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Consumption of a high-fat diet, but not regular endurance exercise training, regulates hypothalamic lipid accumulation in mice

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Key points

- 'Lipotoxicity' refers to the excessive accumulation of lipids in non-adipose tissues that causes cellular dysfunction and, in severe cases, cell death. Lipotoxicity is often present in obesity.
- It is unknown whether lipotoxicity occurs in the hypothalamus of the brain, an area involved in the regulation of feeding behaviour and energy balance.
- We show that high-fat feeding results in increased lipid content in the hypothalamus, including triacylglycerol, diacylglycerol and ceramide, which was not reduced with 6 weeks of exercise training.
- The obese leptin-deficient *ob*/*ob* mouse fed a chow diet had normal hypothalamic lipid content.
- These data show that dietary lipids regulate hypothalamic lipid accumulation, which is not readily reversed by exercise training.

Abstract Obesity is characterised by increased storage of fatty acids in an expanded adipose tissue mass and in peripheral tissues such as the skeletal muscle and liver, where it is associated with the development of insulin resistance. Insulin resistance also develops in the central nervous system with high-fat feeding. The capacity for hypothalamic cells to accumulate/store lipids, and the effects of obesity remain undefined. The aims of this study were (1) to examine hypothalamic lipid content in mice with increased dietary fat intake and in obese *ob*/*ob* mice fed a low-fat diet, and (2) to determine whether endurance exercise training could reduce hypothalamic lipid accumulation in high-fat fed mice. Male C57BL/6 mice were fed a low- (LFD) or high-fat diet (HFD) for 12 weeks; *ob*/*ob* mice were maintained on a chow diet. HFD-exercise (HFD-ex) mice underwent 12 weeks of high-fat feeding with 6 weeks of treadmill exercise training (increasing from 30 to 70 min day^{-1}). Hypothalamic lipids were assessed by unbiased mass spectrometry. The HFD increased body mass and hepatic lipid accumulation, and induced glucose intolerance, while the HFD-ex mice had reduced body weight and improved glucose tolerance. A total of 335 lipid molecular species were identified and quantified. Lipids known to induce insulin resistance, including ceramide (22%↑), diacylglycerol (25%↑), lysophosphatidylcholine (17%↑), cholesterol esters (60%↑) and dihexosylceramide (33%↑), were increased in the hypothalamus of HFD *vs.* LFD mice. Hypothalamic lipids were unaltered with exercise training and in the *ob*/*ob* mice, suggesting that obesity *per se* does not alter hypothalamic lipids. Overall, hypothalamic lipid

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accumulation is regulated by dietary lipid content and is refractory to change with endurance exercise training.

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Abbreviations ALT, alanine transaminase; AST, aspartate transferase; BMP, bis(monoacylglycero)phosphate; CE, cholesterol esters; Cer, ceramide; CNS, central nervous system; COH, free cholesterol; CSF, cerebrospinal fluid; DAG, diacylglycerol; DHC, dihexosylceramide; dhCer, dihydroceramide; FA, free fatty acid; GM3, G_{M3} ganglioside; GTT, glucose tolerance test; HFD, high-fat diet; HFD-ex, high-fat diet exercise; IκBα, nuclear factor of κ light polypeptide gene enhancer in B-cells inhibitor α; JNK, c-Jun N-terminal kinases; LFD, low-fat diet; LPC, lysophosphatidylcholine; MHC, monohexosylceramide; mTOR, mammalian target of rapamycin; MUFA, mono-unsaturated fatty acid; NF-κB, nuclear factor κ-light-chain-enhancer of activated B cells; odd PC, odd chain phosphatidylcholine; PC, phosphatidylcholine; PC(O), alkylphosphatidylcholine; PC(P), alkenylphosphatidylcholine; PE, phosphatidylethanolamine; PE(O), alkylphosphatidylethanolamine; PE(P), alkenylphosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; S6K, p70 S6 kinase 1; SM, sphingomyelin; SM(OH), hydroxysphingomyelin; TAG, triacylglycerol; THC, trihexosylceramide.

Introduction

The term 'lipotoxicity' was coined by Unger and colleagues almost two decades ago and refers to the excessive accumulation of lipids in non-adipose tissues that causes cellular dysfunction and, in severe cases, apoptotic cell death (Lee *et al.* 1994; Unger, 2003). For example, the sphingolipid ceramide induces pancreatic β-cell failure and apoptosis (Shimabukuro *et al.* 1998), several lipid species cause insulin resistance in skeletal muscle and liver (Watt & Steinberg, 2008) and lipid accumulation is related to cardiomyopathy and heart failure (Khan *et al.* 2010). In light of the widely accepted association between excessive lipid accumulation and dysregulated cell function in obesity, it is surprising that few studies have examined whether lipotoxicity extends to the central nervous system (CNS).

Discrete regions of the CNS detect neural, endocrine and metabolic signals from the periphery to monitor whole-body nutrient availability. While not discounting the input of neuronal types located elsewhere in the brain, specialised neurons localised within the arcuate nucleus of the hypothalamus are critical regulators of feeding behaviour and body weight. This is largely attributable to the compromised blood–brain barrier in this region (Peruzzo *et al.* 2000). Plasma fatty acids cross the blood–brain barrier and gain access into the cerebrospinal fluid (CSF) (Miller *et al.* 1987; Freed *et al.* 1994) where CSF fatty acids generally reflect plasma fatty acid levels (Miller *et al.* 1987; Rapoport, 1996). Hypothalamic fatty acid uptake is increased in patients with the metabolic syndrome (Karmi *et al.* 2010); however, the rate of fatty acid β-oxidation is very low in the hypothalamus (Lennox, 1931), indicating that fatty acids do not serve as an important metabolic substrate for ATP production. This apparent mismatch between fatty acid availability/uptake and oxidation suggests that fatty acids are stored in the hypothalamus; however, the capacity for hypothalamic cells to accumulate/store lipids, the resulting type of stored lipid and the effect of these lipids on energy homeostasis remain undefined. In light of the apparent inability of the hypothalamus to modulate fatty acid oxidation when fatty acid delivery is increased, we postulate that the various cell types located in the hypothalamus would also be susceptible to lipotoxic outcomes. Indeed, diets enriched with the fatty acid palmitate (C16:0) promote diacylglycerol accumulation (Benoit *et al.* 2009) and apoptosis (Moraes *et al.* 2009) in the brain. Therefore, the first aim of the present study was to examine the effects of high-fat feeding on hypothalamic lipid species accumulation. Understanding the fate of fatty acids bears relevance for understanding the bases of cellular processes, including energy metabolism, and the pathogenesis of lipid related disease.

