Obesity Weighs down Memory through a Mechanism Involving the Neuroepigenetic Dysregulation of Sirt1

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Aberrant gene expression within the hippocampus has recently been implicated in the pathogenesis of obesity-induced memory impairment. Whether a dysregulation of epigenetic modifications mediates this disruption in gene transcription has yet to be established. Here we report evidence of obesity-induced alterations in DNA methylation of memory-associated genes, including Sirtuin 1 (*Sirt1*), within the hippocampus, and thus offer a novel mechanism by which SIRT1 expression within the hippocampus is suppressed during obesity. Forebrain neuron-specific *Sirt1* knock-out closely recapitulated the memory deficits exhibited by obese mice, consistent with the hypothesis that the high-fat diet-mediated reduction of hippocampal SIRT1 could be responsible for obesity-linked memory impairment. Obese mice fed a diet supplemented with the SIRT1-activating molecule resveratrol exhibited increased hippocampal SIRT1 activity and preserved hippocampus-dependent memory, further strengthening this conclusion. Thus, our findings suggest that the memory-impairing effects of diet-induced obesity may potentially be mediated by neuroepigenetic dysregulation of SIRT1 within the hippocampus.

Key words: DNA methylation; epigenetics; high-fat diet; memory; obesity; Sirt1

Significance Statement

Previous studies have implicated transcriptional dysregulation within the hippocampus as being a relevant pathological concomitant of obesity-induced memory impairment, yet a deeper understanding of the basis for, and etiological significance of, transcriptional dysregulation in this context is lacking. Here we present the first evidence of epigenetic dysregulation (i.e., altered DNA methylation and hydroxymethylation) of memory-related genes, including *Sirt1*, within the hippocampus of obese mice. Furthermore, experiments using transgenic and pharmacological approaches strongly implicate reduced hippocampal SIRT1 as being a principal pathogenic mediator of obesity-induced memory impairment. This paper offers a novel working model that may serve as a conceptual basis for the development of therapeutic interventions for obesity-induced memory impairment.

Introduction

Obesity, a medical condition currently plaguing developed nations, is associated with pathological features that ultimately put obese individuals at risk for numerous negative health outcomes (McArdle et al., 2013). Converging data from epidemiological

and clinical studies suggest that obesity is an independent risk factor for the development of cognitive decline, in general, and memory impairment, in particular, in both middle-aged and old-aged persons (Whitmer et al., 2005; Cournot et al., 2006; Gunstad et al., 2010; Hassenstab et al., 2010; Xu et al., 2011; Bove et al., 2013). Moreover, rodent models of diet-induced obesity (DIO), as well as transgenic models of obesity, present with impairments in hippocampus-dependent spatial memory, abnormalities in hippocampal dendritic spine morphology, deficits in synaptic plasticity, and reduced dentate gyrus neurogenesis (Stranahan et al., 2008a,b; McNay et al., 2010; Stranahan and Mattson, 2011; Valladolid-Acebes et al., 2011; Heyward et al., 2012). Although it has been firmly established that obesity is associated with impaired memory, very little is known about the molecular pathogenesis of this condition.

Recent findings have offered evidence that perturbations in the basal expression of the memory-associated genes within the hippocampus are concomitant with, and potentially involved in, the etiology of obesity-linked memory impairment (Heyward et

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al., 2012; Reichelt et al., 2015). The obesity-linked dysregulation of memory-associated genes is most likely nontrivial, as the consolidation of long-term memory requires lasting neuronal synaptic connections, a process that relies upon an alteration in the expression of memory-associated genes (Kandel, 2001). Additionally, the disturbed basal expression of memory-related genes has been repeatedly observed using models of both aging and neurodegeneration, and thereby underscores the notion that aberrant basal expression of memory-related genes is a principal pathogenic feature of idiopathic, and nonidiopathic, long-term memory impairment (Fischer et al., 2007; Penner et al., 2010, 2011; Gräff et al., 2012). Therefore, we reasoned that the perturbed expression of memory-linked genes may be a principal pathogenic feature of obesity-induced memory impairment.

One viable candidate mechanism through which obesity may impair the expression of memory-associated genes involves the perturbation of epigenetic regulatory mechanisms. Such epigenetic regulatory mechanisms induce stable alterations of gene transcription without altering the associated DNA sequence (Day and Sweatt, 2011). One such epigenetic mechanism, DNA methylation, has been intensely studied for its role in memory-promoting transcriptional regulation within the hippocampus (for review, see Heyward and Sweatt, 2015). DNA methylation involves the covalent attachment of a methyl group to the fifth position (5') carbon within the cytosine pyrimidine ring [5-methylcytosine (5mC)] and has been canonically thought to be restricted to cytosines that constitute palindromic cytosine-phosphate-guanine (CpG) dinucleotides (Ng and Bird, 1999; Bird, 2002; Miranda and Jones, 2007). Moreover, DNA methylation of CpG-rich regions (CpG islands) at gene promoters has been associated with transcriptional repression in most cases (Ng and Bird, 1999; Bird, 2002; Miranda and Jones, 2007). Altered, DNA methylation is a particularly promising candidate to account for obesity-associated hippocampal gene dysregulation due to significant evidence of high-fat diet-induced aberrant DNA methylation in the periphery (Barrès et al., 2009). Furthermore, two reports both using the chronic high-fat diet mouse model of obesity described aberrant DNA methylation at the promoters of the genes encoding both the μ -opioid receptor gene, in the brain's reward related circuit, and the melanocortin-4 receptor in whole brain (Widiker et al., 2010; Vucetic et al., 2011). Yet, to the best of our knowledge, no study has detected obesity-induced epigenetic alterations of any kind within the hippocampus. Therefore, bearing in mind the vital role DNA methylation plays in regulating gene expression within the hippocampus, as well as the precedent for peripheral and central alterations in DNA methylation that occur in an obesity-linked manner, we set out to investigate aberrant DNA methylation of memory-related genes within the hippocampus of mice fed an obesogenic high-fat diet.

Materials and Methods

Animals: standard control/obese comparison. Ten-week-old C57BL/6J male mice from The Jackson Laboratory were shipped to our animal facility in groups of littermates. Upon arrival mice were group-housed ($n = 3$ or 4), with food and water available *ad libitum*, on a 12:12 h light/dark schedule [lights on at 6 A.M.; zeitgeber time (ZT) 0]. Mice were acclimated to the housing conditions for 2 weeks. At 12 weeks of age, cages of mice were randomly assigned to either a control, standard chow, diet (Harlan) or a DIO (60% fat by calories from lard) diet (Research Diets, #D12492) and were maintained on this diet until death. Individual mouse body weights were measured weekly along with cage-wide food consumption.

