이학석사 학위논문

Nicotinamide Adenine Dinucleotide 치료의 시상하부 에너지대사 조절 효과와 작용기전 연구

Effects and Action Mechanisms of Nicotinamide Adenine Dinucleotide Supplement on Hypothalamic Regulation of Energy Metabolism

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ABSTRACT

Background Nicotinamide adenine dinucleotide (NAD)-dependent deacetylase SIRT1 is an important regulator of hypothalamic neuronal function. Thus, adequate hypothalamic NAD content is critical for maintaining normal energy homeostasis.

Methods I investigated whether NAD supplement increases hypothalamic neuron NAD levels and affects energy metabolism in mice. I also explored mechanisms by which exogenous NAD enters hypothalamic neurons and exerts central metabolic effects *in vitro* and *in vivo*.

Results I found that central and peripheral NAD administration suppressed fasting-induced hyperphagia and Iight gain in mice. Extracellular NAD was imported into N1 hypothalamic neuronal cells in a connexin 43-dependent, and CD73-independent manner. Consistent with the *in vitro* data, inhibition of hypothalamic connexin 43 blocked hypothalamic neuronal NAD uptake as well as NAD-induced anorexia. Exogenous NAD effectively suppressed NPY and AgRP transcriptional activity, and this effect was mediated by SIRT1 and FOXO1.

Conclusions Hypothalamic neurons uptake exogenous NAD via a connexin 43-dependent mechanism, which leads to increased NAD content in hypothalamic neurons. Therefore, NAD supplementation is a potential therapeutic method for metabolic disorders accompanied by hypothalamic NAD depletion.

ABBREVATIONS

APCP (adenosine 5′-diphosphate), **AgRP** (Agouti-related protein), **ARC** (arcuate nucleus), **CBN** (carbenoloxolone), **CLAMS** (comprehensive lab monitoring system). **CTA** (conditioned taste aversion), **Cx43** (connexin 43), **CMP** (cytidine 5′-monophosphate), **EE** (energy expenditure), **FITC-NAD** (fluorescein isothiocyanate-conjugated NAD), **GAPDH** (glyceraldehyde-3-phosphate dehydrogenase), **HPLC** (high performance liquid chromatography), **ICV** (intracerebroventricular), **IP** (intraperitoneal), **MBH** (mediobasal hypothalamus), **NPY** (neuropeptide Y), **NAD** (nicotinamide adenine dinucleotide), **NMN** (nicotinamide mononucleotide), Nmnat (nicotinamide mononucleotide adenylyltransferase), **NR** (nicotinamide ribose), **NRK** (nicotinamide ribose kinase), **Nampt** (nicotinamide phosphoribosyltransferase), **PARP** (poly(ADP-ribose) polymerase), **PPARγ** (proliferator-activated receptor-γ), **PGC-1α** (PPARγ coactivator-1α), **POMC** (proopiomelanocortin), **siRNA** (small interfering RNA)

Keywords: hypothalamus, obesity, energy homeostasis, connexin

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INTRODUCTION

1. Obesity: the prevalence and causes.

Obesity has become the most common metabolic disorder in Isternized countries. About one third of adult Americans are obese and a half of American is overIight. The World Health Organization (WHO) reported that at least one billion adults are obese, and this value is expected to continuously increase in the future. Obesity is closely associated with an increased risk of type 2 diabetes (T2D), arteriosclerosis, and coronary heart disease [1, 2]. Obesity results from either or both increased energy intake and decreased energy expenditure which leads to a massive increase in fat storage to a harmful degree. The development of obesity is affected by environmental, metabolic and behavioral factors. Although there have been many efforts to treat obesity, effective and safe anti-obesity treatmet are yet to be discovered.

2. Hypothalamic regulation of energy homeostasis.

The hypothalamus is a critical brain area that regulates energy homeostasis [3-5]. For this purpose, hypothalamus senses the energy status of our body

through hormonal and nutritional metabolic signals from peripheral organs, and coordinates these information and modulates the metabolic rate and feeding behavior to maintain energy balance [6].

The hypothalamus is composed of the hypothalamic nuclei including the arcuate nucleus (ARC), the paraventricular nucleus (PVN), the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN), and the ventromedial nucleus (VMN). Among those, the ARC is the most important area in terms of sensing systemic metabolic conditions. It is located adjacent to the third cerebroventricle and the median eminence (ME), a circumventricular organ where the blood-brain barrier (BBB) allows the free entry of nutrient and hormone from circulation. Two major populations of neurons in ARC play a key role in the regulation of energy balance; i) one population expresses the orexigenic neuropeptide Y (NPY) and *agouti*-related peptide (AgRP); ii) the other population of neurons expresses proopiomelanocortin (POMC) and the cocaine- and amphetamine-regulated transcript (CART). NPY is a representative neuropeptide that causes a positive energy balance by suppressing energy expenditure and increasing food intake. POMC is processed to produce α-melanocyte stimulating factor (α-MSH), an

agonist for the melanocortin receptors MC3R and MC4R that promotes locomotor activity, and energy expenditure but inhibits food intake. AgRP is an inverse agonist for MC3R and MC4R opposing central melanocortin action. Together with downstream target neurons expressing the MC3R and MC4R, both populations constitute the central melanocortin system. This neuronal circuit is known to be crucial for sensing and integrating a number of peripheral signals allow a precise control of food intake and energy metabolis.

3. Cellular NAD biology

Nicotinamide adenine dinucleotide (NAD) is an essential cosubstrate in biochemical reactions catalyzed by the sirtuins, poly (ADP-ribose) polymerase (PARP), and cyclic ADP-ribose cyclase/CD38 [7, 8]. In mammals, cellular NAD levels depend mainly on its salvage pathway, in which NAD is resynthesized from nicotinamide via nicotinamide mononucleotide (NMN) [9, 10]. Alternatively, NAD can be synthesized *de novo* from tryptophan or synthesized from nicotinamide ribose (NR), a trace nutrient in foods. NR is converted to NMN by NR kinase (NRK) and then to NAD by nicotinamide mononucleotide adenylyltransferase [9,

10].

The NAD levels in the liver, skeletal muscle, and white adipose tissues are altered by nutritional conditions. Caloric restriction or fasting increases tissue NAD levels, whereas intake of a high-fat diet (HFD) decreases NAD levels in these metabolic organs [11-13]. Chronic consumption of a high-fat, high-sucrose diet also reduces hypothalamic neuronal NAD content and SIRT1 activity [14].

4. Regulation of NAD-dependent deacethylase sirtuin

The NAD-dependent protein deacetylase sirtuin 1 (SIRT1) in peripheral metabolic organs has been suggested as a key regulator of cellular metabolic processes. SIRT1 deacetylates important metabolic regulators, such as the foxhead box protein O1 (Foxo1), peroxisome proliferator-activated receptor gamma (PPAR-γ), and PPARγ coactivator 1-alpha (PGC-1α), thereby regulating their activities or degradation. Like in the peripheral organs, SIRT1 in the hypothalamic neurons plays an important role in the regulation of the energy balance and normal circadian behaviors [15-18]. Therefore, reduced NAD levels and SIRT1 activity in both peripheral metabolic organs and the hypothalamus

could lead to metabolic dysregulation and, in turn, contribute to the development of metabolic diseases.

Hypothalamus SIRT1 appears to regulate Feeding behavior and food intake, but the exact mechanism currently is unknown. Intracerebroventricular injection of EX527, a specific inhibitor of SIRT1, or siRNA-mediated knockdown of Sirt1 in the Arcuate nucleus inhibits feeding behavior in rodents through a decrease in AgRP and increase in POMC protein [19]. In contrast, intracerebral administration of adenovirus expressing Sirt1 in the midgut hypothalamus has been reported to inhibit food intake [20].

Various experimental results suggest that hypothalamic dysfunction is one of the major causes of abnormal glucose and lipid metabolism in type 2 diabetes and dietary obesity [21, 22]. The hypothalamic SIRT1 is known to prevent the pathology of obesity induced by diet. Deletion of SIRT1 in POMC neurons reduces weight gain and energy expenditure by reducing sympathetic activity and results in a BAT-like modification of WAT in superovulation under HFD [17]. BAT-like remodeling of perigonadal WAT increases mitochondrial content and UCP-1 expression under HFD, resulting in increased energy expenditure against obesity and insulin

resistance [23]. These results suggest that SIRT1 in POMC neurons is required for normal autonomic adaptation to diet-induced obesity and possibly insulin resistance.

5. Gap junction in brain

Intercellular communication in multicellular organisms is important for maintaining homeostasis in tissues and organs, which is mediated through an intercellular channel called gap junction [24-26]. The gap junction channel, encoded by a family of genes called "connexins" [27], links the cytoplasm of two cells and provides a means for the exchange ion, the second messenger [28, 29]. Connexin is a tetramembrane-spanning protein with N-terminal and C-terminal in the cytoplasm [30]. Connexin is transported to the plasma membrane and each connexins is assembled into a hexameric structure called connexons [31, 32]. Among the 19 connexin genes encoding gap junction proteins in mice, Connexin 43 (also known as GJA1) is the most studied and is expressed in a variety of places [33].

Recent studies support a new notion that connexin plays an additional role independent of channel function beyond cellular communication, That is, the

interaction between gene transcription control, cell growth and apoptosis regulatory factors and mechanical roles [34-36].

AIM OF STUDY

Given the important roles of NAD and SIRT1 in normal cellular function and physiology, trials of the treatment of tissue NAD depletion in obese and aged mice have been conducted by providing NAD precursors nicotinamide mononucleotide (NMN) and nicotinamide ribose (NR). These trials proved the beneficial effects of NMN and NR supplementation. Chronic treatment of NMN and NR inhibited HFD- and age-induced Iight gain in mice and humans. As extracellular NAD can be effectively imported into cells and rescue cellular NAD depletion, I investigated the effect of NAD supplementation on food intake and body weight. I also investigated the molecular mechanisms of central metaboli effects of NAD supplement and molecules that mediates hypothalamic NAD uptake.

METHODS

Materials

NAD, carbenoloxolone (CBN), cytidine 5′-monophosphate (CMP), adenosine 5′-diphosphate (APCP), and sirtinol Ire purchased from Sigma-Aldrich (St. Louis, MO, USA). FK866 was obtained from Enzo Life Sciences (Farmingdale, NY, USA) and EX527 from Tocris (Bristol, UK). Fluorescein isothiocyanate- conjugated NAD (FITC-NAD) was obtained from Trevigen (Gaithersburg, MD, USA).

Cell culture

N1 hypothalamic neuron cells Ire maintained in DMEM supplemented with 10% fetal bovine serum and 5% penicillin/streptomycin. SH-SY5Y and AtT-20 cells Ire cultured in DMEM containing 10% fetal calf serum.

Animals

Male C57BL/6N mice Ire fed a standard chow diet (CD; 12.5% of calorie from fat; Cargill Agri Purina, Inc., #38057, Seongnam, Korea) *ad libitum* unless mentioned otherwise. Mice Ire housed under controlled temperature (22 ± 1 °C) with 12 h

light-dark cycle, with light from 8 a.m. to 8 p.m. All procedures Ire approved by the Institutional Animal Care and Use Committee of the Asan Institute for Life Sciences (Seoul, Korea).

