GSK-3α/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-3α, but not GSK-3β, controls production of amyloid[1]. 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-3α-mediated or GSK-3β-mediated control of APP processing in brain *in vivo*.

GSK-3β is believed to be central to the pathogenesis of Alzheimer's disease, linking amyloid and tau pathology[2–5]. Unlike GSK-3β, neither physiological functions nor pathological roles of GSK-3α are well explored[6]. To analyse the function of both GSK-3 kinases in brain, we generated mice that were completely deficient in GSK-3α (denoted as *Gsk3a*^{KO}) as well as mice with neuron-specific GSK-3α or GSK-3β deficiency using Cre/*loxP* as for presenilin 1 (ref. 7).

The contribution of GSK-3α 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-3α-deficient mice, denoted as *Gsk3a*^{KO} and *Gsk3a*$^{n-/-}$ (complete and neuron-specific GSK-3α 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-3α deficiency had no evident impact on GSK-3β 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-3α 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-3α generated serendipitously during the expansion of the *Gsk3a*$^{n-/-}$

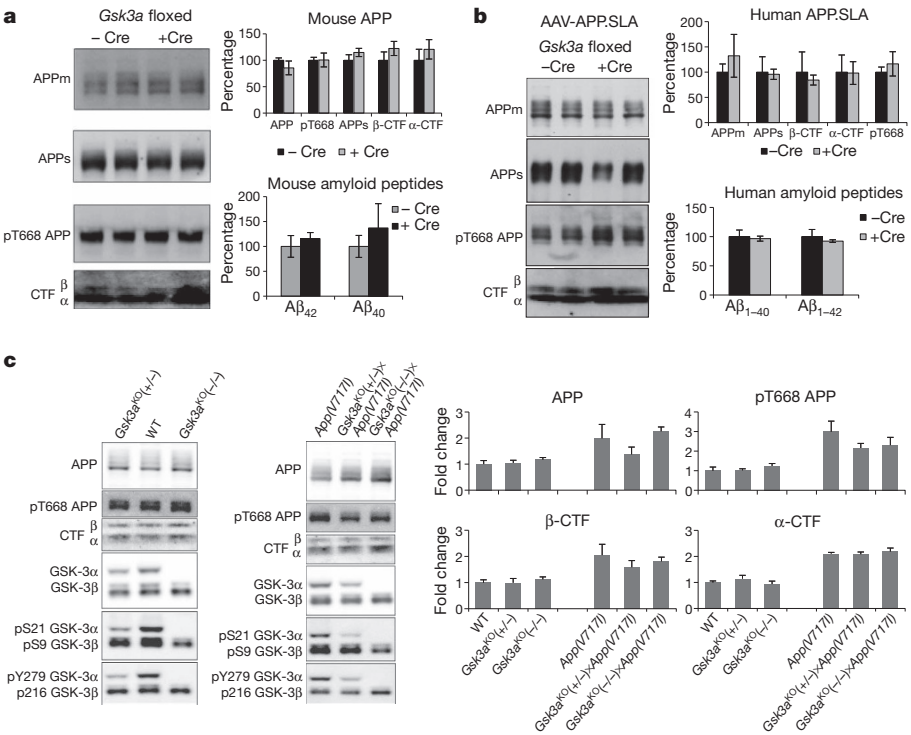

Figure 1 | APP processing in the brains of *Gsk3a*$^{n-/-}$ and *Gsk3a*^{KO} mice. **a**, Biochemical analysis of murine APP metabolites in total brain homogenates by western blotting. Mouse amyloid peptides were measured by specific ELISA. APPm, membrane-bound APP; APPs, secreted APP; Cre, Cre recombinase; CTF, C-terminal fragments; floxed, recombinant genes with inserted *loxP* elements[7]. **b**, Biochemical analysis of hippocampi at 3 weeks after intracerebral injection of AAV-APP.SLA. Human amyloid peptides were measured by specific ELISA[7]. **c**, Analysis of murine APP metabolites in the brains of *Gsk3a*^{KO} mice and of human APP metabolites in *Gsk3a*^{KO}×*App(V717I)* bigenic mice, heterozygous (+/−) or homozygous (−/−) for GSK-3α deficiency. All data are mean ± 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*$^{n-/-}$ 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*$^{n-/-}$ 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*$^{n-/-}$ 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 amyloid[5]. 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].

