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Programmed Hyperphagia secondary to Increased Hypothalamic SIRT1

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Abstract

Small for gestational age (SGA) offspring exhibit reduced hypothalamic neural satiety pathways leading to programmed hyperphagia and adult obesity. Appetite regulatory site, the hypothalamic arcuate nucleus (ARC) contains appetite (NPY/AgRP) and satiety (POMC) neurons. Using in vitro culture of hypothalamic neuroprogenitor cells (NPC) which form the ARC, we demonstrated that SGA offspring exhibit reduced NPC proliferation and neuronal differentiation. bHLH protein Hes1 promotes NPC self-renewal and inhibits differentiation by repressing neuronal differentiation genes (Mash1, neurogenin3). We hypothesized that Hes1/Mash1 and ultimately ARC neuronal differentiation and expression of NPY/POMC neurons are influenced by SIRT1 which is a nutrient sensor and a histone deacetylase. Control dams received ad libitum food, whereas study dams were 50% food-restricted from pregnancy day 10 to 21 (SGA). In vivo studies showed that SGA newborns and adult offspring had increased protein expression of hypothalamic/ARC SIRT1 and AgRP with decreased POMC. Additionally, SGA newborns had decreased expression of hypothalamic neurogenic factors with reduced in vivo NPC proliferation. In vitro culture of hypothalamic NPCs showed similar changes with elevated SIRT1 binding to Hes1 in SGA newborn. Silencing SIRT1 increased NPC proliferation and Hes1 and Tuj1 expression in both Control and SGA NPCs. Although SGA NPC proliferation remained below that of Controls, it was higher than Control NPCs in the absence of SIRT1 siRNA. The direct impact of SIRT1 on NPC proliferation and differentiation were further confirmed with pharmacologic SIRT1 inhibitor and activator. Thus, in SGA newborns elevated SIRT1 induces premature differentiation of NPCs, reducing the NPC pool and cell proliferation.

Keywords

Hypothalamic neuroprogenitor cells; proliferation and differentiation; appetite and satiety neuropeptides; bHLH genes; Sirtinol and Resveratrol

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1. Introduction

Currently, 65% of adults in the United States are overweight and one in five are obese (BMI>30 kg/m²), representing a modern day health crisis. Obesity and its related diseases are the leading causes of death in western society, with associated risks of hypertension, cardiovascular disease, stroke and diabetes. The rapid increase in the prevalence of obesity has been partly attributed to environmental factors, with increasing availability of highly palatable foods and “modern” lifestyles involving less physical work creating a more obesogenic environment. However, as obesity results from an imbalance in caloric intake vs. expenditure, individuals with dysfunctional appetite regulation (i.e., overeating) are at high risk of developing obesity.

Human epidemiologic animal studies have confirmed that the perinatal environment may program the propensity for development of metabolic syndrome. Though the mechanisms and preventative strategies are not yet fully delineated, low birth weight and small for gestational age (SGA) offspring are at markedly increased risk of childhood and adult obesity (Barker, 2002;Barker et al, 2006;Desai and Hales, 1997;Godfrey and Barker, 2001;Nilsson et al, 1997;Ozanne and Hales, 2002;Ravelli et al, 1999;Remacle et al, 2004;Stocker et al, 2005). Animal studies have demonstrated that fetal undernutrition may impact neural, adipose, muscle and hepatic development, via alterations of cell proliferation/differentiation, organ development, and longterm epigenetic regulation (Desai et al, 2013;Osborne-Majnik et al, 2013;Ruchat et al, 2013). Studies in our laboratory and others have demonstrated that SGA rodent offspring express hyperphagia, rapid newborn catch-up growth and adult obesity (Coupe et al, 2009;Desai et al, 2005;Vickers et al, 2000). These offspring demonstrate a reduced anorexigenic response to leptin and sibutramine and impaired signaling responses to leptin in the putative appetite regulatory center, the arcuate nucleus (ARC) (Desai et al, 2007;Ferezou-Viala et al, 2007;Morris and Chen, 2009).

Rodents, rhesus and humans consistently demonstrate a relatively late development of the ARC nucleus, with maturation not achieved until later stages of postnatal development (Grove et al, 2001). Key hypothalamic nuclei only begin to be populated during fetal life, with continued neural development during the neonatal period (Kagotani et al, 1989;Walsh et al, 1982;Walsh and Brawer, 1979). Coinciding with neuronal maturation, ARC projections in rodents are formed beginning in the second postnatal week (Ahima et al, 1998;Bouret et al, 2004;Bouret and Simerly, 2006).

The ARC contains at least two populations of neurons with opposing actions on food intake: primarily medial ARC orexigenic (NPY; neuropeptide Y and AgRP; agouti-related protein) and primarily lateral ARC anorexigenic (POMC; pro-opiomelanocortin and CART; cocaine- and amphetamine-regulated transcript) neurons (Simpson et al, 2009). Explaining hyperphagia, in part, offspring of undernourished dams are SGA and have altered ARC development, with evidence of reduced ARC POMC though not NPY mRNA expression (Delahaye et al, 2008). Our laboratory has explored the development of the ARC in SGA offspring using *in vitro* culture of hypothalamic neuroprogenitor cells (NPC) which form the ARC. SGA offspring demonstrated reduced NPC proliferation as well as differentiation to

both neurons and astrocytes, suggesting impaired function of progenitor cells (Desai et al, 2011a).