The Jackson Laboratory diet-induced obesity model. Premade control and DIO mice were used in Western blot and electrophysiology experiments. Mice that were used involved premade C57BL/6J male DIO mice (The Jackson Laboratory #380050) placed on a high-fat diet (60% fat, Research Diets #D12492) starting at 6 weeks of age, and age-matched control mice on either a standard-chow diet (electrophysiology studies; Harlan; The Jackson Laboratory mice #00064) or a control diet (Western blot studies; 10% fat, Research Diets #D12450B; The Jackson Laboratory mice #380056). Groups of littermates, all from the same The Jackson Laboratory storage area, were ordered and arrived at our animal facility at 24 weeks of age (18 weeks on diet for DIO mice). Upon arrival, the mice were immediately grouped housed ($n = 3$ or 4 per cage), maintained on their respective diets while being provided food and water available *ad libitum*, placed on a 12:12 h light/dark schedule (lights on at 6 A.M.; ZT 0), and acclimated to the housing conditions for 2 weeks before behavioral testing. Individual mouse body weights were measured weekly along with cage-wide food consumption.

Earlier time point diet-induced obesity model. Ten-week-old C57BL/6J male mice from Taconic Farms were shipped to our animal storage facility in groups of littermates. Mice were singly housed and allowed to acclimate for 2 weeks before being assigned to experimental groups. During this time, mice had food and water available *ad libitum*. At 12 weeks of age, mice were randomly assigned to either a control, standard laboratory chow diet (Harlan) or a DIO (60% fat) diet (Research Diets, #D12492) and were maintained on their diet for 13 weeks before behavioral testing.

Resveratrol supplement experiments. Ten-week-old C57BL/6J male mice from Taconic Farms were shipped to our animal storage facility in groups of littermates. Upon arrival, mice were entrained to a reverse 12:12 h light/dark schedule (lights on at 9 P.M.; ZT 0) for 2 weeks before being assigned to experimental groups. During this time, mice were singly housed, with food and water available *ad libitum*. At 12 weeks of age, mice were randomly assigned to either a control, standard laboratory chow diet (Harlan) or a DIO (60% fat) diet (Research Diets, #D12492) and were maintained on their diet for 10 weeks. After being kept on their diets for 10 weeks, half of the control and DIO mice were selected at random and given either a standard-chow (Teklad 7017) or high-fat (60% fat, Research Diets #12492) diet that had been formulated to contain resveratrol (ChromaDex) at a concentration of either 1.73 g (control-resveratrol diet; CR mice) or 3.273 g (DIO-resveratrol; DRES mice) resveratrol/kg of food. Feeding of these diets resulted in an approximate daily dose of 200 mg/kg of body weight per day. Individual mouse body weight was measured weekly along with food consumption. Mice were maintained on their respective diets until death.

General animal protocols. The mice were weighed weekly throughout the experiment. After having been maintained on their respective diets for 20 weeks, mice were subjected to various behavioral tests (described below) before being euthanized. No statistical method was used to predict the optimal sample sizes, but our sample sizes are comparable with those reported in related publications (Chalkiadaki and Guarente, 2012; Gräff et al., 2012, 2013; Heyward et al., 2012). All animals were randomly assigned to their respective groups. All procedures were performed in accordance with the University of Alabama at Birmingham Institutional Animal Care and Use Committee.

Generation of a inducible, forebrain neuron-specific, SIRT1 knock-out mouse. Mice containing a CreER^{T2} (Jax #012362) fusion protein consisting of Cre recombinase that was fused to a triple mutant form of the human estrogen receptor; which does not bind its natural ligand, 17 β -estradiol, at concentrations that would be considered physiological but will bind the synthetic estrogen receptor ligands 4-hydroxytamoxifen (4-OHT) and tamoxifen (which is metabolized to the active metabolite 4-OHT), have been described previously (Indra et al., 1999; Ruzankina et al., 2007; Price et al., 2012; Diaz et al., 2013; Rafalski et al., 2013; Volk et al., 2013). CreER^{T2} is restricted to the cytoplasm and can only translocate into the nucleus following treatment with tamoxifen (Indra et al., 1999; Madisen et al., 2010). CreER^{T2} expression was under the control of the calcium/calmodulin-dependent protein kinase II α promoter (Camk2a), which is expressed throughout forebrain neurons and is robustly expressed within the CA1 pyramidal cell layer in the hippocampus (Cheng

et al., 2003; Madisen et al., 2010). Thus, the mice used to generate forebrain neuron-specific expression of CreER^{T2} were deemed *Camk2a-CreER^{T2}*. These mice were crossed to *SIRT1^{Δex4}* mice (Cheng et al., 2003) to generate *SIRT1^{Δex4}; CamK2a-CreER^{T2}* mice in which exon 4, which contains the active catalytic region, of SIRT1 can be deleted upon treatment with tamoxifen. Male littermates between 8 and 12 weeks of age were subjected to Cre induction. Cre induction was achieved via a 5 d intraperitoneal injection of 0.08 mg/g tamoxifen (Sigma-Aldrich) in corn oil (Sigma-Aldrich) to *SIRT1^{Δex4}; Camk2a-CreER^{T2}* mice. Controls included *SIRT1^{Δex4}* mice injected with tamoxifen or *SIRT1^{Δex4}; Camk2a-CreER^{T2}* injected with vehicle (corn oil). *SIRT1^{Δex4}; Camk2a-CreER^{T2}* mice administered tamoxifen are hereafter referred to as *SIRT1*-inducible and conditional knock-out (SICKO) mice.

Death and serum collection. After having been maintained on their respective diets for 23 weeks, mice were randomly assigned to one of three groups. All groups were euthanized between approximately the same time (ZT 2–5) on consecutive days so as to ensure all blood samples would be collected at comparable times during the circadian cycle. Before death, mice were food deprived starting at ZT 0, as opposed to overnight fasting, so as to mitigate the possibility of inducing changes in gene expression that might be associated with the stress of overnight fasting. Mice were rapidly decapitated; and immediately afterward, trunk blood glucose levels were measured using an Alphasnak glucometer (Abbott). Trunk blood was collected and serum extracted for downstream blood chemistry analysis. After decapitation, bilateral or unilateral (as specified), dorsal-hippocampi were removed by gross dissection, and immediately snap frozen and stored at -80°C .

Behavior: general behavioral conditions. All behavioral testing was conducted during the dark phase, between ZT 13 and ZT 23. Indirect red light was used to aid the experimenter during all behavioral testing. While being transported from their cage storage room and throughout the behavioral testing facility, mice were placed on a cart covered with two light-blocking sheets, as to ensure against light exposure during the dark phase. The test chambers were cleaned with 75% ethanol between trials.

Open field. Animals were handled for at least 4 d before the onset of behavioral testing, and they were transported to the laboratory at least 1 h before the start of each experiment. Locomotion and time in center-square behavior were measured in an open-field arena (43.2 cm $\times$ 43.2 cm $\times$ 30.5 cm) for either 30 min or 15 min, as specific, by an automatic video tracking system (Med Associates).

