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Hypothalamic glycogen synthase kinase 3 β has a central role in the regulation of food intake and glucose metabolism

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Abstract

GSK3 β (glycogen synthase kinase 3 β) is a ubiquitous kinase that plays a key role in multiple intracellular signalling pathways, and increased GSK3 β activity is implicated in disorders ranging from cancer to Alzheimer's disease. In the present study, we provide the first evidence of increased hypothalamic signalling via GSK3 β in leptin-deficient Lep^{ob/ob} mice and show that intracerebroventricular injection of a GSK3 β inhibitor acutely improves glucose tolerance in these mice. The beneficial effect of the GSK3 β inhibitor was dependent on hypothalamic signalling via PI3K (phosphoinositide 3-kinase), a key intracellular mediator of both leptin and insulin action. Conversely, neuron-specific overexpression of GSK3 β in the mediobasal hypothalamus exacerbated the hyperphagia, obesity and impairment of glucose tolerance induced by a high-fat diet, while having little effect in controls fed standard chow. These results demonstrate that increased hypothalamic GSK3 β signalling contributes to deleterious effects of leptin deficiency and exacerbates high-fat diet-induced weight gain and glucose intolerance.

Keywords

adeno-associated virus; arcuate nucleus; Dickkopf 1; food intake; high-fat diet; obesity; synapsin; Type II diabetes

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AUTHOR CONTRIBUTION

Jonas Benzler carried out all of the experiments in Figures 1, 2 and 5, and helped to write the paper and develop the project. Goutham K. Ganjam and Manon Krüger generated the viral construct with technical support from Olaf Pinkenburg and performed the experiments in Figures 3 and 4. Maria Kutschke, Sigrid Stöhr and Juliane Steger provided technical support for all experiments. Christiane Koch helped performing the ipGTTs and Rebecca Ölkug provided support for performing the metabolic measurements. Michael Schwartz helped to write and revise the paper. Peter R. Shepherd helped to write and revise the paper and gave advice on experiments. David R. Grattan helped to write and revise the paper and gave advice on developing the project. Alexander Tups was the principal investigator developed the project, supervised the research and wrote and revised the paper.

INTRODUCTION

Type II diabetes affects more than 165 million individuals and is increasing at an alarming rate [1]. Although a large number of papers has implicated the brain as a critical target for insulin regulation of systemic glucose metabolism [2-7], mechanisms underlying such insulin effects remain incompletely understood. Even less is known about the importance of altered neuronal insulin signalling in the pathogenesis of Type II diabetes. Studies focused on hypothalamic insulin signal transduction via the IRS (insulin receptor substrate)–PI3K (phosphoinositide 3-kinase) pathway suggest that it plays a critical role in CNS (central nervous system) regulation of peripheral glucose homeostasis [5,8,9]. Leptin action in the brain similarly depends on intact IRS–PI3K signalling [9-11], but key neuronal mediators downstream of this pathway remain to be identified.

GSK3 β (glycogen synthase kinase 3 β) is a serine-threonine kinase that is phosphorylated and inhibited by protein kinase B (AKT) [12], a principal target of PI3K signalling. In addition to inhibiting cellular responses to insulin (for example, inhibition of GSK3 β is required for insulin stimulation of glycogen synthesis), this enzyme also influences cell division, growth and development [13] as an endogenous inhibitor of canonical WNT signalling. The finding that systemic inhibition of GSK3 β improves whole-body glucose homeostasis [14-16] implies that GSK3 β exerts a tonic inhibitory effect on glucose metabolism, but neither the mechanism(s) nor the specific tissue(s) involved in this effect are known. Some evidence points to GSK3 β in skeletal muscle as a mediator of impaired glucose metabolism in diabetic mouse models [17,18], but activities of both AKT and GSK3 β are also regulated in mouse brain in response to physiological changes of glucose [19]. In the present study we investigated whether an action in the brain might explain the deleterious effects of GSK3 β signalling on peripheral glucose homeostasis.

MATERIALS AND METHODS

Animals

All experiments used male mice that were purchased from Janvier. The animals were between 2- and 4-months-old and housed individually under standard conditions with a light/dark cycle of 12 h. All procedures were performed in accordance with the guidelines of the German Council of Animal Care. The ambient temperature for mice was 26°C. Apart from the dark phase before the experiments, all animals had access to standard rodent diet, low-fat diet or HFD (high-fat diet) (containing 45% fat) and water *ad libitum*. For the central administration of drugs, cannulae were stereotactically implanted into the left lateral ventricle as described previously [9].

In situ hybridization

To determine the central expression of DKK-1 (dickkopf 1 homologue) and SOCS-3 (suppressor of cytokine signalling 3) we performed *in situ* hybridization on coronal brain sections. As previously described [20], forebrain sections (16 μ m) were collected throughout the extent of the ARC (arcuate nucleus) on to a set of twelve slides, with twelve sections mounted on each slide. Accordingly, the slides spanned the hypothalamic region approximating from –2.8 to –1.22 mm relative to Bregma according to the atlas of the mouse brain [21]. *In situ* hybridizations and analysis was performed as described previously [20].