Cellular NAD uptake

To determine cellular NAD uptake, N1 hypothalamic neuronal cells were plated in 100 mm dishes and grown to approximately 90% confluence. Cells were pretreated with CBN (1 μM), CMP (1 and 10 μM), APCP (1 and 10 μM), FK866 (10 nM) or vehicle (PBS or DMSO) for 1 h and then treated with NAD (10 nM) for 30 min. Cells were washed with PBS twice and then lysed using 100 μl of 1M $HClO_4$ and neutralized by adding 33 μl of 3 M K_2CO_2. After centrifuging (4 °C, 13,000 *g*) for 15 min, 25 μl of supernatant was mixed with 175 μl of 50 mM K_2PO_4/$KHPO_4$ (pH 7.0) and 100 μl of the mixture was loaded onto the High performance liquid chromatography (HPLC) column (Apollo C18, 5 μm, 250 x 4.6 mm, Alltech, Deerfield, IL, USA). HPLC was run at a flow of 1 ml/min. NAD was eluted as a sharp peak at 11 min as described previously [13, 37].

FITC-NAD study

To visualize cellular FITC-NAD uptake, cells were pretreated with CBN (1 μM) or vehicle (PBS) for 1 h before FITC-NAD treatment. One hour after FITC-NAD treatment (250 nM), cells were washed with PBS three times to remove FITC-NAD in the medium. FITC fluorescence was examined by live cell imaging system using confocal microscopy (Leica, Itzlar, Germany). In *in vivo* study, FITC-labeled NAD (250 pmol in 2 μl) was administered via ICV-implanted cannulae. One hour after injection, whole brain was obtained to examine hypothalamic uptake of FITC-NAD.

Promoter analysis

Promoter assay of human NPY, AgRP, and mouse POMC was performed using luciferase reporter constructs containing the NPY promoter (pGL3-hNPY-luc; nucleotides –963 to +67), the AGRP promoter (pGL3-hAGRP-luc; nucleotides –1000 to –1), the POMC promoter (pGL3-mPOMC-luc; nucleotides –2301 to +34) as described in i previous study [30]. Forty-eight hours after transfection, cells Were treated with NAD with or without EX527 (200 nM), sirtinol (100 nM), and FK866 (10 nM) for 2 or 4 h. Cells Were lysed and extracted to measure luciferase activity

by a luminometer (PerkinElmer, Monza, Italy) and normalized for β-galactosidase activity.

Intracerebroventricular cannulation and injection

Intracerebroventricular cannulation and injection was performed as previously described [38]. Mice were implanted with permanent 26-gauge stainless steel cannulae into the 3rd ventricle (1.8 mm caudal to the bregma and 5.0 mm ventral to the sagittal sinus) using a stereotaxic surgery. After one week-recovery period, mice Ire handled daily for 1 week to minimize stress responses. Correct positioning of the cannulae was confirmed by positive dipsogenic responses to angiotensin Ⅱ (50 ng). Only the mice with correctly positioned cannulae were included in the data analysis. NAD, CBN, and CMP were dissolved in normal saline whilst EX527 and sirtinol were dissolved in DMSO-saline (1:10 v/v) before injection. All compounds were administered in a volume of 2 μl.

Feeding study

Mice (8–10 Weeks-old males) were individually housed for 1 week before

study commencement to minimize stress. One day before study commencement, the animals were randomly assigned to groups based on their body Iights in order to match the average body Iight of each group. NAD with or without inhibitors were intracerebroventricularly (ICV) or intraperitoneally (IP) injected in the early light phase (9–10 a.m.) to overnight-fasted mice. Control mice received the same volume of vehicle. Food intake was monitored at 1, 2, 4, 8, and 24 h after injection. Body Iight was measured before and 24 h post-injection.

Conditioned taste aversion study

Mice were habituated to a daily 1-h period of access to water. During this hour, two bottles containing unflavored tap water Were placed in each home cage. After seven days, all mice received two bottles containing 0.15% saccharin instead of water. Immediately following this 1-h period of saccharin exposure, the mice received sequential injections of saline (IP)-saline (ICV), lithium chloride (LiCl, 200 mg/kg, IP; Sigma)-saline (ICV), or saline (IP)-NAD (0.7 ng, ICV). For the next three consecutive days, a two-bottle choice test was conducted; in this test, the mice were alloId 1-h access to one bottle of tap water and another bottle of 0.15%

saccharin solution. Preference ratio was calculated as the intake of saccharin solution/total intake of water and saccharin solution.

Energy expenditure study

Energy expenditure (EE) was measured using a comprehensive lab monitoring system (CLAMS) (Columbus Instruments Inc., Columbus, OH, USA). Mice were placed in the CLAMS chambers for 48 h and fasted overnight before measurement. Either saline or NAD at the indicated doses was administered ICV or IP in the early light phase, and EE was monitored for 4–8 hours following NAD injection.

Measurement of hypothalamic NAD contents

Mice were sacrificed by decapitation 1 h after NAD injections. The mediobasal hypothalamus (MBH) was dissected with the lateral border of half way to lateral sulcus and upper border of half way to anterior commissure as previously described [39] . Hypothalamic blocks were snap frozen in liquid nitrogen, and stored at –70 °C before assay. Hypothalami were Iighed immediately after thawing, lysed in 150 μl of 1 M $HClO_4$, and the neutralized by adding 50 μl of 3 M K_2CO_2. Following

centrifugation (4 °C, 13,000 *g*) for 15 min, 20 μl of supernatant was loaded onto the HPLC column as described above. The amounts of NAD Ire normalized by It tissue Iights as described previously.

Determination of mRNA expression

To determine the effect of NAD administration on the mRNA expression of ARC neuropeptides and sirtuins, mouse hypothalamic ARC was collected at 1 h post-ICV NAD injection using the micropunch technique that used a Harvard brain matrix and blunted 20 gauge-metal needles (inner dimeter 0.91 mm). Bilateral ARC was collected from two consecutive brain slices (1 mm thick). In a separate study, RNA was also obtained from N1 cell, mouse liver, and hypothalamus to compare the expression levels of CD73 and connexin 43 (Cx43). The mRNA expression level was determined by real-time PCR analysis (Life Technologies, Carlsbad, CA) using corresponding primers: NPY (5′-atgctaggtaacaagcgactggg-3′ and 5′-ggctggatctcttgccatatctc-3′), AgRP (5′-acaactgcagaccgagca-3′ and 5′-gacgcggagaacgagact-3′) [38], POMC (5′ caggtcctggagtccgac-3′ and 5′-catgaagccaccgtaacg-3′) [38] , SIRT1 (5′-gttctgactggagctggggt-3′ and 5′-

tctgggaggtctgggaagtc-3′), SIRT3 (5′-gcctgaagacagctccaaca-3′ and 5′-gacatccctggtcagccttt-3′), CD73 (5′-aacccctttcctctcaaatcca-3′and 5′-cagggcgatgatcttattcacat-3′) [40] and Cx43 (5′-acagcggttgagtcagcttg-3 and 5′-gagagatggggaaggacttgt-3′) [41]. PCR primer sequences of NPY, SIRT1, and SIRT3 Were retrieved from the online PrimerBank database. Expression of each mRNA was normalized to that of glyceraldehyde-3-phosphate dehydrogenase (GAPDH). For presenting quantitative real-time PCR data, comparative C_T method was used.

siRNA studies

Small interfering RNA (siRNA) specific to murine Cx43 was purchased from Dharmacon (Chicago, IL, USA). Under anesthesia, Cx43 siRNA (0.5 nmol in 500 nl/each injection site) was microinjected bilaterally into the ARC. Nighttime food intake was measured on the second day after siRNA injection following ICV or IP injection of saline or NAD (0.7 ng for ICV or 1 mg/kg for IP study) 1 h before light-off. In the morning of the 3rd post-injection day, mice Were sacrificed to collect hypothalamic block or whole brain 1 h after ICV or IP injection of NAD or ICV injection of FITC-NAD (650 ng). Knockdown was considered successful if MBH Cx43

mRNA or protein expression was decreased by more than 50% of the average Cx43 expression of controls. Successful knockdown was achieved in about 60% of mice treated with Cx43 siRNA. Only the animals with successful knockdown Were included in data analysis. An equal amount of non-targeting, scrambled control siRNA (Dharmacon) was administered to the control group. To deplete Cx43 expression in the N1 cells, cells Were transfected with Cx43 siRNA (160 pmol/6 Ill).

Immunohistochemistry

Mice were perfused with 50 ml saline folloId by 50 ml 4% paraformaldehyde via the left ventricle. Whole brains were collected, fixed with 4% paraformaldehyde for 24 h, and dehydrated in 30% sucrose solution until brains sank to the bottom of the container. I obtained coronal brain slices (25 μm thick) using cryostat (Leica, Itzlar, Germany) and pre-mounted on charged glass slides (MUTO Pure Chemicals Co., Tokyo, Japan) for immunohistochemistry. Hypothalamic slices were permeabilized in 0.5% PBST for 5 min and blocked with 3% BSA at room temperature for 1 h and then reacted with anti-Cx43 antibody (rabbit, 1:1000, Abcam, Cambridge, UK) at 4°C overnight. After washing, slides were incubated with

the Alexa-Flour 555-conjugated secondary antibodies (1:1000, Invitrogen, Carlsbad, CA) at room temperature for 1 h.

Statistical analysis

Data are presented as means ± SEM. Statistical analysis was performed using SPSS software version 22.0 (SPSS, Chicago, IL, USA). Statistical significance among the groups was tested with one-way ANOVA folloId by a *post hoc* Fishers Least Significant Difference test, or an unpawered Student's t-test when appropriate. Significance was defined as $P < 0.05$.

RESULTS

Hypothalamic neurons uptake NAD through gap junction protein connexin 43

I firstly tested if exogenous NAD can affect cellular NAD levels in N1 hypothalamic neuronal cells. Treatment of N1 cells with a specific Nampt inhibitor, FK866 (10 nM for 2 h), reduced intracellular NAD contents by ~25%, and this drop was rescued by extracellular application of NAD (10 nM for 30 min) [Fig. 1], suggesting that extracellular NAD may enter cells to replenish the cellular NAD content.

Intercellular gap junctions, especially Cx43 hemichannels, have been shown to mediate transmembrane NAD trafficking in 3T3 fibroblasts [42]. Notably, higher expression of Cx43 was found in the hypothalamus than in the liver of normal mice [Fig. 2]. Moreover, when N1 cells were pre-treated with gap junction inhibitor CBN, exogenous NAD-induced increase in cellular NAD levels were significantly blunted [Fig. 3]. Consistently, siRNA-mediated Cx43 knockdown prevented the exogenous NAD treatment-induced elevation of cellular NAD levels [Fig. 4], indicating an involvement of gap junctional protein, especially Cx43 in NAD influx to

hypothalamic neurons.

A recent paper has suggested the possibility that NAD is extracellularly degraded to NMN and then to NR by the CD73 ecto-5′-nucleotidase [43], which is then transported into cells via the nucleoside transporters.