In view of the NPC abnormalities, we sought to determine upstream mechanisms by which maternal/fetal undernutrition programs ARC development. Although an array of extracellular factors, including leptin, insulin and IGF1 modulate NPC function (Arsenijevic et al, 2001), emerging evidence indicates that energy metabolism is a critical regulator of NPC proliferation/differentiation (Rafalski and Brunet, 2011). One such central energy/nutrient sensor is SIRT1, an NAD⁺-dependent histone deacetylase. Among intracellular factors, the bHLH protein Hes1 promotes NPC self-renewal and inhibits differentiation by repressing neuronal differentiation genes (e.g., Mash1, neurogenin) (Kageyama et al, 2007). Hes1 is highly expressed in the ventricular zone and levels decrease as neural differentiation proceeds (Hisahara et al, 2008a). In Hes1-deficient brains, NPCs prematurely differentiate (Hatakeyama and Kageyama, 2006; Kageyama et al, 2007) reducing the NPC pool. We hypothesized that the putative mechanism for nutrient-programming of offspring hyperphagia is via SIRT1 which influences intracellular neurogenic factors (Hes1, Mash1), and ultimately ARC neuronal differentiation and expression of POMC and NYP neurons. We utilized NPC cells from Control and SGA offspring to examine nutrient sensors and signaling responses which program ARC structure and function.

2. Results

2.1 Body Weights

Offspring born to undernourished dams had reduced birth weight as compared to controls (6.6 ± 0.2 vs 7.0 ± 0.2 g, $P < 0.01$; $n = 6$ litters per group), as previously reported (Desai et al, 2005). When nursed by control dams and weaned to an ad libitum normal chow diet, SGA offspring weighed significantly more than did controls at three months of age (502 ± 10 vs 470 ± 9 g, $P < 0.01$). There was no difference in gestational age at birth, litter size and/or gender distribution between SGA and control offspring.

2.2 Hypothalamic Tissue Protein Expression

At one day of age, SGA newborn demonstrated significantly increased hypothalamic SIRT1 protein, though significantly reduced Hes1, Ngn3 and Mash1. In addition, SGA newborn demonstrated an increase in AgRP and a decrease in POMC expression (Figure 1). At three months of age, SGA adult continued to demonstrate an increased level of hypothalamic ARC SIRT1 protein expression, though with elevated ARC Hes1 and no change in Ngn3 and Mash1 expression. Furthermore, SGA offspring showed persistent increased expression of ARC AgRP and decreased ARC POMC expression (Figure 2).

2.3 In Vivo Cell Proliferation

In vivo NPC proliferation was examined from hypothalamic sections obtained at p1. Hypothalamic sections demonstrated co-localization of PCNA and BrdU staining at one day of age. When quantified, SGA offspring evidenced a significant decrease in PCNA positive cells (Figure 3).

2.4 Neurosphere Cultures

When NPCs were cultured in differentiating medium for seven days, SGA offspring demonstrated a marked increase in NPY and AgRP cells both visually and quantitatively (Figure 4A). We were unable to get sufficient cells to quantify POMC. Nonetheless, the triple immunostained images of NPY, POMC and DAPI visually showed reduced POMC and increased NPY per field in SGA offspring.

Under basal conditions, SGA NPC demonstrated significantly increased SIRT1 expression as compared to controls (Figure 5A), and as evidenced visually and quantitatively, cell proliferation (MTT assay) was significantly reduced in SGA NPC (Figures B, C). When both Control and SGA NPC's were transfected with SIRT1 specific siRNA in complete medium, SIRT1 expression decreased as expected (Figure 5A). SIRT1 specific siRNA induced a visually apparent increased colony density and significantly increased cell proliferation in both control and SGA neurospheres, though there was no effect of non-specific siRNA (Figure 5B,C)). Although SGA NPC proliferation remained below that of controls, it rose to levels exceeding that of control neurospheres in the absence of SIRT1 siRNA. Consistent with the expression with cell proliferation measures, Nestin (neuroprogenitor cell marker), Hes1, and Tuj1 (neuronal cell marker) expression was significantly less in SGA as compared to control neurospheres under basal conditions. SIRT1 siRNA increased Nestin, Hes1, and Tuj1 expression in both control and SGA (Figures 5D, E, F, G).

Responses to SIRT1 pharmacologic inhibition with sirtinol were similar to that of SIRT1 siRNA. Sirtinol demonstrated stimulation of NPC proliferation in both control and SGA neurospheres (Figure 6A, B). At 20 μ M, sirtinol in complete medium demonstrated increased Nestin expression in both Control and SGA neurospheres. Although SGA neurosphere Nestin remained significantly less than that of controls, sirtinol treatment in complete medium increased Nestin expression to untreated Control levels (Figure 6D). Sirtinol exposure in complete medium increased SGA Hes1 expression to levels equal to Control, but did not affect Control Hes1 expression (Figure 6E). Although in both Control and SGA, sirtinol exposure in differentiation medium significantly increased Mash1 and Tuj1 expression above respective untreated NPCs, SGA continued to show reduced Mash1 and Tuj1.

Consistent with the effect of SIRT1 inhibition, SIRT1 activator resveratrol markedly inhibited Control and SGA neuroproliferation, both visually (Figure 7A) and as quantified by proliferation index (Figure 7B). Resveratrol in complete medium demonstrated a concentration dependent decrease in Nestin, and Hes1 expression among both control and SGA neurospheres (Figures 7 D, E). In differentiation medium, only higher concentration of resveratrol (20 μ M) was effective in suppressing Mash1 and Tuj1 in both Control and SGA. Overall, resveratrol treated SGA NPC maintained reduced expression of all indices as compared to respective Control NPCs (Figures 7F, G).

PCR results demonstrated nearly a two-fold Hes1 expression among SGA neurospheres after pull-down with anti-SIRT1 antibody. The negative control (anti-insulin antibody) did not pull-down measurable Hes1 (Figure 8).

3. Discussion

A popular view of the etiology of overweight, obesity and morbid obesity is centered around the impression of a lack of individual will power and self control in regards to eating. However, data from a diversity of animal studies demonstrate evidence that programmed hyperphagia and obesity may result from of an excess of orexigenic neurons/gene expression and/or a deficiency of anorexigenic factors which contribute to an enhanced “appetite” (Desai et al, 2007;Li et al, 2013;Morris and Chen, 2009). Thus, overweight individuals may well experience an enhanced orexigenic drive and a reduced satiety sensation, potentially overwhelming conscious dietary regulation. Human epidemiologic studies, including the Dutch winter famine, confirm the potential for programmed hyperphagia and obesity (Lussana et al, 2008;Perala et al, 2013). The results of the present study provide insight into the neurodevelopmental mechanisms by which this dysfunctional appetite network is formed.