Object recognition memory (ORM). The experimental apparatus consisted of four separate identical white square open fields: 25 cm $\times$ 25 cm $\times$ 30 cm (height). On each day of testing, before having been transferred to the room designated for ORM, all mice were initially stored in a nearby storage room within the behavioral facility for at least an hour before the start of the ORM paradigm, to promote acclimation to the test facility environment. After having been acclimated in the storage room, four mice per trial were each placed into their own cage and carted into the ORM testing room. For each trial, the combination of the four mice represented a mixture of the experimental conditions being tested. Each mouse cage was fitted with blackened foam sleeve during the transfer process. Once in the ORM room, mice were habituated to their respective ORM arenas in the absence of objects for 25 min. Habituation was performed for three consecutive days. During the training phase, mice were placed in their arenas in the presence of two new identical objects and were allowed to explore for 15 min. After a 24 h retention period, mice were placed again in the apparatus, where this time one of the objects was replaced by a novel one, and allowed to explore for 5 min. During the ORM test, the two objects used were the head of a toothbrush and a single square Lego. Both variables related to the identity (toothbrush head vs Lego) and the positioning (left side vs right side) of the novel object were balanced between groups.

Object location memory (OLM). Twenty-four hours following the time of the 24 h ORM test marked the beginning of the object location task paradigm. The experimental apparatus contexts were virtually identical to the ones previously used for ORM, except for one important addition: visual cues (electrical tape) were placed on two adjacent arena walls. During the OLM training phase, mice were placed in their respective

arenas in the presence of two identical objects and allowed to explore for 15 min. The objects were all 2-inch-long light bulbs fixed to a stabilizing base. After a 24 h retention period, mice were placed again in the apparatus, where this time one of the objects was displaced to a novel spatial location, and allowed to explore for 5 min. The spatial location of the displaced object was balanced between groups.

All phases of ORM and OLM were recorded using TopScan (Clever Sys). In both tasks, each group's ability to recognize the novel object was determined by dividing the mean time exploring the novel object by the mean of the total time exploring the novel and familiar objects during the test session. This value was multiplied by 100 to obtain a discrimination ratio for the novel object ($\text{T}_{\text{novel}}/[\text{T}_{\text{novel}} + \text{T}_{\text{familiar}}] \times 100$). In both tasks, objects were wiped with ethanol between trials, and before the first trial, to remove residual odors. The first minute of each test phase was hand-scored by a researcher blinded to the experimental conditions, as there is evidence to suggest that the discrimination between two objects is the most discernable during the initial onset of the preference test (Dix and Aggleton, 1999). Total time spent interacting with both objects was measured during the training phase and was accomplished by a researcher blinded to experimental conditions. Mice were deemed to be interacting with an object when facing and sniffing the objects within a maximum distance of 1 cm.

Measurement of endocrine profiles. Insulin analysis was performed via ultra-sensitive mouse insulin ELISA kit (Crystal Chem).

Slice electrophysiology. Electrophysiology was performed in an interface chamber (Fine Science Tools). Oxygenated ACSF (95%/5% O_2/CO_2) was warmed (30°C , TC-324B temperature controller, Warner Instruments) and perfused into the recording chamber at a rate of 1 ml/min. Electrophysiological traces were amplified (model 1800 amplifier, A-M Systems), digitized and stored (Digidata models 1322A with Clampex software, Molecular Devices). Extracellular stimuli were administered (model 2200 stimulus isolator, A-M Systems) on the border of area CA3 and CA1 along the Schaffer collaterals using enameled, bipolar platinum-tungsten (92%/8%) electrodes. fEPSPs were recorded in stratum radiatum with an ACSF-filled glass recording electrode (1–3 $\text{M}\Omega$). The relationship between fiber volley and fEPSP slopes over various stimulus intensities (0.5–15 V, 25 nA to 1.5 μA) was used to assess baseline synaptic transmission. All subsequent experimental stimuli were set to an intensity that evoked an fEPSP that had a slope of 50% of the maximum fEPSP slope. Paired-pulse facilitation was measured at various interstimulus intervals (10, 20, 50, 100, 150, 200, 250, 300 ms). High-frequency stimulus-induced LTP was induced by administering 3 trains, each 20 s apart, of 10 θ bursts (4 pulses per burst) delivered at 5 Hz (or 200 ms between bursts). Synaptic efficacy was monitored 20 min before and 3 h following induction of LTP by recording fEPSPs every 20 s (traces were averaged for every 4 min interval). The experimenter performing the electrophysiology and analysis was blind to genotype or treatment.

Isolation of DNA and RNA. DNA and RNA were isolated from bilateral dorsal hippocampus using the AllPrep DNA/RNA Mini Kit (QIAGEN). Concentrations were determined spectrophotometrically using the NanoDrop 2000c (Thermo Scientific).

Real-time qPCR. RNA DNase treated and purified via a commercial kit (DNA Clean and Concentrator-5, Zymo Research) and DNase I set (Zymo Research) mRNA was reverse transcribed using the iScript RT-PCR kit (Bio-Rad). All primers ensured the amplification of only mRNA as they were designed to span exon boundaries (*Bdnf*, *Pparg1a*, *Ppp1cb*, *Reln*, *Sirt1*, *Hprt1*). Either *Hprt1* or β -actin, as indicated, was used as the internal control for normalization using the $\Delta\Delta\text{Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). Assessment of gene expression was performed using the following primers (annealing temperatures are in parentheses): β -actin: forward 5'-TATGCCAACACAGTGCTGTC-3', reverse 5'-ACCGATCCACACAGAGTACTTG3' (58°C – 62°C); *Bdnf*: forward 5'-CACAGACGGCTCCTGCCAATTTAT-3', reverse 5'-TTTCTGATGCTCAGGAACCCAGGA-3' (62°C); *Homer1a*: forward 5'-TGCAAGGAGAAGTCGCAGGAGAA-3', reverse 5'-CATGATTGCTGAATTGATGTGTACCT-3' (60°C); *Hprt1*: forward 5'-GGAGTCCTGTTGATGTTGCCAGTA-3', reverse 5'-GGGACGCGACCACTGACATTTCTA-3' (60°C); *Pparg1a*: forward 5'-GGTTCCCCATTTGAGAACA-3', reverse 5'-GCCTTGGGTACCAGAACACT-3' (60°C); *Ppp1cb*: forward 5'-AC

GTCCAGAAATTCAGCCCCACCATA-3', reverse 5'-GCGAGTTTGACAATGCTGGTGGTA-3' (60°C); *Reln*: forward 5'-AGTACTCAGATGTGCAGTGGGCAA-3', reverse 5'-AGCGCTCCTCAGGAAAGTCTTCA-3' (60°C); *Sirt1*: forward 5'-AGACCCTCAAGCCATGTTTG-3', reverse 5'-ACACAGAGACGGCTGGAAC-3' (58°C). Probes used to measure gene expression in the 16-week-fed control and DIO mice include the following: Invitrogen Mm00607939_s1 (β -actin), Invitrogen Mm00554690_m1 (*Ppp1cb*) (60°C).

Quantification of global 5mC and 5-hydroxymethylcytosine (5hmC) levels. The quantification of global 5mC and 5hmC levels was performed as previously described (Kaas et al., 2013). High-performance liquid chromatography-electrospray ionization tandem mass spectrometry, with multiple reactions monitoring was used to detect 5mC and 5hmC levels using genomic DNA extracted from bilateral dorsal-hippocampus. Data represent the percentage of 5mC or 5hmC relative to the total cytosine pool (C + 5mC + 5hmC) measured for each experimental sample.