To test this possibility, I co-treated N1 cells with NAD and CD73 inhibitors CMP or APCP. In contrast to gap junction inhibitor, treatment of CD73 inhibitors failed to block an increase in cellular NAD levels following exogenous NAD [Fig. 5]. Moreover, CD73 expression was lower in the hypothalamus compared to the liver [Fig. 2], suggesting that CD73 might have a relatively minor role in NAD metabolism in the hypothalamus.

In order to directly visualize cellular uptake of NAD, I treated N1 cells with FITC labeled-NAD (250 nM) in the presence or absence of CBN. In FITC-NAD, FITC is attached to adenine residue of NAD [Fig. 6], therefore, FITC signal is not detected inside cells if NAD is converted to NMN and NR before cellular uptake. Indeed, FITC signal was found in the cytosolic compartment of N1 cells 1 h after treatment which was significantly reduced by co-treatment of CBN [Fig. 6]. These results support the notion of gap junction-mediated NAD influx in hypothalamic neuronal

cells.

Intracerebroventricular and intraperitoneal injection of NAD regulates energy metabolism

I next investigated if central administration of NAD may affect the food intake, body Iight, and energy expenditure. Young C57 mice were injected with NAD (0.14, 0.7, and 1.4 ng) via ICV cannulae following an overnight fast. Notably, injection of NAD (0.7 ng) significantly suppressed fast-induced feeding and Iight gain compared to saline-injection [Fig. 7]. The anorexigenic effect of NAD persisted for 24 h. To eliminate the possibility that NAD-induced anorexia and Iight loss may be due to toxicity and systemic illness, I conducted a conditioned taste aversion (CTA) test. ICV injection of NAD (0.7 ng) did not affect saccharin preference, while intraperitoneal injection of lithium chloride, a well-known CTA inducer, significantly decreased saccharin consumption [Fig. 8]. Therefore, the effect of centrally-administered NAD is not likely to have been due to sickness or illness caused by the agent.

I also examined the effects of ICV-injected NAD on EE, an important

determinant of energy balance. For this, NAD (0.7 ng) was ICV-administered to freely-fed mice in the early light phase. EE was monitored for 4 h after ICV injections under food-deprived condition to avoid any possible effects of feeding on EE. EE significantly increased during the 2–4 h period post-NAD injection [Fig. 9].

I then investigated the effects of peripheral administration of NAD on energy metabolism, because administration of drugs or chemicals via peripheral routes is more applicable for therapeutic intervention in humans. For this purpose, normal mice were fasted overnight and injected with NAD (0.3, 1, and 3 mg/kg) intraperitoneally in the early light phase. IP injection of NAD (1 and 3 mg/kg) significantly decreased 4 h-food intakes [Fig. 10]. The anorectic effect of IP NAD (1 mg/kg) persisted at 24 h post-injection [Fig. 10]. In addition, EE significantly increased during the 2–4 h post-IP administration of NAD (1 mg/kg) and tended to increase during the 4–8 h post-injection period [Fig. 11].

Hypothalamic Cx43 is critical for the metabolic effects of exogenous NAD

I studied if the hypothalamic gap junction protein Cx43 mediates the metabolic effects of NAD. Consistent with i previous observation on *in vitro* NAD uptake, prior ICV injection of gap junction inhibitor CBN blocked NAD-induced anorexia, whereas CD73 inhibitor CMP injection did not [Fig. 12]. ICV NAD administration (0.7 ng) in mice significantly increased NAD contents in the MBH 1 h after injection, which was inhibited by prior ICV injection of CBN but not by CMP [Fig. 12]. I tested the effects of hypothalamic Cx43 knockdown i*n vivo* by injecting Cx43 siRNA into the bilateral ARC of mice. MBH injection with Cx43 siRNA decreased Cx43 mRNA expression by more than 50% to the average level of control siRNA-injected controls [Fig. 13]. In mice with reduced hypothalamic Cx43 expression, the effects of ICV- and IP-administered NAD on food intake and MBH NAD levels were significantly blunted [Fig. 14 and 15]. Consistently, prior ICV injection of CBN or ARC injection of Cx43 siRNA significantly diminished the hypothalamic uptake of FITC-NAD [Fig. 16 and 17]. All these findings indicate that exogenous NAD is effectively transported into the hypothalamic neurons via Cx43 to induce anorexigenic effect.

Extracellular NAD regulates NPY and AgRP transcription in a SIRT1-dependent manner

I explored the mechanisms by which NAD regulates food intake and body Iight. ICV administration of NAD in overnight-fasted mice decreased the mRNA expression of orexigenic NPY and AgRP mRNA in the ARC [Fig. 18]. Conversely, POMC, SIRT1 and SIRT3 mRNA expression in the ARC Ire not significantly altered by ICV NAD injection [Fig. 18].

I further investigated the effect of extracellular NAD treatment on the transcriptional activities of AgRP, NPY, and POMC *in vitro.* NPY and AgRP promoter activity was significantly decreased by NAD (0.1 and 10 nM) treatment [Fig. 4B]. Consistently, NPY and AgRP promoter activity was increased by FK866 (10 nM) treatment, which inhibits the rate limiting step of NAD salvage pathway and induces intracellular NAD depletion. In contrast, POMC promoter activity was not altered by NAD treatment, but reduced by FK866 [Fig. 19]. These findings suggest that exogenous NAD may modulate energy metabolism, at least in part, through transcriptional suppression of NPY and AgRP.

Considering the important role of hypothalamic sirtuins, especially SIRT1, in

central regulation of energy metabolism [36], I tested the role of hypothalamic sirtuins mediating the NAD actions. Co-treatment of the general sirtuin inhibitor sirtinol and SIRT1-specific inhibitor EX527 completely blocked the NAD-induced decrease on NPY promoter activity [Fig. 20], indicating that transcriptional regulation of NPY by NAD occurs through SIRT1-dependent mechanisms. Consistently, prior ICV administration of sirtinol or EX527 significantly blocked NAD-induced reduction in food intake and Iight gain [Fig. 20].

FOXO1 is an important transcriptional regulator of NPY [30] and FOXO1's transcriptional activity is modulated by SIRT-mediated deacetylation in the peripheral metabolic organs [44]. Notably, NAD treatment was unable to suppress NPY promoter activity when FOXO1 binding sites IRE2 and IRE3 in the NPY promoter region were deleted [Fig. 4F]. These data imply that NAD regulates NPY transcription through FOXO1.

DISCUSSION

During the study of my master degree, I demonstrated using mice that supplementation with NAD effectively increased hypothalamic NAD content. Moreover, central and peripheral administration of NAD suppressed fasting-induced hyperphagia and Iight gain without adverse effects, indicating a therapeutic benefit of NAD supplement in controlling energy metabolism. Of note, the effective dose of IP-administered NAD (1 mg/kg) was more than 100 times lower than the effective dose of IP NMN [13, 39, 45]. Moreover, NAD dose-response studies showed a bell-shaped response curve with the greatest effect seen in very low dose of NAD (1 mg/kg for IP administration and 0.7 ng per animal for ICV administration). The mechanism underlying this observation is yet to be clarified. I assume that supplementation with higher NAD doses could increase intracellular or extracellular NAD levels to the supra-physiologic levels and activate the pathways which degrade NAD to offset the beneficial effects of NAD.

It has been suggested the possibility that exogenous NMN or NAD is extracellularly converted to NR by ecto-5′-nucleotidase CD73 and then enters cells via nucleoside transporters [43]. Therefore, I tested if CD73 is required for

hypothalamic NAD uptake and biological actions of NAD. Blockade of CD73 using CMP and APCP failed to block the effects of exogenous NAD. Moreover, I directly shoId cellular uptake of FITC-NAD; because FITC was bound to the adenine residue of NAD, NMN produced from exogenous NAD was not labeled with FITC and not detected as FITC signal. These findings suggest a direct transport of exogenous NAD to hypothalamic neurons.

Connexin inhibitors Ire shown to block the uptake of isotope-labeled NAD in mammalian cells [46]. Consistently, I found that connexin inhibitor CBN completely blocked the effects of exogenous NAD on hypothalamic NAD levels, food intake, and body Iight. More importantly, I found high expression of Cx43 in the mouse hypothalamus, and inhibition of hypothalamic Cx43 expression effectively blocked hypothalamic uptake of NAD as well as the anorexigenic effects of NAD. Collectively, these data strongly indicate an important role of gap junction, especially Cx43, in NAD trafficking through the plasma membrane of hypothalamus neurons. Interestingly, a recent paper has shown an important role of Cx43 in white adipose tissue browning by mediating cell-to-cell cAMP transfer upon sympathetic activation. Thus, it will be worthwhile to study other physiological functions of

hypothalamic neuronal Cx43.

In my study, ICV injection of NAD significantly decreased hypothalamic NPY/AgRP mRNA expression. Moreover, NAD treatment suppressed the promoter activity of NPY- or AgRP-encoding genes *in vitro*, suggesting that exogenous NAD may influence energy balance through an effect on transcription of key orexigenic neuropeptides NPY and AgRP. SIRT1 is an NAD-dependent protein deacetylase that serves as a cellular energy sensor [47]. Previous studies demonstrated that lack of SIRT1 in hypothalamic neurons leads to impaired function of hypothalamic neurons, indicating a critical role for hypothalamic neuron SIRT1 in normal physiology. In my study, prior ICV administration of sirtuin inhibitor sirtinol and SIRT1 inhibitor EX527 significantly inhibited the effects of exogenous NAD on food intake and body Iight, suggesting that NAD actions Ire mediated through hypothalamic sirtuins. SIRT1 or SIRT2 deacetylates and modulates transcriptional factor FOXO1 in the liver and adipocytes [48]. FOXO1 is an important regulator of NPY transcription via direct binding to IRE sites in the promoter region [38]. Thus, I tested the involvement of FOXO1 in the NAD regulation of NPY transcription. Interestingly, NAD treatment did not suppress NPY promoter activity when FOXO1

binding sites (IREs) in the NPY promoter region Ire deleted. Therefore, FOXO1 binding to the NPY promoter region may be critical for the inhibition of NPY transcription by exogenous NAD.

In conclusion, my study has firstly demonstrated that exogenous NAD is effectively imported into hypothalamic neurons via Cx43 where it increases intra-neuronal NAD content. Therefore, NAD supplement can be used for the treatment of human disorders with NAD deficit in the hypothalamic neurons.

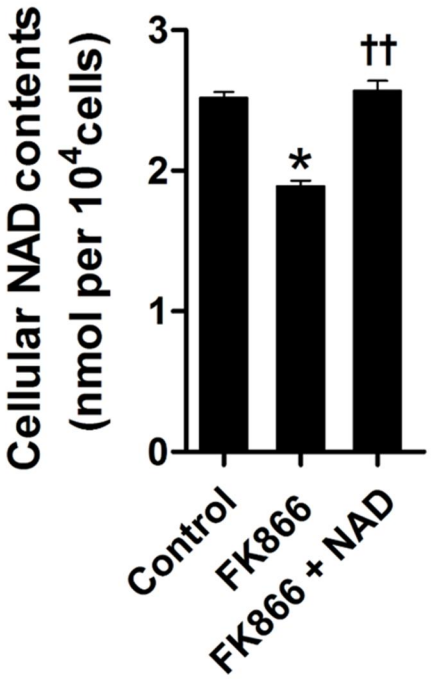


Figure 1. Exogenous NAD reverses cellular NAD depletion by nampt inhibitor FK866

Effects of FK866 (10 nM) with or without NAD (10 nM) on cellular NAD contents in N1 hypothalamic neuronal cells (n = 3) Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs FK866 alone.