Physical activity (exercise) reduces body weight and adiposity, increases daily energy expenditure and is prescribed to enhance fatty acid oxidation, reduce tissue lipid content and enhance insulin action in peripheral tissues (Ross *et al.* 2000; He *et al.* 2004; Hu *et al.* 2004; Goodpaster *et al.* 2010). While these effects are well described in cardiac and skeletal muscle, liver and adipose tissue, the effects of exercise on hypothalamic lipid content remains unresolved. Thus, the second aim of this study was to examine the effect of regular endurance exercise training on lipid accumulation in the hypothalamus. We hypothesised that a high-fat diet and obesity in *ob*/*ob* mice would cause lipid accumulation in the hypothalamus and that regular exercise would reduce hypothalamic lipid levels in the setting of rodent obesity. We addressed these questions by performing unbiased mass spectrometry analysis on hypothalamic lysates obtained from mice fed a low-fat or high-fat diet that were sedentary or subjected to regular endurance exercise training.

Methods

Ethical approval

All experimental procedures were approved by the School of Biomedical Sciences Animal Ethics Committee (Monash University) and conformed to National Health and Medical Research Council (Australia) guidelines regarding the care and use of experimental animals. In addition, the authors have ensured that the experiments comply with the policies of *The Journal of Physiology* outlined by Drummond (2009) and the UK regulations on animal experimentation.

Animal care and husbandry

Male mice (C57BL/6) were purchased from Monash Animal Services. Mice were randomly assigned to their respective diets at 8 weeks of age, which consisted of a low-fat diet ($n = 14$) (5% energy from fat, LFD) or a high-fat micronutrient matched diet ($n = 23$) (59% energy from fat, HFD) *ad libitum* for 12 weeks (Specialty Feeds, Glen Forrest, WA, Australia). The LFD was composed of 15.6% saturated, 45.2% monounsaturated and 39.2% polyunsaturated fatty acids, while the HFD was composed of 60.3% saturated, 32.9% monounsaturated and 6.7% polyunsaturated fatty acids. The fatty acid compositions of the diets are provided in Supplementary Table 1. Body mass was monitored weekly throughout the course of the study. *ob*/*ob* mice were purchased from Monash Animal Services at 8 weeks of age and were maintained on a standard rodent chow (9% energy from fat) until 12–14 weeks of age ($n = 6$).

Treadmill exercise

After 6 weeks of feeding, mice within the HFD group were randomised to either a sedentary ($n = 13$) or endurance exercise trained group ($n = 10$). The exercise programme consisted of treadmill running once daily, five times a week for 6 weeks. The exercise intensity and duration were progressively increased (Supplementary Table 2).

Endurance capacity test

An endurance test was performed on all mice before and after the exercise training period to assess endurance capacity. Mice commenced running on the treadmill at 10 m min^{-1} and the speed was gradually increased to 17 m min^{-1} over 3 min, at a 5% slope until exhaustion, which was defined as sitting at the base of the treadmill and not attempting to re-engage the treadmill with manual prompting from the experimenter.

Glucose tolerance test (GTT)

Glucose tolerance testing was conducted in the last week of training, allowing two exercise free days before the test. Mice were fasted for 4 h and blood glucose was measured at 11.00 h before and after a bolus of glucose (2 g (kg body weight)$^{-1}$, 50% D-glucose in water) was injected intraperitonealy. Blood glucose was measured from the tip of the tail at 0, 15, 30, 45, 60, 90 and 120 min, using a glucometer (Accu-Chek, Roche). As an index of glucose tolerance, the incremental area under the curve was calculated from the blood glucose profiles using the 0 min time point as the baseline.

Analytical methods

Mice were killed 3 days after the last exercise bout to preclude acute effects on the measured variables. Mice were fasted for 4 h (11.00 h), anaesthetized via isoflurane inhalation and killed by decapitation; trunk blood was collected in tubes containing ethylenediaminetetraacetic acid (EDTA). Whole blood was centrifuged for 2 min at 8,000 × g and plasma collected for analysis. Free fatty acids (FAs; Wako Pure Chemical Industries, Osaka, Japan) and triacylglycerol (TAG) were measured by enzymatic colorimetric assays (GPO-PAP reagent, Roche Diagnostics). Alanine transaminase (ALT) and aspartate transferase (AST) were measured using as commercial kits (Thermo Electron, Melbourne, Australia). TAGs were extracted from peripheral tissues using the method of Folch *et al.* (1957) and quantified using an enzymatic colorimetric method (GPO-PAP reagent, Roche Diagnostics).

Immunoblotting

Hypothalamic lysates normalised for protein concentration (BCA method, Pierce Kit, Progen Industries, Darra, QLD, Australia) were solubilised in Laemmeli sample buffer and boiled for 5 min. They were then resolved by SDS-PAGE on 10% polyacrylamide gels, transferred to a polyvinylidenefluoride membrane, blocked with 5% milk for 1 h and probed with the appropriate primary antibody overnight at 4°C. After washing and incubation with a rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, Castle Hill, NSW, Australia), the immunoreactive proteins were detected with enhanced chemiluminescence and quantified by densitometry (ImageJ, NIH). The following primary antibodies from Cell Signaling Technology, Inc. (Danvers, MA, USA) were used: rabbit anti-phospho-JNK 1:1000 (Thr183/Tyr185) (no. 4671), rabbit anti-IκBα 1:500 (no. 4812), and from Sigma-Aldrich Pty, Ltd (Sydney, Australia), rabbit anti-α-actin 1:4000 (no. A5060). Phospho JNK was

corrected for total protein loaded using a Ponceau S stain (Sigma-Aldrich Pty, Ltd, Sydney, Australia).

Electrospray ionisation-tandem mass spectrometry of hypothalamic lipids

The hypothalamus was removed (defined caudally by the mamillary bodies, rostrally by the optic chiasm, laterally by the optic tract, and superiorly by the apex of the hypothalamic third ventricle). The whole hypothalamus (approximately 10–15 mg) was homogenised in modified RIPA buffer (Tris-HCl 50 mM, NaCl 150 mM, EDTA 1 mM, NaF 1 mM, 1 × protein inhibitor (Roche), pH 7.4) and 20–50 μg protein (15–25 μl) was extracted with chloroform–methanol (2:1; 20 vol) with the addition of internal standards (Meikle *et al.* 2011). Samples were spun on a rotary mixer, sonicated, centrifuged and the supernatant dried under N_2. They were then reconstituted in water saturated butanol (50 μl) and MeOH (50 μl) each containing 10 mM ammonium formate and centrifuged for 10 min. Analysis was performed on the supernatant by electrospray ionisation-tandem mass spectrometry (ESI/MS) using an AB Sciex 4000 Q/TRAP mass spectrometer with a turbo-ionspray source and Analyst 1.5 data system. Quantification of individual lipid species was performed using scheduled multiple-reaction monitoring in positive ion mode and Multiquant v1.2. Total lipid concentration of each class was calculated by summing the individual lipid species. The intra-assay coefficient of variation was 13.8 ± 0.5% (median ± SEM, across 325 lipids). The inter-day variability is not relevant because all samples were extracted and analysed on the same day.

