbent assay (ELISA), were unaffected in *Gsk3a*^{n-/-} mice relative to age- and gender-matched control mice with floxed *Gsk3a* genes but not expressing Cre recombinase (Fig. 1a).

We went on to administer AAV-APP.SLA viral vectors (adeno-associated viral vector expressing triple mutant APP with Swedish, London and Austrian mutations) by intra-hippocampal injection into *Gsk3a*^{n-/-} mice, to analyse the processing of human mutant APP.SLA (ref. 9). At 3 weeks after injection, biochemical analysis of hippocampal extracts of AAV-APP.SLA-injected *Gsk3a*^{n-/-} mice revealed no significant differences for any of the APP metabolites (Fig. 1b).

We continued to analyse mice with a complete deficiency in GSK-3 α generated serendipitously during the expansion of the *Gsk3a*^{n-/-}

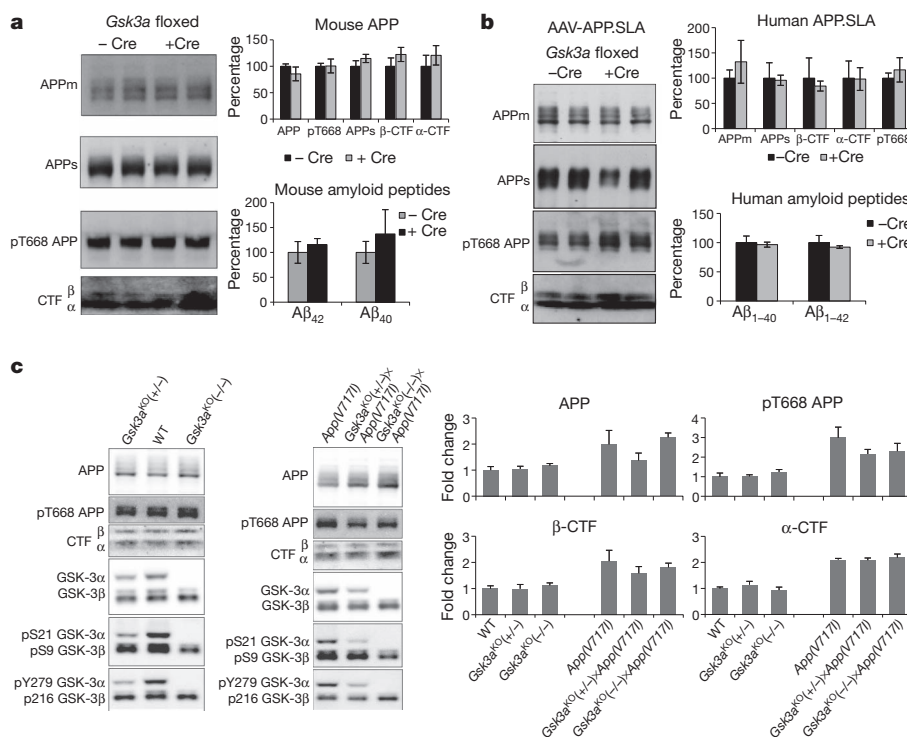


Figure 1 | APP processing in the brains of *Gsk3a*^{n-/-} and *Gsk3a*^{KO} mice. **a**, Biochemical analysis of murine APP metabolites in total brain homogenates by western blotting. Mouse amyloid peptides were measured by specific ELISA. APpm, membrane-bound APP; APPs, secreted APP; Cre, Cre recombinase; CTF, C-terminal fragments; floxed, recombinant genes with inserted *loxP* elements⁷. **b**, Biochemical analysis of hippocampi at 3 weeks after intracerebral

injection of AAV-APP.SLA. Human amyloid peptides were measured by specific ELISA⁷. **c**, Analysis of murine APP metabolites in the brains of *Gsk3a*^{KO} mice and of human APP metabolites in *Gsk3a*^{KO} x *App(V717I)* bigenic mice, heterozygous (+/-) or homozygous (-/-) for GSK-3 α deficiency. All data are mean $\pm$ s.e.m.

colony (data not shown). In addition, we generated bigenic mice by crossing the *Gsk3a*^{KO} mice with our *App*(V717I) mice, a validated model for amyloid pathology^{5,7,9–11}. We carried out a biochemical analysis of metabolites of mouse APP and human mutant APP in brains of *Gsk3a*^{KO} mice and *Gsk3a*^{KO} × *App*(V717I) bigenic mice, respectively. None of the APP metabolites derived from either mouse or human APP was affected by the complete deficiency of GSK-3α (Fig. 1c).