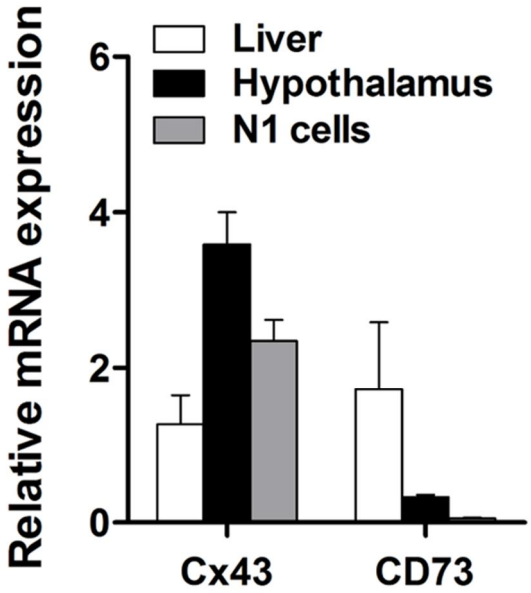


Figure 2. Comparions of mRNA exoression of connnexin 43 and CD73 in the liver, hypothalamus and hypothalamic neuronal cells

The mRNA expression of Cx43 and CD73 in the young mouse liver/hypothalamus and N1 cells. Results are presented as mean ± SEM.

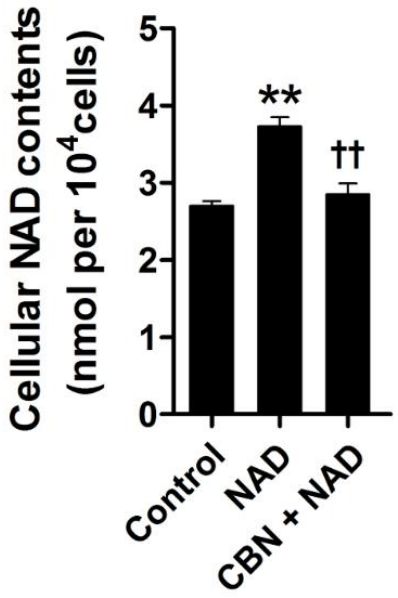


Figure 3. Blockade of NAD-induced increase in cellular NAD contents by gap junction inhibitor CBN

Exogenous NAD increase cellular contents but is inhibited by gap junction inhibitor CBN. Results are presented as mean ± SEM. $^{**}P < 0.01$, $^{††}P < 0.01$ vs. NAD alone.

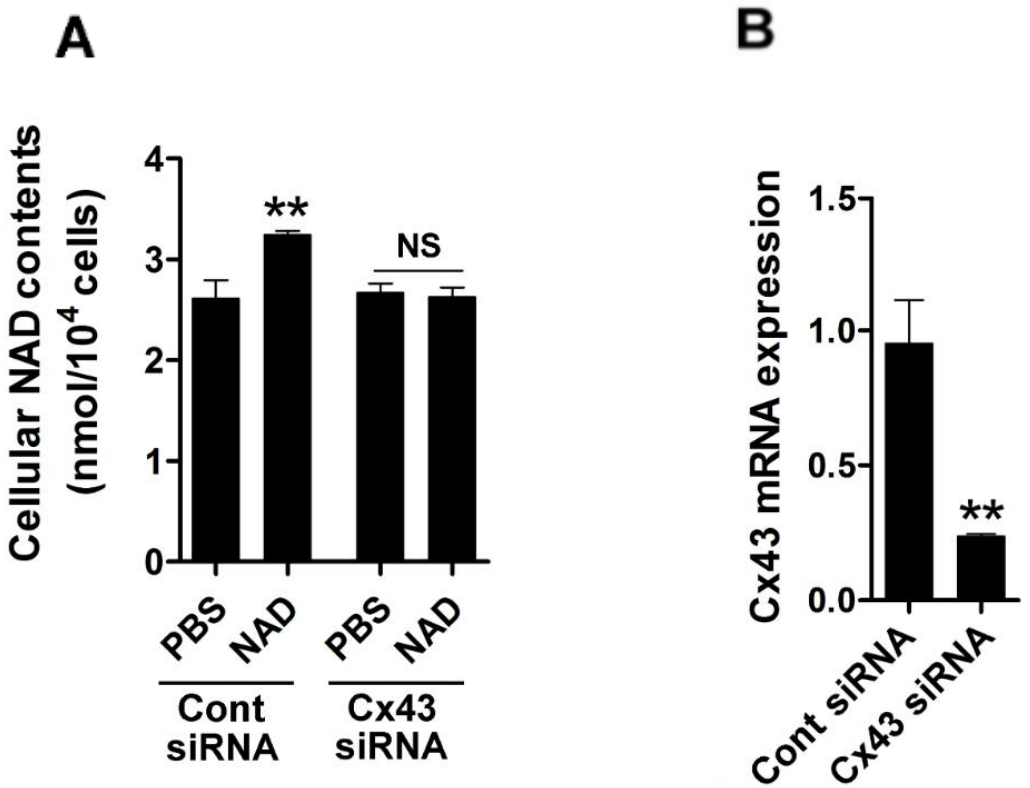


Figure 4. Cx43 siRNA tereatment reduced cellular NAD import

(A-B) Blockade of NAD-induced increase in cellular NAD contents by connexin inhibitor CBN or Cx43 siRNA (n = 3). Cx43 siRNA treatment significantly reduced cellular Cx43 expression. Results are presented as mean ± SEM. $^{**}P < 0.01$ vs. control or NAD, NS: not significant.

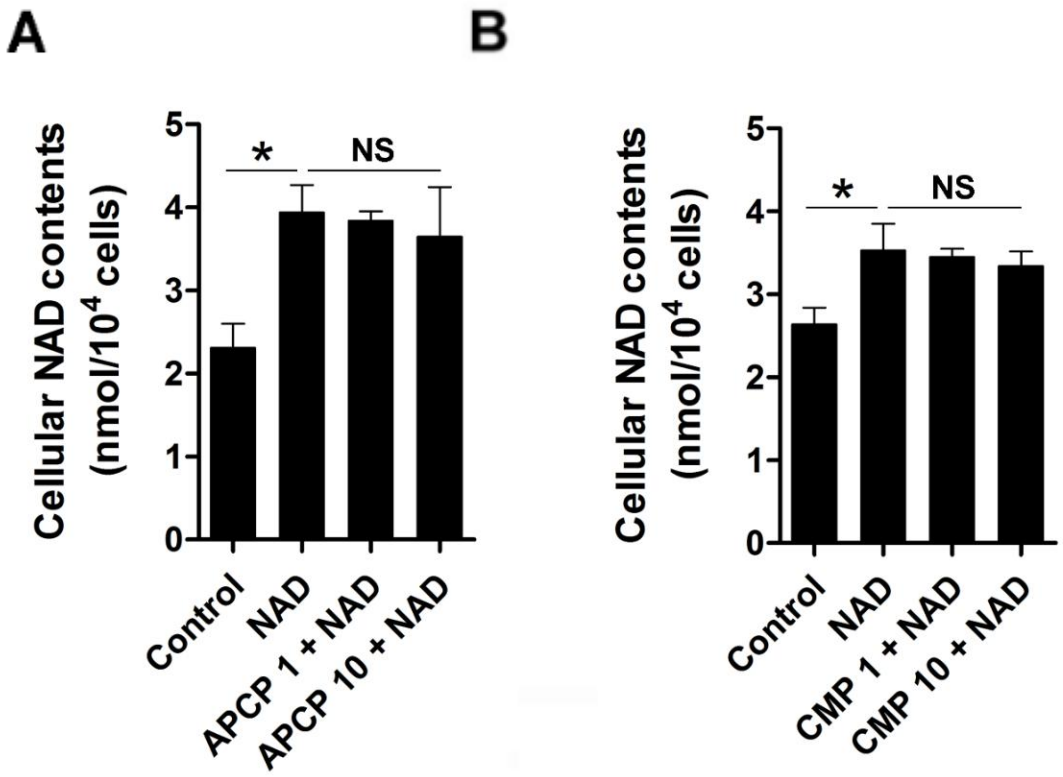


Figure 5. Effect of CD73 inhibitor on exogenous NAD-induced increase in cellular NAD contents

(A-B) Effects of NAD alone or with CD73 inhibitors APCP and CMP on N1 cell NAD contents (n = 3). Results are presented as mean ± SEM. $^{*}P < 0.05$, vs. controlor liver. NS: not significant.