Statistical analysis

All results are expressed as the mean ± SEM. Statistical analysis was performed by employing Student's *t* test for unpaired data, with LFD or HFD mice as the control. Body weight and the glucose tolerance test were analysed using a repeated measures two-way ANOVA with Bonferroni *post hoc* test. Metabolic data was analysed using a one-way ANOVA with a Dunnart's *post hoc* test, with HFD as the control group. Significance was established at the $P < 0.05$ level.

Results

Metabolic characterisation of mice in response to high-fat feeding and exercise training

The body mass of HFD mice was increased compared with LFD and exercise training did not affect absolute body mass in the HFD mice (Table 1). When expressed as a percentage of starting body mass, HFD-ex mice weighed less than HFD sedentary mice after 4 weeks of training, and this was maintained until the cessation of experiments (Fig. 1*A*). The epididymal fat mass was increased in HFD *vs.* LFD; however, exercise training did not significantly decrease epididymal fat mass (Fig. 1*B*). Liver triacylglycerol was increased with HFD and unaffected by exercise training (Fig. 1*C*). Exercise training increased running capacity in HFD mice as demonstrated by a 2.6-fold increase in the time to exhaustion during an endurance running capacity test (Fig. 1*D*). There was no change in running endurance in the LFD and HFD groups. Fasting blood glucose was increased in HFD *vs.* LFD and was reduced by exercise training (Table 1). Glucose tolerance was impaired in HFD *vs.* LFD mice, and was improved with exercise training. (Fig. 1*E*). Liver TAG is strongly correlated with whole body glucose tolerance (r^2=0.571, P=0.007). Surprisingly, fasting plasma FFA and TAG concentrations were decreased in the HFD *vs.* LFD (Table 1). Thus, exercise training induced marked changes in aerobic capacity and glucose tolerance, but mild alterations in blood lipids and body mass. High-fat feeding is accompanied by obesity in mice (Fig. 1*A*). Therefore, to differentiate the effects of obesity *per se* and high-fat feeding we examined lipids in the hypothalamus

Table 1. Body mass, glucose tolerance and plasma metabolites of mice after low-fat or high-fat feeding, and high-fat feeding with regular exercise training

	LFD	HFD	HFD-ex
Initial body weight (g)	23.0 ± 0.5	22.5 ± 0.6	23.6 ± 0.7
Final body weight (g)	31.1 ± 0.7*	36.8 ± 1.2	34.2 ± 1.1
Δ body weight (g)	8.1 ± 0.6*	14.3 ± 1.0	10.8 ± 0.9*
Blood glucose (mmol l^{-1})	8.0 ± 0.3*	10.7 ± 0.4	9.1 ± 0.3*
GTT iAUC	667 ± 99*	1676 ± 192	887 ± 179*
Plasma FFA (mmol l^{-1})	0.67 ± 0.06*	0.43 ± 0.05	0.38 ± 0.04
Plasma TAG (mmol l^{-1})	3.27 ± 0.38*	2.31 ± 0.38	1.98 ± 0.24
Plasma ALT (activity U l^{-1})	10.6 ± 1.3	11.5 ± 1.5	10.1 ± 0.8
Plasma AST (activity U l^{-1})	44.9 ± 7.3	45.5 ± 3.3	42.5 ± 6.1

n = 5–10 per group. GTT iAUC = glucose tolerance test incremental area under the curve, FFA = free fatty acid, TAG = triacylglycerol, ALT = alanine aminotransferase, AST = aspartate aminotransferase. *$P < 0.05$ *vs.* HFD.

of *ob*/*ob* mice fed a chow diet. The *ob*/*ob* mice are obese, possess many characteristics of other murine obesity models including insulin resistance, hyperlipidaemia and hypertension and are routinely used in studies examining obesity (Turpin *et al.* 2009). It is noteworthy that the *ob*/*ob* mouse is leptin deficient and does not faithfully recapitulate the HFD model, which is leptin resistant and hyperleptinaemic.

Hypothalamic lipids increase in response to high-fat feeding, but are not changed in *ob*/*ob* mice or with exercise training

A total of 335 molecular lipid species were identified in hypothalamic extracts. A summary of the lipid species and the most common molecular species within lipid types is given in Table 2.