Glucose tolerance tests

We determined whether activation of GSK3 β might impair glucose tolerance in lean mice. Therefore the WNT antagonist DKK-1 [1 μ g in 1 μ l of aCSF (artificial cerebral spinal

fluid); R&D Systems, 5897-DK/CF] was administered ICV (intracerebroventricular) to one group ($n=4$) of Lep^{+/+} mice, whereas a second group ($n=6$) received an ICV vehicle injection (aCSF). At 15 min later an ipGTT (intraperitoneal glucose tolerance test; 1 g of glucose/kg of body mass) was performed. To determine the blood glucose levels, the vena facialis was punctured and the glucose concentration was measured using a commercially available glucometer (Accu-Check Performa, Roche).

We next determined whether inhibition of GSK3 β might improve glucose homeostasis. Therefore the mice received a specifically designed inhibitor for GSK3 β (AR-A014418, Calbiochem). To test whether central GSK3 β inhibition affects hypothalamic PI3K, four groups of Lep^{ob/ob} mice received two ICV injections (0.5 μ l each), 30 min apart. The first group received vehicle (5% DMSO/aCSF) followed by GSK3 β inhibitor (0.5 nmol in 5% DMSO/aCSF). The second group received isoform-specific PI3K inhibitors (0.1 nmol in 5% DMSO/aCSF, PIK-75/TGX-221), since both isoforms are required for insulin signalling in the hypothalamus [22], followed by vehicle (5% DMSO/aCSF). A third group received isoform-specific PI3K inhibitors, followed by GSK3 β inhibitor, and the last group received two vehicle injections ($n=4-7$). At 15 min after the second injection, an ipGTT was performed (1 g of glucose/kg of body mass) as described above.

To measure glucose tolerance after virus administration we performed two ipGTTs. The first one was performed on day 58 on chow diet (1 g of glucose/kg of body mass), whereas the second ipGTT was conducted at day 18 of the HFD (0.75 g of glucose/kg of body mass) as described above.

Food intake experiment

To test whether inhibition of central GSK3 β affects food intake, we administered a GSK3 β inhibitor (AR-A014418, Calbiochem) ICV in a separate group of 8-week-old Lep^{ob/ob} mice. The mice were fasted for 16 h and weight matched. Mice received either a GSK3 β inhibitor (0.5 nmol in 0.5 μ l of aCSF/5% DMSO) or vehicle injection (aCSF/5% DMSO) 1 h before the beginning of the dark phase. Food intake was measured at 4 h and 24 h after administration ($n=8$ /group).

Immunohistochemistry

First we investigated the phosphorylation of GSK3 β (Ser⁹, catalogue number 9323) in the ARC of 8-week-old wild-type mice fed a HFD (containing 45% fat) for 3 weeks. In addition, we also compared phosphorylation of GSK3 β (Ser⁹) in wild-type and Lep^{ob/ob} mice. To do this the animals were fasted for 16 h and transcardial perfusion was performed. To determine any possible cross-talk between GSK3 β and the IRS-PI3K pathway, we measured the effect of ICV GSK3 β inhibitor on IRS-1 phosphorylation (Ser⁶¹²) and phospho-AKT (Ser⁴⁷³) in the hypothalamic ARC. Accordingly, Lep^{ob/ob} mice ($n=10$ /group) received either GSK3 β inhibitor (AR-A014418, 0.5 nmol in 0.5 μ l aCSF/5% DMSO) or vehicle (aCSF/5% DMSO) ICV 15 min before transcardial perfusion.

Immunohistochemistry was performed using anti-(phospho-IRS-1 Ser⁶¹²) (catalogue number 3203) and anti-(phospho-AKT Ser⁴⁷³) antibodies (catalogue number 4058). Immunohistochemistry was carried out on mouse brain coronal cryosections as described previously [9,22]. All antibodies were purchased from Cell Signaling Technology.

Recombinant adeno-associated viral vector generation and virus production

The human cDNA for GSK3 β was subcloned from the eukaryotic expression vector pcDNA3-GSK3 β HA (Addgene, 14753) into an AAV2-hSyn-EGFP-WPRE vector [23]. GSK3 β cDNA along with an HA (haemagglutinin) tag coding sequence was amplified from the pcDNA3-GSK3 β -HA plasmid by PCR (phusion DNA polymerase) using the forward,

5'-GCTAGCTAATACGACTCACTATAGG-3' and reverse, 5'-TGTACACAATTTAGGTGACACTATCG-3' primers. These primers contain suitable *NheI* and *BsrGI* restriction sites for cloning into the AAV (adeno-associated virus) construct. Amplified PCR product was first cloned into the pGemT easy cloning vector. The *GSK3 β* cDNA fragment from pGemT easy vector was subcloned into *NheI* and *BsrGI* sites of AAV2-hSyn-EGFP-WPRE by replacing EGFP (enhanced green fluorescent protein) cDNA to obtain AAV2-hSyn-GSK3 β -HA-WPRE. In this vector system the expression of GSK3 β is under the control of the human synapsin-1 promoter to restrict the expression to neurons and WPRE [WHV (Woodchuck hepatitis virus) post-transcriptional regulatory element] facilitates long-term expression of the transgene. All molecular cloning procedures were performed in SURE2 bacterial cells to minimize the recombination events.

