ELUCIDATING THE ROLE OF SAD-A KINASE IN REGULATING ENERGY
AND GLUCOSE HOMEOSTASIS

A Thesis in
Laboratory Animal Medicine
by
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ABSTRACT

Obesity is a net result of an imbalance between energy intake and expenditure. AMP-dependent protein kinase (AMPK) is an enzyme that plays a role in energy homeostasis. AMPK is activated in response to an increase in the AMP/ATP ratio under low nutrient conditions. These functions are partly mediated by its role in glucose sensing by hypothalamic neurons. SAD-A, also known as BRSK2 (Brain Specific Kinase 2) is exclusively expressed in the brain and pancreas and is a serine/threonine protein kinase related to the AMPK family of kinases. In order to understand the role of Sad-A in energy metabolism, tissue specific knockout of the Sad-A gene was generated using a Cre-lox mediated approach in Pomc neurons, a cell type primarily involved in food intake and energy metabolism. Pomc-cre^{+/null} mice were bred with Sad-A^{flox/flox} mice to generate a Pomc neuron-specific Sad-A knockout line. The generation of tissue specific Sad-A knockout line was confirmed by genotyping. Metabolic studies were conducted using the genetically modified Sad-A knockout mouse line to determine its role in energy and glucose homeostasis.
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Table 1: PCR steps in amplification of Cre allele

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LIST OF ABBREVIATIONS

AgRP  Agouti-related peptide
AMPK  AMP- dependent protein kinase
ARC   Arcuate nucleus
ATP   Adenosine Tri-Phosphate
BAT   Brown adipose tissue
BRSK2 Brain Specific Kinase 2
BW    Body weight
GLP-1 Glucagon-like peptide-1
GSIS  Glucose-stimulated insulin release
ITT   Insulin tolerance test
MCH   Melanocortin hormone
MCR4  Melanocortin receptor 4
NPY   Neuropeptide Y
OGTT  Oral glucose tolerance test
PPAR-gamma Peroxisome proliferator-activated receptors
PGC-1 PPAR-Gamma coactivating factor
POMC  Pro-opiomeranocortin
UCP   Uncoupled proteins (UCPs-UCP2 and UCP3)
VMH   Ventromedial nucleus
I would like to thank my thesis advisor, Dr. Yuguang Shi, for his support, and allowing me to work on this important project. I would like to offer my sincere thanks to Dr. Ronald Wilson for the encouragement and willingness to devote time and resources toward my project. I would like to thank Dr. Christopher Lynch for his intellectual input into the project and providing access to the metabolic cages.

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With all my heart, a special thanks to my wife, Sushama who has extended all her support throughout my residency training.
CHAPTER I

INTRODUCTION

Obesity is a condition with accumulation of excess body fat to an abnormal quantity that leads to adverse effects on health. It is defined as a body mass index of 30 and above. According to Centers for Disease Control, on average, 25% of population in United States is obese. The prevalence of obesity is high in industrialized nations effecting both adults and children. It is estimated that 300,000 people die each year due to complications of obesity. Obesity leads to other diseases such as diabetes, hypertension, cardiovascular disease, and cancer (1).

Obesity is a net result of energy imbalance between energy intake and expenditure. The energy intake includes composition and calorie of the diet consumed while the energy expenditure includes physical activity, basal metabolism, and adaptive thermogenesis. The central nervous system plays a key role in regulation of energy balance and is coordinated by three mechanisms. 1) effects on appetite control and physical activity 2) effects on basal metabolism 3) effects mediated through neuroendocrine hormones (2).

Both genetic and environmental factors play a role in determining body weight, composition, and storage of energy in adipose tissue. Multiple pathways in the neuroendocrine system controls food intake, energy, and glucose homeostasis. Neuropeptides and neurotransmitters interact closely in the hypothalamus, where hormonal, metabolic, and nutritional signals are
coordinated to regulate physiological processes (3). The peripheral hormones such as insulin, leptin and glucagon-like peptide-1 (GLP-1) play a role in glucose homeostasis and these hormones are transported via blood brain barrier to act on receptors within the brain. Insulin exerts its function both centrally and peripherally via insulin receptor leading to subsequent activation of insulin receptor substrate (IRS), phosphatidylinositol-3 kinase (PI3K), and protein kinase B (PKB) (4). Leptin is another important molecule, secreted by adipose tissue, to regulate energy and glucose homeostasis. It has been demonstrated that deficiency of leptin or its receptor leads to obesity, and insulin resistance (5). Leptin acts centrally in the arcuate nucleus (ARC) by activating two independent pathways: 1) Stat-3 dependent pathway (6) and 2) PI-3 kinase pathway (7). GLP-1 is a hormone secreted by intestine and discrete population of neurons. GLP-1 regulates glucose homeostasis peripherally via pancreatic beta cells to stimulate insulin secretion and biosynthesis (8). Recent evidence indicated that insulin, leptin and GLP-1 exert their functions of glucose homeostasis through pro-opiomelanocortin (Pomc) neurons in the arcuate nucleus of hypothalamus (9). Although these molecules regulate homeostasis through distinct signaling pathways involving several kinases including AMP-dependent protein kinase (AMPK) family of kinases, the detailed molecular mechanism is yet to be discovered (10). This project is intended to understand the molecular mechanism of glucose and energy homeostasis with respect to the SAD-A kinase.
To determine the role of SAD-A kinase in glucose sensing and energy metabolism, the specific aims of this project were:

**Specific aim 1:** To generate a transgenic mouse line with disruption of Sad-A allele specifically within the Pomc-neurons of hypothalamus using Cre-Lox mediated recombination approach.

**Specific aim 2:** To determine the effects of Sad-A knockout on glucose-stimulated insulin secretion (GSIS).

**Specific aim 3:** To determine the effects of Sad-A knockout in Pomc-neurons on metabolic parameters.
CHAPTER II

LITERATURE REVIEW

Role of the hypothalamus in energy balance

The hypothalamus is the central regulator of energy metabolism. The importance of the function of the hypothalamus in the regulation of energy balance was demonstrated in very early studies conducted by ablation of ventromedial hypothalamic areas including the ventromedial (VMH) and arcuate (ARH) nuclei that lead to a hyperphagic and obese phenotype (11). Within the hypothalamus, the glucose sensing neurons are located in the arcuate nucleus (ARC) and ventromedial nucleus.