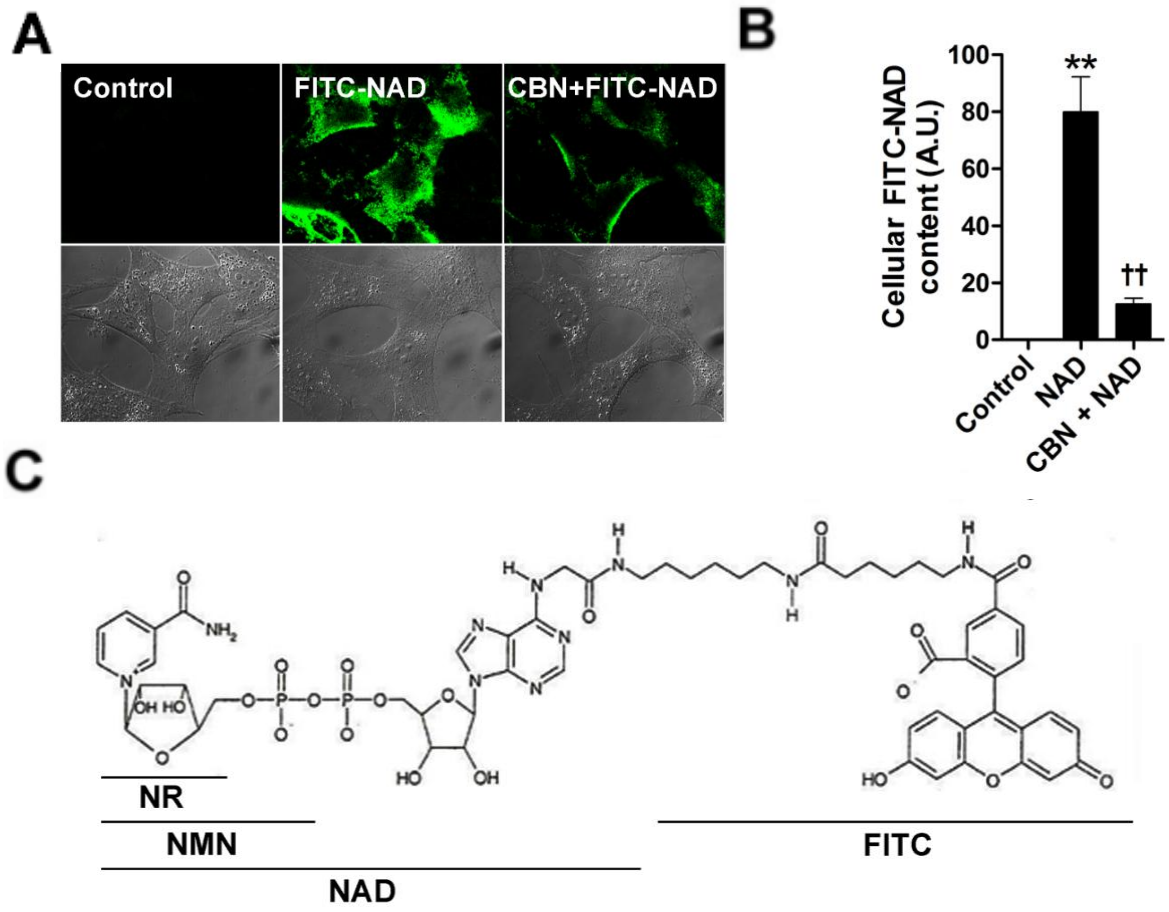
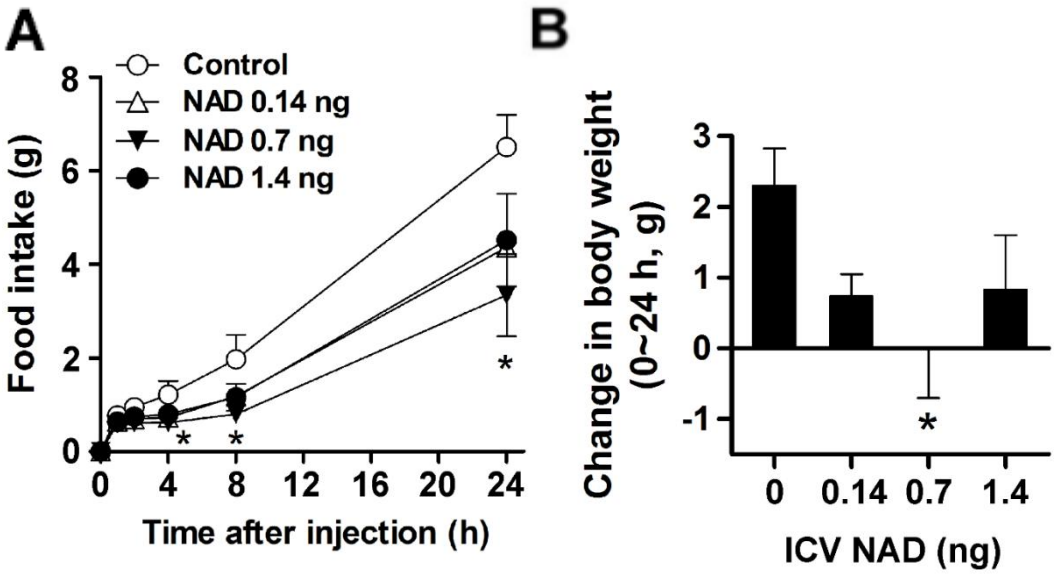


Figure 6. Effect of gap junction inhibitor CBN on cellular FITC-NAD uptake in N1 hypothalamic neuronal cell

(A-C) Cellular uptake of FITC-NAD in N1 cells in the absence or presence of CBN. The molecular structure of FITC-NAD is shown in the loIr panel. NR: nicotinamide ribose, NMN: nicotinamide mononucleotide. Results are presented as mean ± SEM. $^{**}P < 0.01$ vs. control or liver, $^{††}P < 0.01$ vs. NAD alone.



Food intake (A) and changes in body weight (B) for 24 h after ICV injection of NAD at the indicated doses (n = 6). Mice Ire fasted overnight before the injections. Results are presented as mean ± SEM. $^{*}P < 0.05$.

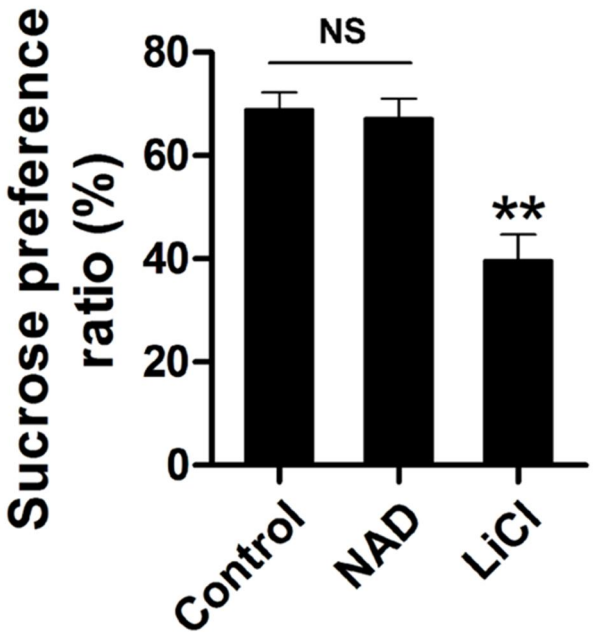


Figure 8. Conditioned taste aversion (CTA) test after NAD and LiCl IntraPeritoneal injection

Effect of ICV injection of NAD (0.7 ng) or IP injection of lithium chloride (200 mg/kg) on saccharin preference ratio. Mice Ire fasted overnight before the injections. Results are presented as mean ± SEM. $^{**}P < 0.01$ vs. control. NS: not significant.

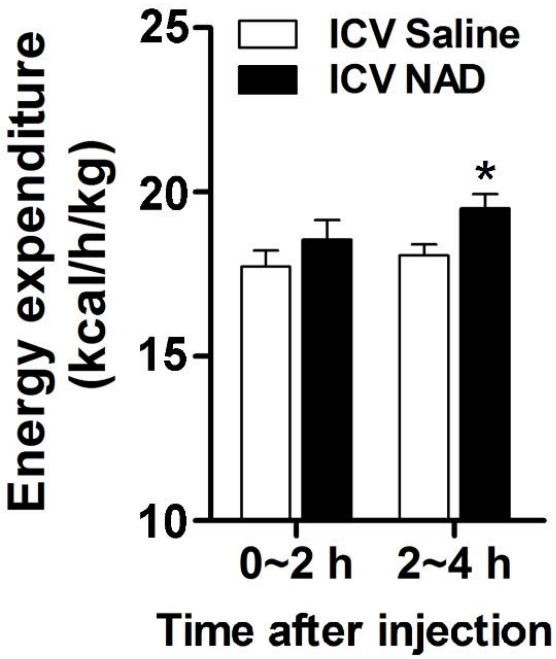


Figure 9. Effects of ICV administration of NAD on energy expenditure

Effects of ICV administration of NAD on energy expenditure for indicated periods (n = 6). Results are presented as mean ± SEM. $^{*}P < 0.05$.

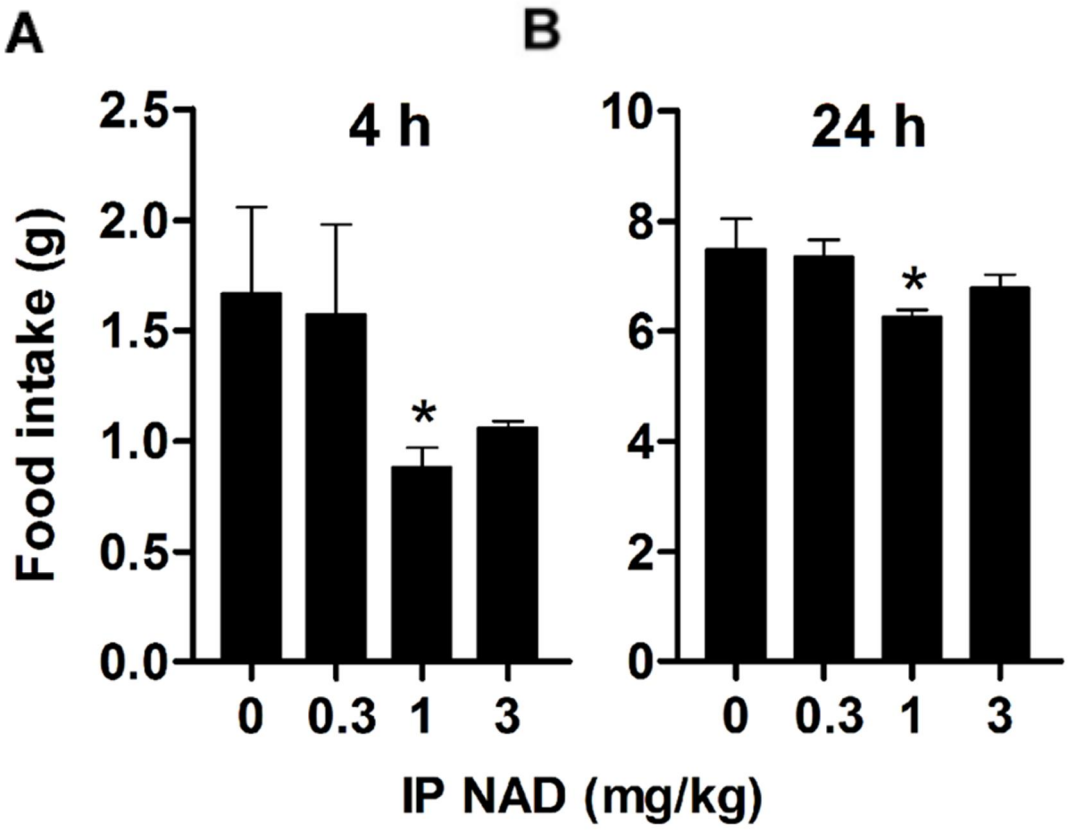


Figure 10. Effect of intraperitoneal injection fo NAD on food intake.

Food intakes for the 4h and 24 h period after IP NAD administration (0.3, 1 and 3 mg/kg) (n = 6). Results are presented as mean ± SEM. $^{*}P < 0.05$, vs. NAD injected control.

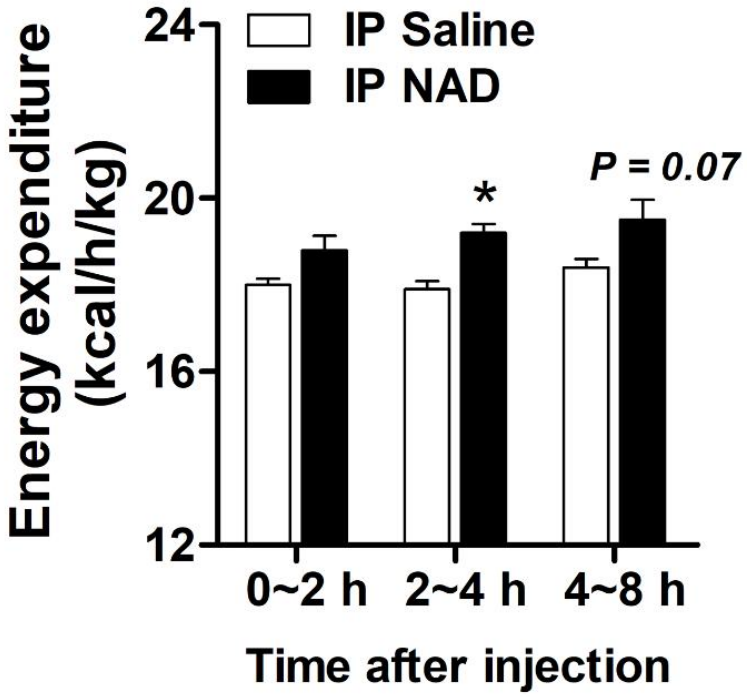


Figure 11. Effect of intraperitoneal injection of NAD on food intake.