Recombinant AAV vectors of serotype 2 were produced by transfecting AAV *cis* plasmids encoding the gene of interest and a viral helper plasmid pDG [24] encoding *rep-2* (replication protein 2) and *cap-2* (capsid protein 2) genes into HEK (human embryonic kidney)-293 cells. Total cell lysates were collected after 48 h of transfection in AAV lysis buffer (50 mM Hepes with 150 mM NaCl, pH 7.6) by repeated freezing in liquid nitrogen and thawing at 37°C. The cell lysates were cleared by centrifugation for 15 min at 4600 g at 4°C to remove the cell debris. Unencapsulated nucleic acids were degraded by treating the cleared cell lysates with 250 units of benzonase (Sigma) for 90 min at 37°C. AAV particles were purified in three step CsCl density gradient ultracentrifugation (rotor type SW41 at 15°C), desalting and concentration by Amicon® Ultra Centrifugal filters (30 K MWCO UFC903008). The viral genome isolated by the Qiagen mini prep plasmid isolation kit was titrated by quantitative real-time PCR using the forward, 5'-CCTCAATCCAGCGGACCTTC-3' and reverse, 5'-ACAGTGGGAGTGGCACCTTC-3' primers.

Protein expression verification in primary cortical neuronal cells

Freshly isolated primary rat cortical neurons were plated at a density of 0.3 million cells on polyethyleneimine pre-coated plates and cultured in MEM $\oplus$ medium as described previously [25]. After 4 h the cells were replenished with neurobasal medium [25] and were infected with 10¹⁰ vector genomes of purified AAV particles for 5 days. Total protein lysate (20 μ g) from the cortical cells was used to verify the expression of GSK3 β by Western blotting with an anti-HA tag antibody (catalogue number 2367, Cell Signaling Technology). The same cell lysates were used to analyse the phosphorylation of Tau protein with an anti-(phospho-Tau Ser³⁹⁶) antibody (catalogue number 9632, Cell Signaling Technology) and total Tau with an anti-Tau antibody (catalogue number 4019, Cell Signaling Technology). The expression of EGFP control virus was verified by fluorescence light microscopy. To prove that the expression of human synapsin promoter controlled GSK3 β is limited to neurons, we infected HEK-293 cells with 10¹⁰ vector genomes of EGFP and GSK3 β viral particles. As a positive control we transfected HEK-293 cells with 5 μ g of pcDNA3-GSK3 β -HA plasmid [26]. After 3 days the total cell lysates were collected as described previously [27]. Total cell lysates (20 μ g) were Western blotted with an anti-HA tag antibody.

Stereotaxic injections

Intracerebral injections were performed under isoflurane anaesthesia as described previously [9]. Stereotaxic co-ordinates to reach the ARC of hypothalamus are 1.5 mm posterior, $\pm$ 0.3 mm lateral and 6.1 mm ventral relative to Bregma. AAV2 particles, containing 4 $\times$ 10¹⁰ vector genomes, were injected into the ARC using a 0.5 μ l Hamilton glass syringe for 2 min. The injection needle remained in place at each injection site for an additional 5 min to

allow for diffusion and prevent backflow. The incision was sutured and the animals were placed under a heating lamp to recover from the surgery.

Body composition

To analyse their body composition, mice were anaesthetized with isoflurane (CP-Pharma) and were analysed via DEXA (dual-emission X-ray absorptiometry)-scan (Lunar PIXImus Densitometer; GE Medical Systems).

Metabolic measurement

We measured the effect on metabolic rate of regular chow and the HFD. Accordingly, carbon dioxide production (VCO_2) and oxygen consumption (VO_2) were measured in metabolic cages (~5 l vol.). Measurements were taken continuously for 2 days with a constant ambient temperature of 23°C. The air flow in the cage was adjusted to ~42 l/h and continuously monitored. The procedure has been described in detail previously [28].

Plasma insulin levels

We investigated whether blood insulin levels were affected by GSK3 β overexpression. Accordingly, blood was collected by decapitation and plasma insulin was measured via a Rat/Mouse insulin ELISA kit (Millipore EZRMI-13K) according to the manufacturer's instructions.