There are two types of glucose sensing neurons that regulate feeding behavior. One set of neurons known as glucose-excited neurons, increase their activity in response to increased local glucose concentrations. The other set of neurons are glucose-inhibited neurons that decrease their activity in response to increased glucose concentrations (12). Within the arcuate nucleus, there are two populations of neurons. One population secretes orexigenic (increased food intake) neuropeptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP) (13). The other population secretes anorexigenic peptides (decreased food intake) α-MSH, derived from proopiomelanocortin (Pomc), and cocaine and amphetamine related transcript (CART) (2). The glucose sensing neurons partly overlap with Pomc
and NPY expressing neuronal populations respectively (12). The pathways involved in food intake and energy expenditure are closely interrelated. The neurons in the arcuate nucleus communicate with other regions of the hypothalamus, the dorsomedial hypothalamic nucleus, ventromedial hypothalamic nucleus (VMH), paraventricular nucleus (PVN) and lateral hypothalamus. The neuronal circuits with axons projecting from the arcuate nucleus as well as from the lateral hypothalamus integrate in the PVN. The neurons in the VMH are interconnected with neurons from PVN, DMH and lateral hypothalamus. This is a region with abundant leptin receptors and it is involved in the regulation of satiety. The neurons in the DMH are interconnected with neurons in PVN, the lateral hypothalamus and the brainstem. THE PVN and DMH coordinate regulation of food intake. This region is rich in insulin as well as leptin receptors (14).

**Peripheral signals in energy balance**

The centrally regulated neuronal signals travel from the hypothalamus to the nucleus of the tractus solitarii (NTS) in the medulla where signals from the gastrointestinal tract are received through vagus nerve. The peripheral stimuli includes insulin that triggers neuronal signals associated with energy metabolism via insulin receptors in the mediobasal hypothalamus (15).

**Pro-opiomelanocortin (Pomc)**

With the arcuate nucleus, Pomc neurons in association with other neuronal population play a major role in feeding behavior and energy
expenditure. Pomc is a polypeptide consisting of 21 amino acids. The gene is expressed in both anterior and intermediate lobes of hypothalamus. Mutations in this gene are known to cause obesity and adrenal insufficiency. The Pomc is enzymatically cleaved into 11 different peptides with various functions. These peptides play a role in regulation of appetite, sexual behavior, and melanin production (16). POMC binds to the MCH receptor, a G-protein coupled receptor encoded by the MC4R gene (17).

**Neuropeptide Y (NPY)**

NPY is known for a wide range of functions including appetite stimulation, decreased energy expenditure, memory processing, regulation of blood pressure and body temperature. NPY promotes positive energy balance by stimulating food intake, as well as decreasing energy expenditure through decreasing non-shivering thermogenesis in brown adipose tissue (BAT) and by promoting triglyceride deposition (18,19).

**Role of AMPK in energy metabolism**

AMPK is a serine/threonine protein kinase involved in the regulation of energy balance. It is known to regulate fatty acid oxidation, glucose uptake, gluconeogenesis and appetite. Several hormones including leptin, adiponectin, insulin, IL-6 and ghrelin regulate metabolic activities through AMPK (19,20).

*Role of AMPK in energy metabolism*
AMPK phosphorylates the Rab-GTPase-activating proteins (GAP) and reduces their activity. This results in release and translocation of glucose transporter type-4 (GLUT-4) to the plasma membrane for uptake of glucose into the cells (20,21).

Role of AMPK in fatty acid metabolism

AMPK phosphorylates ACC1 (acetyl-coA carboxylase) to inactivate it and decrease malonyl-CoA production. With AMPK activation, there is an increase in fatty acid β-oxidation and a decrease in lipogenesis, depending on the tissue. In the obese condition, there is a decreased AMPK activity, as a result there is increased ACC phosphorylation accompanied by increased levels of malonyl-CoA that results in accumulation of lipids in the body (20,22).

Role of AMPK in gluconeogenesis

AMPK is the primary regulator of hepatic gluconeogenesis. AMPK is known to repress the transcription of two gluconeogenic enzymes, phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) by phosphorylation of Ser-171 of the CREB-regulated transcription(20,23).

Role of AMPK in appetite
AMPK regulates appetite via the orixegenic and anorexigenic neuronal signals, orchestrated by a specific set of neurons in the hypothalamus. Overexpression of constitutively active AMPK in the neurons of hypothalamus lead to increased food intake and body weight associated with increased transcript levels of the appetite-stimulating neuropeptides, NPY and AgRP. Similarly, overexpression of a dominant negative form of AMPK in the neurons of the hypothalamus can suppress food intake and reduce body weight associated with decreased transcript levels of the appetite-stimulating neuropeptides, NPY and AgRP (20, 24).

**SAD-A Kinase**

SAD-A, also known as BRSK2 (Brain Specific Kinase 2) is a serine/threonine protein kinase related to the AMPK family of kinases. SAD-A is known to regulate neuronal polarity and axon specification (25, 26). It is known to interact with the cell–cycle checkpoint kinase, Wee1, to regulate the cell cycle (27). SAD-A is exclusively expressed in the pancreas and brain. SAD-A is most closely related to AMPK among the AMPK-family of kinases that are known to play an important role in the central regulation of energy expenditure and glucose homeostasis (28). AMPK is activated in response to an increase in the AMP/ATP ratio under low nutrient conditions. These functions are partly mediated by its role in glucose sensing by hypothalamic neurons (29).
Significance

Obesity is a result of the dysregulation of energy metabolism. Recent studies have elucidated the role of several kinases in the regulation of energy metabolism. Although much progress has been made in the understanding of the neural control of energy balance and glucose homeostasis, many questions remain unanswered. This project will advance our understanding of the intricate pattern of neuronal network involved in regulation of energy metabolism. A complete understanding of the regulation of energy metabolism will help in the design of better therapeutics to treat obesity.
CHAPTER III

