GSK-3α/β kinases and amyloid production *in vivo*

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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 $Gsk3a^{n-/-}$

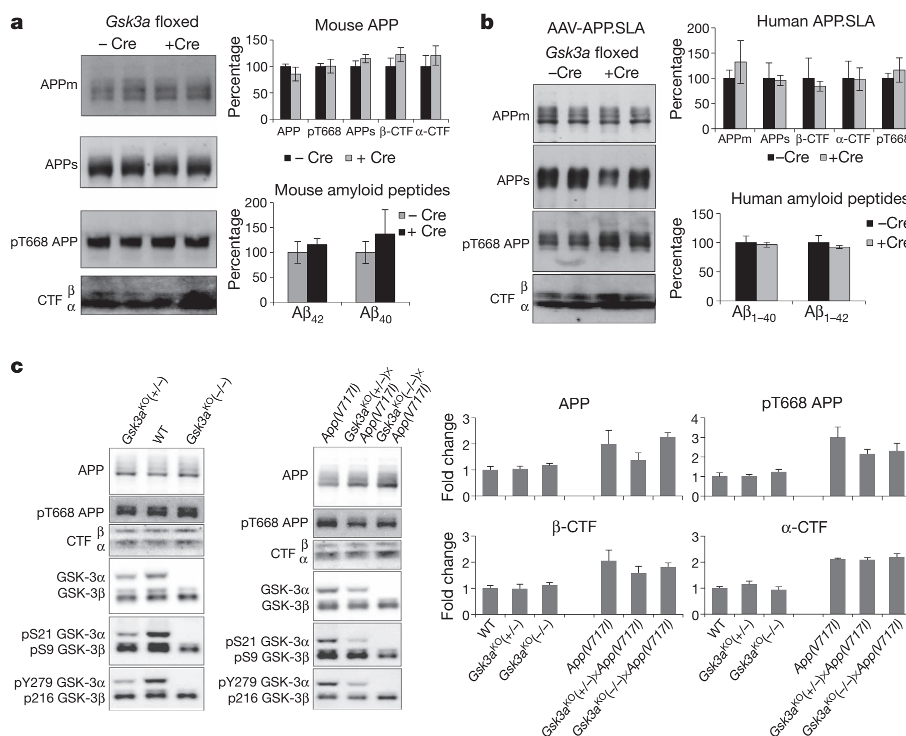

Figure 1 | APP processing in the brains of $Gsk3a^{n-/-}$ and $Gsk3a^{KO}$ mice. **a**, Biochemical analysis of murine APP metabolites in total brain homogenates by western blotting. Mouse amyloid peptides were measured by specific ELISA. APPm, membrane-bound APP; APPs, secreted APP; Cre, Cre recombinase; 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.

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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].

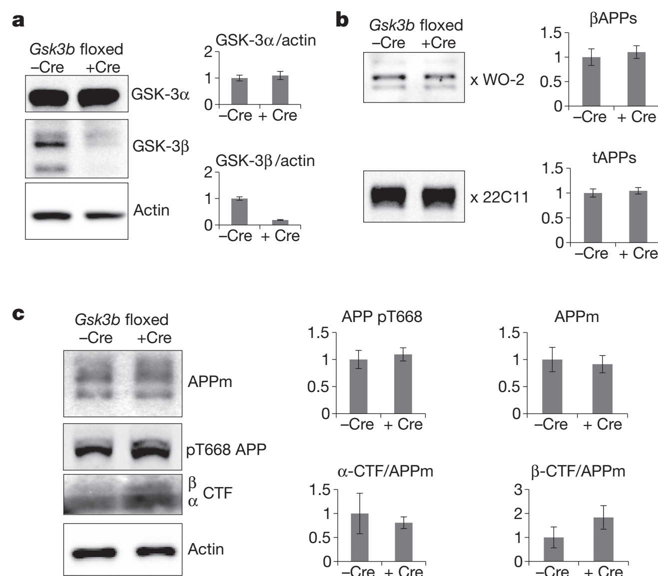

Figure 2 | APP processing in the brains of *Gsk3b*$^{n-/-}$ mice. a, Western blot of GSK-3 in brain homogenates of mice with floxed *Gsk3b* genes, with and without expression of Cre recombinase. **b**, **c**, Biochemical analysis of proteolytic processing of mouse APP in the brains of *Gsk3b*$^{n-/-}$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

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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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Phiel *et al.* reply

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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.

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