Energy expenditure for the indicated periods after IP injection of NAD (1 mg/kg) (n = 4). Mice Ire fasted overnight before the injections. Results are presented as mean ± SEM. $^{*}P < 0.05$ vs. IP saline group.

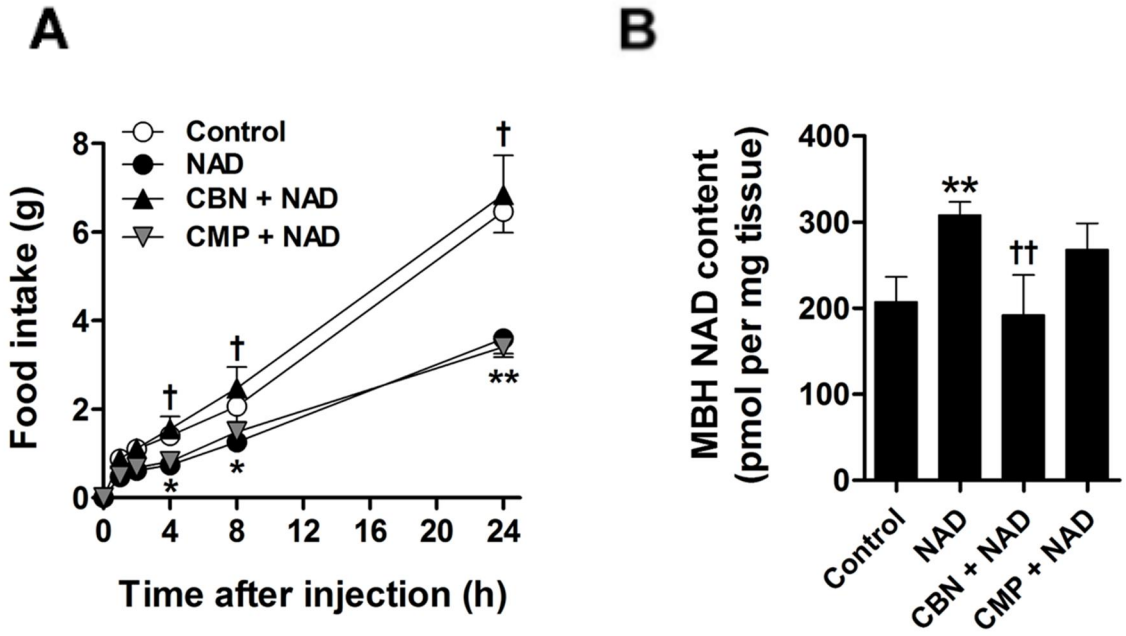


Figure 12. Effects of ICV injection of gap junction inhibitor CBN or CD73 inhibitor CMP on exogenous NAD-induced anorexia and increased MBH NAD content

Gap junction inhibitor CBN (0.1 μg) or CD73 inhibitor CMP (0.2 μg) was injected 30 min prior to ICV NAD (0.7 ng) injection. Food intake was measured for 24 h post-NAD injection. Mice were sacrificed at 24 h post-injection to harvest the hypothalamus for the measurement of hypothalamic NAD contents(n = 5–6). Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. NAD alone.

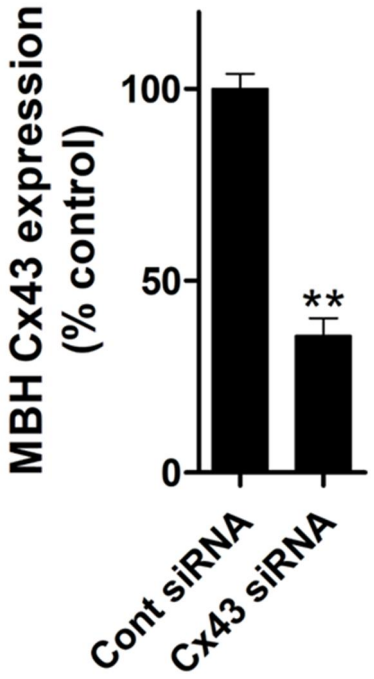


Figure 13. Successful Cx43 knockdown in mediobasal hypothalamus by intrahypothalmic injection of CX43 siRNA

MBH Cx43 mRNA expression was significantly decreased after bilateral intra-ARC injection of Cx43 siRNA (n = 10–12). Results are presented as mean ± SEM, $^{**}P < 0.01$ vs. control.

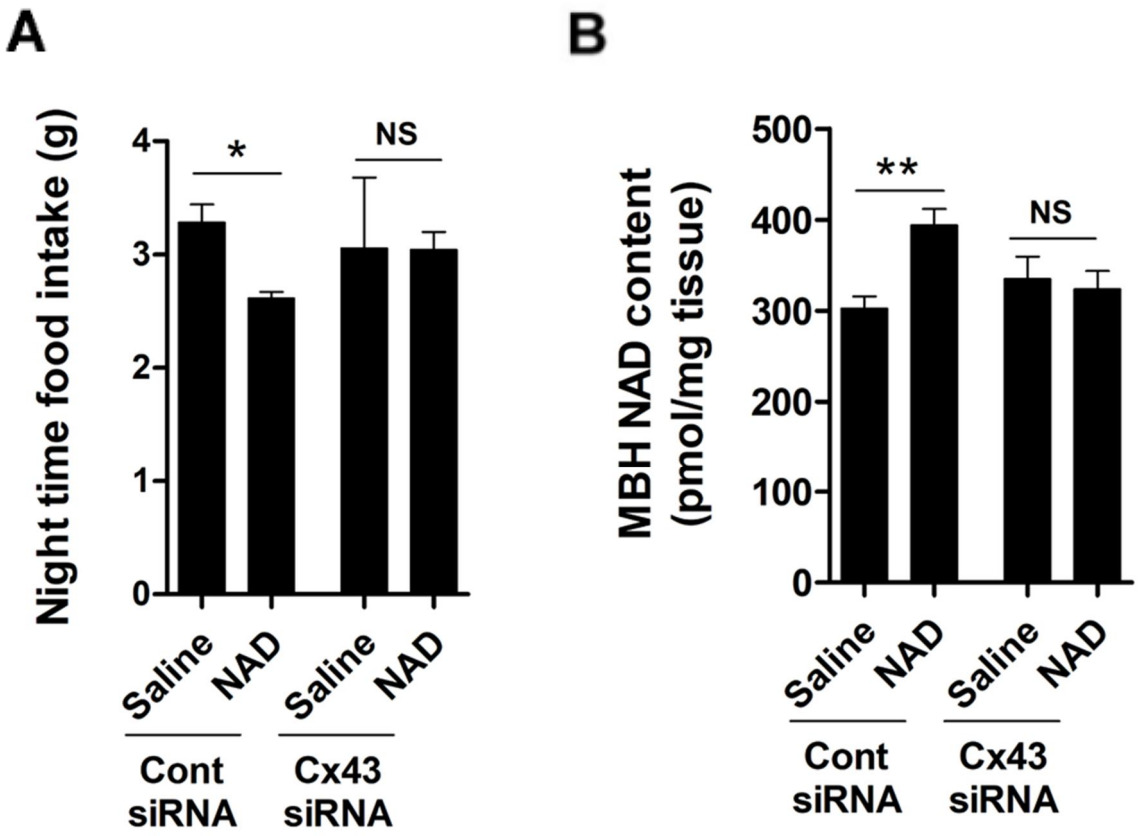


Figure 14. Effects of hypothalamic Cx43 knockdown on ICV NAD-induced anorexia and increased MBH NAD content

(A-B) Effects of ICV NAD (0.7 ng) on nighttime food intake and MBH NAD contents in mice with bilateral intra-ARC injection of control siRNA or Cx43 siRNA (n = 5–6). Results are presented as mean ± SEM. $^{*}P < 0.05$, , $^{**}P < 0.01$ between indicated groups. NS: not significant.

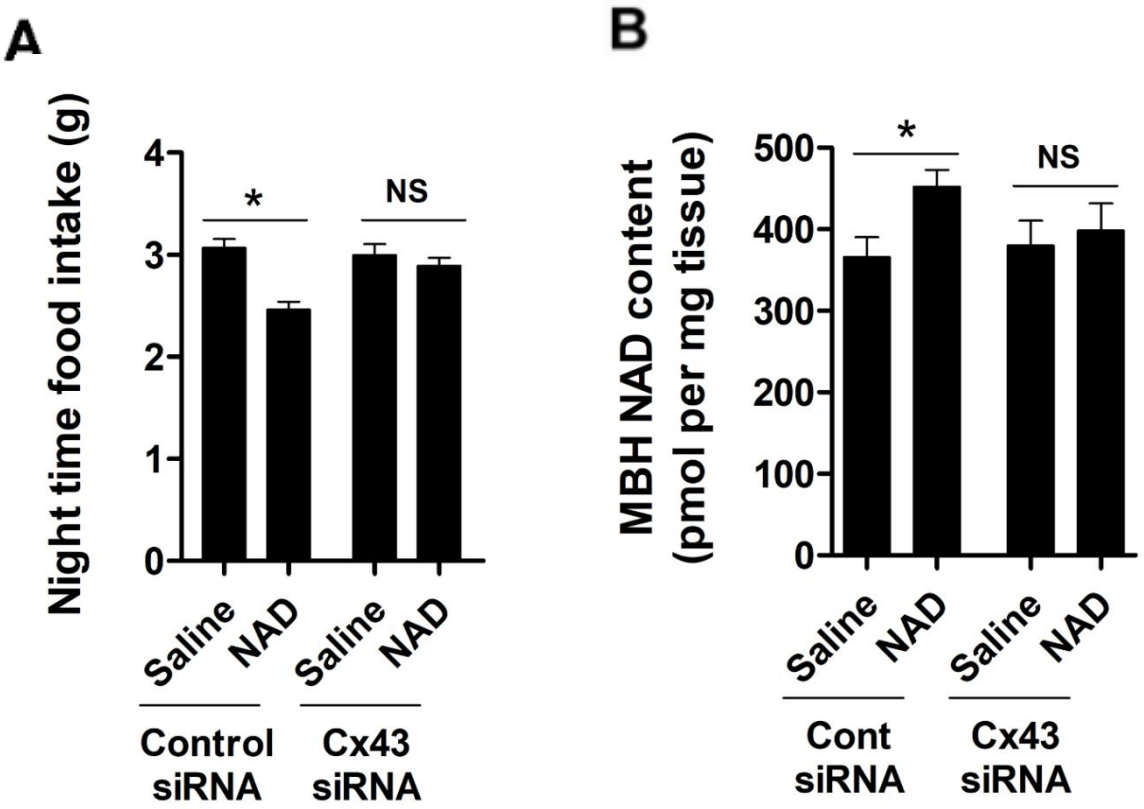

A
Night time food intake (g)
4
3
2
1
0
*
NS
Saline
NAD
Saline
NAD
Control siRNA
Cx43 siRNA
B
MBH NAD content (pmol per mg tissue)
500
400
300
200
100
0
*
NS
Saline
NAD
Saline
NAD
Cont siRNA
Cx43 siRNA

Figure 15. Effects of hypothalamic Cx43 knockdown on IP NAD-induced suppression of food intake and increase in MBH NAD contents

Effects of IP NAD (1 mg/kg) on nighttime food intake and MBH NAD contents in mice with bilateral intra-ARC injection of control siRNA or Cx43 siRNA (n = 5–6). Results are presented as mean ± SEM. $^{*}P < 0.05$ between indicated groups. NS: not significant.