Methylated and hydroxymethylated DNA immunoprecipitation. Both methylated DNA immunoprecipitation (MeDIP) and hydroxymethylated DNA immunoprecipitation (hMeDIP) were performed using either a 5mC antibody (4 μ g per sample, mouse monoclonal, Epigentek #A-1014) or 5hmC antibody (4 μ g per sample, mouse polyclonal, Active Motif #39791) as described previously (Day et al., 2013). Briefly, genomic DNA was extracted (All Prep DNA/RNA Mini Kit, QIAGEN), treated with RNase A (Zymo Research), and quantified (NanoDrop), 200 ng of DNA per sample was removed and sonicated (Fisher Sonic Dismembrator 120) to 200 to 800 bp DNA fragments for methylation analysis. Sonicated DNA was incubated for 1 h with 4 μ l 5mC antibody, or 2 h with the 5hmC antibody, and then methylated DNA was collected with protein A-coated magnetic beads (Invitrogen), washed (Bind Wash Buffer, Epimark kit, New England BioLabs), extracted for 2 h at 65°C with proteinase K in TE buffer with 1% SDS, and purified (QIAGEN DNA micro kit). Methylation at selected DNA regions was assayed via qPCR on an iQ5 real-time PCR system (Bio-Rad). C_t values for IP samples were normalized to unprocessed (input) DNA. *Hprt1*, which did not change across samples, was used as an internal normalization control. qPCR used was as follows (annealing temperatures are in parentheses): *Hprt*: forward 5'-AGATCATGAGGAGGAGAGAAA-3', reverse 5'-TGACTAGGTGGGCGCTGATAA-3' (60°C); *Pparg1a*: forward 5'-CAAAGCTGGCTTCAGTCA CA-3', reverse 5'-TTGCTGCACAACTCCTGAC-3' (64.8°C); *Ppp1cb*: forward 5'-GGGAGGGAGTGACGCTGA-3', reverse 5'-CTCTCCACC TCCTCTCG-3' (66°C); *Reln*: forward 5'-ATGTAGAAGTAACTCGG ACCT-3', reverse 5'-CTTCGCCGACTCTGTATTT-3' (58°C); *Sirt1*: forward 5'-CAAGGCAGGTGGAGGAGTT-3', reverse 5'-GGTGGTTC AAGTTGCGATG-3' (64.8°C).

Western blots. Snap-frozen unilateral dorsal hippocampus tissue samples, stored at -80°C , were homogenized using 500 μ l of ice-cold hypotonic lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 2.5 mM sodium pyrophosphate, 1 mM PMSF, 1 mM β -glycerophosphate, 1% NP-40, protease inhibitor tablets (Roche) and 1 mM sodium orthovanadate), as described previously (Maddox and Schafe, 2011). Protein samples were separated by 8% SDS-PAGE and transferred to Immobilon-FL PVDF membranes (Millipore). The membrane was blocked in Li-Cor Odyssey blocking buffer for 1 h at room temperature and incubated with the appropriate antibody. The specific antibody treatment parameters were as follows: SIRT1 (Millipore, 07–131, 1:500, 2 h room temperature), Ac-p53 (Lys379) (Cell Signaling Technology, 2570, 1:500, overnight 4°C), and actin (Abcam, ab3280, 1:1000, 1 h room temperature). After primary antibody treatment, membranes were washed 3 times in 0.1% TBS-T, followed by incubation for 1 h in either goat anti-rabbit or goat anti-mouse, AlexaFluor-800 (1:15,000, Li-Cor), washed 3 times in 0.1% TBS-T, followed by 2 times with TBS, and imaged on the Li-Cor Odyssey fluorescent imaging system.

Statistics. Differences in body weight between control and DIO mice were examined using two-tailed Student's *t* tests. Comparisons between two groups were analyzed using unpaired two-tailed Student's *t* test, unless stated otherwise, in which case a one-tailed Student's *t* test will have been used when appropriate. Other comparisons between multiple groups were analyzed using a one-way ANOVA followed by Tukey's *post*

hoc tests where appropriate. All data are represented as mean $\pm$ SEM. Statistical significance was designated at $\alpha = 0.05$ for all analyses. Statistical and graphical analyses were performed with GraphPad software (Prism 6).

Results

Diet-induced obesity leads to impaired hippocampus-dependent spatial memory

As a means of assessing the impact of diet-induced obesity on hippocampal DNA methylation, we fed adult mice either a control, standard-chow, diet or a high-fat diet (60% fat) for 20 weeks before measuring memory performance (Fig. 1*A,B*). As expected, DIO mice exhibited hyperinsulinemia, a condition typically associated with chronic DIO (Fig. 1*C*). DIO mice traveled a significantly less distance compared with control mice when evaluated in an open field test of ambulation (Fig. 1*D*). Mindful of the obvious physical limitations of the DIO mice, as well as the unconfirmed, albeit conceivable, potential for heightened stress responsiveness of obese mice (Pasquali et al., 2006), we sought to access the memory of DIO mice using a behavioral paradigm that has a low degree of physical demand and is minimally stressful. The object memory class of memory tests properly addressed these concerns while also offering the benefit of being sensitive to subtle memory deficits (Heyward et al., 2012; McNulty et al., 2012; Vogel-Ciernia and Wood, 2014). DIO mice exhibited intact novel object-recognition memory, albeit impaired OLM, and thus recapitulated previous findings that DIO mice present with a selective deficit in hippocampus-dependent spatial memory (Heyward et al., 2012; Reichelt et al., 2015) (Fig. 1*E,F*). Importantly, no difference between groups was apparent in terms of total object interaction time during the OLM training trial, thus dismissing the possibility that the perceived deficit in OLM demonstrated by DIO mice was due to a performance deficit, rather than impaired hippocampus-dependent spatial memory (Fig. 1*G*).

Diet-induced obesity leads to impaired synaptic plasticity

Long-term memory formation requires the establishment of persistent neuronal synaptic connections within the hippocampus (Heyward, 2015). Therefore, the observation that DIO mice exhibited a deficit in long-term hippocampus-dependent spatial memory inspired us to determine whether we might detect a corresponding deficit in synaptic plasticity. To test this idea, we used high-frequency stimulation to induce long-term potentiation at Schaffer collateral synapses in acute slices taken from either DIO or control mice. Despite having normal baseline synaptic transmission (measured by input/output curves and paired-pulse facilitation; Fig. 1*H,I*), DIO mice exhibited a deficit in both early- and late-phase LTP (Fig. 1*J*).