ELUCIDATING THE ROLE OF SAD-A KINASE IN REGULATING ENERGY AND GLUCOSE HOMEOSTASIS

ABSTRACT

Obesity is a net result of an imbalance between energy intake and expenditure. AMPK is an enzyme that plays a role in energy homeostasis. AMPK is activated in response to an increase in the AMP/ATP ratio under low nutrient conditions. These functions are partly mediated by its role in glucose sensing by hypothalamic neurons. SAD-A, also known as BRSK2 (Brain Specific Kinase 2) is exclusively expressed in the brain and pancreas and is a serine/threonine protein kinase related to the AMPK family of kinases. In order to understand the role of SAD-A in energy metabolism, tissue specific knockout of the Sad-A gene was generated using a Cre-lox mediated approach in Pomc neurons, a cell type primarily involved in food intake and energy metabolism. Pomc-cre<sup>+/null</sup> mice were bred with Sad-A<sup>lox/lox</sup> mice to generate a tissue specific Sad-A knockout line. The generation of tissue specific Sad-A knockout line was confirmed by genotyping. Metabolic studies were conducted using the transgenic Sad-A knockout mouse line to determine its role in energy and glucose homeostasis.
INTRODUCTION

Genetic and environmental factors play a role in determining body weight, composition, and storage of energy in adipose tissue. Multiple pathways in the neuroendocrine system control food intake, energy, and glucose homeostasis. Neuropeptides and neurotransmitters interact closely in the hypothalamus, where hormonal, metabolic, and nutritional signals are coordinated to regulate physiological processes (3). The peripheral hormones such as insulin, leptin and glucagon-like peptide-1 (GLP-1) play a role in glucose homeostasis and are transported across the blood brain barrier to act on receptors within the brain. Insulin exerts its function both centrally and peripherally via insulin receptors leading to the subsequent activation of insulin receptor substrate (IRS), phosphatidylinositol-3 kinase (PI3K), and protein kinase B (PKB) (4). Leptin is another important molecule, secreted by adipose tissue, to regulate energy and glucose homeostasis. It has been demonstrated that deficiency of leptin or its receptor leads to obesity, and insulin resistance (5). Leptin acts centrally in the arcuate nucleus (ARC) by activating two independent pathways 1) Stat-3 dependent pathway (6) and 2) PI-3 kinase pathway (7). GLP-1 is a hormone secreted by the intestine and discrete population of neurons. GLP-1 regulates glucose homeostasis peripherally via pancreatic beta cells to stimulate insulin secretion and biosynthesis (8). Recent evidence indicated that insulin, leptin and GLP-1 exert their functions of glucose homeostasis through pro-opiomelanocortin (Pomc) neurons in the arcuate nucleus of the hypothalamus (9). Though
these molecules regulate homeostasis through distinct signaling pathways involving several kinases including the AMPK family of kinases, the detailed molecular mechanism is yet to be discovered (10). This project is intended to understand the molecular mechanism of glucose and energy homeostasis with respect to the SAD-A kinase.
MATERIALS AND METHODS

Animal Studies

Transgenic mice Cg.Tg (Pomc1-cre)16Lowl/J of 6 weeks-old were obtained from Jackson laboratories. All mice were housed under specific pathogen free condition in a barrier facility in individually ventilated microisolator cages. Cg.Brsk2\textsuperscript{tm1San} (designated as Sad-A\textsuperscript{floxelox}) mice were obtained from Harvard University (gift from Dr. Joshua Sanes), quarantined for 6 weeks before moving to barrier facility. All mice had \textit{ad libitum} access to feed (2018 Global Rodent Diet, Harlan Teklad, Indianapolis, IN) and water and maintained at an ambient temperature of 21 ± 0.5 °C and humidity range between 30-70% on a 12-h light and dark cycle. All the animal procedures were in accordance with the \textit{Guide for Care and Use of Laboratory Animals} and were approved by the Institutional Animal Care and Use Committee. Euthanasia was performed using carbon dioxide asphyxiation.

Isolation of genomic DNA

Tail snips were obtained from the mice between 18-21 days. Local anesthesia was performed by immersing tail in ice-cold isopropyl alcohol for 10 seconds. Tail snip of approximately 2-3 mm size was obtained using a sterile sharp scalpel or razor blade. The tail tips were stored at -80°C. Each tail tip was placed in an Eppendorf tube with in a pre-chilled mixture of 600 μL of Nuclei Lysis Solution (Promega, Madison, WI) (NLS)/0.5M EDTA (pH 8.0) in 5:1 ratio respectively. Proteinase K (20 mg/mL) (Sigma Aldrich, St. Louis,
MO) of 17.5 µL volume was added to each tube and tubes were incubated at 55°C overnight. Rnase solution (4 mg/mL) (Sigma Aldrich, St. Louis, MO) of 3 µL volume was added to the nuclear lysate and incubated at 37°C for 30 min. The samples were later allowed to cool down to room temperature for 5 min. Two hundred micro liters of protein precipitation solution (Promega, Madison, WI) was added and vortexed vigorously at high speed for 30 seconds. The samples were chilled on ice for 5-10 min. The samples were later centrifuged for 10 min at 14,000 rpm. Next, supernatant was transferred to clean Eppendorf tube containing 600 µL isopropanol (100%). The tube was mixed well until the white thread-like strands of DNA form a visible mass. Next the tubes were centrifuged for 10 min at 14,000 rpm at room temperature and supernatant was discarded. The tubes were washed with 70% ethanol followed by centrifugation at 14,000 rpm for 5 min and rehydrated with 50 µL water.

**PCR based genotyping**

500 ng of genomic DNA was used for PCR amplification. Forward primer: GCG GTC TGG CAG TAA AAA CTA TC, Reverse primer GTG AAA CAG CAT TGC TGT CAC TT, Internal control forward primer: CTA GGC CAC AGA ATT GAA AGA TCT, Internal control reverse primer: GTA GGT GGA AAT TCT AGC ATC ATC C were used for PCR amplification. DNA polymerase from Go Taq green (Promega, Madison, WI) was used for the amplification.

