

A high-fat-diet-induced cognitive deficit in rats that is not prevented by improving insulin sensitivity with metformin

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Abstract

Aims/hypothesis We previously demonstrated that animals fed a high-fat (HF) diet for 10 weeks developed insulin resistance and behavioural inflexibility. We hypothesised that intervention with metformin would diminish the HF-feeding-evoked cognitive deficit by improving insulin sensitivity.

Methods Rats were trained in an operant-based matching and non-matching to position task (MTP/NMTP). Animals received an HF (45% of kJ as lard; $n=24$), standard chow (SC; $n=16$), HF + metformin (144 mg/kg in diet; $n=20$) or SC + metformin (144 mg/kg in diet; $n=16$) diet for 10 weeks before retesting. Body weight and plasma glucose, insulin and leptin were measured. Protein lysates from various brain areas were analysed for alterations in intracellular signalling or production of synaptic proteins.

Results HF-fed animals developed insulin resistance and an impairment in switching task contingency from matching to non-matching paradigm. Metformin attenuated the insulin resistance and weight gain associated with HF feeding, but had no effect on performance in either MTP or NMTP tasks. No major alteration in proteins associated with insulin signalling or synaptic function was detected in response to HF diet in the hypothalamus, hippocampus, striatum or cortex.

Conclusions/interpretation Metformin prevented the metabolic but not cognitive alterations associated with HF feeding.

The HF diet protocol did not change basal insulin signalling in the brain, suggesting that the brain did not develop insulin resistance. These findings indicate that HF diet has deleterious effects on neuronal function over and above those related to insulin resistance and suggest that weight loss may not be sufficient to reverse some damaging effects of poor diet.

Keywords Alzheimer's · Brain · Dementia · Diet · Insulin · Metformin · Signalling

Abbreviations

AMPK	AMP-activated protein kinase
APP	Amyloid precursor protein
CNS	Central nervous system
ERK	Extracellular regulated kinase
FIRI	Fasting insulin resistance index
GFAP	Glial fibrillary acidic protein
GSK3	Glycogen synthase kinase-3
HF	High fat
LC	Liquid chromatography
MCI	Mild cognitive impairment
mTOR	Mammalian target of rapamycin
MTP	Matching to position
NMTP	Non-matching to position
nNOS	Neuronal nitric oxide synthase
p70S6K	70 kDa ribosomal S6 kinase
PI3K	Phosphatidylinositol 3-kinase
PKB	Protein kinase B
SC	Standard chow
TBS	TRIS-buffered saline

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Introduction

Epidemiological evidence suggests a higher risk of Alzheimer's disease associated with type 2 diabetes [1–6]. This

implies related molecular pathologies affecting the progression of symptoms associated with each. Hence current diabetes therapeutic(s) may have value in treating Alzheimer's disease. Potential molecular connections include abnormal expression/activity of insulin-degrading enzyme [7, 8], glycogen synthase kinase-3 (GSK3) [9, 10], cholesterol biosynthesis enzymes [11] and activated glycation end-products [12, 13], and cerebrovascular alterations [14]. However the major *clinical* commonality in epidemiological studies is hyperinsulinaemia (indicative of insulin resistance). The increased risk of dementia associated with insulin resistance is particularly worrying, as it is rarely diagnosed but is present in two million people without type 2 diabetes in the UK. Obesity is the greatest risk factor for insulin resistance, while mid-life obesity increases risk of Alzheimer's disease later in life [15]. In addition, obesity in adolescents is associated with cognitive deficits [16], suggesting that poor diet in early life reduces cognitive performance during the formative years.

The mechanisms underlying effects of poor diet on the brain are not understood, but may be related to alterations in cerebral glucose/insulin signalling. Rats fed on a diet high in saturated fat (20% wt/wt beef tallow/soy oil) were impaired in a complex test of hippocampal function [17]. Performance was improved after glucose administration before testing only in those on the high-fat (HF) diet [17]. Similarly, young rats fed a diet rich in saturated (butter/corn oil; 38%kJ) fat performed worse in a spontaneous alternation task [18]. Direct administration of insulin to the hippocampus of control, but not HF-fed, rats improved performance in this task, a phenomenon mediated via activation of the phosphatidylinositol 3-kinase (PI3K) signalling pathway. Insulin administration also caused an increase in local glucose transport and glycolytic metabolism. Together these studies suggest that neuronal insulin resistance and/or alterations in glucose metabolism may underlie a decline in cognitive function associated with HF diets.

Insulin receptors are present on astrocytes and neurons in the brain, and insulin influences many aspects of neuronal biology. Similar pathways to those characterised in more classical insulin target tissues are active in the brain [19]. This includes insulin receptor phosphorylation of IRSs, activation of PI3K [20], phosphoinositide-dependent kinase-1 and protein kinase B (PKB) [21], and modulation of multiple downstream pathways (e.g. mammalian target of rapamycin [mTOR]–70 kDa ribosomal S6 kinase [p70S6K] and GSK3 [22]). In the periphery, this network controls glucose homeostasis, while, in the brain, GSK3 activity is linked to pathology of Alzheimer's disease [23]. In addition, insulin activates growth-promoting pathways, including RAS–Raf–ERK (extracellular regulated kinase), and immediate early genes such as *c-fos* (also known as *FOS*) and *c-jun* (also known as *JUN*) [24].