Diet-induced obesity leads to aberrant gene expression and DNA methylation

We next sought to identify target genes exhibiting basal obesity-induced transcriptional dysregulation to further explore the possibility that neuroepigenetic dysregulation of memory-related genes within the hippocampus underlies the hippocampus-dependent memory impairment and synaptic plasticity deficit detected in DIO mice. *Pparg1a*, *Ppp1cb*, *Reln*, and *Sirt1* all exhibited reduced gene expression within in hippocampus of DIO mice, a finding that recapitulates and extends previous findings (Heyward et al., 2012; Reichelt et al., 2015) (Fig. 1*K*).

DNA methylation at gene promoter regions is a neuroepigenetic modification canonically thought to be associated with gene suppression. Having identified target genes whose basal expression

Figure 1. Diet-induced obesity leads to impaired hippocampus-dependent spatial memory, synaptic plasticity, and aberrant basal hippocampus gene expression. **A**, Schematic depicting the experimental time course. The mice subjected to DIO were fed a high-fat diet for 20 week, whereas control mice were maintained on a standard chow diet. Mice were then subjected to behavioral testing, before down-stream serum and molecular analysis. **B**, Representative experiment of diet-induced body weight gain of DIO and control mice throughout the 20 week time period. DIO mice weighed significantly more than their chow-fed control counterparts in the weeks immediately following the onset of high-fat feeding, as determined by body weight ($n = 12-14$ per group, Student's t test, $t_{(24)} = 7.209$, $*p < 0.0001$ (t test)). **C**, DIO mice had a significantly higher concentration of serum insulin than controls ($n = 12-14$ per group, Student's t test, $t_{(24)} = 4.735$, $****p < 0.0001$ (t test)). **D**, During the 30 min open field trail, DIO mice ambulated for a significantly lesser distance than control mice ($n = 12-14$ per group, Student's t test, $t_{(24)} = 2.302$, $*p < 0.05$ (t test)). **E**, Control and DIO mice did not significantly differ in terms of their performance on the 24 h test of ORM. **F**, DIO mice exhibited impaired OLM that was significantly different from that of control mice ($n = 12-14$ per group, Student's t test, $t_{(24)} = 3.186$, $**p < 0.01$ (t test)). **G**, Impaired performance in OLM exhibited by DIO mice is not attributed to a deficit in object exploration during the OLM training trial, as DIO mice did not differ significantly from control mice. **H**, **I**, Control and DIO mice do not differ in terms of their baseline synaptic transmission, measured by input/output curves and paired-pulse facilitation. **J**, DIO mice show decreased levels of early- and late-phase LTP after θ burst stimulation (TBS; Control: $n = 48$ slices from 6 mice; DIO: $n = 50$ slices from 6 mice; one-tailed Student's t test, $*p < 0.05$ at $t = 20, 40, 120, 140, 160, 180$ min). **K**, Diet-induced obesity decreases basal hippocampus expression of peroxisome proliferator-activated receptor gamma coactivator 1- α (*Ppargc1a*) mRNA ($n = 6-9$ per group, Student's t test, $t_{(13)} = 2.591$, $*p < 0.05$), protein phosphatase 1, catalytic subunit, β isozyme (*Ppp1cb*) ($n = 6-9$ per group, Student's t test, $t_{(13)} = 3.020$, $**p < 0.01$), reelin (*Reln*) ($n = 6-9$ per group, Student's t test, $t_{(13)} = 2.854$, $*p < 0.05$), sirtuin 1 (*Sirt1*) ($n = 6-9$ per group, Student's t test, $t_{(13)} = 2.948$, $*p < 0.05$), compared with that detected in chow-fed controls. **E**, **F**, The perforated lines indicate performance indicative of random chance (50%). Error bars indicate SEM.

Figure 2. Evidence of high-fat diet induced aberrations in basal hippocampus DNA methylation. **A**, Gene targets for MeDIP assay. Lines below genes indicate loci for genomic DNA primer pairs. TSS, Transcription start site. **B**, MeDIP reveals high-fat diet-induced alterations in DNA methylation at *Ppp1cb* ($n = 8$ per group, Student's t test, $t_{(14)} = 4.773$; $**p < 0.001$), *Reln* ($n = 8$ per group, Student's t test, $t_{(14)} = 2.265$; $*p < 0.05$), and *Sirt1* ($n = 8$ per group, Student's t test, $t_{(14)} = 2.197$; $*p < 0.05$). **C**, No difference in the percentage of global 5mC genomic DNA was detected.

Figure 3. Evidence of high-fat diet-induced aberrations in basal hippocampus DNA hydroxymethylation. **A**, hMeDIP reveals high-fat diet-induced alterations in DNA hydroxymethylation at *Sirt1* ($n = 8$ per group, Student's t test, $t_{(13)} = 2.983$; $*p < 0.05$). **B**, No difference in the percentage of global 5hmC genomic DNA was detected.

within the hippocampus is decreased during DIO, we next attempted to detect evidence of increased DNA methylation at the promoter regions of these genes. To accomplish this, we performed methylated DNA immunoprecipitation (MeDIP), a technique used to detect site- and gene-specific changes in the status of methylation, as previously described (Day et al., 2013). Gene-specific primers were designed to assay methylation at promoter targets for the genes (*Hprt1*, *Ppargc1a*, *Ppp1cb*, *Reln*, *Sirt1*), all of which, except *Ppargc1a*, contain at least one CpG island upstream of the transcriptional start site (Fig. 2A). Intriguingly, all of the genes that have been implicated in high-fat diet-induced memory impairment exhibited a significant degree of hypermethylation (*Ppp1cb*, *Reln*, *Sirt1*; Fig. 2B). Wondering whether the hypermethylation detected at the promoter regions of various target genes was gene-specific in nature, we then measured global hippocampus DNA methylation using high-performance liquid chromatography-mass spectrometry, as previously described (Kaas et al., 2013). The levels of global 5mC within the hippocampus of DIO did not differ from that of control mice (Fig. 2C).

Along with DNA methylation, DNA hydroxymethylation at CpGs (hmCpG) has received increased attention as an epigenetic modification that regulates gene expression, with transcriptional activity being associated with hmCpG enrichment in adult mouse frontal cortex (Lister et al., 2013). Therefore, we next attempted to detect evidence of decreased DNA hydroxymethylation at the promoter regions of these genes using hydroxymethylated DNA immunoprecipitation (hMeDIP). Interestingly, *Sirt1* was the only gene that exhibited evidence obesity-induced hypohydroxymethylation at its gene promoter (Fig. 3A). Furthermore, much like global 5mC levels, global 5hmC levels within the hippocampus did not differ between groups (Fig. 3B). Therefore, these findings reveal that high-fat diet-induced obesity leads to hypermethylation of select memory-related gene promoters, and hypohydroxymethylation of the *Sirt1* gene promoter, observations that complement the observed reductions in gene expression that were observed within the hippocampus of DIO mice.