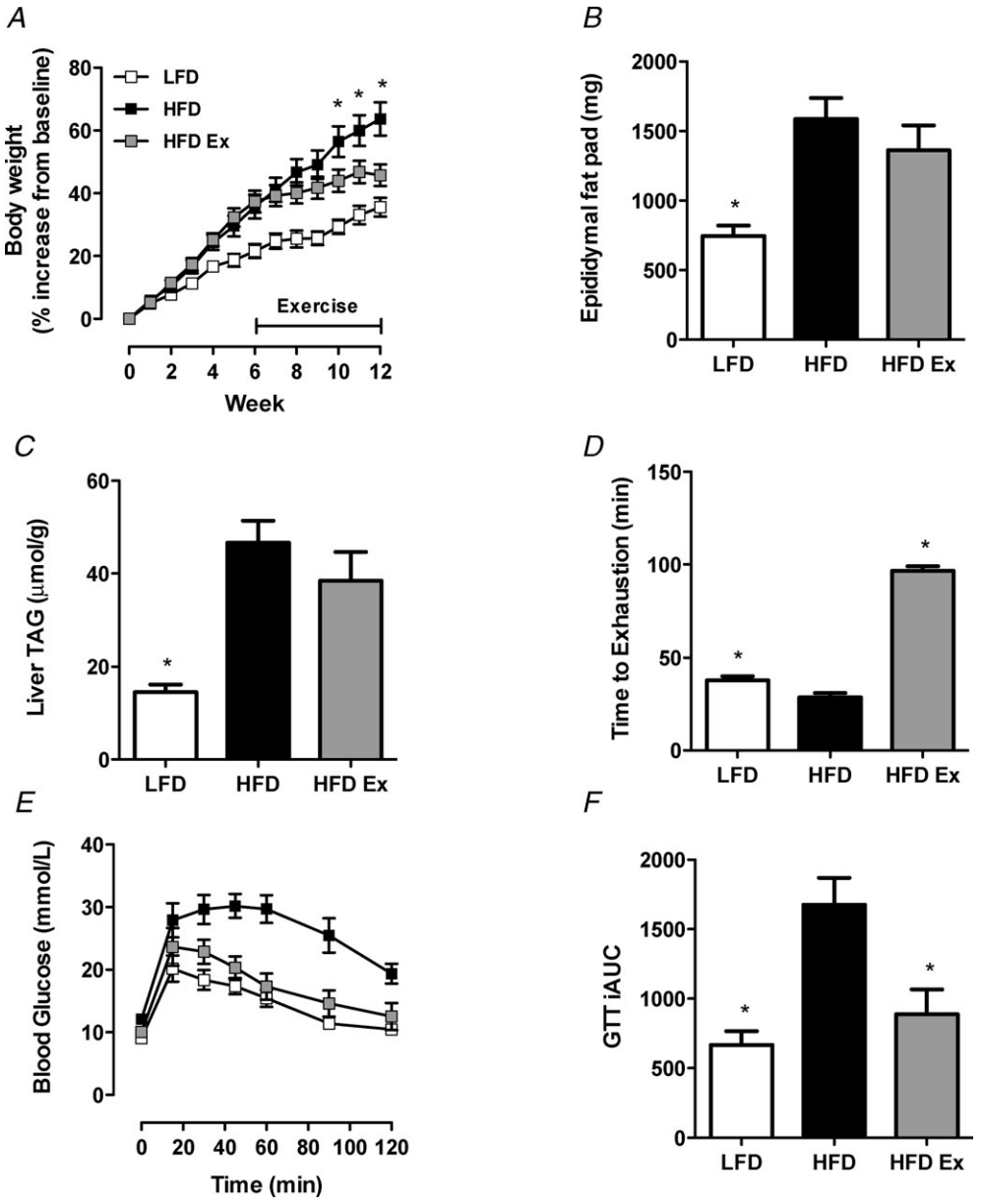

Figure 1. Metabolic characterisation of mice in response to high-fat feeding and exercise training
A, changes in body weight presented as a percentage of starting body weight. $n = 10$ per group, $^*P < 0.05$ HFD *vs.* HFD-ex at specific time point. *B*, epididymal fat pads were excised and weighed at time of killing. $n = 10$, per group $^*P < 0.05$ *vs.* HFD. *C*, livers were excised from LFD, HFD and HFD-ex mice and TAG content analysed. $n = 8$ per group, $^*P < 0.05$ *vs.* HFD. *D*, an endurance test was performed on all mice before and after the 6 weeks of exercise training. LFD ($n = 10$), HFD ($n = 10$) HFD-ex ($n = 5$), $^*P < 0.05$ *vs.* HFD. *E*, glucose tolerance tests were performed for LFD, HFD and HFD-ex mice at the end of the exercise period. $n = 5$ per group. *F*, the incremental area under the curve was calculated from the GTT data. $n = 5$ per group, $^*P < 0.05$ *vs.* HFD.

Table 2. Overview of lipid species in hypothalamic analysis

Species Name	Abbreviation	No. of species identified	Most abundant species	% of total lipid class
Free cholesterol	COH	1	—	—
Phosphatidylcholine	PC	31	34:1	17
Diacylglycerol	DAG	25	16:0 18:0	39
Phosphatidylserine	PS	7	40:6	43
Phosphatidylinositol	PI	20	38:4	46
Phosphatidylethanolamine	PE	31	18:0/22:6	21
Monohexosylceramide	MHC	6	24:1	58
Sphingomyelin	SM	10	18:0	71
Alkenylphosphatidylethanolamine	PE(P)	12	18:0/22:5	19
Lysophosphatidylcholine	LPC	21	16:0	45
Triacylglycerol	TAG	41	16:0 16:0 18:0	44
Ceramide	Cer	6	18:0	81
Alkylphosphatidylethanolamine	PE(O)	13	40:4	25
Alkenylphosphatidylcholine	PC(P)	14	38:6	61
Alkylphosphatidylcholine	PC(O)	23	34:1	35
Odd chain phosphatidylcholine	Odd PC	17	35:1	35
Hydroxysphingomyelin	SM(OH)	8	22:1	51
Cholesterol ester	CE	21	16:0	24
Phosphatidylglycerol	PG	4	16:0 18:1	72
Dihexosylceramide	DHC	6	18:0	53
G_{M3} ganglioside	GM3	6	18:0	84
Bis(monoacylglycero)phosphate	BMP	1	18:1 18:1	—
Dihydroceramide	dhCer	6	18:0	74
Trihexosylceramide	THC	2	18:0	91

Phospholipids. Phospholipids constitute ∼60% of the plasma membrane and >90% of some organelle membranes such as mitochondria (McMurchie, 1988). The most abundant phospholipid indentified was phosphatidylcholine (PC), followed by phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylethanolamine (PE), alkenylphosphatidylethanolamine (PE(P)), lysophosphatidylcholine (LPC), alkylphosphatidylethanolamine (PE(O)), alkenylphosphatidylcholine (PC(P)), alkylphosphatidylcholine (PC(O)), odd chain phosphatidylcholine (odd PC), phosphatidylglycerol (PG) and bis(monoacylglycero)phosphate (BMP) in descending order. PC(O) ($P = 0.03$, ↑13%) and BMP ($P = 0.04$, ↑24.6%) contents were increased in the hypothalamus of HFD *vs.* LFD mice, and LPC ($P = 0.07$, ↑12.8%), PC ($P = 0.09$,↑ 13.2%), PC(P) ($P = 0.08$, ↑11.7%) and PG ($P = 0.09$ ↑10.8%) tended to increase (Fig. 2*A*). Phospholipids were largely unaltered in *ob*/*ob vs.* LFD (Fig. S1*A*) or HFD-ex *vs.* HFD mice with the exception of PG ($P = 0.06$, ↓13.1%) (Fig. S2*A*).

Sterol lipids. Sterol lipids are important components of biological membranes and can act as hormones and signalling molecules. The most abundant sterol lipid quantified was free cholesterol (COH) followed by cholesterol esters (CE). CE tended to be increased in the hypothalamus of HFD *vs.* LFD mice ($P = 0.09$, ↑82.4%) (Fig. 2*B*). CE and COH were unchanged in the hypothalamus of *ob*/*ob vs.* LFD (Fig. S1*B*) and HFD-ex *vs.* HFD mice (Fig. S2*B*).

Sphingolipids. Sphingolipids play important roles in signal transmission and cell recognition, while sphingolipid metabolites, such as ceramide, participate in numerous signalling cascades that result in apoptosis, proliferation and inflammation. The most abundant sphingolipid identified was monohexosylceramide (MHC), followed by sphingomyelin (SM), ceramide (Cer), hydroxysphingomyelin (SM(OH)), dihexosylceramide (DHC), G_{M3} ganglioside (GM3), dihydroceramide (dhCer) and trihexosylceramide (THC) in descending order. dhCer ($P = 0.05$, ↑25.4%) and DHC ($P = 0.03$, ↑39.8%) contents were increased in the hypothalamus of HFD *vs.* LFD mice while Cer ($P = 0.08$, ↑25.4%), SM ($P = 0.07$, ↑12.9%) and MHC ($P = 0.08$, ↑22.7%) tended to increase (Fig. 2*C*). THC content was the only sphingolipid lipid increased in *ob*/*ob vs.* LFD mice ($P < 0.0001$, ↑132%) (Fig. S1*C*). Sphingolipids were unchanged in the hypothalamus of HFD-ex *vs.* HFD mice (Fig. S2*C*).