Diets rich in fat and cholesterol promote a decline in the insulin sensitivity of liver, muscle and adipose tissue [25–29], so it seems plausible that similar effects on the insulin sensitivity of neurons could occur in the obese population. HF feeding of mouse models of Alzheimer's disease (e.g. overproduction of mutant amyloid precursor protein [APP] or presenilin-1 leading to generation of amyloid pathology) induces more severe behavioural deficits and extensive pathology [30, 31]. Similarly, crossing these models with models of diabetes exacerbates cognitive problems [32]. This is consistent with the concept that the generation of insulin resistance accelerates the progression of Alzheimer's disease. Insulin protects retinal neurons from apoptosis induced by cellular stress, possibly via the PI3K–PKB pathway [33] or the mTOR–p70S6K pathway [34]. Therefore loss of responsiveness to insulin could render neurons more susceptible to neurotoxic insults (e.g. β -amyloid or inflammation), leading to decreased survival of neurons in brain areas associated with insulin action [35]. Peripheral hyperinsulinaemia reduces insulin transport across the blood/brain barrier, generating insulin deficiency in the brain (for a review, see Craft [36]), and thus reducing the protective actions of insulin on neurons. Conversely, hyperinsulinaemia in the periphery has been reported to promote hyperinsulinaemia in the central nervous system (CNS) [37]. In contrast with the protective effects of physiological insulin, hyperinsulinaemia can sensitise neurons to toxin and stress-induced insults in culture [38]. Also, peripheral high-dose insulin injection in mice causes a rapid and dose-dependent increase in tau phosphorylation in the CNS [39], a hallmark of Alzheimer's disease. In this way, too much *or* too little insulin in the brain could enhance neurodegenerative processes. Distinguishing whether any of these processes contributes to cognitive impairment is vital if we are to develop effective therapeutics.

We previously reported that HF-fed rats, lacking any specific risk factor for Alzheimer's disease development, exhibited a marked impairment in behavioural flexibility in a food-reinforced operant task in which the animals were required to switch (reversal learning) contingency [40]. This result was taken as evidence for perseveration in the HF-fed animals. The present study used co-treatment with metformin to investigate whether this deficit is associated with the peripheral insulin resistance observed in HF-fed animals. Additional experiments assessed the effect of HF feeding on the basal activity of key nodes of insulin signalling and the production of a number of Alzheimer-related proteins across several brain areas.

Methods

Animals All experiments were performed using male Wistar rats (Harlan, Bicester, UK), initial body weight 125–150 g.

Animals were housed in cages of four (12 h light/dark cycle) at $22\pm 1^\circ\text{C}$ and 50% humidity, with ad libitum access to food unless stated. Weight was monitored weekly, and in vivo experiments were performed in accordance with UK Home Office regulations under the auspices of Project Licence PIL60/3766.

Diet Rats were randomly assigned after initial training to receive standard chow (SC; kJ composition: 7.4% fat, 17.5% protein, 75.1% carbohydrate), an HF diet (SDS 824053; kJ composition: 45% fat, 20% protein, 35% carbohydrate), standard rat chow plus metformin (SC+M, 1,800 ppm) or HF diet plus metformin (HF+M, 1,800 ppm). All diets were supplied by SDS Diets (Grangemouth, Falkirk, UK). More animals received the HF diet than SC, as previous studies suggest a variable response to HF feeding.

Plasma analysis Blood samples were taken from the saphenous vein of the hind limb after an overnight fast and at least 3 days before behavioural testing. At the end of study, rats were fasted overnight, anaesthetised by isoflurane, and killed by cervical dislocation. Glucose was measured immediately using an Accuread hand-held monitor and blood collected into lithium/heparin-coated microvette tubes (Sarstedt, Leicester, UK). Plasma insulin and leptin were measured by ELISA (insulin: Ultra-sensitive Rat Insulin ELISA Kit from Crystal Chem, Downers Grove, IL, USA; leptin: R&D Systems, Abingdon, UK). The metformin levels in plasma were quantified by isotope dilution liquid chromatography (LC)-MS/MS [41] with some modifications. In brief, protein was precipitated from 10 μl rat plasma in the presence of metformin- D_6 , and 5 μl supernatant fraction was analysed on a Dionex Ultimate 3000 LC interfaced with a Thermo TSQ Quantum Ultra triple-quadrupole mass spectrometer (Thermo Fisher Scientific, Horsham, UK). Data were processed using LCQuan software. The recovery of metformin from low-, mid- and high-quality control samples ranged between 87% and 89%. Lower and upper limits of quantification were 25 and 500 ng/ml.

Apparatus, training and testing Behavioural tests were performed in a bank of eight operant chambers (Med Associates, Middlesex, UK) under food restriction (85% of the free feeding daily intake). Six weeks of training [40] was generally required for rats to reach the criterion (80 correct responses over 3 consecutive days) on the matching to position (MTP) task. Briefly, a single (sample) lever was presented, and depression of this caused its retraction and the start of an intra-trial interval (5 s). The first nose poke of the central food hopper after the intra-trial interval initiated the choice phase where both levers were extended, and a correct response (i.e. the sample lever) was rewarded with a sucrose pellet. An incorrect response resulted in no reward

and a 5 s ‘time out’ period during which both levers were retracted and the house light illuminated. Upon reaching the criterion, rats were tested for 5 days to provide baseline measurements. Animals were retested during week 10/11 on their respective diets. After 5 days retesting on the MTP task, the contingency was switched to a non-matching to position (NMTP) paradigm, which was identical apart from during the choice phase, when a response to the opposite lever was rewarded. Animals were tested for a further 5 days [40].

Tissue harvest A separate cohort of animals on SC or HF diets for 12 weeks had brain tissue isolated after a 14 h fast. One hemisphere was dissected on ice to give hippocampus, striatum, parietal cortex, frontal cortex and hypothalamus. Individual brain regions, liver and skeletal muscle were snap-frozen in liquid nitrogen.