We next sought to better understand the etiology of high-fat diet-induced memory impairment by studying the temporal properties of its development and progression. In short, we wished to determine whether chronic obesity was a contributing factor and therefore whether memory performance and gene expression did not decline in DIO mice at a time point that occurred much earlier than one used in the experiments just described. To accomplish this end, we assessed memory performance and measured hippocampal gene expression and DNA methylation/hydroxymethylation, at time points that occurred 7 weeks earlier (13 weeks and 16 weeks, respectively) than those used in the previous experiments (i.e., 20 weeks and 23 weeks, respectively). Interestingly, at an earlier time point, despite possessing traits indicative of obesity such as increased weight gain

Figure 4. Evidence that obesity-induced transcriptional and epigenetic modifications are temporally regulated. **A**, Sixteen-week-fed DIO mice weighed more at the time of tissue collection ($n = 7$ or 8 per group, Student's t test, $t_{(13)} = 10.35$; one Control weight was not recorded). **** $p < 0.0001$. **B**, Sixteen-week-fed DIO mice exhibit hyperinsulinemia ($n = 7$ or 8 per group, Student's t test, $t_{(13)} = 3.614$; one DIO insulin sample was lost during processing). ** $p < 0.01$. **C**, Thirteen-week-fed Control and DIO mice did not significantly differ in terms of their performance on the 24 h test of object location memory. **D**, Measurement of gene expression within the hippocampus of DIO and control mice maintained on their respective diets for the shorter time period of 16 weeks revealed that diet-induced obesity decreases basal hippocampus expression of *Ppargc1a* mRNA ($n = 8$ per group, Student's t test, $t_{(14)} = 2.499$) but failed to alter the expression of other genes. * $p < 0.05$. **E**, MeDIP failed to reveal significant high-fat diet-induced alterations in DNA methylation at any of the target gene loci in the 16 week DIO mice compared with control mice. **F**, In the DIO group, the only gene promoter with evidence of reduced hydroxymethylation was *Ppargc1a* ($n = 8$ per group, Student's t test, $t_{(14)} = 2.255$). * $p < 0.05$.

(Fig. 4A) and corresponding hyperinsulinemia (Fig. 4B), DIO mice did not present with a statistically significant impairment on the OLM task (Fig. 4C). Additionally, although *Ppargc1a* exhibited a significant reduction in gene expression at the earlier time point, *Reln*, *Sirt1* and *Ppp1cb* exhibited mRNA levels that, while trending downward, were not different from control mice (Fig. 4D). Additionally, the aforementioned evidence of an obesity-induced elevation in DNA methylation and decreased DNA hydroxymethylation of memory-related genes was completely absent in the earlier 16 week time point, with the exception of decreased hydroxymethylation at the *Ppargc1a* promoter of obese mice (Fig. 4E,F). The observation that after 13 weeks DIO mice had intact OLM despite *Ppargc1a* having been found to be significantly reduced calls into question the likelihood that reduced *Ppargc1a* solely mediates the deleterious effects of high-fat diet-obesity on memory performance, and certainly suggests that a significant reduction in *Ppargc1a* mRNA is insufficient to recapitulate the memory impairment exhibited by mice fed a high-fat diet for 20 weeks. Moreover, the observation that both *Sirt1* and *Ppp1cb* mRNA were found to be reduced after 23 weeks in DIO mice that exhibit impaired memory, yet were unaltered in DIO mice after the shorter time period of 16 weeks, raises the possibility that *Sirt1* and *Ppp1cb* may each be involved in the pathogenesis of high-fat diet-induced memory impairment. Last, the time-dependent development of hypermethylation across vari-

ous genes, and hypohydroxymethylation of *Sirt1*, suggests that epigenetic dysregulation of gene expression develops in an insidious manner that tracks with and is potentially owed to the progressive exacerbation of obesity's pathological concomitants.

Sirt1 depletion within the forebrain is sufficient to recapitulate the memory deficit exhibited by DIO mice

Of the genes linked to high-fat diet-induced memory impairment, *Sirt1*, which codes for, a multifaceted NAD⁺-dependent deacetylase, was the most intriguing because of its established role in energy expenditure and fat mobilization, as well as its apparent depletion and dysfunction in obesity (Pfluger et al., 2008; Costa Cdos et al., 2010; Chalkiadaki and Guarente, 2012; Price et al., 2012). Moreover, SIRT1 is clearly permissive for memory consolidation, as early postnatal whole-brain deletion of *Sirt1* impairs memory and synaptic plasticity (Gao et al., 2010; Michán et al., 2010). Thus, *Sirt1* may conceivably lie at the nexus of high-fat diet-induced molecular pathology and memory-impairment. Therefore, we sought to interrogate the possible role of *Sirt1* in high-fat diet-induced memory impairment. Using obese mice from The Jackson Laboratory, we determined that SIRT1 protein expression within the hippocampus was significantly diminished compared with that of control mice (Fig. 5A). Moreover, acetylated-p53 (Ac-p53), a bona fide substrate of SIRT1, and a common proxy for SIRT1's deacetylase activity

Figure 5. *Sirt1* depletion within the forebrain is sufficient to recapitulate the memory deficit exhibited by DIO mice. **A**, SIRT1 protein expression was measured from the hippocampus of mice rendered obese at The Jackson Laboratory (DIO) and their chow-fed counterparts (control). SIRT1 protein expression is reduced in the hippocampus of DIO mice relative to that of control mice ($n = 6$ or 7 per group, Student's t test, $t_{(11)} = 2.250$, $p < 0.05$). **B**, There is also a corresponding reduction in expression the SIRT1 substrate Ac-p53 in the hippocampus of DIO mice compared with that of controls ($n = 6$ or 7 per group, Student's t test, $t_{(11)} = 2.312$, $p < 0.05$). We generated mice in which we could inducibly and conditionally delete exon 4 of the *Sirt1* gene, thus forming a truncated, less stable, and catalytically inactive form of *Sirt1* (*sicko*). **C**, To disrupt *Sirt1* function in forebrain neurons, Camk2a-driven CreERT2 expressed selectively in the forebrain was used to excise the *Sirt1*, exon 4, catalytic domain upon a 5 d of IP tamoxifen injection. **D**, Using qPCR primers, specific for exon 4 of *Sirt1*, it was shown that *sicko* mice have reduced *Sirt1* mRNA expression in dorsal hippocampus compared with their control littermates ($n = 10$ –12 per group, Student's t test, $t_{(20)} = 8.339$). **** $p < 0.0001$. **E**, Western blot analysis of SIRT1 in hippocampus tissue isolated from control and *sicko* mice revealed that *sicko* mice have reduced SIRT1 protein expression compared with their control littermates ($n = 10$ –12 per group, Student's t test, $t_{(20)} = 2.220$). * $p < 0.05$. **F**, During the 30 min open field trail, control and *sicko* mice ambulated for a similar distance ($n = 14$ –19 per group, Student's t test, $t_{(31)} = 0.9593$, $p = 0.3463$). **G**, Control and *sicko* mice did not significantly differ in terms of their performance on the 24 h test of ORM ($n = 14$ –19 per group, Student's t test, $t_{(31)} = 0.0123$, $p = 0.9903$). **H**, *sicko* mice exhibited impaired OLM that was significantly different from that of control mice ($n = 14$ –19 per group, Student's t test, $t_{(31)} = 2.058$). * $p < 0.05$. **B**, **C**, The perforated lines indicate performance indicative of random chance (50%). Error bars indicate SEM.

