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Adult hippocampal neurogenesis can be enhanced by cold challenge independently from beigeing effects

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Adult hippocampal neurogenesis can be enhanced by cold challenge independently from beigeing effects

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ABSTRACT

Adult hippocampal neurogenesis can be enhanced by cold challenge independently from beigeing effects

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Ambient cold challenge has been known to improve metabolism, and this metabolic improvement arises from the control of glucose metabolism and lipid homeostasis; lipid homeostasis is caused by changes in adipose tissue, called the 'beigeing effect'. Many studies have investigated the relationship between metabolic changes and the adult hippocampal neurogenesis (AHN) process and have found that metabolic improvement enhances AHN and hippocampal function, whereas metabolic disorders have been found to impair them. In this study, we hypothesized that metabolic changes induced by cold challenge from beigeing effects could influence AHN and the hippocampus. In addition, we examined changes in AHN-related gene expression and signaling pathways in the hippocampus.

AHN was assessed at weeks 1 and 4 of cold challenge (6 ± 2°C). Metabolic amelioration by cold challenge was confirmed by beigeing effect of white adipose tissue, based on hematoxylin and eosin staining, as well as uncoupled protein-1 (UCP-1), a marker of beigeing effects, immunohistochemical staining. Additionally, we observed the effects of CL 316,243 as a chemical beigeing inducing agent on metabolic improvements and AHN; peripheral beigeing induced by cold challenge having a positive effect on AHN was ruled out. CL 316,243, which is not permeable to the blood-brain barrier, was administered 1 mg/kg subcutaneous to mice for either 1 week or 4 weeks.

Cold challenge for 1 and 4 weeks was found to induce beigeing effects in inguinal white adipose tissue (iWAT), but not in epididymal WAT (eWAT). UCP-1 was also increased in iWAT, but not in eWAT. Cold challenge for 1 and 4 weeks increased neural progenitor cells (NPCs), proliferative neural stem cells (NSCs), and neuroblasts in the dentate gyrus compared to the control group. In addition, cold challenge for 4 weeks significantly increased the integration into mature neurons in the granule cell layer of dentate gyrus. Cold challenge for 1 and 4 weeks increased genes related to AHN, including notch signaling, G protein-coupled receptor signaling, and β-adrenergic signaling. In addition, cold challenge for 1 and 4 weeks increased early growth response 1 (Egr1), achaete-scute homolog 1 (Ascl1), histone deacetylase 4 (Hdac4), dopamine receptor D2 (Drd2), glutamate ionotropic receptor N-methyl-D-aspartate type subunit 1 (Grin2), cholinergic receptor muscarinic 2 (Chrm2), and erb-B2 receptor tyrosine kinase 2 (Erbb2). In signaling pathways, cold challenge increased Ascl1, Notch1, Drd2, transforming growth factor-β

(Tgf-β), and Chrm2 at both 1 and 4 weeks of cold challenge. Beta1-adrenergic receptor (Adbr1) for β-adrenergic signaling was increased at both 1 and 4 weeks of cold challenge. Cold challenge for 1 week, not 4 weeks, increased gene expression, such as in Hes family basic helix-loop-helix transcription factor 1 (Hes1), midkine (Mdk), cAMP responsive element binding protein 1 (Creb1), and fibroblast growth factor 2 (Fgf2). In contrast, cold challenge for 4 weeks, not 1 week, increased hairy/enhancer-of-split related with YRPW motif-like protein (Heyl), paired box gene 3 (Pax3), neurotrophin3 (Ntf3), and neuregulin 1 (Nrg1). In this study, we confirmed transient cold challenging could influence the AHN process and gene expression changes in the hippocampus with beigeing effect.

To rule out peripheral beigeing effects on AHN, CL 316,243 was used to induce beigeing in adipose tissue. CL 316,243 showed more potent beigeing effects in iWAT and eWAT than those of cold challenge. CL 316,243 for 1 and 4 weeks could induce beigeing effects both in iWAT and eWAT, which were confirmed by an increased expression of UCP-1. However, CL 316,243 administration for 1 and 4 weeks did not show any significant changes in populations of NPCs, proliferative NSCs, and neuroblasts in the hippocampus. In addition, CL 316,243 administration for 4 weeks did not show any effect on integration into granule cells in the hippocampus. Upon analysis of mRNA expression in the hippocampus, we found that CL 316,243 had no effect on AHN-related gene expression, signal transduction, or β1-adrenergic receptor.