Protein lysate preparation Frozen tissue was homogenised in $2 \times \text{vol.}$ lysis buffer (50 mmol/l TRIS/HCl [pH 7.4], 0.1 mmol/l EGTA, 1 mmol/l EDTA, 1% [vol./vol.] Triton X-100, 1 mmol/l Na_3VO_4 , 50 mmol/l NaF, 5 mmol/l NaPPi, 0.27 mol/l sucrose, 0.1% [vol./vol.] β -mercaptoethanol, complete protease inhibitor tablet [Roche, Lewes, UK]). Insoluble material was removed by centrifugation at 18,900 g. Protein concentration was determined by the Bradford method. Lysates were frozen in liquid nitrogen and stored at -80°C .

Western blotting Equal amounts of protein were resolved on BioRad 4–15% (wt/vol.) precast gels. Proteins were transferred to nitrocellulose (Hybond-C extra, Amersham Biosciences, Little Chalfont, UK), blocked in TRIS-buffered saline containing 0.2% (vol./vol.) Tween 20 and 3% (wt/vol.) non-fat dried milk for 1 h before incubation with primary antibodies (see electronic supplementary material [ESM] Table 1) overnight at 4°C followed by 3×10 min washes in TRIS-buffered saline/Tween 20. Immunodetection was performed using IRDye800-conjugated anti-rabbit IgG (Rockland Immunochemicals, Gilbertsville, PA, USA) and Alexa Fluor-conjugated goat anti-mouse (Invitrogen, Paisley, UK) on the Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE, USA). For quantification, fluorescence for each antibody (directly related to amount of target protein) was normalised to actin or tubulin on the same membrane, using the Odyssey software. Phospho-AMPK (AMP-activated protein kinase), GSK3 and ERK quantification was normalised to the endogenous protein.

Tyrosine-phosphorylated proteins were immunoprecipitated from 500 μg lysate overnight at 4°C using 5 μg PY20 antibody coupled to 10 μg protein G–Sepharose. Immunoprecipitates were washed in lysis buffer containing 0.5 mol/l NaCl and twice in lysis buffer. Pellets were resuspended in 40 μl SDS-PAGE sample buffer and heated to 100°C for 10 min.

PKB assay Total PKB was immunoprecipitated from 100 µg lysate overnight at 4°C using pan-PKB antibodies (1 µg anti-PKB-α, PKB-β and PKB-γ [42]) coupled to 5 µg protein G–Sephrose. Immunoprecipitates were washed in lysis buffer containing 0.5 mol/l NaCl and twice in assay buffer (50 mmol/l TRIS/HCl [pH 7.4], 0.03% [vol./vol.] Brij-35, 0.1% [vol./vol.] β-mercaptoethanol). Pellets were incubated with 30 µmol/l ‘crosstide’ peptide (GRPRTSSFAEF), 10 mmol/l MgCl₂ and 0.1 mmol/l [γ -³²P]ATP (0.5 × 10⁶ cpm/nmol) in assay buffer at 30°C with shaking for 15 min. Reaction was terminated by adsorption on to Whatman p81 paper (GE Healthcare, Little Chalfont, UK) and washing in 75 mmol/l phosphoric acid. Phosphate incorporation was measured by scintillation counting. One unit of kinase activity is that amount catalysing the incorporation of 1 nmol phosphate into substrate in 1 h.

Data analysis Fasting insulin resistance index (FIRI) was defined as (fasting glucose × fasting insulin)/25. Behavioural data were analysed by two-way ANOVA for repeated measures (main factors of diet and drug) or one-way ANOVA when appropriate. Data for blood biochemistry were not homogeneous among groups, and therefore a square root transform was used for statistical analysis. Biochemical data for brain were analysed by one-way ANOVA, with brain region as the repeated measure. Spearman rho statistics were used for correlation analyses. Significance was set at $p < 0.05$. All statistical analyses were performed using SPSS.

Results

Body weight and hormonal changes Body weight, fasting plasma glucose, plasma insulin and FIRI were comparable between the groups at baseline before the dietary and pharmacological interventions. HF diet significantly increased body weight gain ($p < 0.01$) within 3 weeks of the start of the study through to termination (Fig. 1a). Supplementation of the diet with metformin reduced body weight gain ($p < 0.01$; Fig. 1a) irrespective of the diet. The reduction in body weight with metformin was not due to differences in food or fluid intake (ESM Fig. 1).

After 12 weeks on the diet, fasting plasma insulin ($p < 0.01$; Fig. 1b) but not glucose (Fig. 1c) was significantly higher in the HF group, resulting in significant alteration in FIRI ($p < 0.01$; Fig. 1d). Metformin supplementation reduced fasting plasma insulin with both HF and SC diets ($p < 0.05$; Fig. 1b) and hence FIRI ($p < 0.05$; Fig. 1d), but had no effect on fasting glucose (Fig. 1c). There were no significant interactions between metformin and the diet, confirming that metformin had an equivalent effect in both SC and HF groups. Fasting plasma leptin was significantly increased in HF-fed

animals ($p < 0.01$; Fig. 1e). Supplementation of the diet with metformin significantly reduced leptin levels. This was more obvious in the SC group (Fig. 1e), although it was not a selective effect. The mean level of metformin in the plasma at the end of the study was 220.7 ± 27.8 pg/ml in SC animals and 198.9 ± 18.0 pg/ml in HF animals ($p = 0.497$).

Behavioural testing There was no significant difference in accuracy (% of correct presses) between groups at baseline. After 11 weeks on the respective diets, all groups exhibited a fall in accuracy, but there was significant improvement over the 5 days of testing (ESM Fig. 2a; $p < 0.01$). There was a significant decrease in the % of correct responses ($p < 0.01$; ESM Fig. 2a) in the HF group, and this was sustained until the final day of testing (Fig. 2a). Metformin supplementation of the diets had no significant effect on % of correct responses (Fig. 2a). The groups fed HF also displayed a significant reduction in the total number of presses ($p < 0.01$; ESM Fig. 2b), which is most obvious on the last day of testing (Fig. 2b). However, metformin had no significant effect on total presses (Fig. 2b).