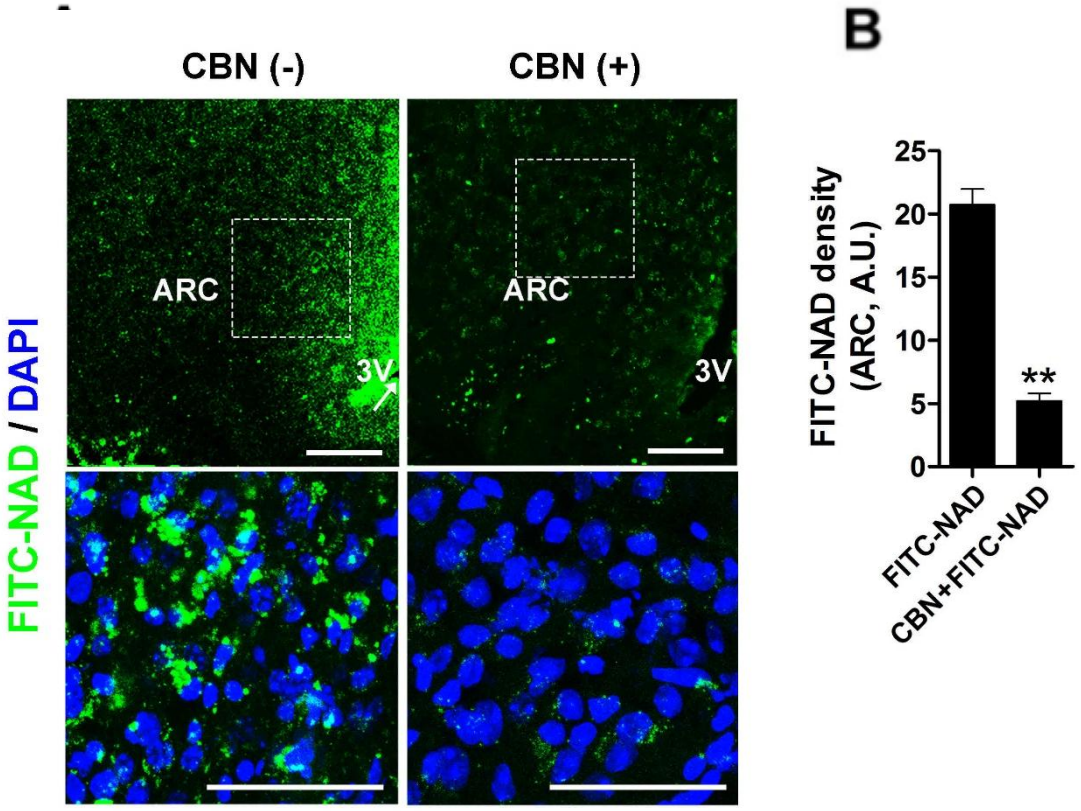


Figure 16. ICV coadministration of gap junction inhibitor CBN reduced Hypothalamic uptake of FITC-NAD

Images (A) and quantification (B) of FITC-NAD signals in the ARC of mice with ICV injection of FITC-NAD alone or together with CBN (0.1 μg). Scale bar: 50 μm. Results are presented as mean ± SEM. $^{**}P < 0.01$ vs. control.

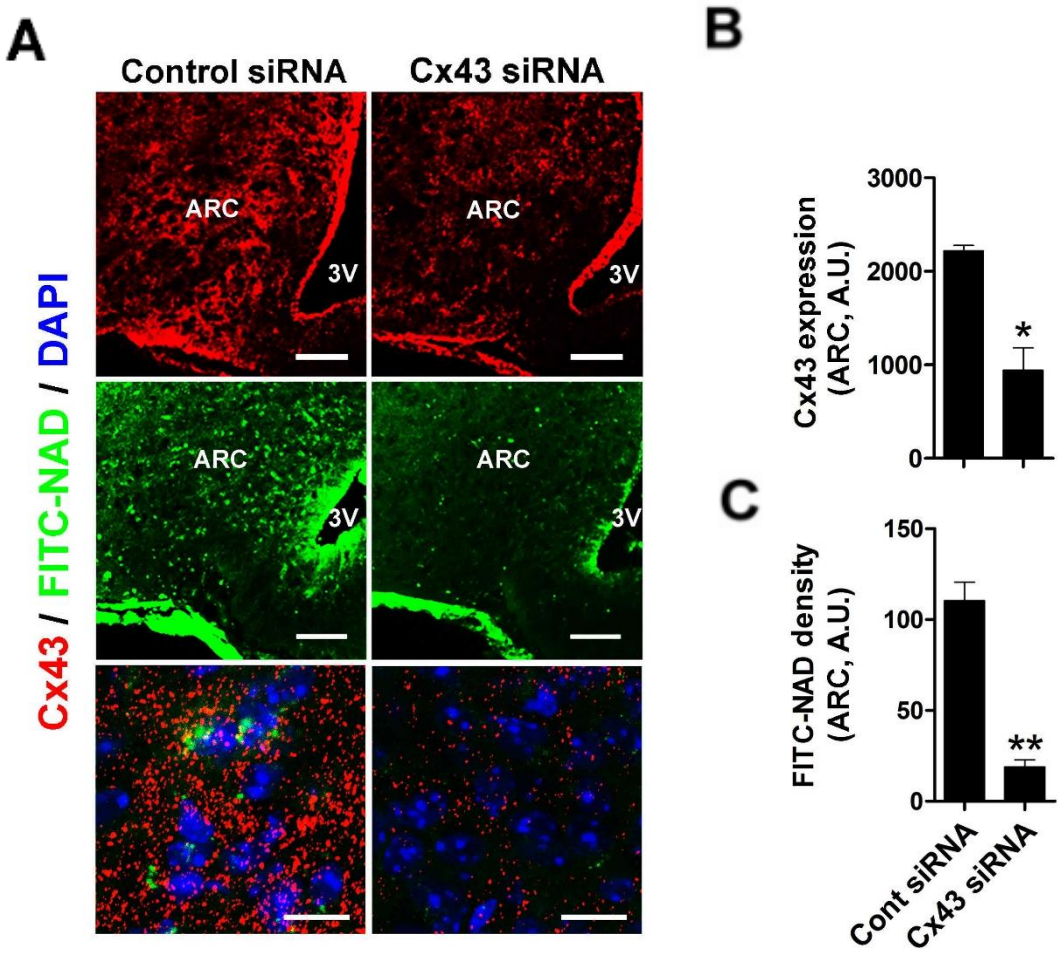


Figure 17. Hypothalamic Cx43 knockdown inhibits hypothalamic uptake of FITC-NAD

(A-C) Cx43 immunohistochemistry and FITC-NAD signals in the ARC of mice injected with control siRNA or Cx43 siRNA in bilateral ARC (n = 4–5). Scale bar: 50 μm. Results are presented as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. control.

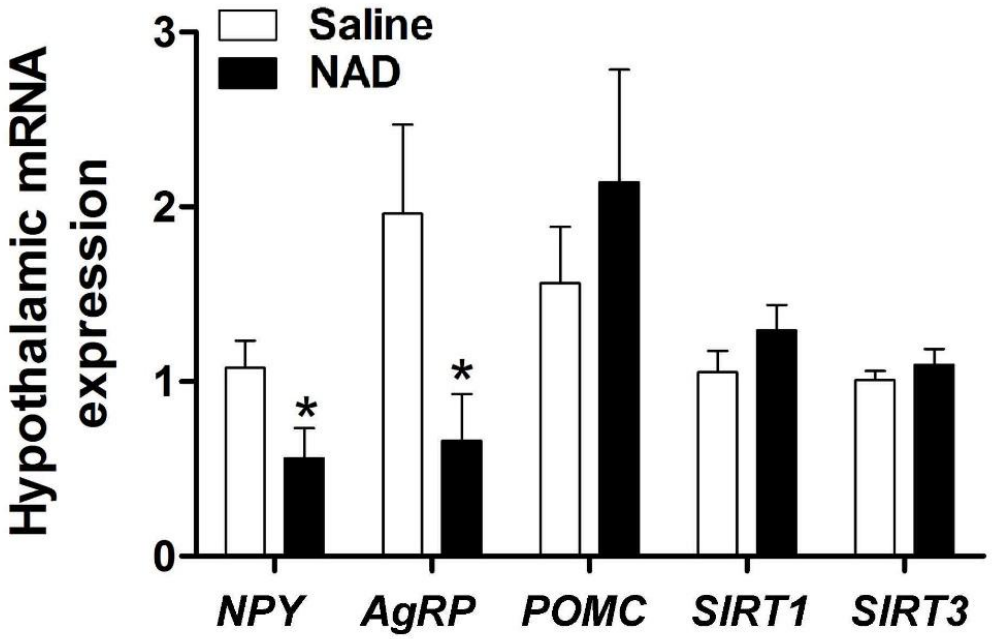


Figure 18. Effect of ICV injection of exogenous NAD on the mRNA expression levels of hypothalamic appetite-regulating neuropeptides

Effect of ICV injection of NAD (0.7 ng) on the mRNA expression levels of appetite regulating neuropeptides and sirtuins in the hypothalamic ARC (n = 8). Results are presented as mean ± SEM. *P < 0.05 vs. NAD alone.

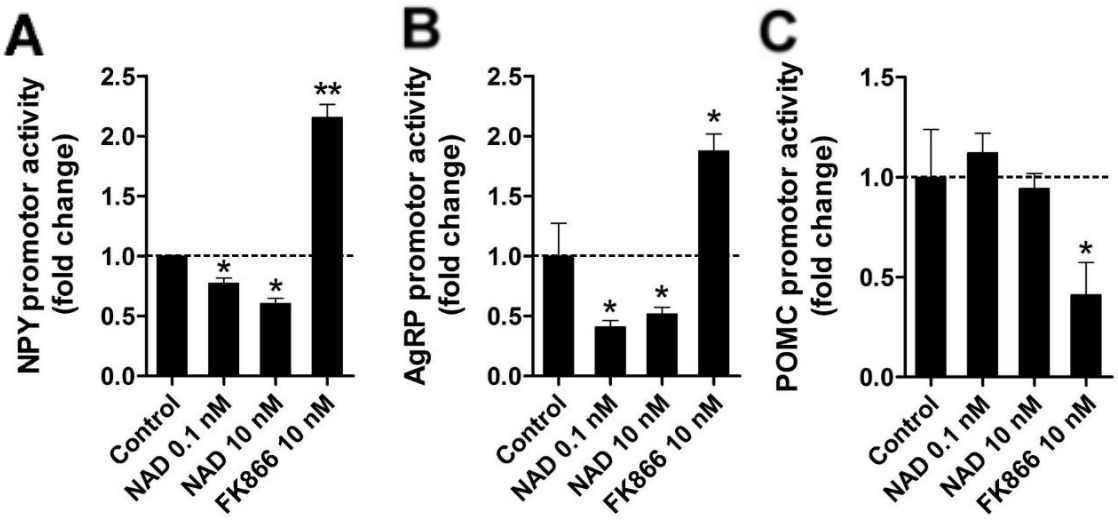


Figure 19. Exogeous NAD decreased NPY and AgRP promotor activities

Effects of NAD and FK866 treatment on the transcriptional activities of NPY (A), AgRP (B), and POMC (C) was examined using N1 hypothalamic cells. Results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01 vs. control.