**PCR steps in amplification of cre gene**
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<th>Step #</th>
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<td>1</td>
<td>94</td>
<td>3 min</td>
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<tr>
<td>2</td>
<td>94</td>
<td>30 s</td>
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<td>3</td>
<td>51.7</td>
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<tr>
<td>4</td>
<td>72</td>
<td>1 min</td>
<td>Steps 2-4 for 35 cycles</td>
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<tr>
<td>5</td>
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**Table 1:** PCR steps in amplification of Cre gene

**PCR steps in amplification of Sad-A<sup>lox/lox</sup> gene**

500 ng of genomic DNA was used for PCR amplification. Forward primer: AGT ATG TGG GGC CCT ACC GGC TGG A Reverse primer: ATG TCC TGG GGC TGC ACC CGC CCT CC were used for PCR amplification. Taq DNA polymerase (Promega, Madison, WI), betadine (Sigma-Aldrich, St. Louis, MO) was used for the amplification.
Table 2: PCR steps in amplification of $Sad-A^{floxflox}$ gene

RNA isolation

To isolate RNA, tissue was homogenized and mixed with trizol reagent (Invitrogen, Carlsbad, CA). For 1 mL of trizol mix, 0.2 mL of chloroform (Fisher Scientific, Pittsburgh, PA) was added and mixed vigorously and allowed to incubate at room temperature for 15 min. The mix was then centrifuged at 12,000 rpm for 15 min at 4°C. The supernatant aqueous phase was transferred to a clean tube and RNA was precipitated by adding 0.5 mL of isopropanol (100%) (Sigma-Aldrich, St. Louis, MO) and mixing well. The solution was allowed to incubate for 10 min at room temperature. This was followed by centrifugation at 12,000 rpm for 8 min at 4°C. The supernatant was discarded and the pellet was washed with 1 mL of 75% ethanol and centrifuged at 7,500 rpm for 15 min. The supernatant was discarded and the pellet was allowed to dry for 10 min under the fume hood. The pellet was resuspended in 50 μL of RNAse free DEPC water (Invitrogen, Carlsbad, CA), the concentration was measured and stored at -80°C.

cDNA Synthesis

A total of 2 μg of RNA was used to synthesize the cDNA. 2 μL of Random hexamers (Invitrogen, Carlsbad, CA) was used and the volume was brought up to 11 μL with RNAse free DEPC water. The mix was incubated at 70°C for 5 min followed by 5 min on ice. A mix of 5x-first strand buffer (4 μL/sample), DTT (2 μL/sample), dNTP (2 μL/sample) and 1 unit of superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was prepared. 9 μL of mix
per sample was added to the RNA mix previously incubated on ice. The final mix was placed in the PCR machine and incubated at 25°C for 10 min followed by 42°C for 50 min. The enzyme was finally inactivated by heating at 65°C for 10 min.

**RT-PCR**

The forward and reverse PCR primers were designed to be intron spanning, and their sequences and conditions are listed in Table 3-1. To eliminate false positives, no reverse transcriptase, positive, or blank controls were included.

**Western blot**

*Sodium Dodecylsulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE)*

Proteins from cell lysates were separated by SDS-PAGE performed using a mini gel electrophoresis apparatus (Bio-Rad, Hercules, CA). 10% polyacrylamide gels were used to run the protein gel. Lysate samples were diluted in 2X sample Laemmli loading dye (Bio-Rad, Hercules, CA). Composition: 62.5 mM Tris-HCl, pH6.8, 2% SDS, 25% Glycerol, 0.01% Bromophenol Blue containing. 50 µL of β-mercaptoethanol for every 950 µL of 2X sample buffer). The electrophoresis buffer (1X TGS buffer) consisted of 25 mM Tris, 192 mM Glycine, 0.1 % SDS, pH 8.3, (Bio-Rad, Hercules, CA). The protein was extracted using cell lysis buffer composed of 150 mM sodium chloride, 1mM EDTA, 0.5% NP-40, 50 mM Tris, pH 7.5 and protease inhibitor cocktail (Roche diagnostics, Indianapolis, IN).Equal volumes of the sample lysate and 2x sample loading dye were mixed and boiled at 95°C for 5 min.
Precision plus protein ladder (Bio-Rad, Hercules, CA) was used as a standard. The electrodes of the apparatus were connected to the power pack (Bio-Rad, Hercules, CA) and run at 80 V for 1-2 hours.

**Western transfer and blotting**

Transfer buffer with 15% methanol (100 mL 1x TGS buffer, 150 mL methanol, and 750 mL ddH2O) was used to transfer the proteins onto the polyvinylidene fluoride (PVDF) membrane. A transfer sandwich was prepared by encasing the gel and nitrocellulose membrane between the filter paper and sponge (sponge – filter paper – gel – PVDF membrane – filter paper – sponge). Then, the transfer sandwich was placed in the electrophoresis tank. The apparatus was run at 80 volts for 1.5 hours. The membrane was washed in 1x TBST by combining 100 mL 10x TBS, 1 mL Tween-20, and 899 mL ddH2O. The membrane was then blocked with blocking buffer using TBST with 5% non-fat dry milk (50 mL TBST + 2.5 g dry milk) for 1 hour at room temperature or at 4°C overnight with gentle rotation. The primary antibody was diluted (rabbit polyclonal anti-HA (1:5000) or anti-cre antibody (1:1000; Novus Biologicals, Littleton, CO) in 10 mL of blocking buffer with 5% non-fat dry milk and incubated at room temperature for 1 hour or at 4°C overnight with gentle rotation. The membrane was washed with approximately 30 mL 1x TBST buffer for 15 min followed by 2 washes for 10 min each. The membrane was then incubated with the anti-rabbit secondary antibody (Amersham Biosciences, Piscataway, NJ) with HRP conjugation (1:10,000) in blocking buffer with 5% non-fat dry milk and incubated at room temperature for 0.5-1 hour with gentle rotation. The membrane was finally washed with 30 mL 1x
TBST buffer for 15 min, followed by 2 washes for 10 min each. The membrane was dried by blotting the corner on a paper towel for 5 seconds. 1mL each of western blotting detection ECL reagents 1 and 2 (Amersham Biosciences, Piscataway, NJ) was mixed. The membrane was placed on parafilm or polyvinyl wrap and covered evenly with western blotting reagent mixture and incubated at room temperature for 1-2 min. The x-ray film was developed in the dark room by exposing it for varying times in order to visualize the bands of interest.