Immediately after the task contingency was switched to NMTP, all groups exhibited poor accuracy (ESM Fig. 2c), but significantly improved over 5 days of testing. However, HF groups had reduced improvement over time and remained significantly impaired on the final day of testing (Fig. 2c). There was no significant effect of metformin on accuracy in animals receiving either diet, and, in fact, the performance of the SC group was actually slightly worse on day 5. As with the MTP task, the HF diet significantly reduced the total number of presses (correct plus incorrect, $p < 0.01$; Fig. 2d), and this effect was not significantly influenced by metformin.

Correlations of behavioural task with insulin sensitivity, leptin or body weight Accuracy for each task (% correct responses) for the SC ($n = 19$) and HF ($n = 24$) animals only did not correlate with insulin sensitivity (as determined by FIRI) or insulin concentrations, despite the HF animals having higher insulin and performing worse in the tasks compared with the SC animals. In contrast, there was a significant negative correlation between weight change after 12 weeks on the diet and accuracy, in both the MTP ($r^2 = -0.43$; $p < 0.01$) and the NMTP ($r^2 = -0.26$; $p < 0.01$) task.

Molecular analysis We assessed production of insulin signalling proteins (insulin receptor β-subunit, IRS1, ERK1/2, GSK3) in several brain areas dissected from SC- and HF-fed animals (Table 1 and ESM Fig. 3). In addition, we assessed the phosphorylation of ERK1/2, GSK3 and p70S6 kinase at residues associated with enzyme activity and regulated by insulin. Finally, we quantified the phosphorylation of AMPK, a target of metformin, at a residue vital for its activity. Production of

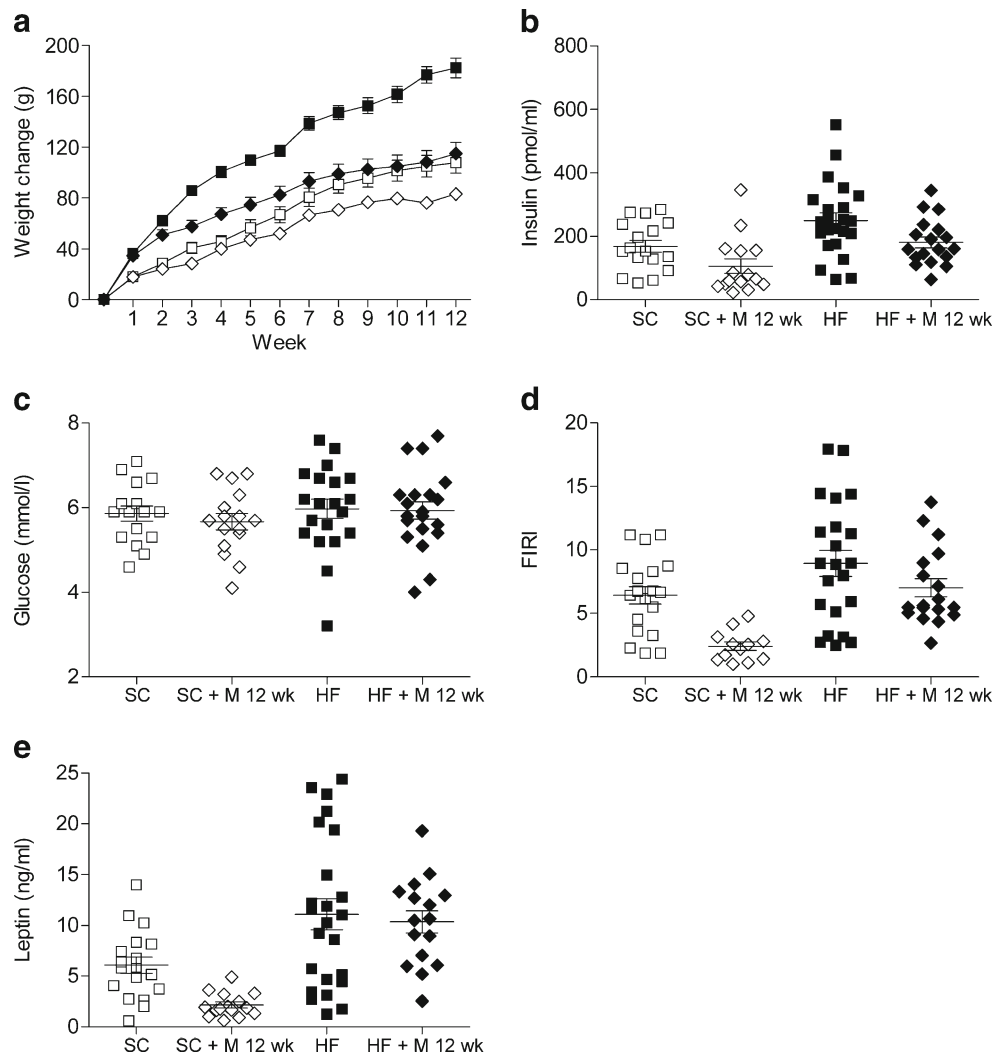


Fig. 1 Body weight and hormonal changes following 12 weeks (wk) of dietary intervention. After 12 weeks on the diet: (a) HF animals had gained significantly more weight than SC animals (diet: $F[1,75]=71.72$; $p<0.01$). Supplementing the diet with metformin (drug: $F[1,75]=11.91$; $p<0.01$) reduced body weight gain on a background of both SC and HF diets (diet \times drug; $p=0.36$). (b) Fasting plasma insulin was significantly greater in HF-fed animals (diet: $F[1,75]=11.93$; $p<0.01$) and was reduced (both SC and HF) by supplementation with metformin (drug: $F[1,75]=8.02$; $p<0.01$). (c) Plasma glucose was unaltered by diet ($F<1$) or drug ($F<1$). The FIRI (d) was significantly higher in