Collectively, cold challenge as an extrinsic stimulus showed beigeing effects and enhanced the potency of the AHN process. However, CL 316,243, a peripheral beigeing inducer, did not show any significant effects on AHN. These results suggest that cold challenge promotes AHN, not through peripheral beigeing effects, but through the possible central activation of AHN-related genes, signal transduction, and β1-adrenergic receptors. Considering the increase in AHN and gene expression changes, cold challenge may have unique modulation of cold signaling pathway in the hippocampus. These results provide an understanding to the process of AHN and therapeutic approaches with cold challenges.

Keywords: Cold challenge, neurogenesis, hippocampus, beigeing effects, β adrenergic signaling

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LIST OF ABBREVIATION

Ache	Acetylcholinesterase
Ascl1	Achaete-scute homolog 1
Adbr1	Adrenergic beta receptor 1
Adbr2	Adrenergic beta receptor 2
Adbr3	Adrenergic beta receptor 3
AHN	Adult hippocampal neurogenesis
BAT	Brown adipose tissue
BeAT	Beige adipose tissue
BrdU	5-bromo-2'-deoxyuridine
BDNF	Brain-derived neurotrophic factor
Bmp2	Bone morphogenetic protein 2
Bmp4	Bone morphogenetic protein 4
Bmp8	Bone morphogenetic protein 8
Creb1	cAMP responsive element binding protein 1
Chrm2	Cholinergic receptor muscarinic 2

CNS	Central nervous system
DCX	Doublecortin
DG	Dentate gyrus
Drd2	Dopamine receptor D2
eWAT	Epididymal WAT
Egr	Early growth response protein
Erbb2	Erb-B2 receptor tyrosine kinase 2
Fgf2	Fibroblast growth factor 2
GCL	Granule cell layer
GPCR	G protein coupled receptor
Grin1	Glutamate ionotropic receptor *N*-methyl-D-aspartate type subunit 1
Hdac4	Histone deacetylase 4
Hes1	Hes family basic helix-loop-helix transcription factor 1
Heyl	Hairy/enhancer-of-split related with YRPW motif-like protein
HPA	Hypothalamic-pituitary-adrenal

iWAT	Inguinal white adipose tissue
LC	Locus coeruleus
LTP	Long-term potentiation
MoL	Molecular layer
NE	Norepinephrine
Neurod1	Neurogenic differentiation 1
Neurog1	Neurogenin 1
Neurog2	Neurogenin 2
NPCs	Neural progenitor cells
NSCs	Neural stem cells
Notch1	Notch homolog 1
Nrg1	Neuregulin 1
Ntf3	Neurotrophin 3
No	Numbers
Olig2	Oligodendrocyte transcription factor 2
Pax3	Paired box gene 3

Pax5	Paired box gene 5
Pax6	Paired box gene 6
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pou4f1	POU domain, class 4, transcription factor 1
PSD95	Postsynaptic density protein 95
S100b	S100 calcium-binding protein B
SEM	Standard error of means
Sod1	Cu,Zn-superoxide dismutase
SGZ	Subgranular zone
SVZ	Subventricular zone
T2DM	Type 2 diabetes mellitus
Tgf-β	Transforming growth factor-β
UCP-1	Uncoupling protein-1
WAT	White adipose tissue