We subsequently extended the study to the GSK-3β isozyme by generating *Gsk3b*^{n/n} mice with a neuron-specific deficiency of GSK-3β. GSK-3β deficiency had no evident impact on the expression of the GSK-3α isozyme, further corroborating their independent regulation (Fig. 2). Notably, no significant deviation was noted in the brain levels of mouse APP metabolites in *Gsk3b*^{n/n} mice (Fig. 2).

In addition, we attempted to generate mice deficient in both GSK-3 isozymes in their central neurons. The combination of mice containing both floxed *Gsk3* genes and the *Thy1*-Cre recombinase transgene yielded 354 offspring over a time span of 16 months that were all genotyped for the five genes of interest: wild-type and floxed GSK-3 isozymes and *Thy1*-Cre. None of the pups contained the wanted combination with both *Gsk3* genes recombined homozygously. The outcome proved, not unexpectedly, that complete deficiency of GSK-3 activity in central neurons is lethal for mice.

We conclude that the GSK-3 isozymes do not contribute significantly to the processing of APP in mouse brain *in vivo*. Of interest, the combination of *Gsk3b*^{n/n} with *App*(V717I) did not yield viable offspring (data not shown), in contrast to viable offspring yielded by the *Gsk3a*^{KO} × *App*(V717I) combination (Fig. 1c). This underlines

the substantial functional difference of the GSK-3 isozymes in brain; a difference that we believe is not, however, related to APP processing. The combined outcome substantiates the notion that GSK-3β is physiologically and pathologically the dominant isozyme, notwithstanding the fact that both are activated by amyloid⁵. The latter point testifies that both kinases contribute, downstream of APP, to tauopathy and cognitive defects in bigenic and viral mouse models^{3,5,12,13} (data not shown) and by extrapolation in human disease^{2,4,13}.

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- Phiel, C. J., Wilson, C. A., Lee, V. M. & Klein, P. S. GSK-3α regulates production of Alzheimer's disease amyloid-β peptides. *Nature* **423**, 435–439 (2003).
- Ishiguro, K. *et al.* Glycogen synthase kinase 3β is identical to tau protein kinase I generating several epitopes of paired helical filaments. *FEBS Lett.* **325**, 167–172 (1993).
- Spittaels, K. *et al.* GSK3β phosphorylates protein tau and rescues the axonopathy in the central nervous system of human four-repeat tau transgenic mice. *J. Biol. Chem.* **275**, 41340–41349 (2000).
- Takashima, A. GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J. Alzheimers Dis.* **9** (Suppl.), 309–317 (2006).
- Terwel, D. *et al.* Amyloid activates GSK-3β to aggravate neuronal tauopathy in bigenic mice. *Am. J. Pathol.* **172**, 786–798 (2008).
- Kaidanovich-Beilin, O. *et al.* Abnormalities in brain structure and behavior in GSK-3α mutant mice. *Mol. Brain.* **2**, 35 (2009).
- Dewachter, I. *et al.* Neuronal deficiency of presenilin 1 inhibits amyloid plaque formation and corrects hippocampal long-term potentiation but not a cognitive defect of amyloid precursor protein [V717I] transgenic mice. *J. Neurosci.* **22**, 3445–3453 (2002).
- Perez-Costas, E., Gandy, J. C., Melendez-Ferro, M., Roberts, R. C. & Bijur, G. N. Light and electron microscopy study of glycogen synthase kinase-3β in the mouse brain. *PLoS ONE* **5**, e8911 (2010).
- Jaworski, T. *et al.* AAV-tau mediates pyramidal neurodegeneration by cell-cycle re-entry without neurofibrillary tangle formation in wild-type mice. *PLoS ONE* **4**, e7280 (2009).
- De Strooper, B. *et al.* Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391**, 387–390 (1998).
- Moechars, D. *et al.* Early phenotypic changes in transgenic mice that overexpress different mutants of amyloid precursor protein in brain. *J. Biol. Chem.* **274**, 6483–6492 (1999).
- Tanghe, A. *et al.* Pathological hallmarks, clinical parallels, and value for drug testing in Alzheimer's disease of the APP[V717I] London transgenic mouse model. *Int. J. Alzheimers Dis.* **2010**, 417314 (2010).
- Jaworski, T. *et al.* Alzheimer's disease: old problem, new views from transgenic and viral models. *Biochim. Biophys. Acta* **1802**, 808–818 (2010).