**Diet-induced obesity model:**

6-week old mice (Wild type, *Pomc-cre*+/null, *Sad*-A knockout mice and *Sad*-A<sup>flx/flx</sup> mice were placed either on *ad libitum* high-fat diet (Rodent Purified Diet w/60% Energy From Fat – D12492, Research Diets, New Brunswick, NJ) or regular chow (2018 Global Rodent Diet, Harlan Teklad, Indianapolis, IN) for 14 weeks. Body weights were monitored bi-weekly.

**Metabolic cages**

The TSE LabMaster System (TSE Systems, Chesterfield, MO) was used to measure the metabolic performance, animal activity as well as the drinking and feeding behavior. The LabMaster System has calorimetric components that measure the oxygen consumption, carbon dioxide consumption, respiratory exchange rate (RER) and heat. The system also has the sensor frames to measure the activity. The sensor frames consisting of infrared emitter and infrared receivers are supplied with the infrared photo-
beams in the X, Y and Z level intersecting in the animal cage. The X and Y measure the animal movements in 2-dimensional horizontal plane where as the Z sensor detects the vertical activity. The sensors records animal movements based on the interrupted light beams. The feeding and drinking patterns are measured by sensitive sensors located in the feeder and nipple respectively. The metabolic cages were calibrated with various percentages of oxygen and carbon dioxide gases. Various oxygen concentrations at 19.99%, 20.51%, and 20.89% were used for calibration. Similarly, various carbon dioxide concentrations at 0.050%, 0.483%, and 0.9526% were used for calibration. To access the metabolic parameters, the mice (n=4) were singly housed in a TSE LabMaster System metabolic cages and acclimatized for 48 h. The body mass and lean body mass were measured using spectroscopy before placing the mice in the metabolic cages. The metabolic parameters were monitored for 72 hrs. The body mass and lean body mass were re-measured using spectroscopy soon after removing the mice from the metabolic cages.

**Spectroscopy**

Body fat and lean body mass were measured using a QNMR system (Bruker Corp, Billerica, MA).

**Oral glucose tolerance test**

The Sad-A knockout mice and Sad-A<sup>flox/flox</sup> mice (n = 4 for each group) were fasted overnight for 12 hours. To prepare glucose solution, 1 gram of dextrose (Fisher Scientific) was dissolved in 10 mL of distilled water to make
100 mg/mL solution. After 12 hours of fasting, a fasting glucose level was obtained using glucometer. Tail tip was pricked with needle to draw drop of blood. The mice were weighed. The mice were gavaged with 2 mg/g body weight glucose solution. Blood glucose values were obtained at 5, 15 30, 60, and 120 min.

**Insulin tolerance test (ITT)**

Sad-A knockout mice and Sad-A$^{floxflox}$ mice were fasted for 6 hours. Insulin (0.75 mU/g bd wt) was administered intraperitoneally and blood glucose was measured at 0, 15, 30, 60, 90 and 120 min after injection (n=4).

**Statistical Analysis**

Statistical significance was analyzed by independent sample t-test and two-way analysis of variance (ANOVA) using a GraphPad Prsim Software Version 5.0 (Sandiego, CA) and requiring a $P$ value <0.05 for the data to be considered statistically significant.
RESULTS

Breeding and genotyping

Pomc-cre\(^{+/null}\) and Sad-A\(^{\text{flox}/-}\) mice were bred and selected for Pomc-cre\(^{+/null}\); Sad-A\(^{\text{flox}/+}\). The Pomc-cre\(^{\text{null}/null}\); Sad-A\(^{\text{flox}/+}\) were bred together to select for Pomc-cre\(^{\text{null}/null}\); Sad-A\(^{\text{flox}/\text{flox}}\). Pomc-cre\(^{+/null}\); Sad-A\(^{\text{flox}/+}\) mice were bred with Pomc-cre\(^{\text{null}/null}\); Sad-A\(^{\text{flox}/\text{flox}}\) to select for Pomc-cre\(^{+/null}\); Sad-A\(^{\text{flox}/\text{flox}}\). Pomc-cre\(^{+/null}\); Sad-A\(^{\text{flox}/\text{flox}}\) mice were bred with Pomc-cre\(^{\text{null}/null}\); Sad-A\(^{\text{flox}/\text{flox}}\) to select for Pomc-cre\(^{+/null}\) x Sad-A\(^{\text{flox}/\text{flox}}\) (Sad-A knockout).

The genotyping analysis for detection of Cre allele using PCR method yielded a PCR product of ~100 bp. The internal positive control primers yielded a PCR product of 324 bp (Fig. 1A). PCR analysis of Sad-A\(^{\text{flox}/+}\) allele yielded a product 360 bp for flox allele while the wild type allele yielded a product of 300 bp (Fig. 1B).
Figure 1. Genotyping Cre and flox alleles. (A) PCR analysis with Cre specific primers yielded a 100 bp product (B) PCR analysis with Sad-A-lox specific primers yielded a 360 bp transgene product or a 300 bp wild type product. Lanes 1-16 demonstrates PCR products obtained from various samples of genomic DNA.

Determination of Sad-A gene knockout in Pomc neurons

The analysis of gene expression of cre was determined using western blotting and RT-PCR. In order to test the sensitivity and specificity of the anti-cre antibody, protein extracts from a Hela cell line expressing cre with HA tag was run on polyacrylamide gel. Immunoblotting with anti-HA antibody yielded a 37 kDa band with a strong signal (Fig. 2A, left panel) while immunoblotting with anti-cre antibody did not yield any band (Fig. 2A, right panel). This demonstrated the poor sensitivity of the anti-cre antibody.

Next, the expression of cre gene was analyzed at mRNA transcript level. For this, total RNA was isolated from hypothalamus of Pomc-cre+/null mice and quantified. 2 µg of RNA was used to convert to cDNA. PCR analysis using cre specific primers yielded a 100 bp product from cDNA samples derived from Pomc-cre+/null mice while no bands were observed in samples from wild type mice. Genomic DNA samples from Pomc-cre+/null mice was used as positive control.
Figure 2. Analysis of expression of Cre gene. (A) Western blot analysis of Cre expression in a cell line over expressing cre with HA tag using anti-HA antibody (left panel) and anti-cre antibody (right panel). (B) RT-PCR analysis of cre expression using cre specific primers. Lanes (1-7). Lane 1: cDNA from hypothalamus of Pomc-cre<sup>+/null</sup> mice 1, Lane 2: cDNA from hypothalamus of Pomc-cre<sup>+/null</sup> mice 2, Lane 3: Genomic DNA, Lane 4: cDNA from hypothalamus of wild type mice, Lane 5: NO RT control for Pomc-cre<sup>+/null</sup> mice 1, Lane 6: No RT control for Pomc-cre<sup>+/null</sup> mice 2, Lane 7: Water.