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INTRODUCTION

Adult hippocampal neurogenesis (AHN) is the process of continuous generation of neural stem cells in the subventricular zone of the lateral ventricle, and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus. Neural stem cells (NSCs) in the SGZ go through the maturation process in the granular cell layer (GCL) (Gage, 2000, 2002; Kempermann *et al.*, 1997; Palmer *et al.*, 1997). A large number of studies have investigated the changes in extrinsic factors during the AHN process, including environmental conditions, diet compositions, and regulation factors of metabolism (Leuner *et al.*, 2002; Stangl and Thuret, 2009). Metabolic disturbances have also been found to cause impairments in AHN. For example, high-fat diet fed mice showed impairments in AHN, along with increased lipid peroxidation and decreased brain-derived neurotrophic factor (BDNF) (Park *et al.*, 2010). In addition, a genetically engineered mouse model of type 2 diabetes mellitus (T2DM) and a diet-induced T2DM mouse model showed impaired AHN with dementia (Ramos-Rodriguez *et al.*, 2014). These studies determined that metabolic disorders damage the AHN process, and decrease NSCs and their lineages. In contrast, metabolic improvement had been found to promote AHN. For example, physical exercise has improvement effects on metabolism (Laing *et al.*, 2016), enhanced AHN, synaptic plasticity, and hippocampus-related cognitive function (Brandt *et al.*, 2010; Vivar *et al.*, 2013; Yau *et al.*, 2011). In addition, calorie restriction has shown improvement in AHN with increased BDNF and other neurotrophins (Bondolfi *et al.*, 2004; Lee *et al.*, 2002a; Lee *et al.*, 2002b). These results suggest that metabolic changes influence hippocampal function,

as well as AHN.

Several studies have found that cold challenge as an extrinsic stimulus could prevent and improve obesity and diabetes (Harms and Seale, 2013; Jeremic *et al.*, 2017; Ravussin *et al.*, 2014). As a metabolic improvement factor, cold challenge has been known to improve glucose metabolism and lipid homeostasis, coming from changes in adipose tissue. Adipose tissue is divided into two types; brown adipose tissue (BAT) and white adipose tissue (WAT) (Gil *et al.*, 2011). Metabolic changes induced by cold challenge comes from thermogenic effects of adipose tissue (Shabalina *et al.*, 2013). Cold challenge generates heat by activation of BAT (Gordon, 2012; Nicholls *et al.*, 1978) and beige adipose tissue (BeAT) from WAT so called 'beige' (Ishibashi and Seale, 2010), 'brite' (Petrovic *et al.*, 2010), or 'beigeing'(Ohno *et al.*, 2012).

It has been reported that "beigeing effects" induced by cold challenge improves metabolic disorders and metabolism in mice (Ravussin *et al.*, 2014). Acute cold challenge activates BAT and elicits shivering activity in humans (Ouellet *et al.*, 2012). In an experiment on obese and lean people, they found that mild cold challenge (16°C) activated BAT and increased energy expenditure (van Marken Lichtenbelt *et al.*, 2009). In other studies, cycles of cold challenge showed improvement in insulin sensitivity (Lee *et al.*, 2014), and the amount of BAT showed correlation with body mass index, glucose level, age, and sex (Cypess *et al.*, 2009). Moreover, it has been found that cold challenge improves systemic metabolism, based on the activation of BAT and formation of BeAT. In addition,

cold challenge can influence AHN and the hippocampus by direct effects of cold challenge or indirect retrograde effects of activation of BAT and BeAT.

Metabolic improvement induced by cold challenge comes from thermogenic function via the activation of BAT and formation of BeAT. This regulation is mediated by activation of uncoupling protein-1 (UCP-1) accompanied by functional upregulation of mitochondria (Shabalina *et al.*, 2013). The roles of epigenetic factors peroxisome proliferator-activated receptor-γ and proliferator-activated receptor γ coactivator 1α are as critical regulators of BAT activation and formation of BeAT (Lo K and Sun, 2013). Many extrinsic and intrinsic factors are known to induce beige adipocyte recruitment via various mediators (Kajimura *et al.*, 2015). In signaling pathways, BAT activation and beigeing effects are through β3 adrenergic signaling (Peng *et al.*, 2015).

There are few studies about the effect of cold challenge on the brain, specifically the hippocampus. Cold challenge coordinates insulin sensitivity and glucose homeostasis via sympathetic nervous system outflow with thermogenic changes (Nakamura and Morrison, 2007). Correlation and interaction of peripheral adipose tissue with the brain is usually investigated in cytokines from adipose tissue (adipokines; e.g., leptin), and mainly related to the hypothalamic-pituitary-adrenal (HPA) axis (Malendowicz *et al.*, 2007). Moreover, the neuronal projection of the hypothalamus to peripheral adipose tissue has been investigated to elucidate the relationship between the brain and adipose tissue. For example, leptin and proopiomelanocortin neurons activated by insulin in the hypothalamus, as well

as genetic inhibition of agouti-related peptide neurons promote beigeing adipocyte biosynthesis (Dodd *et al.*, 2015; Ruan *et al.*, 2014). In the study of behavioral and electrophysiological parameter linked hippocampal function, cold challenge was found to partially improve spatial learning scores, but showed impaired long-term potentiation (LTP) (Elmarzouki *et al.*, 2014).