Glycerolipids. Diacylglycerol (DAG) ($P = 0.006$, ↑23.1%) and triacylglycerol (TAG) ($P = 0.04$, ↑23.9%) contents were increased in the hypothalamus of HFD *vs.* LFD mice (Fig. 2*D*). Glycerolipids were unchanged in the hypothalamus of *ob*/*ob vs.* LFD (Fig. S1*D*) and HFD *vs.* HFD-ex mice (Fig. S2*D*).

High-fat feeding causes remodelling of hypothalamic lipid species known to cause insulin resistance

The increase in DAG accumulation in the hypothalamus of HFD mice (Fig. 2*D*) was attributed to an increase in both saturated and mono-unsaturated fatty acids (MUFAs) (Fig. 3*A*), which was unexpected because the HFD contains a smaller percentage of MUFAs compared with the LFD (32.9% *vs.* 45.2%, respectively). This suggests post-prandial modification of lipids prior to storage. Similarly, the increased TAG accumulation in the hypothalamus of HFD mice (Fig. 2*D*) was attributed to an increase in both saturated FAs and MUFAs (Fig. 3*D*). There were no further changes observed in the fatty acid content of DAGs or TAGs in obese *ob*/*ob* or HFD-ex hypothalami (Fig. S3*B–D*). The composition of intracellular fatty acids in various lipid pools is often reflective of the dietary fatty acid composition (e.g. increasing dietary PUFAs increases intracellular PUFA storage) (Lee *et al.* 2006). However, the changes in the fatty acid composition of TAG, DAG and phospholipids within the hypothalamus were not reflected by the marked differences in the fatty acid composition between the LFD and HFD (Fig. S4).

The accumulation of ceramides and DAGs had been implicated in the development of insulin resistance in peripheral tissues; therefore we looked more closely at individual species changes of these lipids in the hypothalamus. Ceramide species 18:0 ($P = 0.05$, 24.8%), 22:0 ($P = 0.01$, 29.1%) and 24:0 ($P = 0.02$, 28.9%) were increased in the hypothalamus of HFD *vs.* LFD mice (Fig. 4*A*), while ceramide species 20:0 ($P = 0.06$,

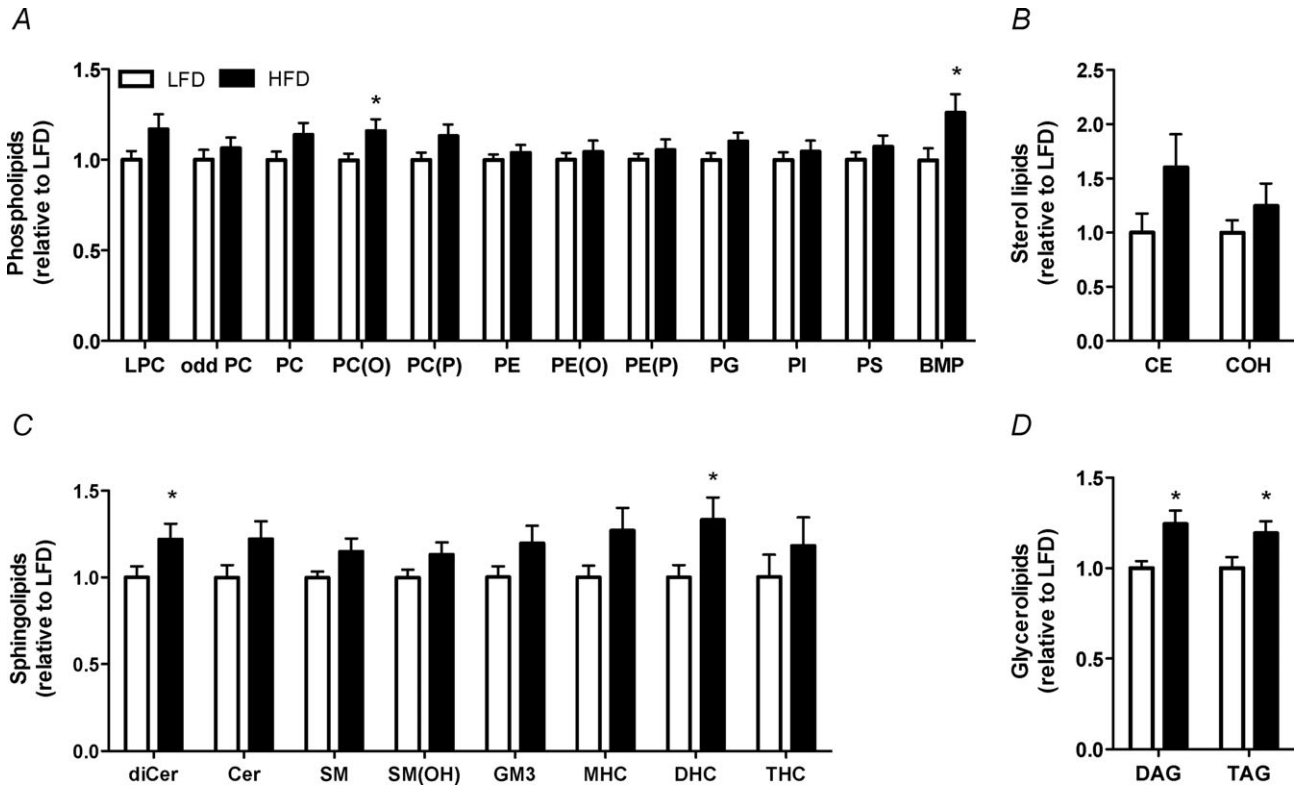

Figure 2. Hypothalamic lipid accumulation in response to high-fat feeding
The hypothalamus was excised from LFD and HFD mice and analysed for total lipid content. *A*, phospholipid content, lysophosphatidylcholine (LPC), odd chain phosphatidylcholine (odd PC), phosphatidylcholine (PC), alkylphosphatidylcholine (PC(O)), alkenylphosphatidylcholine (PC(P)), phosphatidylethanolamine (PE), alkylphosphatidylethanolamine (PE(O)), alkylphosphatidylethanolamine (PE(P)), phosphatidylglycerol (PG), phosphatidylinositol (PI) and phosphatidylserine (PS). bis(monoacylglycero)phosphate (BMP) *B*, sterol lipid content, cholesterol ester (CE) and cholesterol (COH). C Sphingolipids dihydroceramide (dhCer), ceramide (Cer), sphingomyelin (SM), hydroxyphingomyelin (SM(OH)), G_{M3} ganglioside (GM3), monohexosylceramide (MHC), dihexosylceramide (DHC) and trihexosylceramide (THC). D Glycerolipid content, diacylglycerol (DAG) and triaclyglycerol (TAG). LFD, white bars, HFD black bars. $n = 13–14$ per group, $^*P < 0.05$ *vs.* LFD.