Analysis of body weight of mice fed high-fat diet

In order to determine if Sad-A gene plays a role in energy homeostasis, 6-week old mice (Wild type, Pomc-cre<sup>+/null</sup>, Sad-A knockout) and Sad-A<sup>lox/lox</sup> were fed either regular chow or a high-fat diet for 14 weeks. Body weights were monitored bi-weekly. For the mice fed high-fat diet, the average
body weights of wild type, *Pomc-cre<sup>+/null</sup>*, *Sad-A* knockout and *Sad-A<sup>flox/flox</sup>* male mice were 49.8 ± 0.98, 52.16 ± 0.5, 45.5 ± 3.09, 47.8 ± 3.0 grams (g) respectively (Fig. 3A). There was no statistically significant difference in the body weights among the groups. Similarly the wild type, *Pomc-cre<sup>+/null</sup>* , *Sad-A* knockout and *Sad-A<sup>flox/flox</sup>* female mice measured an average body weights of 33.8 ± 2.65, 32.5 ± 0.86, 38 ± 3.3, 37.8 ± 2.15 g respectively with no significant difference among the groups (Fig. 3A).

*Sad-A* knockout mice were also fed high-fat diet or regular chow for 14 weeks, and the weights were monitored biweekly. The average body weight of *Sad-A* knockout male mice fed regular chow or high-fat diet were 32.4 ± 1.15 g and 45.5 ± 3.09 g respectively while female mice fed regular chow or high-fat diet were 26.66 ± 1.21 g and 38.0 ± 3.32 g respectively (Fig. 3B). The male and female *Sad-A* knockout mice fed high-fat diet were 24% and 20% higher respectively compared to the group fed regular chow indicating a significant difference between mice fed on high-fat vs regular chow.

A second trial with higher number of *Sad-A* knockout and *Sad-A<sup>flox/flox</sup>* (n=17) mice fed high-fat diet for 14 weeks measured an average weights of 36.52 ± 2.27 and 38.05 ± 2.62 grams respectively. While the female *Sad-A* knockout mice and *Sad-A<sup>flox/flox</sup>* female mice measured an average weight of 28.70 ± 3.26, 26.70 ± 3.12 grams respectively (Fig. 3C). There was no significant difference in body weights between the two groups.
A

Males on HF diet

- Sad-A KO
- Sad-A^{flox/flox}
- Wild type
- Poma-cre^{+/null}

Body wt (g)

Time in weeks on HF diet

Females on HF diet

- Sad-A KO
- Sad-A^{flox/flox}
- Wild type
- Poma-cre^{+/null}

Body wt (g)

Time in weeks on HF diet
B

Males Sad-A KO

- HF diet
- Regular chow

Females Sad-A KO

- HF diet
- Regular chow

Body wt (g)

Time in weeks
Figure 3. Comparative analysis of body weights (A) Body weights of mice fed high-fat diet belonging to all four groups (Wild type, Pomc-cre+/null, Sad-A knockout mice and Sad-A flox/flox) mice (n=6). (B) Sad-A knockout mice fed high-fat diet or regular chow. (C) Sad-A knockout mice and Sad-A flox/flox mice on high-fat diet on trial 2 (n=17). * P < 0.05; ** P< 0.01.
Average basal blood glucose levels between the groups

In order to determine the effect of Sad-A gene on basal blood glucose levels after feeding with high-fat diet, random blood glucose levels were measured on Sad-A knockout mice and Sad-A\(^{\text{flox/flox}}\) fed high-fat diet for 10 weeks. The average random blood glucose levels of Sad-A knockout mice and Sad-A\(^{\text{flox/flox}}\) was 154 ± 31.23 and 162.87 ± 30.35 respectively with no significant difference between the groups.

**Figure 4.** Blood glucose levels. Analysis of average blood glucose levels in Sad-A knockout mice and Sad-A\(^{\text{flox/flox}}\) fed high-fat diet.

Oral glucose tolerance test

In order to determine the effect of Sad-A gene knockout on the ability to respond to elevated blood glucose, the oral glucose tolerance test was compared between Sad-A knockout and Sad-A\(^{\text{flox/flox}}\) mice fed high-fat diet for
14 weeks. The *Sad-A* knockout *mice* and *Sad-A*\(^{flox/flox}\) mice \((n = 4\) for each group) were fasted overnight for 12 hours. The mice were gavaged with 2mg/g body weight glucose solution. Glucose levels were measured at 0, 15, 30, 60, 90 and 120 min post gavage. Comparative analysis between *Sad-A* knockout mice and *Sad-A*\(^{flox/flox}\) mice female mice did not show significant difference in blood glucose handling ability measured at 0, 15, 30, 60, 90 and 120 min post gavage (Fig. 5A).

In order to determine the effect of high-fat diet on the *Sad-A* knockout mice on glucose handling ability, OGTT was performed on *Sad-A* knockout mice fed either high-fat diet or regular chow. The *Sad-A* knockout male and female mice \((n = 4\) for each group) were fasted overnight for 12 hours. The mice were gavaged with 2mg/g body weight glucose solution. Glucose levels were measured at 0, 15, 30, 60, 90 and 120 min post gavage. There was decreased ability of male and female *Sad-A* knockout mice fed high-fat diet to handle the blood glucose compared to the mice fed regular chow (Fig. 5B).
Figure 5. Oral glucose tolerance test (OGTT). (A) OGTT between Pomc-cre<sup>+/null</sup>, Sad-A<sup>flox/flox</sup> mice and Sad-A<sup>flox/flox</sup> mice fed high-fat diet (n=4). (B) OGTT of Sad-A knockout mice fed either high-fat diet or regular chow (n=4). * P < 0.05; ** P < 0.01.
**Insulin tolerance test:**

In order to determine the effect of *Sad-A* gene on insulin tolerance, insulin tolerance test was performed comparing the *Sad-A* knockout mice with *Sad-A*<sup>flx/flx</sup> mice fed high-fat diet for 14 weeks. The *Sad-A* knockout mice and *Sad-A*<sup>flx/flx</sup> mice (*n* = 4 for each group) were fasted for 6 hours. The mice were injected i.p with 0.75U/kg body weight glucose solution. Glucose levels were measured at 0, 30, 60, 90 and 120 min post gavage. Comparative analysis was done between *Sad-A* knockout mice and *Sad-A*<sup>flx/flx</sup> mice. Both male and female mice did not show a significant difference in insulin tolerance measured at 0, 30, 60, 90 and 120 min (Fig. 6).