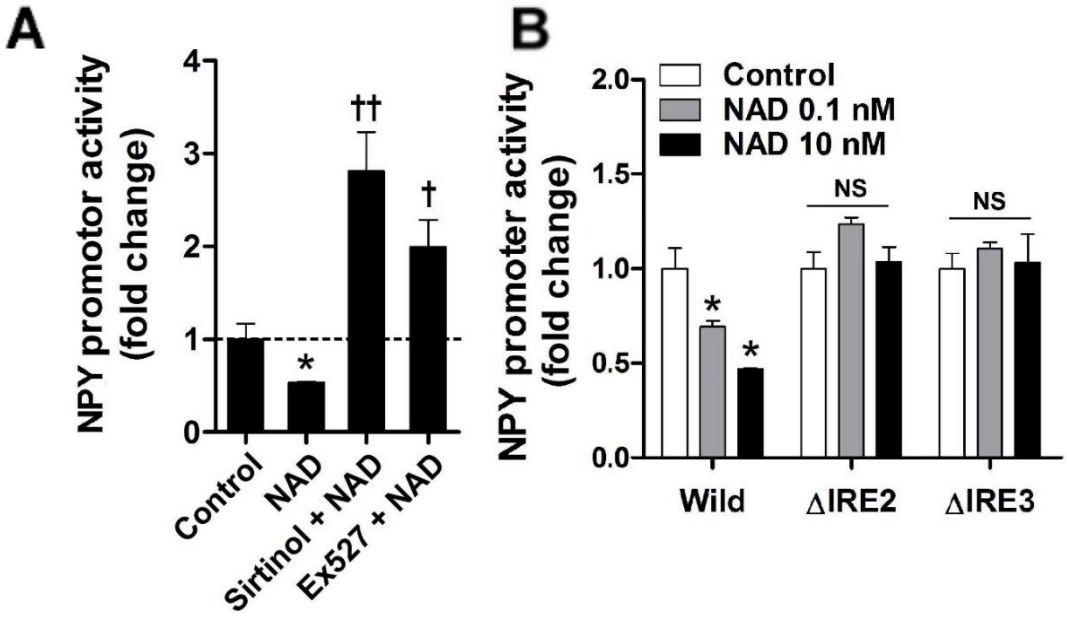


Figure 20. Sirtuin inhibitors and FOXO1 binding site mutation block exogenous NAD-induced suppression in NPY pormotor activity

(A) Effect of treatment of NAD alone (10 nM) or with sirtinol (200 nM) or EX527 (100 nM) on NPY promoter activity. (B) Effect of treatment of NAD on wild type NPY promoter or NPY promoter with deletion of FOXO1 binding sites IRE2 (ΔIRE2) and IRE3 (ΔIRE3). Results are presented as mean ± SEM. $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$ vs. NAD alone.

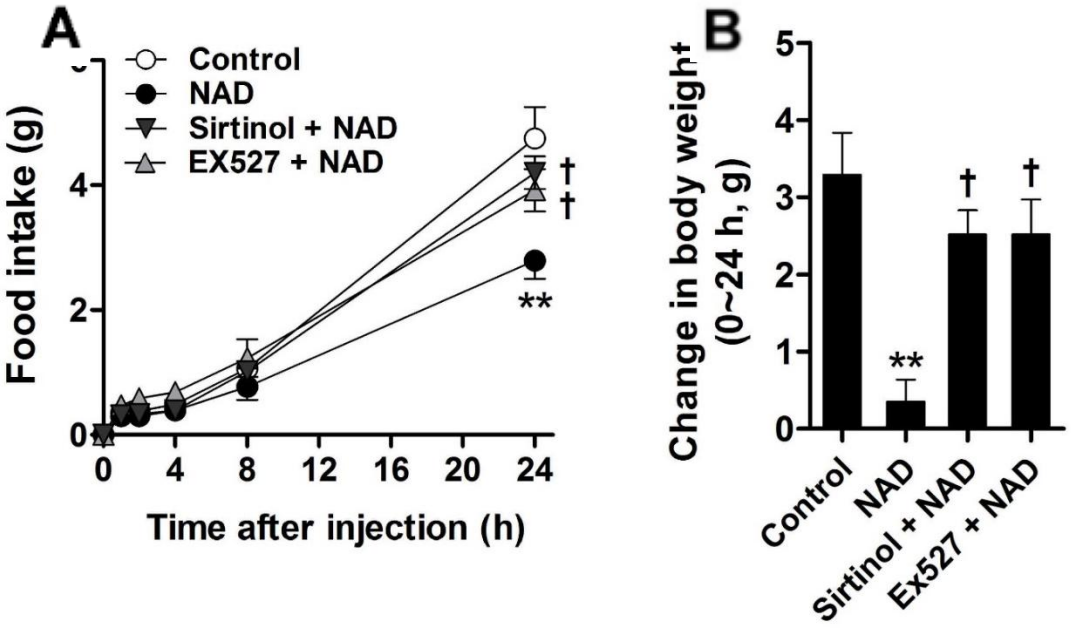


Figure 21. Blockade of NAD-induced anorexia and weight loss by sirtuin inhibitors sirtuiol and EX527.

Food intakes (A) and body weight changes (B) for the 24 h period following ICV administration of NAD (0.7 ng) alone or together with sirtinol (200 ng) or EX527 (100 ng) (n = 6). Results are presented as mean ± SEM. **P* < 0.05, ***P* < 0.01 vs. control, †*P* < 0.05, ††*P* < 0.01 vs. NAD alone. NS: not significant.

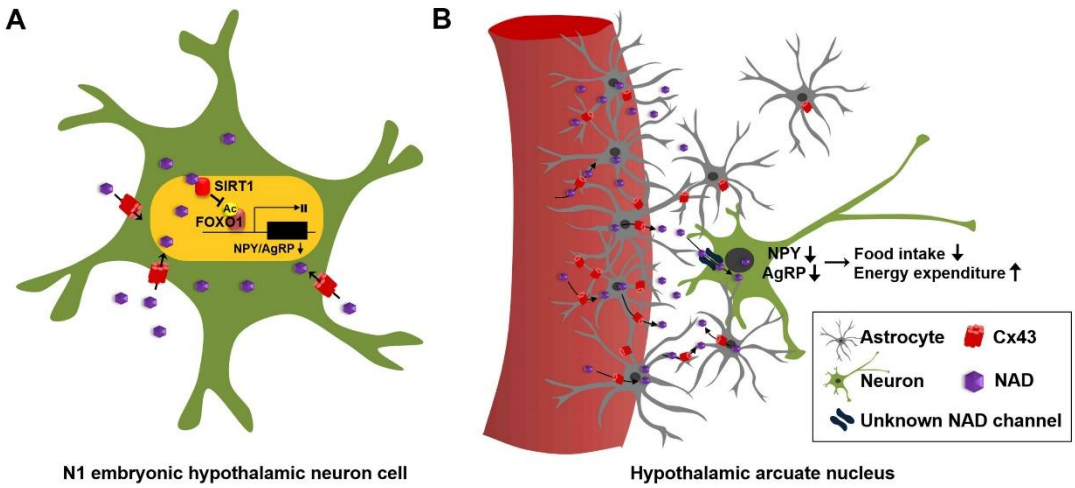


Figure 22. A schematic representation of the role for Cx43 in exogenous nicotinamide adenine dinucleotide (NAD) trafficking and metabolic effects in the hypothalamus.

(A) In the embryonic hypothalamic neuron cells, extracellular NAD is imported to the cells via Cx43 hemichannels, thereby increasing cellular NAD levels and repressing neuropeptide Y/Agouti-related protein transcription through SIRT1 and FOXO1. (B) In mice, exogenous NAD may be transported to hypothalamic extracellular space via Cx43 expressed at the blood-brain barrier (between perivascular astrocyte endfeet) and the brain-cerebrospinal fluid barrier (between ependymal cells). Subsequently, NAD may enter arcuate nuclear neurons via unknown channels and regulates energy metabolism.

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ABSTRACT IN KOREAN

목적: 니코티나마이드 아데닌 디뉴클레오타이드 (NAD)-의존적 탈아세틸화효소 SIRT1 은 시상하부 뉴론의 기능 조절에 중요한 역할을 하기 때문애 시상하부에서 충분한 NAD 의 양은 에너지항상성을 유지하는데 매우 중요하다. 따라서 본인은 석사학위 과정 중 외부에서 NAD 를 투여하여 시상하부 NAD 양을 증가시킬 수 있는지, 그리고 NAD 투여가 먹이섭취 및 에너지대사에 미치는 영향을 분석하고, 외부에서 투여한 NAD 가 시상하부로 들어가는 기전을 연구하였다.

방법: 시상하부 뉴런 세포에 NAD 를 투여하거나 마우스의 뇌실과 복강 내로 NAD 를 투여하고 세포 혹은 시상하부 NAD 양이 증가하는지 관찰하였다. 그리고 NAD 의 세포 혹은 시상하부로 유입에 gap junction protein 인 connexin 43 이 관여하는지, CD73 5'ecto-nucelotidase 이 관여하는지 연구하였다. 또한 정싱 마우스의 복강과 노실로 NAD 를 투여 후 먹이섭취, 에너지대사 및 체중에 미치는 영향을 관찰하였다. 그리고 NAD 투여가 시상하부 식욕 조절 신경펩타이드 발현과 전사활성에 미치는 양향을 관창하였다. 마지막으로 NAD 에 의한 대사 조절 작용에 시상하부 sirtuin 특히 SIRT1 과 전사조절인자인 FOXO1 이 관여하는지를 연구하였다.

결과: 외부에서 투여한 NAD 는 시상하부 뉴런세포와 마우스 사상하부의 NAD 의 양을 효과적으로 증가시켰다. 외부에서 투여한 NAD 가 시상하부 뉴런인 N1 세포로 들어가는 유입 경로는 CD73 이 아닌 Cx43 을 통해 들어간 다는 것을 발견했으며 In vitro 실험에서도 일치한다는 것을 확인하였다. 마우스의 복강과 뇌실내로 NAD 주입은 먹이섭취와 체중 증가를 억제하고 에너지소모를 촉진시켰다. 그리고 이러한 작용은 PY/AGRP 전사 활성의 억제를 통하여 이루어지며, 이러한 효과는 SIRT1 과 FOXO1 에 의해 매개되었다.

결론: 마우스에 외부 NAD 를 투여하여 효과적으로 시상하부 NAD 함량을 증가시키고, 에너지대사를 조절할 수 있었다. 따라서, NAD 투여는 시상하부 NAD 결핍과 함께 나타나는 대사 장애에 대한 치료 방법이 될 수 있다는 것을 시사한다.