SIRT1, a recognized nutrient sensor, increases in response to nutrient deprivation in a diversity of tissues, particularly metabolically active sites including the hypothalamic ARC (Ramadori et al, 2008). SIRT1 is an NAD⁺-dependent protein deacetylase which removes acetyl groups from histones and non-histone proteins (Guo et al, 2012). Deacetylation of histones alters chromatin structure reducing gene transcription. In the present study, newborn hypothalamic tissue protein expression demonstrated an increased level of SIRT1, consistent with maternal/fetal undernutrition and reduced newborn birthweight. Assuming SIRT1-mediated epigenetic modifications, SGA hypothalamic tissue expressed reduced levels of Hes1, Ngn3 and Mash1. It is known that SIRT1 binds to the transcription factor Hes1 and subsequently inhibits pro-neuronal Mash1 (Libert et al, 2008;Prozorovski et al, 2008). Importantly, SGA offspring demonstrate increased AgRP and reduced POMC protein expression, suggesting a shift toward an increased appetite to satiety ratio, potentially predisposing to the offspring hyperphagia, as previously reported in this model (Gumus et al, 2007). Of note, due to the small size of the newborn, these values represent total hypothalamic protein expression, though AgRP and POMC are primarily localized to the ARC. Hes1 represents a bHLH transcriptional repressor which modulates cell proliferation and differentiation during embryogenesis. Primarily expressed within NPCs, Hes1 acts to maintain NPC proliferation and inhibit premature neurogenesis. An additional cascade of bHLH genes determine neuronal versus glial fate, and ultimately the neuronal expression of POMC versus NPY/AgRP neurons. Mash1 has proneural function acting upstream of Ngn3 (McNay et al, 2006). Thus a SIRT-mediated reduction of Mash1 (Teng et al, 2009;Zhang et al, 2011) leads to a loss of Ngn3 transcripts in ARC progenitors, explaining in part the results of the present study. Ngn3 promotes the development of POMC and inhibition of NPY neurons (Arai et al, 2010;Pelling et al, 2011) provides insight into the programmed reduction of anorexigenic POMC expression.

At three months of age, SGA offspring were significantly heavier than controls. However, ARC protein expression continued to demonstrate an excess of SIRT1. Whether the elevated SIRT1 reflects reduced energy within the ARC, despite the increased offspring weight gain, or is secondary to posttranslational modification effects (eg, phosphorylation, methylation) is unknown. It is also possible that the elevation in SIRT1 does not correlate with SIRT1

activity at three months. In contrast, ARC Hes1 demonstrated increased expression. As Hes1 contribute to the maintenance of neural stem/progenitor cells in the adult brain, these results suggest that ARC neuronal regeneration may be occurring and thus raises the potential for plasticity of appetite circuitry through early adulthood. Consistent with the likely completed ARC development, SGA three month old offspring demonstrated no change in Ngn3 or Mash1 expression. However, these offspring continue to evidence an imbalance in appetite to satiety neuropeptides, with elevated AgRP and reduced POMC protein. Thus, the increased protein expression of appetite/satiety neuropeptide ratio evidenced at one day of age persisted into adulthood.

Hypothalamic neurogenesis occurs in the fetal/newborn brain (periventricular zone of third ventricle). During development, NPCs undergo extensive proliferation (two daughter NPCs), self-renewal (one NPC and one differentiated cell), and terminal division into cells destined for neuron or glial fate (Miller and Gauthier, 2007). Temporally and stochastically orchestrated cellular signaling prevents premature NPC differentiation, assuring an adequate NPC pool. We propose that maternal undernutrition-induced SIRT1 and Hes1 responses results in a premature differentiation of NPC to neuronal or glial cells, thus prematurely reducing the periventricular pool of NPCs. As undernutrition was experienced from 10 days to term, this premature differentiation may ultimately be expressed as reduced NPC proliferation, consistent with the decreased number of PCNA/BrdU cells in the periventricular region. It ultimately would be of value to examine hypothalamic NPC proliferation at each stage of fetal development to confirm evidence of premature NPC differentiation.

Remarkably, culturing of NPCs from control and SGA newborns in differentiating medium demonstrated terminal differentiation to NPY and AgRP cells. The positive NPCs staining for NPY or AgRP indicate the potency and intrinsic property of periventricular NPCs to form orexigenic neurons. Despite the removal of the cells from the *in vivo* environment, NPC cultures initially in control media and then in differentiating media for seven days, retain the intrinsic properties by which they form an excess of orexigenic neurons. Thus, short term changes in the developmental nutrient environment may have persistent effects on neuronal development and differentiation.

We further explored the critical role of SIRT1 utilizing gene silencing (siRNA) and pharmacologic modification. As evidenced in Figure 5 both non-specific and SIRT1-specific siRNA were examined in Control and SGA NPCs. In response to non-specific siRNA, SIRT1 expression remained elevated in SGA NPC culture consistent with the hypothalamic tissue protein expression at day one and three months. SIRT1-specific siRNA reliably decreased SIRT1 expression to approximately 50% of basal values in both Control and SGA NPCs. Affirming the role of SIRT1-mediated responses in NPC proliferation SIRT1 siRNA significantly increased colony density in both Control and SGA NPCs. These findings are consistent with our hypothesis that elevated SIRT1 induces premature differentiation of NPCs, reducing the NPC pool and cell proliferation. Consistent with the visual increase in NPC cell growth, SIRT1 siRNA increased proliferation (MTT) and nestin expression in both Control and SGA NPCs while the increase in Tuj1 indicates accelerated neurogenesis. The

increase in Hes1 expression in both Control and SGA NPCs, reflects the epigenetic reduction in Hes1 expression by SIRT1 histone deacetylase action (Hisahara et al, 2008b).