Author Contributions Generation of transgenic and knockout mice, B.L., D.D., H.D., S.P. and J.R.W.; generation of AAV vectors and intracerebral injections, T.J., D.D. and S.K.; brain analysis, T.J., I.D., B.L., M.G., A.K. and P.B.; design of experiments, data analysis and figures, T.J., I.D., P.B. and F.V.L.; writing of manuscript, T.J. and F.V.L. T.J., I.D. and B.L. contributed equally to this work.

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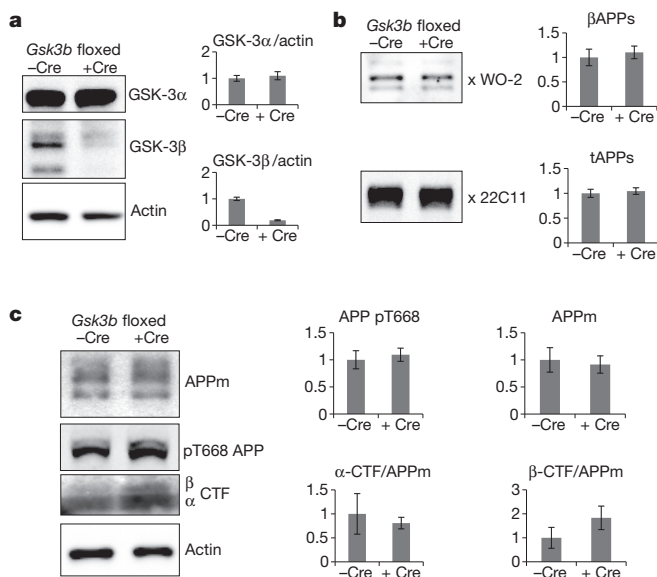


Figure 2 | APP processing in the brains of *Gsk3b*^{n/n} mice. **a**, Western blot of GSK-3 in brain homogenates of mice with floxed *Gsk3b* genes, with and without expression of Cre recombinase. **b**, **c**, Biochemical analysis of proteolytic processing of mouse APP in the brains of *Gsk3b*^{n/n} mice. All data are mean ± s.e.m. The y axes of graphs in **a–c** show relative percentage. βAPPs, β-secretase cleaved secreted APP; tAPPs, total secreted APP; WO-2, monoclonal antibody specific for the amyloid sequence in APP and amyloid peptides; 22C11, monoclonal antibodies specific for N-terminus of APP.

Phiel *et al.* replyREPLYING TO T. Jaworski *et al.* *Nature* **480**, doi:10.1038/nature10615 (2011)

GSK-3 has been implicated in the pathogenesis of Alzheimer's disease through regulation of tau phosphorylation, cellular responses to amyloid- β and processing of amyloid precursor protein (APP) to amyloid- β . We previously reported¹ that inhibition of GSK-3 reduces the accumulation of A β ₄₀ and A β ₄₂ (amyloid- β peptides of 40 and 42 amino acids) in mouse brain and cell culture and that knockdown of *Gsk3a* reduces amyloid- β accumulation in CHO cells, indicating that *Gsk3a* contributes to the processing of APP in this context¹. At about the same time, others reported that knockdown of *Gsk3b* reduces amyloid- β production and overexpression of active *Gsk3b* enhances amyloid- β production^{2–4}. A reasonable explanation for these findings, as suggested previously^{2,5,6}, was that both of these highly similar enzymes contribute to APP processing and their respective contributions depend on cell type, relative abundance and assay conditions. Jaworski *et al.*⁷ now show that APP can be processed in mouse brain in the absence of either *Gsk3a* or neuronal *Gsk3b*.