HF-fed animals (diet: $F[1,75]=8.93$; $p<0.01$) and reduced in animals receiving metformin (drug: $F[1,75]=4.24$; $p<0.05$). There was no diet \times drug interaction, confirming that metformin improved measures of insulin and insulin sensitivity irrespective of diet type (diet \times drug: insulin, $p=0.918$; FIRI, $p=0.820$). (e) Fasting plasma leptin levels were significantly higher in HF-fed animals (diet: $F[1,75]=22.31$; $p<0.01$) and reduced by the addition of metformin to the diet (drug: $F[1,75]=4.02$; $p<0.05$). White squares, SC ($n=19$); white diamonds, SC + M ($n=16$); black squares, HF ($n=24$); black diamonds, HF + M ($n=20$)

these proteins (relative to actin) varies across different brain regions (Table 1), but there were no major changes in the basal (fasted) production or phosphorylation of any signalling proteins after 12 weeks on the HF diet. Interestingly, there was a significant reduction in GSK3 β production in the hippocampus, albeit only a 15% change ($p<0.005$). This was not observed in any other brain area. Basal PI3K signalling was quantified by PKB immunoprecipitation and assay from lysates prepared from all brain areas, liver and muscle (Fig. 3). Interestingly, there was high PKB activity in brain compared with more classical insulin-sensitive tissues. However, there was no

significant effect of diet on basal PKB activity. Similarly, we did not detect IRS1 (Fig. 4a) or insulin receptor (data not shown) in phosphotyrosine immunoprecipitates from cortex or hippocampus. Conversely, if we immunoprecipitated IRS1, we detected very low tyrosine phosphorylation, but no difference between SC- and HF-fed animals (Fig. 4b, c). Finally, as IRS1 serine phosphorylation is implicated in obesity-induced antagonism of insulin signalling, we examined the phosphorylation of IRS1 at Ser616 (Fig. 4d). Although phosphorylation of this residue was detected, it did not differ between lysates prepared from SC- and HF-fed animals.

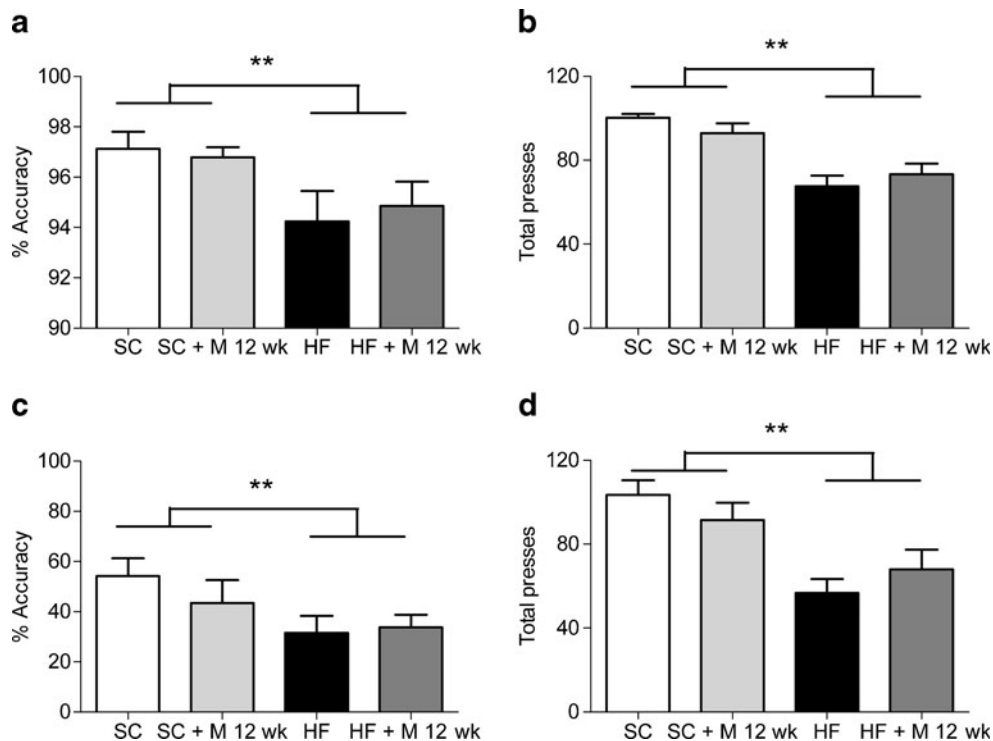


Fig. 2 Effect of dietary intervention on final day of testing for either the MTP or NMTP operant task. **(a)** Accuracy (% correct presses) on day 5 of testing on the MTP test. There was a significant reduction in % correct presses in HF-fed animals compared with those receiving the SC diet (diet: $F[1,75]=20.89$; $p<0.01$). Supplementation of the diet with metformin had no impact ($F<1$). The total number of presses (correct and incorrect) on day 5 of the MTP task **(b)** was also significantly lower in HF-fed animals (diet: $F[1,75]=36.34$; $p<0.01$), but metformin

had no effect ($F<1$). On day 5 of the NMTP task, the % correct presses **(c)** was also significantly impaired in HF- compared with SC-fed animals (diet: $F[1,75]=20.59$; $p<0.01$). Again there was no significant effect of metformin ($F<1$). There was also a significant reduction in the total number of presses **(d)** following the HF diet on day 5 of testing (diet: $F[1,75]=240.9$; $p<0.01$), but no significant effect of metformin (both $F<1$). SC, $n=18$; SC + M, $n=18$; HF, $n=24$; HF + M, $n=20$. ** $p<0.01$. wk, week