Central inhibition of GSK3 β decreased hepatic glucose production

To test whether inhibition of GSK3 β in the brain affects gluconeogenesis, we performed pyruvate tolerance tests. Lep^{ob/ob} mice received a single ICV injection of either GSK3 β inhibitor (AR-A014418, Calbiochem, 0.5 nmol in 0.5 μl of aCSF/5% DMSO, $n=5$) or vehicle (0.5 μl of aCSF/5% DMSO, $n=11$). At 60 min before pyruvate was administered, blood glucose levels were measured as described above. To analyse the protein level of PEPCCK (phosphoenolpyruvate carboxykinase 2) in the liver, three groups of Lep^{ob/ob} mice ($n=4-5/\text{group}$) underwent the treatment regimen shown above with the exception that livers were removed 90 min after ICV treatment. Immunoblotting with 5 μg of total liver protein lysate was performed as described elsewhere [27] and normalized to β -actin.

Statistics

The data were analysed by one- or two-way ANOVA followed by a Holm–Sidak comparison test, as appropriate, using SigmaStat statistical software (Jandel). Where the data failed equal variance or normality tests, they were analysed by one-way ANOVA on ranks followed by Dunn's multiple comparison test. The results are presented as means $\pm$ S.E.M. and differences were considered significant if $P < 0.05$.

RESULTS AND DISCUSSION

As a first step, we investigated whether activity of GSK3 β is increased in the ARC, a key brain region for neuronal control of energy and glucose homeostasis, during diet-induced obesity and leptin deficiency. It has been comprehensively established that activity of GSK3 β is mediated via phosphorylation at Ser⁹, a post-translational modification that is critical to inactivate the enzyme [12,29-32]. Furthermore, intact insulin signalling appears to involve inhibition of GSK3 β phosphorylation at Ser⁹ in the muscle [12,33]. Using an antibody specific against Ser⁹ phosphorylation, we found that the number of phospho-GSK3 β immunoreactive cells in this brain area was reduced in both models of impaired glucose homeostasis (mice fed an HFD and Lep^{ob/ob} mice) compared with their respective controls, suggesting that local GSK3 β activity is increased in these animals (Figures 1a and

1b, $n=5-6/\text{group}$, $P=0.007$ and $P=0.026$ respectively). For a proof of concept, in extremely glucose intolerant $\text{Lep}^{\text{ob/ob}}$ mice we investigated whether central inhibition of GSK3 β affects glucose homeostasis. Consistent with this hypothesis, glucose tolerance in these animals was markedly improved following a single ICV injection of a GSK3 β inhibitor (AR-A014418) relative to the ICV vehicle (Figure 1c, $n=4-7/\text{group}$, $P=0.016$). This effect cannot be attributed to changes of food intake or energy balance, since ICV injections were performed 15 min before ipGTT and the animals were not provided food during this time.

Since central insulin action is required for whole-body glucose homeostasis [2-4,8,9,34] via a hypothalamic mechanism involving signal transduction via the PI3K pathway, we next asked whether pharmacological inhibition of central GSK3 β restores impaired PI3K signalling in the ARC of $\text{Lep}^{\text{ob/ob}}$ mice. This was accomplished by histochemical analysis of the effect of ICV administration of a GSK3 β inhibitor (AR-A014418) on the phosphorylation of IRS-1 (Ser⁶¹²) and AKT (Ser⁴⁷³) in the ARC of $\text{Lep}^{\text{ob/ob}}$ mice. These markers were selected because serine phosphorylation of IRS-1 impairs signalling via PI3K, whereas phospho-AKT (Ser⁴⁷³) is a marker of PI3K activation [9]. Following ICV injection, the GSK3 β inhibitor acutely (within 15 min) decreased the number of phospho-IRS-1 (Ser⁶¹²) immunoreactive cells within the ARC of $\text{Lep}^{\text{ob/ob}}$ mice by ~20% relative to vehicle-treated mice (Figure 2a, $P=0.028$, $n=10/\text{group}$), while increasing the number of phospho-AKT (Ser⁴⁷³) immunoreactive cells in the ARC by ~3-fold, compared with the ICV vehicle (Figure 2b, $P=0.001$, $n=10/\text{group}$). Thus the glucose-lowering effects of central GSK3 β are associated with increased hypothalamic IRS-PI3K signalling.

To determine whether the beneficial effect of central GSK3 β inhibition depends on intact PI3K signalling, we determined if its effect on glucose tolerance is blocked by pharmacological inhibition of PI3K. Animals received an ICV injection of selective inhibitors of the PI3K catalytic subunits, p110 α and p110 β (PIK75 and TGX221) [22] with and without the GSK3 β inhibitor, which was given 30 min later, followed by an ipGTT. Our findings that the metabolic improvement induced by the GSK3 β inhibitor was fully blocked by co-administration of the PI3K p110 α - and β -selective inhibitors (Figure 2c, $n=4-7/\text{group}$) implicate increased PI3K signalling as a mediator of this beneficial effect.