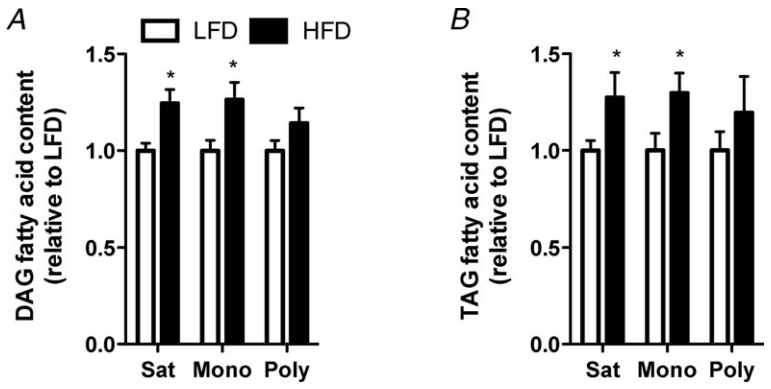

Figure 3. Hypothalamic DAG and TAG fatty acid content in response to high-fat feeding
Hypothalamic DAG (*A*) and TAG (*B*) content was analysed for the total amount of saturated (Sat), monounsaturated (Mono) and polyunsaturated (Poly) fatty acids. LFD, white bars; HFD, black bars; $n = 13$–14 in each group, $^{*}P < 0.05$ *vs.* LFD.

↑12.2%) and 24:1 ($P = 0.09$, ↑38.5%) tended to increase. The 10 most abundant DAG species were analysed. DAG species 14:0 16:0 ($P = 0.004$, 13.9%), 18:1 18:1 ($P = 0.005$, 31.4%), 16:0 20:0 ($P = 0.003$, 22.7%), 18:0 18:1 ($P = 0.02$, 25.0%), 16:0 18:1 ($P = 0.008$, 24.8%), 16:0 16:0 ($P = 0.0002$, 20.2%), 18:0 18:0 ($P = 0.004$, 25.5%), and 16:0 18:0 ($P = 0.0005$, 24.5%) were all increased in the hypothalamus of HFD *vs.* LFD mice (Fig. 4*B*).

Hypothalamic serine/threonine kinase signalling in response to high-fat feeding, obesity and exercise training

High-fat feeding and obesity are associated with low grade inflammation and activation of pro-inflammatory serine/threonine kinases (Wellen & Hotamisligil, 2005). The NF-κB pathway was activated in the hypothalamus of HFD *vs.* LFD (Fig. 5*A*) and *ob*/*ob* mice (Fig. 5*B*) as demonstrated by reduced IκBα expression. IκBα

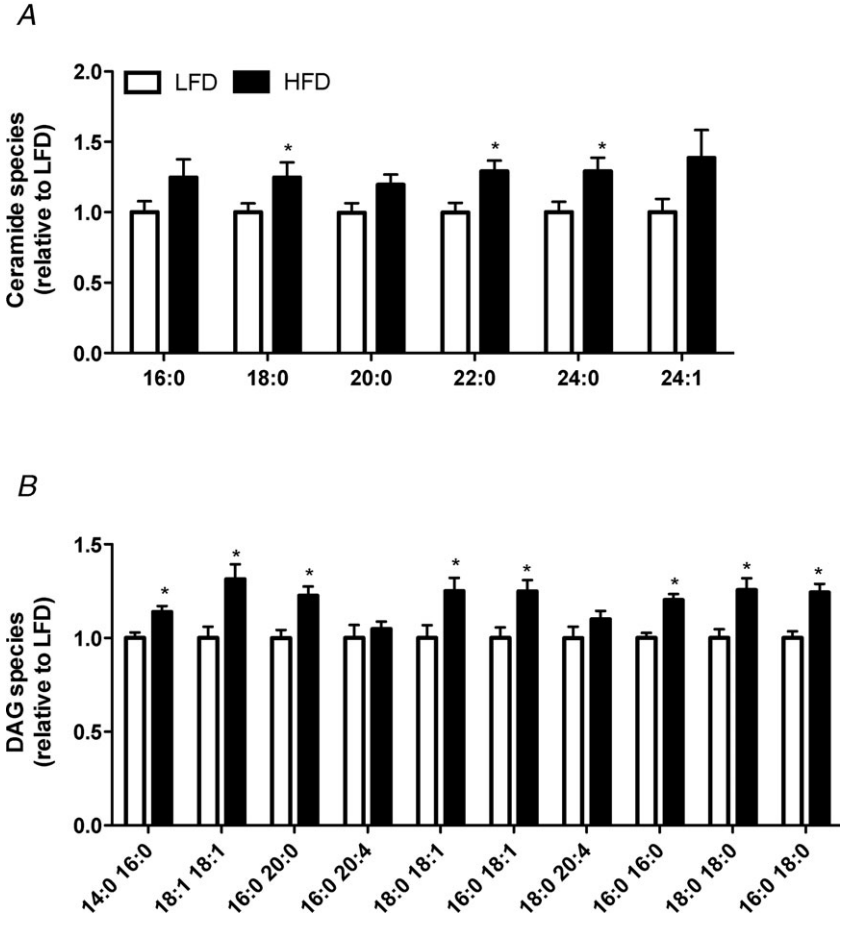

Figure 4. Individual species of hypothalamic bioactive lipids in response to high-fat feeding
A, individual ceramide species. *B*, the 10 most abundant diacylglycerol (DAG) species. LFD, white bars; HFD, black bars; $n = 13$–14 in each group, $^{*}P < 0.05$ *vs.* LFD.

expression was increased with exercise training in high-fat fed mice (Fig. 5*C*), indicating that regular exercise training was able to partially attenuate hypothalamic pro-inflammatory signalling induced by high-fat feeding. In contrast, hypothalamic JNK signalling was not affected by obesity or exercise training (Fig. 5*D*–*F*).

Discussion

The excessive storage of lipids in cell types other than adipocytes creates cellular stress leading to cellular dysfunction and sometimes apoptotic cell death (e.g. lipotoxicity), processes that underpin the pathogenesis of disease states such as non-alcoholic steatosis, atherosclerosis and type 2 diabetes. ESI/MS-facilitated lipidomics has provided the opportunity to quantify the lipidome of tissues and thereby enhance the understanding of human disease. Here, we have utilised this technology to demonstrate that increasing dietary fat in mice increases the content of several lipid species in the hypothalamus and that regular exercise training is unable to ameliorate these effects.