The pharmacologic inhibitor Sirtinol mimicked the results of SIRT1 siRNA, confirming a direct impact of SIRT1 on neuronal proliferation and differentiation. Sirtinol demonstrated a concentration response increase in NPC proliferation and, at the 20 μ M concentration an increase in nestin and Tuj1 expression. Sirtinol increased Hes1 expression in SGA though not Control offspring and increased Mash1 expression in both groups.

The SIRT1 activator Resveratrol displayed results opposite that of the Sirtinol. Resveratrol demonstrated a concentration response decrease in cell proliferation with reduced nestin, Tuj1, Hes1 and Mash1 expression. Together these findings confirm a critical and likely direct role of SIRT1 on NPC proliferation and differentiation.

Recently using SIRT1 knockin mice, Sasaki et al demonstrated protection against age-associated weight gain via increased leptin sensitivity. However, the effects of conditional SIRT1 overexpression in POMC or AgRP neurons on body weight and food intake were evident only in older mice consuming normal diet though not in mice fed a high-fat, high-sucrose diet (Sasaki et al, 2014). In contrast, selective knockout of SIRT1 in hypothalamic AgRP neurons was shown to decrease food intake, fat mass and body weight (Dietrich et al, 2010). The results of the present study on SIRT1-mediated suppression of neurogenesis is consistent with recent studies demonstrating adverse effects of SIRT1 on NPC proliferation and differentiation (Prozorovski et al, 2008; Rafalski et al, 2013). SIRT1 is activated by NAD⁺, which predominates during caloric restriction and oxidative conditions (Bordone and Guarente, 2005; Libert et al, 2008). Prozorovski et al showed that under oxidative conditions SIRT1 expression is increased, SIRT1-Hes1 complex is activated and Mash1 is suppressed (Prozorovski et al, 2008). Collectively, this suggests that SIRT1 which has deacetylase activity may not always confer beneficial effects in all cell types.

To examine the potential mechanism of SIRT1 epigenetic modification of Hes1, we utilized ChIP procedure. Anti-SIRT1 antibody pulled down significantly greater amounts of Hes1 in SGA as compared to Control NPCs consistent with an increased SIRT1 binding to Hes1. There was no difference in the pull down of anti-insulin, as a negative control.

In summary, these results provide a plausible biologic mechanism by which maternal and thus fetal undernutrition programs offspring hyperphagia and obesity. Limiting calorie availability *in utero* result in elevated SIRT1 expression and binding to Hes1 chromatin structure in the offspring NPC. The SIRT1 histone deacetylase activity reduces expression of Hes1 in the periventricular region, resulting in premature differentiation of neural progenitor cells and a reduced pool of proliferative cells. SIRT1 epigenetic modifications reduce Ngn3 and Mash1 expression shifting ARC neural differentiation in favor of appetite vs. satiety neurons. Were these findings to be extended to cortical neuronal development, it may explain reports of altered neurobehavior and cognitive function among SGA offspring.

As appetite is both a subconscious and conscious physiologic function, and is influenced by a diversity of social and environmental cues, it has been difficult to determine if “enhanced appetite” contributes to human hyperphagia and obesity. It would not be surprising that there

is diversity among humans such that there are varying degrees of appetite and satiety. Our prior studies demonstrated that SGA offspring exhibit increased food intake and reduced anorexigenic signaling responses, both contributing to obesity (Desai et al, 2007). The present results provide a basis to explain programmed appetite pathways. Although these findings remain to be demonstrated in humans, an understanding of this timing and mechanism of programmed appetite and the potential for neuronal remodeling may provide the opportunity for directed interventions.

4. Experimental Procedure

4.1 Maternal Diet

Studies were approved by the Animal Research Committee of the Los Angeles Biomedical Research Institute at Harbor-UCLA (LABioMed), and were in accordance with the American Association for Accreditation of Laboratory Care (AALC), and National Institutes of Health (NIH) guidelines. The rat model utilized for maternal food restriction during pregnancy and lactation has been previously described (Desai et al, 2005). Briefly, first time pregnant Sprague Dawley rats (Charles River Laboratories, Hollister, California) were housed in a facility with constant temperature and humidity and controlled 12:12 hour light/dark cycle. From day 10 of pregnancy to term (21 days), rats were provided either an ad libitum (Control) diet of standard laboratory chow (protein 23%, fat 4.5%, metabolizable energy 3030 kcal/kg; Lab Diet 5001, Brentwood, MO), or a 50% food restricted diet determined by quantification of normal intake in ad libitum fed rats, to create small for gestation age newborns (SGA). After birth, litters were culled to four males and four females and all SGA newborns were cross-fostered and nursed by Control dams and weaned to control diet at day 21 (Lab Diet 5001) till 3 months of age. Similarly, all Control pups were cross-fostered amongst Control dams to normalize the study design.

The total number of Control and SGA dams studied were N=15 dams per group. 6 additional Control dams were utilized for cross-fostering of SGA pups.

4.2 Hypothalamic/ARC Dissection

For hypothalamus, semi-spheres adjunct to two sides of hypothalamus were cut, the dorsal part removed, and the ventral part (~2 mm) used. The ARC was microdissected using the fornix and third ventricle as landmarks (the area adjacent to the bottom of the third ventricle is dissected parallel to the border of the ventricle).