We next investigated the effects of central inhibition of GSK3 β on food intake in $\text{Lep}^{\text{ob/ob}}$ mice. Relative to the vehicle, ICV injection of the GSK3 β inhibitor 30 min before the beginning of the dark phase induced a modest, but significant, reduction of 24 h food intake in these animals (–15% compared with the vehicle, $n=8/\text{group}$, $P=0.032$) (Figure 2d). These observations are consistent with published evidence that leptin administration increases hypothalamic IRS-PI3K signalling, and that leptin's ability to reduce food intake is prevented by central blockade of PI3K [10].

The above data collectively suggest that GSK3 β is overactive in the hypothalamus of leptin-deficient mice, contributing to the diabetic state of these animals. Given the known role of the WNT pathway to inactivate GSK3 β , we next investigated whether expression of DKK-1, a potent antagonist of the WNT pathway that activates GSK3 β in neurons [35], might be up-regulated in the ARC of diabetic $\text{Lep}^{\text{ob/ob}}$ mice. As predicted, *DKK1* mRNA, as measured by *in situ* hybridization using an antisense riboprobe specific for DKK-1, was increased 3-fold in the ARC of diabetic $\text{Lep}^{\text{ob/ob}}$ mice (Figure 2e, $P=0.008$) compared with wild-type controls. This observation suggests that increased hypothalamic GSK3 β activity may be a consequence of increased DKK-1 in these animals. If this hypothesis is correct, interventions that increase neuronal DKK-1 signalling in normal animals should impair glucose homeostasis. To test this hypothesis, we administered DKK-1 as an acute ICV injection to wild-type mice 15 min before an ipGTT was performed. Remarkably, the marked impairment of glucose homeostasis induced by ICV injection of DKK-1 ($n=4-6$,

$P < 0.001$ compared with the controls) was comparable with that observed in $Lep^{ob/ob}$ mice (Figure 2f). On the basis of these findings, we infer that hypothalamic GSK3 β activity in leptin-deficient mice: (i) arises at least in part from increased DKK-1 signalling and (ii) contributes to their impaired glucose metabolism. Further, metabolic benefit arising from reduced hypothalamic GSK3 β action depends upon intact hypothalamic PI3K signalling, and increased brain signalling via either DKK-1 or GSK3 β impairs systemic glucose homeostasis.

As local inhibition of GSK3 β in the brain improved glucose homeostasis, the neuroanatomical identity of the underlying phenomenon remained limited due to ICV administration of the GSK3 β inhibitor. Therefore we generated a viral construct enabling neuron-specific overexpression of functional GSK3 β in the ARC, a key brain region for neuronal control of energy and glucose homeostasis. This approach furthermore enables GSK3 β overexpression in adult healthy mice, thereby circumventing potential defects in embryogenesis due to the ontogenic capacity of this enzyme. This was accomplished by cloning human *GSK3 β* cDNA into the AAV2 vector. In contrast with adenoviruses, AAV2s do not cause adverse local immune responses and they have a higher transfection rate in the brain than lentiviruses [36,37]. To direct GSK3 β expression selectively to neurons, transcription was directed by the human synapsin-1 promoter (Figure 3a). The ability of AAV2 vectors to drive selective neuronal expression of a transgene was validated by infecting both primary cortical neurons (Figure 3c) and a peripheral cell line (HEK-293; Figure 3d) with AAV2 vectors expressing the fluorescent reporter EGFP (Figure 3b). As expected, viral-induced GSK3 β overexpression occurred in neurons, but not HEK-293 cells. Following intraparenchymal injection of the AAV2–GSK3 β vector into the ARC of wild-type mice, local overexpression of GSK3 β was confirmed by *in situ* hybridization (Figure 3f), and increased GSK3 β kinase activity was verified by measuring phospho-Tau content, a target of GSK3 β in the brain [38]. In cultured primary cortical neurons, AAV2-mediated overexpression increased phospho-Tau protein 5-fold (Figure 3e, P 0.001).