Physiological variations of plasma FA concentrations can be detected and integrated by FA sensing hypothalamic neurons to regulate feeding behaviour and substrate metabolism (Lam *et al.* 2005). In this way, it is proposed that FA fluxes signal the metabolic state of the organism. Short term intracerebroventricular (I.C.V.) infusion of palmitate induces inflammatory stress (Posey *et al.* 2009), endoplasmic reticulum stress, insulin and leptin resistance (Benoit *et al.* 2009; Kleinridders *et al.* 2009; Posey *et al.* 2009) and apoptosis (Moraes *et al.* 2009). However, it is uncertain whether the accumulation of intra-hypothalamic lipid(s) mediates these responses because CNS ablation of the toll-like receptor adaptor protein, MyD88 prevents many of these effects (Kleinridders *et al.* 2009). Moreover, the effects of palmitate are unique for this the type of fatty acid (Coll *et al.* 2008), and from a physiological perspective, should be interpreted with caution when a mixture of saturated

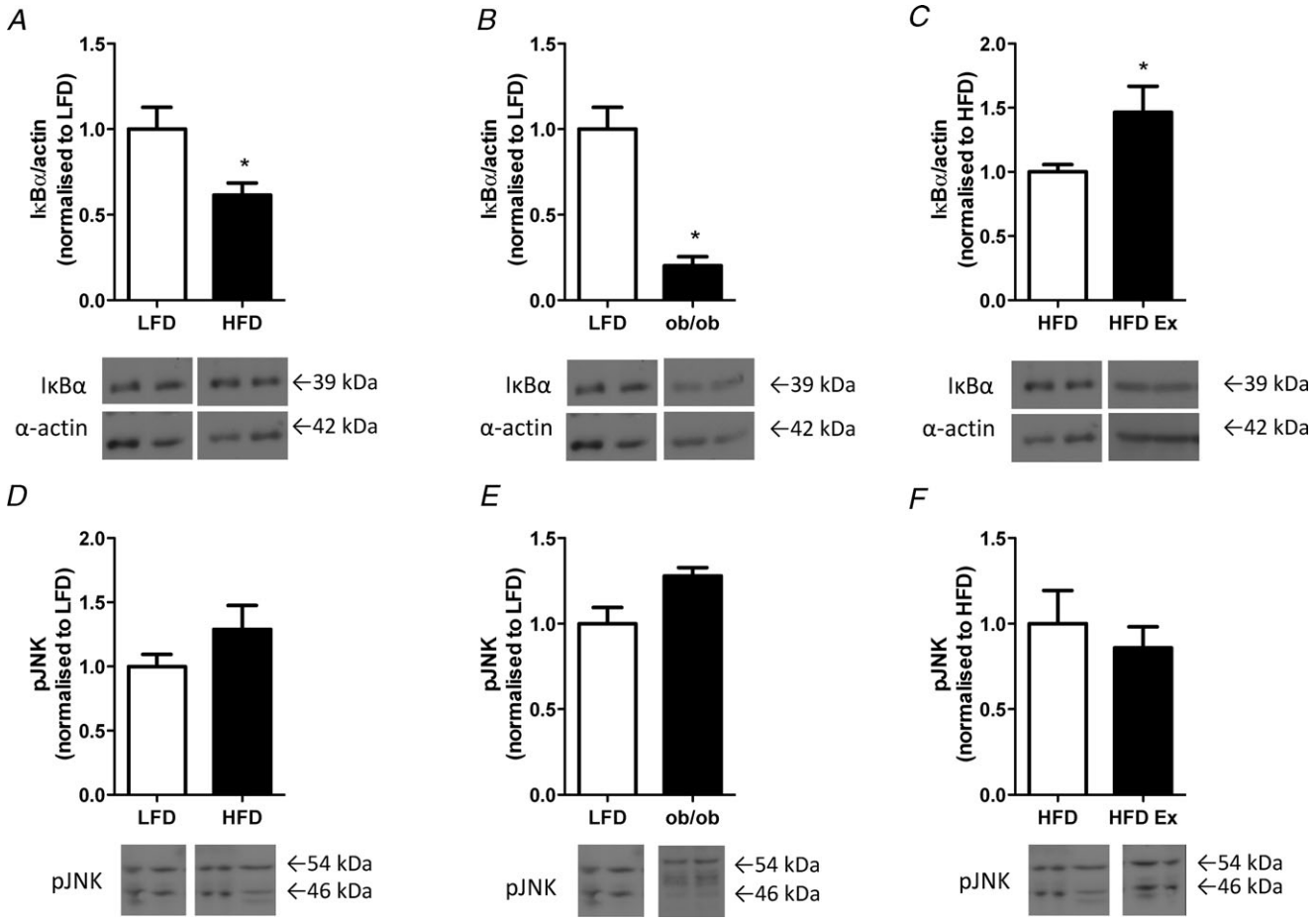

Figure 5. Hypothalamic stress signalling in response to high-fat feeding, obesity and exercise training
The hypothalamus was excised and analysed for IκBα protein expression in LFD *vs.* HFD (*A*), LFD *vs. ob/ob* (*B*), and in HFD *vs.* HFD-ex (*C*). The hypothalamus was also analysed for pJNK protein expression in LFD *vs.* HFD (*D*), LFD *vs. ob/ob* (*E*) and HFD *vs.* HFD-ex (*F*). $n = 3$–11 per group, $^*P < 0.05$ *vs.* control.

and unsaturated fatty acids naturally perfuse the brain (Watt *et al.* 2012). Hence, we asked whether prolonged increases in dietary fat would alter hypothalamic lipid composition. Our experiments demonstrate that high fat feeding increases the total contents of several neutral lipid species such as the phospholipid PC(O) and TAG, whilst also increasing signalling lipids, such as the sphingolipids dhCer and DHC, and the glycerolipid DAG.