![Insulin tolerance test](image)

**Figure 6.** Insulin tolerance test (ITT) between *Sad-A* knockout mice and *Sad-A*<sup>flx/flx</sup> mice fed high-fat diet (*n*=4).
Body composition of mice

The mice fed high-fat diet for 14 weeks were placed in nuclear a magnetic spectrometer to measure body composition. The male Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice have fat composition of 29.05 ± 0.86% and 27.32 ± 0.61% respectively while the lean composition of male Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice was 54.52 ± 1.94% and 53.37 ± 0.50% respectively. The female Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice have fat composition of 25.9 ± 4.11% and 30.4 ± 2.57% respectively while the lean composition of female Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice have 55.6 ± 4.27% and 51.7 ± 2.78% respectively (Fig. 7A). There was no significant difference in body composition between the Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice fed high-fat diet. In order to determine the effect of high-fat diet on body composition, the wild type mice fed either high-fat diet or regular chow was measured for fat and lean percentage. As expected, the wild type mice fed high-fat had significantly higher body fat composition of 67.47 ± 1.29% vs 52.52 ± 1.76% and lower lean composition of 12.92 ± 1.04% vs 28.12 ± 1.39% (Fig. 7B).
A

Body composition males on HF diet- Males

![Graph showing body composition of males on HF diet.]

B

Body composition on HF diet- Females

![Graph showing body composition of females on HF diet.]

B

Body composition in WT mice

![Graph showing body composition in WT mice.]

Figure 7. Body composition. (A) Body composition of male and female Sad-A knockout mice and Sad-A$^{flox/flox}$ mice fed high-fat diet (n=4). (B) Body weight composition of wild type mice fed either high-fat diet or regular chow (n=4). * P < 0.05; ** P< 0.01.

Measurement of metabolic parameters:

To determine the effect of Sad-A gene knockout in the Pomc-cre neurons on energy metabolism, Sad-A knockout mice and Sad-A$^{flox/flox}$ mice (n=4) were placed in metabolic cages and food intake, water intake, physical activity, energy expenditure and respiratory exchange ratio was measured over 24 h. Food intake: The male Sad-A knockout mice and Sad-A$^{flox/flox}$ mice (n=4) had food intake of 1.47 ± 0.66 g/d and 2.20 ± 0.56 g/d respectively. The female Sad-A knockout mice and Sad-A$^{flox/flox}$ mice had an average food intake of 2.48 ± 0.42 g/d and 2.0 ± 0.16 g/d respectively (Fig. 8A). There was no significant difference in amount of food intake between the Sad-A knockout and Sad-A$^{flox/flox}$ mice both in males and females. In order to determine the effect of high-fat diet on energy metabolism, the food intake was measured in wild type male mice fed high-diet or regular chow for 14 weeks. After 14 days on high-fat diet, the mice were moved to metabolic cages and fed regular chow after 48 h acclimation on regular chow. The high-fat fed mice group had an average food intake of 1.60 ± 0.42 g/day while the regular chow mice group had an average food intake of 3.27 ± 0.36 g/day, a total of 50% higher compared to high-fat group (Fig. 8B).

Water intake: The male Sad-A knockout mice and Sad-A$^{flox/flox}$ mice (n=4) had water intake of 3.24 ± 0.13 mL/24 h and 3.04 ± 0.17 respectively.
The female *Sad-A* knockout mice and *Sad-A*\textsuperscript{flox/flox} mice had an average water intake of $2.86 \pm 0.65$ and $2.09 \pm 0.22$ mL/h respectively (Fig. 8C). There was no significant difference in food intake between the *Sad-A* knockout mice and *Sad-A*\textsuperscript{flox/flox} mice both in males and females.
Figure 8. Metabolic cage studies for measurement of food and water intake (A) Food intake by Sad-A knockout mice and Sad-A$^{flox/flox}$ mice
(n=4). (B) Food intake by wild type mice fed either high-fat diet or regular chow (n=4). (C) Water intake by Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice (n=4). * P < 0.05; ** P< 0.01.

**Physical activity:** The male Sad-A knockout mice exhibited an average physical activity of 68472 ± 8168.31 counts/day while male Sad-A\textsuperscript{flox/flox} mice exhibited an average physical activity of 56359 ± 10115.77 counts/day. The female Sad-A knockout mice exhibited an average physical activity of 49901.5 ± 7923.23 counts/day while female Sad-A\textsuperscript{flox/flox} mice exhibited an average physical activity of 69137.25 ± 21415.86 counts/day (Fig. 9A). There was no significant difference in physical activity between the Sad-A knockout mice and Sad-A\textsuperscript{flox/flox} mice both in males and females.

In order to determine the effect of high-fat diet on energy metabolism, physical activity was measured in wild type male mice fed high-diet or regular chow for 14 weeks. After 14 days on high-fat diet, the mice were moved to metabolic cages and fed regular chow after 48 h acclimation on regular chow. The high-fat fed mice group had an average physical activity measured 32247.75 ± 4608.34 counts per day while the regular chow mice group had an average physical activity measured 57799.0 ± 1500.91, a total of 56% higher compared to high-fat group (Fig. 9B).
Figure 9. Metabolic cage studies for measurement of physical activity (A) Physical activity of Sad-A knockout mice and Sad-A<sup>flox/flox</sup> mice measured in counts per day (n=4). (B) Physical activity of wild type mice fed either high-fat diet or regular chow measured in counts per day (n=4). ** P< 0.01.
Energy expenditure: To determine the effect of Sad-A gene knockout in the Pomc-cre neurons on energy metabolism, Sad-A knockout mice and Sad-A^{lox/lox} mice (n=4) were placed in metabolic cages and energy expenditure was measured over 24 h. The male Sad-A knockout mice exhibited a energy expenditure of 15.29 ± 2.05 kcal/kg/h while male Sad-A^{lox/lox} mice exhibited an expenditure of 14.17 ± 2.13 kcal/kg/h. The female Sad-A knockout mice exhibited an energy expenditure of 11.62 ± 1.04 kcal/kg/h while male Sad-A^{lox/lox} mice exhibited an expenditure of 11.20 ± 0.56 kcal/kg/h (Fig. 10A). There was no significant difference in energy expenditure between the Sad-A knockout and Sad-A^{lox/lox} mice both in males and females.