4.3 In Vivo Cell Proliferation

We assessed *in vivo* hypothalamic NPC proliferation by labeling of DNA with 5-bromodeoxyuridine (BrdU) which is incorporated into nuclei during the DNA synthetic phase of the cell cycle (S-phase). BrdU (50mg/kg/day, i.p.) was administered to pregnant dams from e17-e19 (N=3 per group). After birth, brains were collected from 1 day old SGA and Control newborn males. Three brains per litter were frozen, and three sections per brain (5µm thickness) were immunostained for BrdU and proliferating cell nuclear antigen (PCNA; expression increases during G1-phase, peaks at the S-phase, and declines during G2/M-phases of the cell cycle) and thereafter with secondary antibodies (donkey anti-rabbit

IgG conjugated with Alexa 488; (Invitrogen, Carlsbad, CA). Photomicrographs (fluorescence microscopy, Zeiss, Axioskop 40) were captured at $\times 20$ magnification and Image PRO software (version 5.1) was used to determine cell proliferation by counting PCNA/BrdU positive cells in third ventricle per field (Mosmann, 1983).

4.4 Neurosphere Cultures, SIRT1 silencing (siRNA) and Treatment with SIRT1 Modulators

For NPC culture studies, 1 day old newborn males were sacrificed (6 per group), hypothalamus dissected and pooled from each litter (4 per litter). NPC cultures were prepared as previously reported by us using modified standard method (Desai et al, 2011b; Erickson et al, 2008; Kelly et al, 2009).

Briefly, hypothalamus was dissected in DMEM/F12 medium, cells dissociated by trypsin, centrifuged and cells seeded at 5×10^4 cell/ml in complete medium [NeurobasalTM Medium containing 1% anti-anti (Invitrogen), 2% B27 (GIBCO, Cat#17504-044), 20ng/ml FGF2 (Sigma), 20ng/ml EGF (Sigma), 1 μ g/ml heparin (Lylli), and 2.5 μ g/ml L-glutamine (Invitrogen)]. After 8 days in culture (passage 0), centrifuged neurospheres were dissociated into single-cell by trypsinization and reseeded at same cell density (passage 1) in complete medium.

For induction of differentiation, dissociated cells were re-suspended in differentiating medium (DM; in absence of FGF2, EGF and heparin) and seeded in culture dishes pre-coated with 0.01% poly-L-lysine (Sigma).

For SIRT1 silencing (siRNA), hypothalamic NPCs were cultured in complete medium. At day 1 of passage 1 NPC seeding, the NPCs were transfected with rat SIRT1-specific siRNA (20 nM) or control siRNA (negative control) using siPORTTM NeoFXTM Reagent (Ambion). The transfected cells were reseeded in complete or differentiating medium. Cell proliferation and protein expression (Western blot) was determined at day 5 of siRNA transfection.

For treatment with SIRT1 Modulators, hypothalamic NPCs were cultured in complete or differentiating medium. At day 1 of passage 1 NPC seeding, the NPCs were treated with SIRT1 inhibitor (20 μ M sirtinol; a specific inhibitor for SIRT1) or SIRT1 activator (10, 20 μ M resveratrol) for 5 days. Cell proliferation rate and protein expression (Western blot) of untreated and treated NPCs was determined at day 5 of siRNA transfection.

4.5 *In Vitro* NPC Cell Proliferation

This was determined by MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl tetrazolium bromide; Sigma) colorimetric assay (McLenachan et al, 2009). Cell proliferating index was expressed as value of OD 570nm.

4.6 Chromatin Immunoprecipitation (ChIP)

Passage 1 NPCs were cultured in complete media for 7 days and chromatin isolated for ChIP (ab500, Abcam, Cambridge, MA). $\sim 10^6$ NPCs were cross-linked (1% formaldehyde), chromatin extracted and equivalent amount of chromatin was used for immunoprecipitation by specific antibodies anti-SIRT1 and anti-insulin (as negative control). The pulled down

chromatins were purified for DNA and used as template for qPCR reactions and the PCR primer specific to Hes1 (249 bp length) promoter regions. PCR product densities were normalized by relative input control density.

4.7 Western Blot

For tissue studies, excess 1 day old newborns and 3 month old male adult, brain were collected and frozen (N=6 per group per age), and subsequently dissected for hypothalamus (1 day newborn) or ARC (3 month adult). Westerns were performed as previously reported by our group (Desai et al, 2008). Sample lysates (lysis buffer with protease and phosphatase inhibitors) together with positive control lysate or available recombinant protein were used for each gel. Specificity of antibody binding was verified by mixing available blocking peptide with lysate samples. Data are normalized to GAPDH/ β -actin.

4.8 Double Immunofluorescence

For staining of NPC NPY and AgRP, Passage 0 disassociated NPCs were seeded in differentiating medium (passage 1). After 7 day culture, the cells were fixed in 4% paraformaldehyde in PBS for 0.5 h and then immunostained with rabbit anti-NPY or anti-AgRP (Abcam, 1:500). Goat anti-rabbit IgG-Alexa 488 was used to visualize positive cells. All cells were stained by DAPI. In each case 1000 DAPI cells were counted and positive labeled cells were counted using Image J program.

For triple immunostaining, NPCs fixed in 4% paraformaldehyde were immuno-labeled with goat anti-POMC and rabbit anti-NPY and then visualized with donkey anti-goat-IgG-Alexa 568 and donkey anti-rabbit-IgG-Alexa 488. The cellular nuclei were stained with DAPI.

4.9 Data Analysis

Differences between SGA and the Control males were compared using either unpaired t-test or ANOVA with Dunnett's post hoc tests.