To investigate the effects of ARC-directed GSK3 β overexpression on energy metabolism, we injected 10^{10} genomic units of either AAV2–GSK3 β or a control AAV2 virus into the ARC of 8-week-old wild-type mice. On a standard chow diet, GSK3 β overexpression induced a mild increase in body mass that was only intermittently and transiently significant over a time period of 78 days after injection (Figure 4a). Despite its minor effects on body mass, ARC-directed overexpression of GSK3 β impaired glucose tolerance significantly relative to controls injected with AAV2–EGFP, based on an ipGTT performed on day 58 (Figure 4b, $P=0.04$). After 78 days, both groups of mice were switched to an HFD for 18 days, which led to an increased body mass in both groups. However, the increase of body mass induced by the HFD was strikingly increased in AAV2–GSK3 β mice compared with the controls, an effect that became significant within 2 days after the diet switch and reached a value 17% greater than the controls by day 18 (Figure 4a). An ipGTT after 18 days on the HFD revealed that glucose tolerance had deteriorated markedly in the AAV2–GSK3 β mice compared with the controls (Figure 4b, P 0.001). DEXA analyses performed immediately after the ipGTT revealed significant reductions of relative lean mass (P 0.001) and increases of relative fat mass (P 0.001) in AAV2–GSK3 β mice fed the HFD, but not in mice fed standard chow (Figure 4c). Cumulative food intake (kJ/day) was increased in AAV2–GSK3 β mice compared with the AAV2–EGFP controls regardless of the diet (Figure 4d, P 0.05), whereas energy expenditure was unaltered (Figure 4e). Lastly, the respiratory quotient (an indicator of substrate utilization) was reduced in AAV2–GSK3 β mice on both the standard chow diet and HFD (Figure 4f, P 0.001), suggesting preferential oxidation of lipid as a fuel.

Taken together, these data provide clear evidence that GSK3 β signalling potently affects energy and glucose homeostasis in a diet-sensitive manner via effects in the mediobasal hypothalamus. The striking increase in the body mass differential between AAV2–GSK3 β and AAV2–EGFP mice after being fed the HFD probably involves increased food intake, whereas the metabolic rate was unchanged by hypothalamic GSK3 β overexpression in the former mice. By comparison, the effects of neuronal GSK3 β overexpression on the regular chow diet were modest, a discrepancy that might be explained by redundancy in components of the evolutionarily conserved WNT pathway, which may have partially compensated for the effects of increased GSK3 β signalling on the chow diet. Interestingly, growing evidence indicates that consuming an HFD induces pro-inflammatory responses in the hypothalamus [39]. Combined with evidence that mediators of peripheral tissue inflammation [e.g. JNK (c-Jun N-terminal kinase)] also activate GSK3 β [40], it is plausible that inflammation triggered by the HFD disrupts compensatory mechanisms that might be functional on the chow diet, thereby revealing potent deleterious effects of increased GSK3 β activity.

This interpretation is strengthened by evidence of increased hypothalamic expression of SOCS3, which is induced during inflammation and suppresses leptin and insulin signalling [41,42], in AAV2–GSK3 β mice compared with the controls while being fed an HFD (Figure 4g, $P=0.02$). The possibilities that increased hypothalamic GSK3 β signalling exacerbates inflammation and leptin resistance during HFD feeding and/or that hypothalamic inflammation increases local GSK3 β activity are each consistent with the results of the present study and warrant additional study.

Our findings show that although activation of central GSK3 β exacerbates the deleterious effects of HFD feeding on whole-body energy and glucose metabolism, there was only a trend towards increased serum insulin levels in AAV2–GSK3 β mice relative to the controls (Figure 4h). A large number of papers suggest that hypothalamic insulin signalling is required for the inhibition of hepatic glucose production [3,4] and both hypothalamic insulin and leptin signalling appear to improve liver insulin sensitivity via a mechanism involving the vagus nerve [43]. To investigate whether central GSK3 β activity opposes these effects and increases hepatic glucose output, we employed a strategy that involves an indirect measurement of hepatic glucose production using pyruvate as a substrate for gluconeogenesis, such that the observed glucose excursion reflects hepatic glucose output. Lep^{ob/ob} mice received either GSK3 β inhibitor or vehicle ICV 60 min before pyruvate was injected intraperitoneally and blood glucose levels were measured (Figure 5a). Leptin-deficient mice treated with the GSK3 β inhibitor showed a significantly reduced glucose excursion compared with the vehicle ($P=0.001$, $n=5-11$ /group). To further analyse the impact of central GSK3 β inhibition on liver glucose metabolism, we measured the hepatic levels of PEPCK, which is rate-limiting for gluconeogenesis. Lep^{ob/ob} mice received either GSK3 β inhibitor or vehicle (aCSF) ICV 90 min before the liver was removed and immunoblotting was performed (Figure 5b) and normalized to the β -actin content. Central inhibition of GSK3 β administration significantly decreased the amount of PEPCK protein in the liver compared with the vehicle-treated group ($P=0.003$, $n=4-5$ /group), which is consistent with data obtained from hepatoma cells [44]. These results support a model in which GSK3 β in the brain tonically favours increased plasma glucose levels via a mechanism that involves increased hepatic gluconeogenesis, a phenomenon that can be induced by local hypothalamic inhibition of PI3K [3] and is also observed in diabetic animals and humans [45,46].