However, *Gsk3a* and *Gsk3b* are highly homologous and frequently redundant genes^{8,9}. For this reason, it is not possible to exclude a role for GSK-3 with single gene knockouts. Furthermore, structurally and mechanistically diverse GSK-3 inhibitors reduce amyloid- β accumulation *in vivo* and in cell culture, including lithium, kenpaullone, bisindolylmaleimide I, FRAT peptide, the TDZD-related NP12 and kinase-dead GSK-3 (refs 1–6, 10, 11). These compounds inhibit both GSK-3 α and GSK-3 β and therefore get around the issue of redundancy. Although off-target effects for these diverse GSK-3 inhibitors could, formally, explain their effects on APP processing, a far more plausible explanation is that these compounds act through GSK-3 inhibition.

Jaworski *et al.*⁷ show that APP can be processed in mouse brain in the absence of either *Gsk3a* or neuronal *Gsk3b*, but do not examine redundant functions of *Gsk3a* and *Gsk3b* in APP processing and do not consider the substantial body of work showing that acute inhibition of GSK-3 reduces amyloid- β accumulation *in vivo*. The simplest explanation for these findings^{2,5,6} is that both *Gsk3a* and *Gsk3b* can contribute to APP processing and that inhibition of GSK-3 reduces amyloid- β in Alzheimer's disease models.

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1. Phiel, C. J., Wilson, C. A., Lee, V. M. & Klein, P. S. GSK-3 α regulates production of Alzheimer's disease amyloid- β peptides. *Nature* **423**, 435–439 (2003).
2. Ryder, J. *et al.* Divergent roles of GSK3 and CDK5 in APP processing. *Biochem. Biophys. Res. Commun.* **312**, 922–929 (2003).
3. Li, B. *et al.* FRAT1 peptide decreases A β production in swAPP₇₅₁ cells. *FEBS Lett.* **553**, 347–350 (2003).
4. Su, Y. *et al.* Lithium, a common drug for bipolar disorder treatment, regulates amyloid- β precursor protein processing. *Biochemistry* **43**, 6899–6908 (2004).
5. Huang, H. C. & Klein, P. S. Multiple roles for glycogen synthase kinase-3 as a drug target in Alzheimer's disease. *Curr. Drug Targets* **7**, 1389–1397 (2006).
6. Huang, H. C., O'Brien, W. T. & Klein, P. S. Targeting glycogen synthase kinase-3 in Alzheimer's disease. *Drug Discov. Today Ther. Strateg.* **3**, 613–619 (2006).
7. Jaworski, T. *et al.* GSK-3 α / β kinases and amyloid production *in vivo*. *Nature* **480**, doi:10.1038/nature10615 (2011).
8. Doble, B. W. & Woodgett, J. R. GSK-3: tricks of the trade for a multi-tasking kinase. *J. Cell Sci.* **116**, 1175–1186 (2003).
9. Doble, B. W., Patel, S., Wood, G. A., Kockeritz, L. K. & Woodgett, J. R. Functional redundancy of GSK-3 α and GSK-3 β in Wnt/ β -catenin signaling shown by using an allelic series of embryonic stem cell lines. *Dev. Cell* **12**, 957–971 (2007).
10. Sun, X. *et al.* Lithium inhibits amyloid secretion in COS7 cells transfected with amyloid precursor protein C100. *Neurosci. Lett.* **321**, 61–64 (2002).
11. Serenó, L. *et al.* A novel GSK-3 β inhibitor reduces Alzheimer's pathology and rescues neuronal loss *in vivo*. *Neurobiol. Dis.* **35**, 359–367 (2009).

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