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- Increased hypothalamic/NPC appetite to satiety ratio in SGA offspring is via SIRT1
- Elevated SIRT1 reduces NPC proliferation induces premature NPC differentiation
- SGA offspring have elevated hypothalamic/NPC SIRT1 and reduced neurogenic factors
- SGA offspring show reduced in vivo and in vitro NPC proliferation
- SIRT1 silencing and pharmacologic modulation confirm role of SIRT1 in neurogenesis

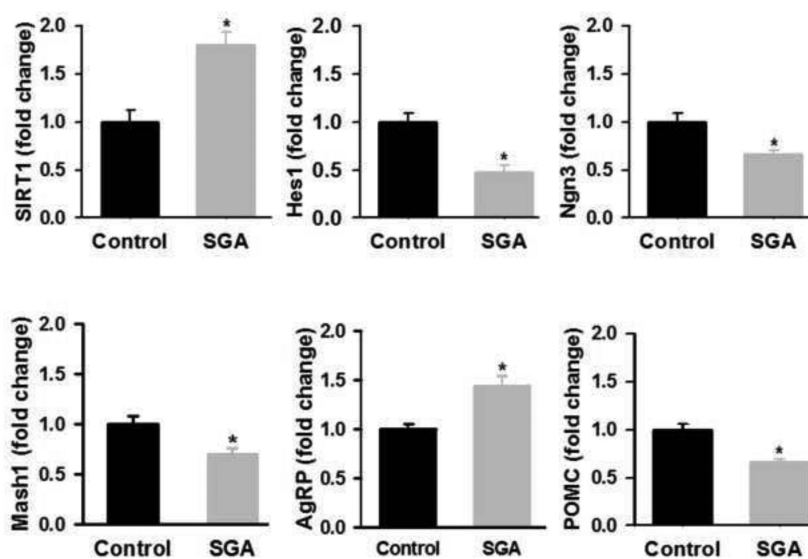


Figure 1. Newborn Hypothalamic Tissue Protein Expression

Hypothalamic protein expression of SIRT1, Hes1, Ngn3, Mash1, AgRP and POMC from 1 day old Control (■) and SGA (■) males. * $p < 0.05$ vs Control; $N = 6$ male pups from 6 litters were studied in each group.

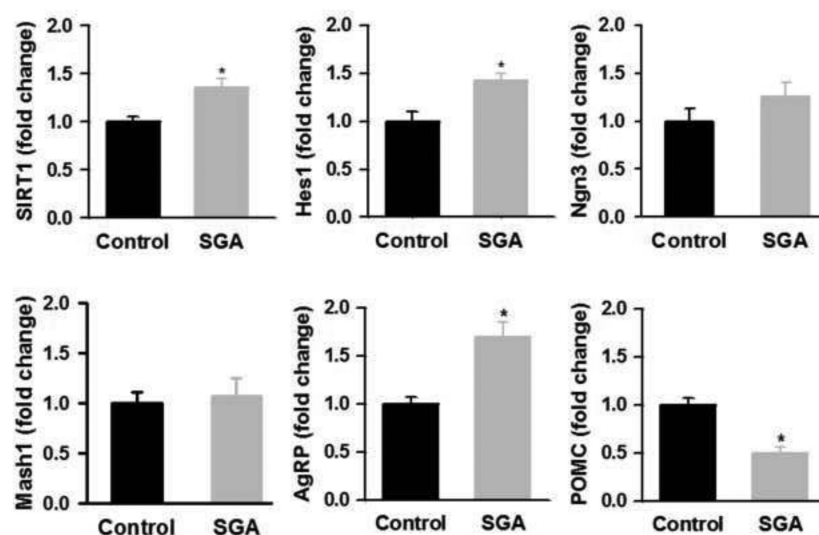


Figure 2. Adult Hypothalamic Tissue Protein Expression

Hypothalamic protein expression of SIRT1, Hes1, Ngn3, Mash1, AgRP and POMC from 3 month old Control (■) and SGA (■) males. * $p < 0.05$ vs Control. N=6 males from 6 litters were studied in each group.

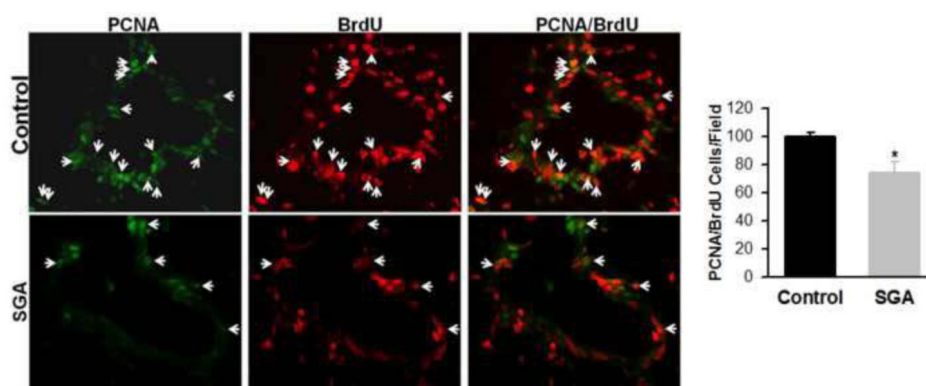


Figure 3. In Vivo NPC Proliferation

Hypothalamic third ventricle NPCs from p1 Control (■) and SGA (■) males. (A) immunostained for PCNA (green) and BrdU (red), and (B) quantified PCNA /BrdU positive cells. * $P < 0.05$. 3 litters were studied in each group. From each litter, N=3 male pups were studied.