Taken together, our data provide the first evidence that increased GSK3 β signalling within the CNS drives hyperglycaemia and exerts deleterious effects on whole-body energy balance and glucose metabolism in the adult mouse, particularly in the setting of an HFD or genetic leptin deficiency. This, together with the notion that this enzyme sensitizes insulin signalling

in the hypothalamus, further reinforces the concept that the brain is an essential organ in the maintenance of peripheral glucose homeostasis. Aberrant GSK3 β has also been associated with the pathogenesis of many human diseases, such as osteoporosis [47], atherosclerosis [48], cancer and Alzheimer's disease [49]. The proposed role of GSK3 β in the brain in regulating peripheral glucose homeostasis might constitute an important link between Type II diabetes and the pathogenesis of these other severe diseases.

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Abbreviations used

AAV	adeno-associated virus
aCSF	artificial cerebral spinal fluid

ARC	arcuate nucleus
CNS	central nervous system
DEXA	dual-emission X-ray absorptiometry
DKK-1	dickkopf 1
EGFP	enhanced green fluorescent protein
GSK3β	glycogen synthase kinase 3 β
HA	haemagglutinin
HEK	human embryonic kidney
HFD	high-fat diet
ICV	intracerebroventricular
ipGTT	intraperitoneal glucose tolerance test
IRS	insulin receptor substrate
PEPCK	phosphoenolpyruvate carboxykinase 2
PI3K	phosphoinositide 3-kinase
SOCS-3	suppressor of cytokine signalling 3
WPRE	WHV (Woodchuck hepatitis virus) post-transcriptional regulatory element

Figure 1. Phospho-GSK3β in the ARC during obesity and pharmacological inhibition of this enzyme

(a) Immunohistochemistry was performed on brain sections of mice fed an HFD compared with low-fat diet (LFD) mice. Inserts depict representative images of phospho-GSK3β (Ser⁹) immunoreactivity in the ARC (phosphorylation at Ser⁹ inactivates the enzyme). The histogram shows the counted phospho-GSK3β (Ser⁹) cells in the ARC ($n=5-6$ / group). (b) The same experiment, mentioned above, was performed on brain sections of wild-type and Lep^{ob/ob} mice. The histogram shows the counted phospho-GSK3β (Ser⁹) cells in the ARC ($n=5-6$ / group). (c) A central injection of a GSK3β inhibitor improves glucose tolerance in Lep^{ob/ob} mice. ipGTT was performed 15 min after administration of GSK3β inhibitor (○) or vehicle (●) into the lateral ventricle ($n=4-7$ each group). Results are means±S.E.M., * P 0.05 and ** P 0.01. AUC, area under curve.

Figure 2. Pharmacological manipulation of GSK3β in the brain: effects on food intake and interaction with hypothalamic insulin signaling

(a) Immunohistochemistry was performed on brain sections of *Lep^{ob/ob}* mice after central administration of a GSK3β inhibitor (Inhib.) or vehicle, 15 min before transcardial perfusion. Images are representative of phospho-IRS-1 (Ser⁶¹²) immunoreactivity in the ARC. The histogram shows the counted cells in the ARC that were immunoreactive for phospho-IRS-1 and were down-regulated after ICV injection of the GSK3β inhibitor ($n=10$ /group). (b) An additional set of brain sections of the experiment presented in (a) was analysed for phospho-AKT (Ser⁴⁷³) immunoreactive cells in the ARC. The number of phospho-AKT (Ser⁴⁷³) immunoreactive cells was increased after ICV injection of the GSK3β inhibitor. (c) Central improvement of glucose homeostasis by the GSK3β inhibitor in *Lep^{ob/ob}* mice was blocked by pre-treatment with isoform-specific PI3K inhibitors. The PI3K-inhibitor was injected ICV 30 min before GSK3β inhibitor injection (ICV) ($n=4-7$ each group). (d) A single central injection of a GSK3β inhibitor decreased food intake in

$Lep^{ob/ob}$ mice within 24 h by approximately 15%. $Lep^{ob/ob}$ mice received a GSK3 β inhibitor or vehicle injection ($n=8$ each group) into the lateral ventricle and food intake was analysed after 4 h and 24 h. (e) Autoradiographs of mouse brain sections after *in situ* hybridization to an antisense ^{35}S -labelled riboprobe binding to the WNT antagonist DKK-1. Within the ARC gene expression was up-regulated in $Lep^{ob/ob}$ mice compared with the $Lep^{+/+}$ mice. The upper panels depict autoradiographs of the respective genes, whereas the lower panels show a histogram generated from quantification of the signal in the ARC ($n=5-6$ animals in each group). (f) ICV administration of DKK-1 protein impairs glucose tolerance in wild-type mice ($Lep^{+/+}$ mice). DKK-1 protein (○, $n=4$) or aCSF (●, $n=6$) was administered 15 min before the ipGTT. Results are means $\pm$ S.E.M., * P 0.05, ** P 0.01 and *** P 0.001. AUC, area under curve.