Next we investigated whether HF feeding altered the production of proteins with a confirmed link to Alzheimer's disease pathology (clusterin, synaptophysin, glial fibrillary acidic protein [GFAP], APP, tau, prion, neuronal nitric oxide synthase [nNOS]). In addition, we assessed the phosphorylation of 202/205 (AT8), 396 and 404 residues of tau, all of which are highly phosphorylated in Alzheimer's disease. We did not detect major alteration in the production or phosphorylation of these proteins in any brain area studied in response to the HF diet (Table 2). However, a significant increase in APP production (35%, $p<0.005$) was identified in parietal cortex, and a significant decrease in APP production (18%, $p<0.005$) observed in hippocampus. There was a 55% increase in synaptophysin production in parietal cortex of HF-fed animals in comparison with SC-fed rats ($p<0.05$).

Discussion

We have confirmed that HF feeding generates a deficit in behavioural flexibility, but we now establish that this is not

reliant on a decline in insulin sensitivity. We provide three pieces of evidence in support of this conclusion. First, intervention with metformin prevents the generation of insulin resistance, but has no effect on cognitive performance; second, there is no statistical correlation between insulin sensitivity and severity of cognitive deficit; third, key molecules of insulin signalling in different brain areas are not affected (at least in the fasted state) by the exposure to HF diet. Taken together, the data establish that HF diets have detrimental effects on brain function independently of changes in insulin sensitivity.

Previous studies have shown that glucose and/or insulin administration can alter performance in behavioural tasks [17, 18, 43], which, when taken together with the correlations between insulin sensitivity and decline in cognitive function in human populations, argues that insulin is a key regulator of cognitive function. The HF-fed rats in our study exhibited the greatest impairment when the task was switched, with perseveration responding to the previously rewarded lever. Patients with mild cognitive impairment (MCI, an early warning sign of dementia) have profound problems with perseverative errors in a modified Wisconsin

Table 1 Quantification of western blot analysis of signalling

Protein analysed	Hypothalamus		Hippocampus		Striatum		Parietal cortex		Frontal cortex	
	SC	HF	SC	HF	SC	HF	SC	HF	SC	HF
AMPK p-T172	0.35±0.01	0.35±0.01	1.20±0.02	1.33±0.02	1.48±0.07	1.53±0.04	1.51±0.06	1.49±0.03	1.10±0.04	1.21±0.02
Total ERK	94.5±6.4	85.5±3.9	80.7±2.6	77.2±1.6	123.0±8.7	123.6±5.3	129±12.8	125±7.8	22.2±1.7	23.4±0.8
ERK p-T202Y204	10.4±0.21	10.18±0.16	10.63±0.18	11.2±0.22	4.09±0.09	4.33±0.05	6.56±0.21	6.45±0.19	6.37±0.27	6.59±0.18
Total GSK	31.8±2.36	31.4±1.43	65.7±2.57	55.6±1.6 ^a	61.2±3.9	59.33±2.4	54.9±6.57	67.8±3.99	51.7±1.56	46.8±1.6
GSK3β p-S9	1.51±0.17	1.49±0.09	0.19±0.01	0.20±0.01	0.35±0.03	0.33±0.02	0.39±0.03	0.39±0.03	0.48±0.04	0.55±0.03
S6K1 p-T389	0.07±0.01	0.15±0.02	10.0±0.76	9.8±0.59	3.81±0.45	3.74±0.30	9.44±0.87	9.95±1.07	6.45±0.43	6.11±0.28
IRβ subunit	1.28±0.1	0.95±0.06	5.87±0.36	5.87±0.22	1.55±0.19	1.72±0.12	3.89±0.3	4.44±0.12	ND	ND
STAT3	3.2±0.27	3.5±0.16	ND	ND	11.1±1.4	10.6±0.87	17.1±0.82	19.2±0.5	8.72±0.52	10.0±0.32
S6, total	0.26±0.05	0.28±0.03	1.99±0.24	2.43±0.24	1.71±0.25	1.63±0.24	4.16±0.5	3.63±0.3	2.85±0.22	2.67±0.10
IRS1	ND	ND	0.33±0.02	0.36±0.02	0.11±0.02	0.11±0.01	0.33±0.01	0.39±0.03	0.13±0.01	0.14±0.01

CNS tissue was isolated after an overnight fast. Proteins of interest in each brain area were visualised by western blot of protein lysates using the specific antibodies listed in ESM Table 1 (representative blots are provided in ESM Fig. 3). All proteins were normalised to a loading control present on the same membrane. Phosphorylated GSK3 and ERK were normalised to total GSK3 and total ERK, respectively, and all others were normalised to β-actin. Values represent the mean ± SEM of $n=7$ for SC and $n=19$ for HF. Diet significantly affected the total GSK3 production (diet × region $F[4,96]=3.32$; $p<0.05$) in a regionally selective way

^aBrain regions where there was a significant difference between HF and SC groups

ND, not determined

Card Sorting Test [44]. This type of deficit is characteristic of frontal lobe dysfunction, but, in the case of MCI, it may result from alterations to basal forebrain cholinergic function or disruption of the tracts connecting parietal and frontal cortices. Total lever presses were reduced in HF-fed rats, possibly indicating reduced motivation. However, there was no evidence of a general decrease in locomotor activity or any reduction in effort to obtain a sucrose reward due to diet [40]. In addition, a reduced rate of response did not influence the performance in a task requiring discrimination of a large and small reward, or its reversal (ADM & CAS; unpublished data).