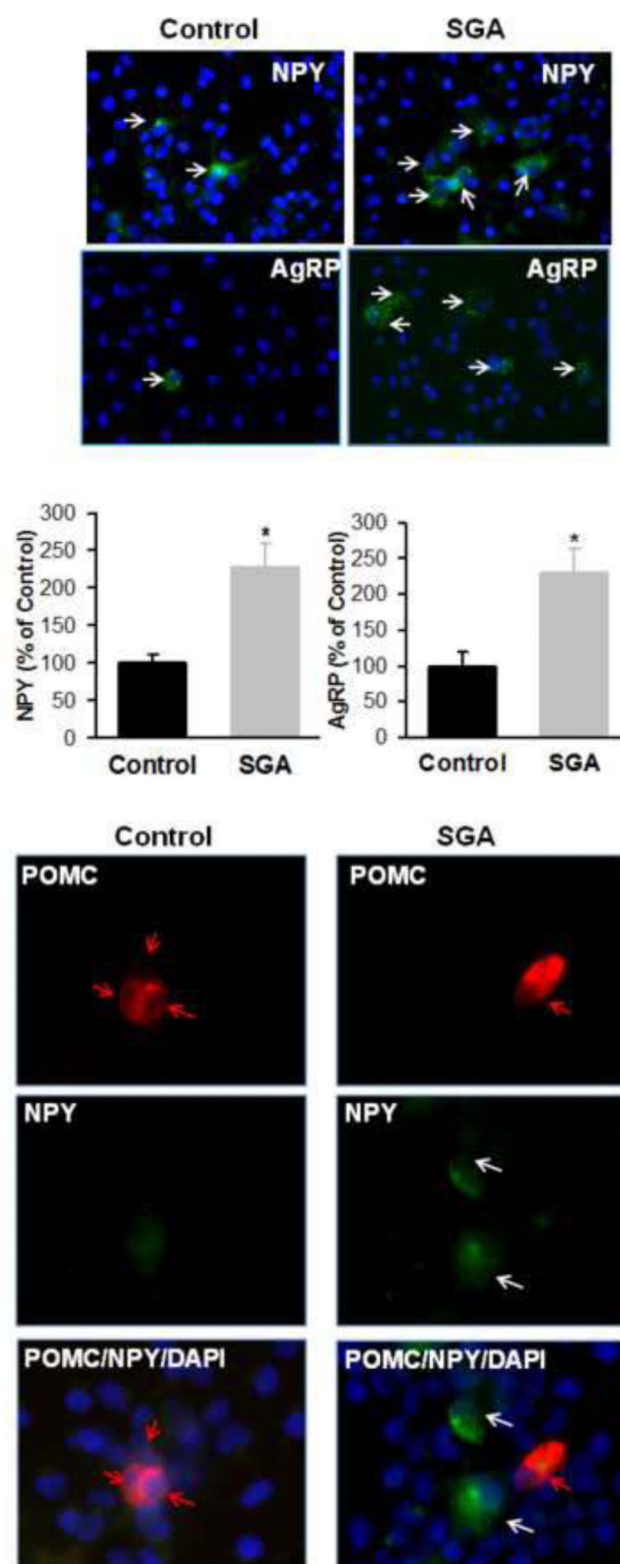


Figure 4. Hypothalamic NPC

(A) Immunostaining of NPY and AgRP Neurons NPCs from Control (■) and SGA (■) newborns were cultured in differentiating medium for 7 days. NPY or AgRP positive (green) and DAPI stained cells (blue) were quantified. In each case 1000 DAPI cells were counted from 6 separate cultures per group. Values (mean±SE) are percentage of Control. * $P < 0.05$ vs Control. (B) NPCs from Control (■) and SGA (■) newborns were cultured in differentiating medium for 7 days. Representative image of positive POMC (red), NPY (green) and DAPI (blue) stained cells are shown. 6 litters were studied in each group. From each litter, hypothalami from N=4 male pups were pooled representing N=1.

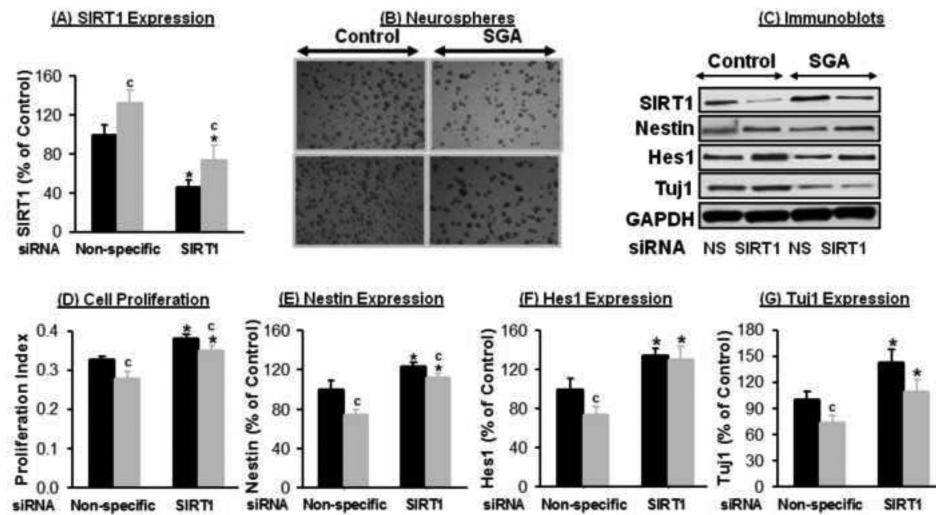


Figure 5. SIRT1 Silencing (siRNA) Effect on Neurosphere Proliferation and Neuronal Differentiation

NPCs from Control (■) and SGA (■) were transfected with non-specific or SIRT1-specific siRNA (20 nM). (A) SIRT1 expression, (B) images of neurospheres (x20), (C) immunoblots (NS=non-specific siRNA), (D) NSC proliferation index, and protein expression of (E) Nestin, (F) Hes1 and (G) Tuj1. * $p < 0.05$ vs non-specific siRNA and ^c $p < 0.05$ SGA vs Control. 6 litters were studied in each group. From each litter, hypothalami from N=4 male pups were pooled representing N=1.