In order to determine the effect of high-fat diet on energy metabolism, energy expenditure was measured in wild type male mice fed high-fat diet or regular chow for 14 weeks. After 14 days on high-fat diet, the mice were moved to metabolic cages and fed regular chow after 48 h acclimation on regular chow. The high-fat fed mice group had an average energy expenditure of 8.62 ± 0.81 kcal/h/day while the regular chow mice group had an average energy expenditure measured 10.46 ± 0.57, a total of 16% higher compared to high-fat group (Fig. 10B).
Figure 10. Energy expenditure (A) Sad-A knockout mice and SAD-^flox/flox^ mice measured in kcal/h/kg (n=4) or (B) Energy expenditure of wild type mice fed either high-fat diet or regular chow measured in kcal/h/kg (n=4). * P < 0.05.
**Respiratory exchange ratio:** The male and female Sad-A knockout mice exhibited an average respiratory exchange ratio measured over 24 h of 0.79 ± 0.02 and 0.72 ± 0.03 respectively. While the male and female Sad-A\textsuperscript{flox/flox} mice exhibited an average respiratory exchange ratio measured over 24 h of 0.78 ± 0.03 and 0.70 ± 0.02. There was no significant difference in RER between the Sad-A knockout and Sad-A\textsuperscript{flox/flox} mice both in males and females (Fig. 11A and 11B).
Figure 11. Respiratory exchange ratio (A) Male Sad-A knockout mice and Sad-A^{flox/flox} mice and (B) Female Sad-A knockout mice and Sad-A^{flox/flox} mice measured in light and dark phases (n=4). * P< 0.05.

Oxygen Consumption: The male Sad-A knockout mice and Sad-A^{flox/flox} mice exhibited an average oxygen consumption of 2688.66 ± 246.22 and 2522.95 ± 243.08 mL/kg/h respectively while female Sad-A knockout mice exhibited an average oxygen consumption of 2512.67 ± 157.60 and 2688.66 ± 246.22 respectively (Fig. 12A). There was no significant difference in VO_{2}
between the Sad-A knockout mice and *Sad-A* \(^{\text{flox/flox}}\) mice both in males and females.

In order to determine the effect of high-fat diet on energy metabolism, energy expenditure was measured in wild type male mice fed high diet or regular chow for 14 weeks. After 14 days on high-fat diet, the mice were moved to metabolic cages and fed regular chow after 48 h acclimation on regular chow. The high-fat fed mice group had an average VO\(_2\) measured 1821.41 ± 163.36 mL/kg/h while the regular chow mice group had an average VO\(_2\) measured 2220.01 ± 170.67 mL/kg/h, a total of 20% higher compared to high-fat group (Fig. 12B).
Figure 12. Oxygen consumption (A) Sad-A knockout mice and Sad-A$_{flox/flo}$ mice measured in mL/kg/h (n=4) or (B) Wild type mice fed either high-fat diet or regular chow measured in kcal/kg/h (n=4). * p<0.01, ** P< 0.01.
DISCUSSION

SAD-A is a protein kinase related to AMPK family of kinase and its expression is limited to the pancreas and brain. Previous studies have shown that SAD-A is involved in neuronal polarization and neuronal cell differentiation. Previous unpublished studies have shown the role of SAD-A in glucose-stimulated insulin release in pancreatic beta cells. However, its role in the brain remains unknown. The whole body knockout of Sad-A demonstrated a phenotype of alternation in glucose and energy metabolism. In order to delineate the precise role of SAD-A in glucose and energy metabolism, Sad-A gene was knocked out in Pomc neurons of hypothalamus using cre-lox recombination technology. The generation of Sad-A knockout mice was confirmed by PCR based genotyping. Phenotypically, there were no significant differences in metabolic parameters between Sad-A knockout mice and Sad-A^{flox/flox} mice as measured for glucose handling ability and energy metabolism. There were technical challenges that hindered the proof of the hypothesis that SAD-A kinase is required for energy and glucose homeostasis.

The demonstration of cre-mediated recombination for Sad-A gene knockout has been a technical challenge at several fronts. SAD-A is expressed within the hypothalamus as well as several parts of the brain. The global expression of SAD-A in brain neurons made it extremely difficult to quantify the gene knockout in Pomc neurons which comprise a small population either using real time PCR or western blot. The lack of good
antibody with high sensitivity and specificity has severely hampered the
demonstration of gene knockout. Alternative approaches to demonstrate cre-
mediated recombination includes a breeding strategy with LacZ or GFP
reporter mice (30). Besides determining the cre-lox recombination, the
efficiency of recombination of Sad-A knockout in pomc neurons is a key
element for significant knockout of Sad-A gene. Despite high recombination
efficiency, the demonstration of phenotype is highly essential (31). The
phenotypic difference in Sad-A knockout mice depends on how important the
role of SAD-A is in energy metabolism. Are there any homologues playing
similar role or other genes in the same or different neuronal circuits that play
a compensatory role in the hypothalamus? (32).

Despite Sad-A knockout, are other neuronal circuits playing a
compensatory role in maintaining energy homeostasis to counteract the loss
of SAD-A function in pomc neurons? The phenotype could also vary with the
strain background. The mice used for Sad-A knockout have a mixed
background, any subtle phenotype could be masked due to lack of
homogeneity (33). Backcrossing to wild type C57BL/6 for 4-5 generations
could resolve this issue.

Future experiments designed to solve the above questions may lead to
conclusive evidence of the role of Sad-A gene in Pomc neurons in glucose
sensing and energy metabolism.
REFERENCES