There is consensus that HF diet alters several aspects of cognitive ability; however, it seems likely that different HF-evoked cognitive deficits may arise by distinct mechanisms. Our work establishes that insulin resistance is not required for this deficit in behavioural flexibility and also questions whether exposure to HF diet alters signalling pathways in the brain, at least in the fasted state. It does not rule out the possibility that insulin resistance generates other cognitive problems (although we did not observe deficits previously in the Morris water maze [40]). We had hypothesised that insulin-sensitising drugs such as metformin could be an addition to the armoury in the fight against cognitive decline. Indeed, others have found that a different class of insulin-sensitising agent (rosiglitazone), as well as the insulin secretagogue, glibenclamide (known as glyburide in the USA and Canada), significantly improve cognitive performance in patients with type 2 diabetes, and this improvement correlated with better control of fasting blood glucose [45]. There are studies currently underway to examine potential benefits of metformin on cognitive tasks in patients with type 2 diabetes and MCI. Metformin has been used in the treatment of diabetes for decades, but its mechanism of action remains the subject of intense study. In humans, metformin has not been reported to induce weight loss, but in our study it prevented weight gain. This was not due to changes in food or water intake, but metformin is a metabolic poison and could thus alter metabolic rates. In addition, it reduces hepatic fat in fatty liver disease, while reducing hepatic glucose production and lowering fasting plasma insulin levels in type 2 diabetes [46], but the

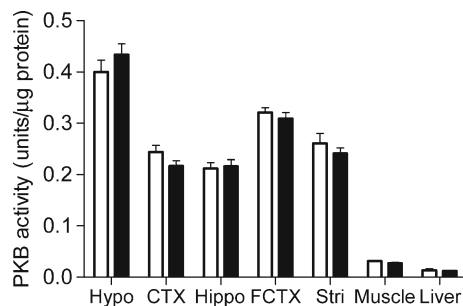


Fig. 3 Effect of diet on PKB activity in different brain areas. All three isoforms of PKB were simultaneously immunoprecipitated from 100 μg protein lysate generated from the tissues indicated after an overnight fast. The purified kinase was incubated in vitro with cross-tide and radiolabelled ATP, and inherent activity assessed by rate of phosphate incorporation into crosstide substrate. One unit of kinase activity is defined as that amount catalysing the incorporation of 1 nmol phosphate into crosstide in 1 h. Hypo, hypothalamus; CTX, cortex; Hippo, hippocampus; FCTX, frontal cortex; Stri, striatum. White bar, SC diet; black bar, HF diet

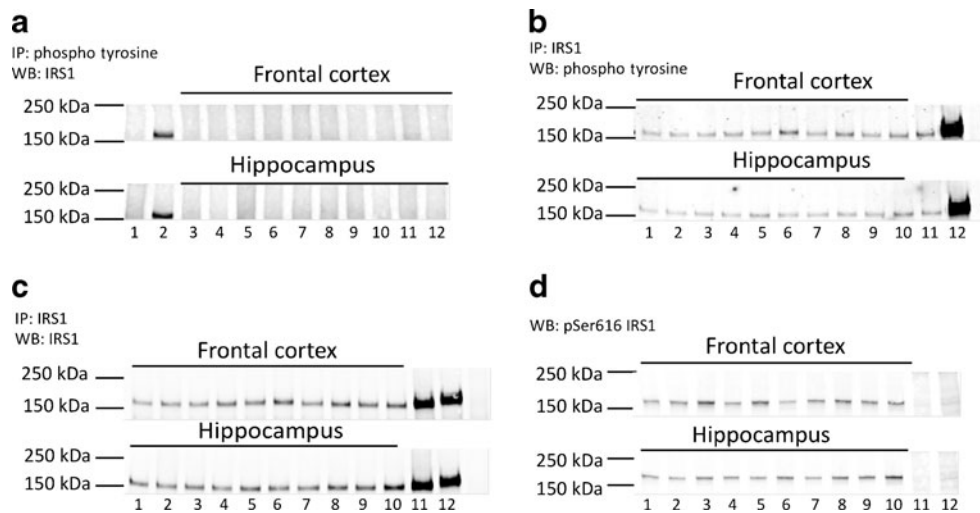


Fig. 4 Effect of diet on IRS1 phosphorylation. **(a)** Proteins phosphorylated on tyrosine residues were immunoprecipitated from 500 μ g protein lysate generated from the tissues indicated after an overnight fast (five separate animals from the SC and HF groups). Purified proteins were subjected to SDS-PAGE and immunoblotted for IRS1. **(b, c)** IRS1 was immunoprecipitated from 1 mg protein lysate generated from the tissues indicated after an overnight fast and immunoblotted for phosphotyrosine **(b)** or IRS1 **(c)**. **(d)** Lysates generated from

the tissues as indicated (five separate animals from the SC and HF groups) were immunoblotted using an antibody that recognises IRS1 only when phosphorylated at Ser616. Lysates generated from rat hepatoma cells (H4IIE) incubated with or without insulin for 30 min were included as a positive control. Lanes in **(a)**: 1, liver control; 2, liver insulin; 3–7, SC; 8–12, HF. Lanes in **(b–d)**: 1–5, SC; 6–10, HF; 11, liver control; 12, liver insulin. IP, immunoprecipitate; WB, western blot

molecular dissection of these actions remains incomplete [47]. Metformin can reduce tau phosphorylation in the brain of a mouse model of Alzheimer's disease [48], suggesting it crosses the blood/brain barrier and enters neurons and could have potential in the treatment of Alzheimer's disease. In our studies, metformin lowered fasting plasma insulin

without altering fasting blood glucose in either group, although the HF rats were not hyperglycaemic. This allowed us to dissociate potential effects on cognition due to improved insulin sensitivity from that of improved glycaemic control, and establish that improved insulin sensitivity in controls did not improve performance on this task.