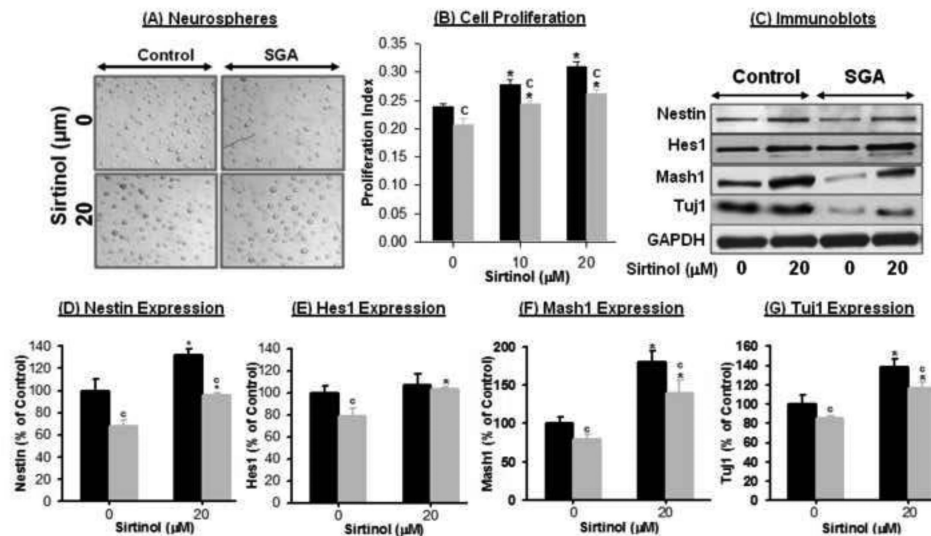


Figure 6. SIRT1 Inhibitor (Sirtinol) Effects on Neurosphere Proliferation and Neuronal Differentiation

Control (■) and SGA (■) p1 hypothalamic neurospheres were cultured treated with sirtinol for 5 days. (A) Images of neurospheres (x20). (B) NPC proliferation index. (C) Immunoblots. Protein expression of (D) Nestin, (E) Hes1, (F) Mash1 and (G) Tuj1. * $p < 0.05$ vs untreated; ^c $p < 0.05$ SGA vs Control. 6 litters were studied in each group. From each litter, hypothalami from N=4 male pups were pooled representing N=1.

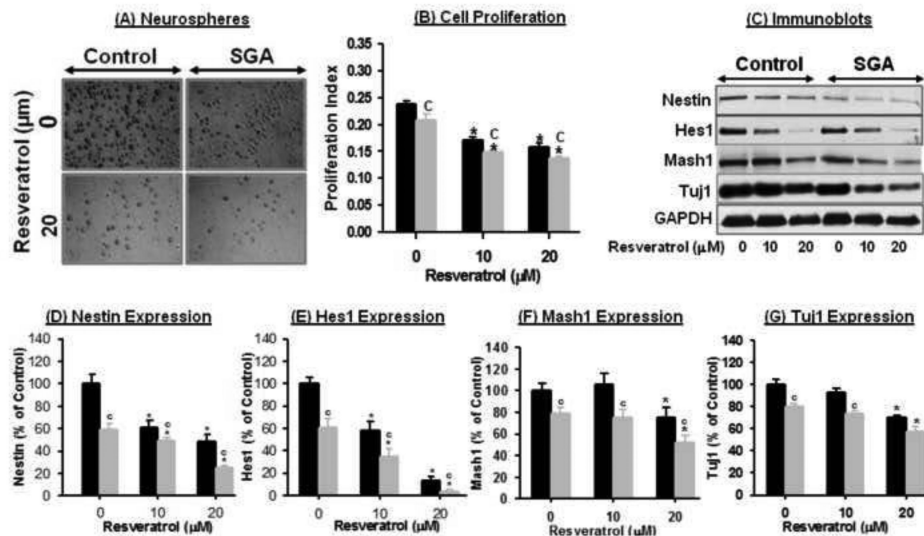


Figure 7. SIRT1 Activator (Resveratrol) Effects on Neurosphere Proliferation

Control (■) and SGA (■) p1 hypothalamic neurospheres were cultured and treated with resveratrol for 5 days. (A) Images of neurospheres (x20). (B) NSC proliferation index. (C) Immunoblots. Protein expression of (D) Nestin, (E) Hes1 and (F) Tuj1. *P<0.05 vs untreated; ^c p<0.05 SGA vs Control. 6 litters were studied in each group. From each litter, hypothalami from N=4 male pups were pooled representing N=1.

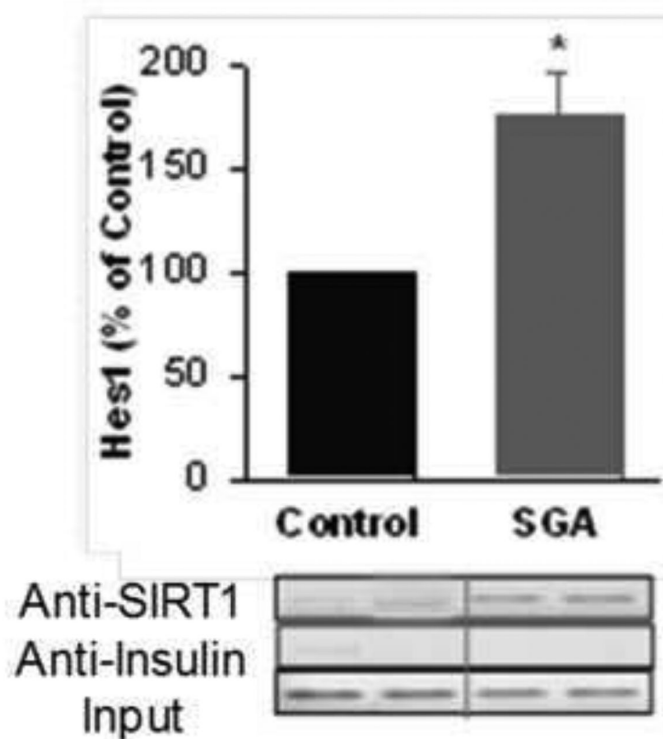


Figure 8. Interaction between Hypothalamic NPC SIRT1 and Promoter Hes1

NPCs from Control (■) and SGA (■) were cultured for 7 days and chromatin isolated for ChIP. Antibodies used for ChIP were anti-SIRT1 and anti-insulin (as negative control). Pulled-down chromatin was purified and used as template for PCR primer specific to Hes1 promoter regions. * $p < 0.05$ vs Control. 6 litters were studied in each group. From each litter, hypothalami from N=4 male pups were pooled representing N=1.