(Cheng et al., 2003), was significantly increased within the hippocampus of obese mice, suggestive of reduced SIRT1 enzymatic activity within the hippocampus of obese mice (Fig. 5B).

We next sought to test whether reduced forebrain neuron SIRT1 alone is sufficient to reduce memory performance in adult mice. Many of the previous studies that determined the role of

SIRT1 in learning and memory deleted SIRT1 in a early postnatal, whole-brain, manner (Gao et al., 2010; Michán et al., 2010). Alternatively, for our studies, we sought to model adult forebrain neuron deletion of *Sirt1* using mice expressing the CaMKII-driven CreERT2, which upon tamoxifen injection deletes exon 4, the catalytic domain, of *Sirt1* (Cheng et al., 2003; Price et al.,

Figure 6. Resveratrol-fed obese mice (DRES) exhibit increased hippocampus SIRT1 activity and preserved hippocampus-dependent memory. **A**, Representative experiment of diet-induced body weight gain of all groups throughout the experiment. Arrow indicates the start of resveratrol diet supplementation. **B**, Dietary resveratrol administration did not alter serum insulin levels of obese mice ($n = 7$ – 10 per group; one-way ANOVA: main effect of diet on insulin expression; $F_{(3,30)} = 5.314$, $p = 0.0047$). * $p < 0.05$, significant differences between control compared with either DIO or DRES mice (Tukey *post hoc* tests). ** $p < 0.01$, significant differences between control compared with either DIO or DRES mice (Tukey *post hoc* tests). **C**, Dietary resveratrol administration in obese mice led to a reduction in a surrogate marker whose expression is inversely related to Sirt1 activity ($n = 4$ per group; one-way ANOVA: main effect of diet on acetyl-p53 levels; $F_{(2,9)} = 12.80$, $p = 0.0023$). * $p < 0.05$, significant differences between either control or DRES mice (Tukey *post hoc* tests). ** $p < 0.01$, significant differences between DIO compared with either control or DRES mice (Tukey *post hoc* tests). **D**, Distance traveled in the 15 min open field test of locomotion was reduced in the resveratrol-treated mice ($n = 12$ – 14 per group; one-way ANOVA: main effect of diet on distance traveled; $F_{(3,48)} = 3.984$, $p = 0.0130$). * $p < 0.05$, significant differences between control and DRES mice (Tukey *post hoc* tests). **E**, Neither of the groups differed in their performance on the 24 h test of ORM. **F**, Dietary resveratrol administration prevented the development of memory impairment observed in obese mice ($n = 12$ – 14 per group; one-way ANOVA: main effect of diet on OLM performance; $F_{(3,48)} = 3.562$, $p = 0.0208$). * $p < 0.05$, significant differences between DIO compared with either control or DRES mice (Tukey *post hoc* tests). **E**, **F**, The perforated lines indicate performance indicative of random chance (50%). Error bars indicate SEM. n.s., Not significant.

2012) (Fig. 5C). These *sicko* mice, upon tamoxifen exposure, exhibit a significant reduction in both *Sirt1* mRNA (measured using cDNA primers that span the exon 4/exon 5 junction) and protein within the hippocampus (Fig. 5D,E). *sicko* mice did not exhibit deficits in locomotion compared with control mice (Fig. 5F). Importantly, *sicko* mice exhibited intact ORM, albeit impaired OLM, thereby successfully recapitulating the selective deficit in hippocampus-dependent spatial memory exhibited by DIO mice (Fig. 5G,H).

Resveratrol-fed obese mice exhibit increased hippocampal SIRT1 activity and preserved hippocampus-dependent memory

Taking into account the aforementioned evidence that SIRT1 may be involved in high-fat diet-induced memory impairment, we attempted to further test the hypothesis that reduced SIRT1 activity within the hippocampus drives obesity-linked memory impairment. Aware of the precedent for the activation of SIRT1 to mitigate the memory impairment in a mouse model of neurodegeneration, we sought to confirm whether chemical activation of SIRT1 might preserve the memory of DIO mice (Gräff et al., 2013). Control and DIO mice were maintained on their respec-

tive diets for 10 weeks, at which time half of the mice had their diets supplemented with the 200 mg/kg of the SIRT1-activating molecule resveratrol, after which mice were maintained on their respective diets for another 10 weeks before being subjected to our tests of memory (Fig. 6A). During this experiment, food intake was measured weekly to ensure that an average dose of 200 mg/kg of resveratrol per day was consumed across mice (data not shown). DIO and DIO plus resveratrol (DRES) mice exhibited increased serum insulin concentrations compared with standard chow-fed controls (Fig. 6B). Importantly, whereas the hippocampus of DIO mice exhibited elevated Ac-p53 compared with control mice, DRES mice revealed Ac-p53 levels that were significantly lower than those of DIO mice, evidence that resveratrol supplementation led to increased Sirt1 activity within the hippocampus of obese mice (Fig. 6C). Obese mice traveled a significantly lesser distance than control mice during the open field test of ambulation, with DIO mice strongly trending toward reduced ambulation and DRES mice exhibiting a significant reduction in ambulation (Fig. 6D). There was no appreciable difference between the groups in terms of their performance on the ORM task (Fig. 6E). Remarkably, while DIO mice exhibited a significant impairment in their performance on the OLM para-

digm relative to control mice, DRES mice exhibited an object location memory that was significantly elevated relative to their DIO counterparts and was on par with that of control mice (Fig. 6F). Importantly, control mice administered resveratrol (CR) did not show evidence of enhanced memory, a finding that suggests that resveratrol supplementation regimen does not lead to a general enhancement in memory. These findings are evidence that resveratrol administration has a neuroprotective effect for mice already rendered obese and can preserve their hippocampus-dependent spatial memory and SIRT1 function within the hippocampus.

Discussion

To the best of our knowledge, the present data provide the first evidence that high-fat diet-induced obesity leads to the time-dependent development of aberrant epigenetic modifications within the hippocampus, as well as corresponding reductions in the expression of various memory-related genes. Previous studies have observed obesity-induced alterations in mRNA expression within the hippocampus of enzymes known to alter a subset of epigenetic regulators controlling histone acetylation, namely, the Class III (*Sirt1*) and Class IIa (*Hdac5* and *Hdac9*) histone deacetylases (Heyward et al., 2012; Wang et al., 2014). Although intriguing, these findings only went so far as to allude to the existence of obesity-induced alterations in epigenetic modifications via a proposed dysregulation in the functioning of chromatin-modifying enzymes. Yet, until now, we are unaware of there having been direct evidence of altered epigenetic modifications, in general, and DNA methylation and hydroxymethylation, in particular, within the hippocampus of obese mice.