Table 2 Quantification of western blot analysis of Alzheimer's disease-related proteins

Protein analysed	Hypothalamus		Hippocampus		Striatum		Parietal cortex		Frontal cortex	
	SC	HF	SC	HF	SC	HF	SC	HF	SC	HF
Prion	1.51±0.31	1.32±0.09	6.65±0.75	5.57±0.40	2.81±0.40	3.63±0.72	2.57±0.31	2.62±0.16	2.61±0.25	4.63±0.68
GFAP	9.26±1.65	8.92±1.09	9.38±0.84	9.45±0.59	0.57±0.09	0.80±0.14	1.76±0.3	1.76±0.13	6.60±0.68	6.44±0.47
Clusterin	4.67±0.19	4.09±0.12	10.67±0.26	10.59±0.3	0.65±0.04	0.66±0.02	3.54±0.27	3.35±0.12	2.22±0.07	1.94±0.1
APP	3.38±0.31	3.77±0.19	12.83±0.63	10.4±0.4 ^a	7.58±0.71	7.01±0.43	2.39±0.23	3.22±0.14 ^a	2.17±0.18	1.98±0.11
Synaptophysin	2.26±0.47	2.28±0.29	16.25±1.15	17.5±0.72	10.1±0.79	8.79±0.49	3.93±0.92	6.1±0.57 ^a	5.6±0.44	5.73±0.28
Tau5	5.95±1.5	12.5±0.90	103.7±6.08	103.3±3.7	17.9±2.86	16.0±1.74	104.2±4.8	102.8±2.9	19.5±1.71	19.5±1.04
Tau ph396	2.02±0.47	3.00±0.30	8.73±1.08	9.83±0.67	5.67±0.83	5.37±0.52	12.4±1.49	14.2±0.95	4.90±0.51	4.72±0.31
Tau ph404	ND	ND	2.02±0.13	2.35±0.08	ND	ND	6.34±0.45	6.26±0.29	2.07±0.15	1.89±0.1
Tau AT8	ND	ND	ND	ND	1.08±0.23	1.19±0.75	ND	ND	ND	ND
nNOS	ND	ND	17.87±2.04	19.5±1.24	0.09±0.01	0.07±0.01	10.0±0.95	8.7±0.6	17.30±1.8	19.3±1.1

CNS tissue was isolated after an overnight fast. Proteins of interest in each brain area were visualised by western blot of protein lysates using the specific antibodies listed in ESM Table 1 (representative blots are provided in ESM Fig. 3). All proteins were normalised to a loading control present on the same membrane. GFAP, APP and prion were normalised to tubulin, and all others were normalised to β -actin. Values represent the mean \pm SEM of $n=7$ for SC and $n=19$ for HF. Diet significantly affected APP (diet \times region $F[4,96]=6.33$; $p<0.001$) and synaptophysin (diet \times region $F[4,92]=2.65$; $p<0.05$) in a regionally selective way

^a Brain regions where there was a significant difference between HF and SC groups

ND, not determined

Since it is unlikely that neuronal insulin resistance or deficiency in response to HF diet would develop in the absence of peripheral insulin resistance, it is likely that neuronal insulin resistance is not responsible for the metformin-insensitive cognitive deficit observed. Although we did see a significant decrease in GSK3 β production in the hippocampus after HF feeding, this was a relatively small change, and no alteration in the phosphorylation of GSK3 β was observed. Furthermore, this small reduction in GSK3 did not affect tau phosphorylation (a GSK3 substrate) in the hippocampus (Table 1), consistent with normal IRS1–PI3K–PKB signalling (Figs 3 and 4). We have included a measure of a number of different insulin signalling pathways (albeit basal activity). Most of the signalling proteins examined play key roles in the response of cells to multiple extracellular stimuli (e.g. IGF1, brain-derived neurotrophic factor, nerve growth factor and insulin). Hence monitoring basal signalling of these molecules is not examining activity directly in response to insulin. However, it was not possible to obtain insulin-specific responses using the current experimental protocol, and indeed this is not trivial to achieve in different areas of the CNS. Interestingly, others have found that measurement of muscle (but not liver) insulin sensitivity varies with length of fasting before assessment [49], therefore it is conceivable that shorter periods of fasting could uncover differences in basal signalling, although we did maintain differences in fasting insulin during the 14 h fast. Taken together, our data argue against the development of major changes in basal insulin signalling pathways in the brain in response to HF feeding, and supports a CNS mechanism that is independent of insulin sensitivity underlying the loss of behavioural flexibility in the HF-fed animals. The small, isolated changes in APP and synaptophysin production in response to HF feeding may indicate changes in neuronal function, but more sophisticated approaches are required to confirm that the changes observed have a physiological outcome. An alternative possibility is that a component of the diet, or the weight gain, directly induces neuroinflammation or oxidative stress, due to release of cytokines and/or adipokines from the adipose or from exposure to high levels of triglyceride/fatty acids. If so, antioxidant and/or anti-inflammatory intervention should prevent this behavioural deficit. One could also speculate that the HF-diet-induced deficits in behavioural flexibility could provide an additional barrier to interventions aimed at reversing weight gain through lifestyle modifications. Importantly, the reduced weight gain in the metformin-treated animals did not reverse the deficit, thus weight gain is not mechanistically related to the maintenance of the deficit and hence weight loss may not, in itself, reverse the deficit.

In summary, we provide evidence that HF diet can be detrimental to brain function independently of alterations in

insulin sensitivity or glycaemic control. It remains to be established what component of the diet is responsible and the mechanism involved, but this work lays a foundation for a more widespread investigation of the effects of poor diet on cognition over and above weight change and insulin sensitivity.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

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