The accumulation of ceramides and DAGs had been implicated in the development of insulin resistance in peripheral tissues such as the liver and skeletal muscle (Holland *et al.* 2007). We examined the molecular species of these lipids in the hypothalamus of high-fat fed mice. The majority of ceramide species were increased in the hypothalamus with high-fat feeding (Fig. 4*A*). High-fat feeding causes central insulin resistance as evidenced by decreased insulin signal transduction and an inability of insulin to suppress food intake when delivered directly into the brain (Ono *et al.* 2008; Posey *et al.* 2009). The molecular underpinnings of hypothalamic insulin resistance are unclear, although several mechanisms have been proposed including activation of the IKKβ/NF-κB pathway (Posey *et al.* 2009), PKCθ (Benoit *et al.* 2009), JNK (Belgardt *et al.* 2010), p70 S6 kinase 1 (S6K; the major downstream effector of the mammalian target of rapamycin (mTOR; Ono *et al.* 2008), and endoplasmic reticulum stress (Ropelle *et al.* 2010) all of which can directly interfere with components of the insulin signalling cascade. Here, we show that ceramide accumulates in the hypothalamus of high-fat fed mice, and together with previous observations (Gao *et al.* 2011; Holland *et al.* 2011) supports a putative role for ceramide in the development of central insulin resistance. DAG is an intermediate of both TAG and phospholipid metabolism, accumulates in the muscle and liver with diet-induced obesity and is postulated to be a key lipid intermediate linking nutrient excess to insulin resistance (Montell *et al.* 2001; Itani *et al.* 2002). DAG was elevated in the hypothalamus of high-fat fed mice and increases were detected in eight of the 10 species analysed, many of which contain saturated fatty acids (Fig. 5*B*). Itani *et al.* (2002) showed that lipid induced insulin resistance was caused by increase DAG accumulation and membrane-associated PKC-βIII and -δ and a decrease in IκBα. DAG is a potent allosteric activator of both conventional and novel PKC isoforms (Bronfman *et al.* 1988), suggesting a possible role in the aetiology of CNS insulin resistance.

Having confirmed lipid accumulation in the hypothalamus with high-fat feeding, we examined the hypothalamic lipid profile of the *ob*/*ob* mouse, a monogenic obesity model characterised by leptin deficiency, severe peripheral insulin resistance and ectopic lipid accumulation. We anticipated that the ectopic lipid accumulation would extend to the hypothalamus of the *ob*/*ob* mice. Unexpectedly, lipids were not increased in the hypothalamus of *ob*/*ob* mice, with the exception of a marked increase in THC. The discrepancies between these models of obesity (high-fat feeding and the *ob*/*ob* mouse) might reflect the differences in dietary composition, with *ob*/*ob* mice consuming a low-fat diet. While the absence of leptin may account for the differences in hypothalamic lipid storage between obesity models, it appears that obesity *per se* may not drive hypothalamic lipid accumulation; rather diets enriched in fatty acids may mediate this process.

Exercise training has been used as a mode to reduced lipid accumulation in the liver and skeletal muscle of humans (Bruce *et al.* 2006; Goodpaster *et al.* 2010) and rodents (Mitchell *et al.* 2004; Petridou *et al.* 2005). We investigated its use as a means of reducing lipid content in the hypothalamus of high-fat fed mice. Surprisingly, exercise training did not reduce the total lipid content of the hypothalamus in mice fed a high-fat diet (Fig. S2). Unlike studies showing exercise-mediated plasticity of the phospholipid pool in rodent and human muscle (Andersson *et al.* 1998; Mitchell *et al.* 2004), and liver (Petridou *et al.* 2005) and reductions in ceramide, DAG and TAG (Bruce *et al.* 2006; Goodpaster *et al.* 2010), the hypothalamic lipidome was essentially unaltered with exercise training (Fig. S2). Exercise training is associated with increased oxidation of fatty acids in peripheral tissues that are postulated to limit lipid accumulation (Bruce *et al.* 2006). It is unknown whether the rate of hypothalamic fatty acid oxidation is altered with exercise training. In this regard, 1 week of endurance exercise training does not alter the expression of several proteins associated with fatty acid metabolism including carnitine palmitoyltransferase 1B, carnitine palmitoyltransferase 1C, medium-chain acyl-CoA dehydrogenase, nuclear respiratory factor 1, peroxisome proliferative-activated receptor-γ coactivator-1α, uncoupling protein 2 (all involved with fatty acid oxidation), fatty acid translocase, glycerol-3-phosphate acyltransferase, and diacylglycerol acyltransferase 1 (fatty acid uptake/storage) (data not shown). Indeed, oxidation rates may never be high enough to limit lipid accumulation in the setting of lipid oversupply.

Sustained excessive energy intake adversely influences cognitive function, and a sedentary lifestyle exacerbates these adverse effects of overeating (Mattson *et al.* 2010). Several neurological disorders are characterised by defective lipid metabolism, and increase in prevalence with obesity. For example, long chain ceramides (C_{18-24}) are increased in the brain in Alzheimer's disease, HIV, arteriosclerosis, stroke and ageing (Han *et al.* 2002; Cutler *et al.* 2004; Haughey *et al.* 2004; Han, 2005; Sawai *et al.* 2005) and ceramide accumulation is detrimental to neuronal cell function via the induction of apoptosis (Hannun & Obeid, 2002). From a metabolic viewpoint, hypothalamic ceramide accumulation is linked to insulin resistance

(Holland *et al.* 2011). The evidence presented in our studies supports a role for altered lipid metabolism in the development of hypothalamic insulin resistance, but does not support a generalised role of 'obesity' *per se*.

There were several considerations/assumptions made in the analysis and interpretation of the lipidomics data: (1) the lipids represent the sum of all hypothalamic cell types that include various hypothalamic nuclei, astrocytes, oligodendrocytes, ependymal cells and radial glia, but it has been suggested that the gross lipid composition of neurons and astrocytes are quite similar (Norton *et al.* 1975); (2) the lipidomics analysis provides a 'snapshot' of cellular lipid levels and does not assess fluxes; (3) the number of internal standards are limited and assume that the one standard for each class of lipids is representative of all species in that class; and (4) there may be some degradation of lipids during the hypothalamic extraction. However, this is unlikely because brains were sectioned on ice and snap-frozen within 1 min of decapitation. Despite these limitations, the relative changes between groups are accurate as all samples were treated the same. It should also be noted that the biological implications of the changes in the lipid compositions are likely to be complex and difficult to predict on the basis of lipidomics data alone. Indeed, the biological effects of lipids depend on their location (membrane *vs*. cytosolic *vs*. nuclear) and amount (Schievella *et al.* 1995; Simopoulos, 2006) and these detailed questions will be examined in future studies.

In conclusion, we have demonstrated that high-fat feeding results in lipid accumulation in the hypothalamus of mice and that hypothalamic lipids remain elevated despite regular endurance exercise training. Furthermore, the hypothalamic lipids remain unchanged in genetically obese mice fed a chow diet. Together, these data suggest that dietary lipids regulate hypothalamic lipid accumulation and this is not readily reversed by an exercise intervention.

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Author contributions

M.L.B. and M.J.W. conceived the conceptual framework of the study and designed the experiments. S.O. performed the exercise training. Tissue collection was performed by M.L.B. and M.J.W. Biochemical and lipidomic analysis was performed by M.L.B., S.O., J.W. and P.J.M. M.L.B. wrote the manuscript with M.J.W. and it was reviewed/edited by P.J.M. All authors approved the final version of the submitted manuscript. All experiments were performed at Monash University; the lipidomics analysis was performed at the Baker IDI.

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