Figure 3. Neuron-specific AAV2-mediated overexpression of GSK3β in the ARC

(a) Schematic representation of *cis* and helper plasmid maps transfected into HEK-293 cells to produce AAV2–GSK3β (upper panels). Important elements of the viral genome are depicted in the lower panel. GSK3β transcription was controlled by the neuron-specific synapsin promoter. (b) Confocal image showing primary cortical neurons expressing EGFP after 5 days of AAV2–EGFP infection. (c) Confirmation of AAV2–GSK3β over-expression by immunoblotting of viral HA tag specific to AAV2–GSK3β in primary cortical neurons 5 days after infection. (d) In immunoblots of peripheral HEK-293 cells infection with neuron-specific AAV2–GSK3β was absent. Only cells transfected with pcDNA3-GSK3β led to an increase in the HA tag protein. (e) Immunoblot showing increased phospho-Tau (Ser³⁹⁶)/total Tau protein ratio in cortical neurons infected with AAV2–GSK3β. (f) Overexpression of AAV2–GSK3β *in vivo* was confirmed by *in situ* hybridizations. Shown are representative autoradiographs of mouse brain sections after *in situ* hybridization to an antisense ³⁵S-labelled riboprobe binding to GSK3β of AAV2–EGFP and AAV2–GSK3β mice. For

validation each cell culture experiment was repeated three times and statistical analysis is represented in the histograms. Results are means $\pm$ S.E.M. ** P 0.01 and *** P 0.001. TB, transcriptional blocker; hSyn1, human synapsin1 promoter; hGSK3 β , human GSK3 β tagged with HA epitope; pA, poly A sequence from bovine growth hormone; ITR, inverted terminal repeat; IB, immunoblot.

Figure 4. Neuron-specific AAV2-mediated overexpression of GSK3β in the ARC: effects on whole-body energy and glucose metabolism in mice

(a) Wild-type mice were stereotactically injected into the bilateral halves of the ARC with 2×200 nl of AAV2 virus expressing EGFP ($n=7$) as a control and GSK3β ($n=8$). Shown are the body masses of EGFP- and GSK3β-overexpressing animals maintained on a chow diet (*ad libitum*) for 11 weeks followed by 18 days on an HFD (*ad libitum*). (b) Glucose tolerance tests 1 (GTT1) and 2 (GTT2) (upper panel) and associated area under the curve (AUC; lower panel). GTT1, performed on day 58 on chow diet after surgery, revealed impaired glucose tolerance in AAV2–GSK3β mice compared with AAV2–EGFP mice. After mice were switched to an HFD for 18 days GTT2 revealed exacerbated enhanced glucose intolerance in mice treated with AAV2–GSK3β compared with HFD-induced

glucose intolerance in AAV2-EGFP mice. (c) DEXA scan images of representative animals with overexpression of AAV2-EGFP or AAV2-GSK3 β mice on a chow diet and an HFD (upper panel). Body lean and fat mass did not change after treatment with AAV2-GSK3 β virus on the chow diet. On the HFD, body lean mass was significantly reduced and body fat mass was increased accordingly after AAV2-GSK3 β overexpression (lower panels). Values are expressed as the percentage of body mass. (d) AAV2-GSK3 β over-expression in the ARC led to an increase in cumulative food intake in animals fed a chow diet or an HFD. Food intake is shown in kJ/day over the whole period of chow diet or an HFD. HFD led to a significant increase in energy expenditure (e), whereas GSK3 β overexpression did not alter energy expenditure regardless of diet; respiratory quotient was reduced on both the chow diet and HFD after GSK3 β overexpression (f). (g) GSK3 β over-expression led to an increase in *SOCS3* gene expression in the ARC. Representative autoradiographies of coronal brain sections exposed to ³⁵S-labelled riboprobe against SOCS3. Insets depict localization within the ARC of mice treated with AAV2-EGFP (left-hand) or AAV2-GSK3 β (right-hand). Semi-quantitative analyses of gene expression of *SOCS3* is represented in the histogram as the percentage of AAV2-EGFPb. (h) Plasma insulin levels as measured by ELISA were unaltered regardless of treatment with AAV20-EGFP or AAV20-GSK3 β . Results are means $\pm$ S.E.M., * *P* 0.05, ***P* 0.01 and ****P* 0.001.

Figure 5. Central GSK3 β regulates hepatic glucose production

(a) Shown are glucose concentrations (left-hand panel) and associated area under the curve (AUC; right-hand panel) during intraperitoneal pyruvate tolerance tests. A central injection of a GSK3 β inhibitor decreased hepatic glucose production in *Lep^{ob/ob}* mice. Pyruvate tolerance tests were performed 60 min after the administration of GSK3 β inhibitor (○) or vehicle (●) into the lateral ventricle ($n=5-11$ /group). (b) Hepatic PEPCK levels were semi-quantitatively assessed by immunoblotting using a specific antibody against PEPCK 90 min after the central administration of either GSK3 β inhibitor ($n=5$) or vehicle ($n=11$) and were normalized by β -actin. Results are means $\pm$ S.E.M., ** P 0.01 and *** P 0.001.