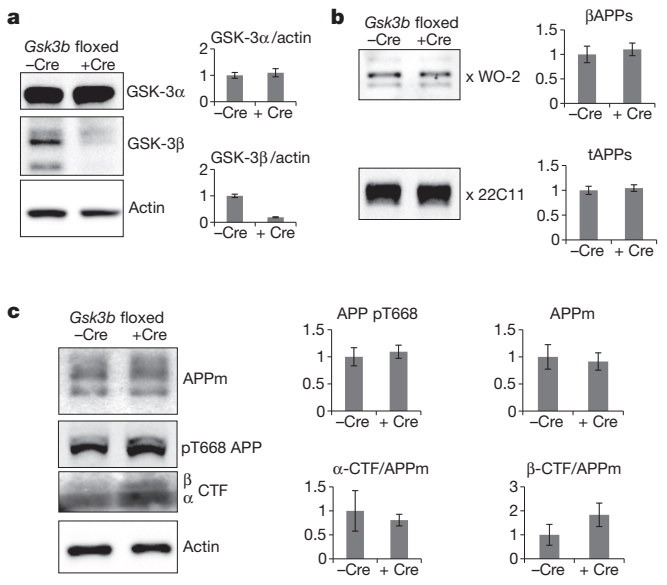

Figure 2 | APP processing in the brains of *Gsk3b*$^{n-/-}$ mice. **a**, Western blot of GSK-3 in brain homogenates of mice with floxed *Gsk3b* genes, with and without expression of Cre recombinase. **b**, **c**, Biochemical analysis of proteolytic processing of mouse APP in the brains of *Gsk3b*$^{n-/-}$ 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.

Tomasz Jaworski[1], Ilse Dewachter[1]†, Benoit Lechat[1], Maarten Gees[1]†, Anna Kremer[1], David Demedts[1], Peter Borghgraef[1], Herman Devijver[1], Seb Kügler[2], Satish Patel[3], Jim R. Woodgett[3] & Fred Van Leuven[1]

[1]Experimental Genetics Group, KU Leuven, Leuven, Belgium.
e-mail: fred.vanleuven@med.kuleuven.be
[2]Department of Neurology, Georg-August-University, Göttingen, Germany.
[3]Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Canada.
†Present addresses: FARL-Institute of Neurosciences, UC Louvain, B-1000 Brussels, Belgium (I.D.); Laboratory of Ion Channel Research, KU Leuven, B-3000 Leuven, Belgium (M.G.).

Received 1 July; accepted 15 September 2011.

1. 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).
2. 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).
3. 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).
4. Takashima, A. GSK-3 is essential in the pathogenesis of Alzheimer's disease. *J. Alzheimers Dis.* **9** (Suppl.), 309–317 (2006).
5. Terwel, D. *et al.* Amyloid activates GSK-3β to aggravate neuronal tauopathy in bigenic mice. *Am. J. Pathol.* **172,** 786–798 (2008).
6. Kaidanovich-Beilin, O. *et al.* Abnormalities in brain structure and behavior in GSK-3α mutant mice. *Mol Brain.* **2,** 35 (2009).
7. 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).
8. 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).
9. 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).
10. De Strooper, B. *et al.* Deficiency of presenilin-1 inhibits the normal cleavage of amyloid precursor protein. *Nature* **391,** 387–390 (1998).
11. 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).
12. 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).
13. 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.

Competing financial interests: declared none.

doi:10.1038/nature10615

Phiel *et al.* reply

REPLYING 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 reported[1] that inhibition of GSK-3 reduces the accumulation of $A\beta_{40}$ and $A\beta_{42}$ (amyloid-ß 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 context[1]. 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-3α and GSK-3β 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.

Christopher J. Phiel[1], Christina A. Wilson[2], Virginia M.-Y. Lee[3] & Peter S. Klein[4]

[1]Center for Molecular and Human Genetics, The Research Institute at Nationwide Children's Hospital, Columbus, Ohio 43205, USA.
[2]Department of Neurology, 3 West Gates Building, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA.
[3]Center for Neurodegenerative Disease Research, Department of Pathology and Laboratory Medicine, 3rd Floor Maloney Building, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA.
[4]Department of Medicine, Division of Hematology–Oncology, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6148, USA.
e-mail: pklein@mail.med.upenn.edu

1. 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).
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 Aβ production in $swAPP_{751}$ 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-3α/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-3α and GSK-3β 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-3β inhibitor reduces Alzheimer's pathology and rescues neuronal loss *in vivo*. *Neurobiol. Dis.* **35,** 359–367 (2009).

doi:10.1038/nature10616