Long-term memory formation requires the establishment and maintenance of synaptic connections within the CNS, a process that is dependent on alterations in the transcription of memory-related genes (Sweatt, 2013). Epigenetic modifications, which have the potential to persistently regulate the transcriptional machinery, have been heavily implicated in modulating the cellular information storage program so as to enable the preservation of memory-subserving synaptic connections (Sweatt, 2013). Importantly, empirical evidence accumulated throughout the past decade points to experience-associated alterations in the DNA methylation of memory-related genes within the hippocampus as being indispensable for long-term cellular information storage (Day and Sweatt, 2010). Through stably regulating the transcription of various memory-related genes, DNA methylation is hypothesized to directly promote relatively long-lived alterations in gene transcription and, in turn, synaptic plasticity (Heyward and Sweatt, 2015).

Recently, evidence of perturbations in the epigenetic regulation of genes subserving synaptic plasticity, and memory, have been demonstrated in a mouse model of neurodegeneration, pointing to the possibility that epigenetic dysregulation of genes involved in synaptic plasticity may underlie the memory disorders associated with various disease models (Gräff et al., 2012). Therefore, a working model, whereby various adverse health conditions lead to memory impairments by way of epigenetic dysregulation of memory-related genes, can be formulated and tested. The findings from the present study certainly corroborate this working model, as obesity (an adverse health condition), leads to memory impairment, and it appears to do so via an epigenetic dysregulation (i.e., hypermethylation) of genes that are indispensable for proper memory formation.

The present findings also implicate the epigenetic regulator SIRT1 as playing a central role in the pathogenesis of obesity-

linked memory impairment. Using a novel transgenic model, we determined that deletion of the SIRT1 catalytic domain within the forebrain of adult mice closely mimics the specificity of the memory deficit exhibited by obese mice, and thus revealed the sufficiency of reduced SIRT1 to recapitulate obesity-linked memory disturbances. Conversely, administering the SIRT1-activating compound resveratrol averted the manifestation of memory impairment in obese mice, thereby providing further evidence that SIRT1 depletion is a central pathogenic feature of high-fat diet-induced memory impairment. It should be noted that, although resveratrol's ability to directly activate SIRT1 is known (Hubbard et al., 2013), whether resveratrol selectively activates SIRT1 is still an open debate. Therefore, whereas the findings from our resveratrol rescue experiment are in no way conclusive, they are consistent with a model whereby a reduction in SIRT1 activity mediates obesity-induced memory impairment while pointing to SIRT1 as a promising candidate therapeutic target for the treatment of obesity-induced memory impairment.

That diet-induced obesity leads to epigenetic and molecular perturbation in the brain, a region that is relatively isolated from the pathological epicenter of obesity, namely, intra-abdominal adiposity, illustrates the point that the pathogenic concomitants of obesity exert their effects in a systemic fashion (McArdle et al., 2013). Obesity concomitants suspected to have a pernicious system-wide effect include, but are not limited to, insulin resistance, chronic low-grade inflammation, leptin resistance, hypercortisolism, dyslipidemia, and hyperglycemia. Intriguingly, saturated fatty acids and the proinflammatory cytokine tumor-necrosis factor α , both of which are elevated systemically within the context of obesity (Rosen and Spiegelman, 2014), when administered *in vitro* to human myotubes, resulted in hypermethylation of *Ppargc1a* (Barrès et al., 2009). Moreover, recent findings using *db/db* mice have determined that the inflammatory cytokine IL-1 β secreted from fat depots in the periphery mediates the obesity-linked memory impairment (Erion et al., 2014). Therefore, it is conceivable that various obesity concomitants may mediate high-fat diet-induced alterations in the epigenetic landscape within the CNS. Future experiments should seek to determine the various obesity concomitants that underlie obesity-linked alterations in hippocampal DNA methylation. Moreover, the current findings raise the enticing possibility that changes in DNA methylation that can be observed via noninvasive means could hold prognostic potential foretelling early-stage obesity-linked cognitive impairment.

An issue not directly studied in these experiments, but of epidemiological importance, is the potential for obesity to increase the risk for developing age-related cognitive decline, dementia, and Alzheimer's disease. Studies of these diseases of aging imply that they occur in an insidious fashion. Interestingly, the etiology of many obesity-linked health complications parallels that of obesity-linked memory impairment both in animal and human models. For instance, in this study, it was found that 13 week high-fat diet exposure failed to produce a significant deficit in memory or reduced *Sirt1* expression, whereas both were observed after 7 additional weeks of high-fat diet exposure. This suggests that the length of exposure to the obesity milieu confers its pathological effects on the hippocampus in an insidious, length of exposure-dependent, fashion. Therefore, these findings raise the possibility that the behavioral, cellular, and molecular perturbations resulting from high-fat diet-induced obesity may be exacerbated with exposure times that exceed those used in the present study. There is also the possibility that obesity-linked changes in DNA methylation within the hippocampus can persist indefinitely, thereby leading to persistent molecular and cellular perturbations that, in time, may set the stage for eventual age-

related cognitive decline. For these reasons, it would prove valuable to evaluate the role of obesity-induced alterations in DNA methylation in late age.

Although the present study strongly implicates SIRT1 in the etiology of obesity-induced memory impairment, a lingering issue not presently addressed by this study has to do with the putative memory-disrupting effect conferred by SIRT1 depletion. Clues to this question can be found in the earlier studies that identified the role of SIRT1 in memory consolidation; specifically, there is evidence that SIRT1 indirectly positively regulates the expression of BDNF (Gao et al., 2010). If detected, evidence of reduced BDNF expression within the hippocampus of obese mice would prove extremely satisfying and fit well within an already established model for the role of SIRT1 in the CNS. Indeed, there is disagreement in the field regarding the existence of altered BDNF due to obesity. Other studies involving rats placed on a high-fat, high-sucrose, diet or a more profoundly obese, diabetic, mouse model have observed reduced BDNF protein and mRNA expression, respectively (Molteni et al., 2002; Wosiski-Kuhn et al., 2014). However, other studies have found that *Bdnf* mRNA is not altered due to obesity using the DIO model we used in the current study (Heyward et al., 2012; Reichelt et al., 2015). Whether or not the length of exposure to the obesogenic diet, the high-fat diet composition, or the diabetic nature of the obesity model, dictates the observation of reduced BDNF remains to be thoroughly studied and seems plausible upon face value. BDNF expression appears to be regulated in an activity-dependent manner within the hippocampus (Lubin et al., 2008). It follows that, although the present study did not observe a reduction in basal BDNF gene expression, an impairment in neuronal activity-induced BDNF mRNA expression might still be occurring in our model. This raises the important point that a distinction should be made between basal alterations in gene expression versus activity-dependent alterations in gene expression, and highlights the need to investigate the latter with respect to obesity-linked memory impairment.

In conclusion, the present findings are concordant with those from clinical studies suggesting a link between obesity and disruption of memory performance. Future studies harnessing the power of high-throughput genome-wide transcriptomic analysis leveraging the ability to obtain a comprehensive assessment of the diversity of gene expression perturbations concomitant with obesity-linked memory impairment are needed to find promising therapeutic targets for potential intervention. Hopefully, this and future studies will serve as a basis for the assessment of SIRT1 as a potential target for human therapeutic intervention.

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