CL 316,243, a β3-selective adrenergic agonist, has the potential to activate BAT and form BeAT (Guerra *et al.*, 1998), without any permeability through the brain-blood barrier (Mirbolooki *et al.*, 2011; Mirbolooki *et al.*, 2015). Chronic administration of CL 316,243 has been found to increase glucose uptake in brown adipocytes in non-obese rats (de Souza *et al.*, 1997). Formation of BeAT induced by CL 316,243 has also been found to regulate the decrease of glucose levels with increased insulin (MacPherson *et al.*, 2014). In MKR mice, which is an animal model of T2DM, CL 316,243 administration restored the diabetes-induced downregulation of gene expression levels related to fatty acid oxidation (Kumar *et al.*, 2015). In transgenic diabetic mice and rat models, CL 316,243 showed anti-diabetic effects with decreases in glucose, serum fatty acids, and insulin levels (Ghorbani *et al.*, 2012; Liu *et al.*, 1998). To activate BAT and formation of BeAT by extrinsic stimuli, CL 316,243 has been used for peripheral adipose tissue changes, without direct stimulation to the brain. In this study, we aimed to rule out whether AHN is activated or beigeing effects are induced by cold challenge.

Correlation of cold challenge and AHN has not been fully understood; in this study, we

questioned the direct effects of cold challenge on the hippocampus, NSCs, and lineage of NSCs. Immunohistochemistry for markers of NSCs and their lineage was conducted to identify metabolic changes from the activation of BAT and formation of BeAT induced by CL 316,243, and if they could affect the hippocampus. In addition, mRNA expression was assessed after 1 or 4 weeks of CL 316,243 treatment.

In the present study, we hypothesized that ambient cold challenge would cause beigeing effects, and possibly changes in the population of NSCs, the lineage of NSCs, and AHN-related gene expressions in the hippocampus. In our study, we used two groups: a cold challenge ($6 \pm 2°C$) and a control group ($20 \pm 2°C$). The duration of cold challenge was for either 1 or 4 weeks to assess the proliferation of neuroblasts and integration into mature neurons during AHN. In addition, mRNA expressional changes were analyzed for a further understanding of direct cold effects on the hippocampus.

MATERIALS AND METHODS

Experimental animals

Seven-week-old male C57BL/6N mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). The animals were housed in a specific pathogen-free animal facility at 22°C with 60% humidity, a 12 h/12 h light/dark cycle, and ad libitum access to food and tap water until the start of experiment at the age of 8 weeks. The handling and care of the animals conformed to guidelines established in compliance with current international laws and policies (NIH Guide for the Care and Use of Laboratory Animals, NIH Publication No. 85-23, 1985, revised 1996) and was approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University (SNU-160111-2). All experiments were conducted with an effort to minimize the number of animals used and the suffering caused by study procedures.

Cold challenge condition

At 8 weeks of age, mice were divided into four groups (n = 10 in each group): 1 week control group (CON Cd 1W), 1 week cold challenge group (Cd 1W), 4 weeks control group (CON Cd 4W), and 4 weeks cold challenge group (Cd 4W), depicted in Figure 1. Cold challenge was conducted by maintaining the cage at 6 ± 2°C, while control mice were kept at 20 ± 2°C during experimental periods(Figure 1).

CL 316,243 administration

At 8 weeks of age, mice were divided into four groups (n = 10 in each group); 1 week control group (CON CL 1W), 1 week CL 316,243 treated group (CL 1W), 4 weeks control group (CON CL 4W), and 4 weeks cold challenge group (CL 4W), depicted in Figure 1. To induce beigeing in adipose tissues, the animals received subcutaneous injections of 1 mg/kg CL 316,243 every day during the experimental period. The same volume of saline was subcutaneously injected into mice for the control group during the experimental period (Figure 1).

Labeling of newly generated cells

To label newly generated cells in the hippocampus, intraperitoneal injections of 5-bromo-2'-deoxyuridine (BrdU; 50 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) were administered to mice, except polymerase chain reaction (PCR) experimental animals.

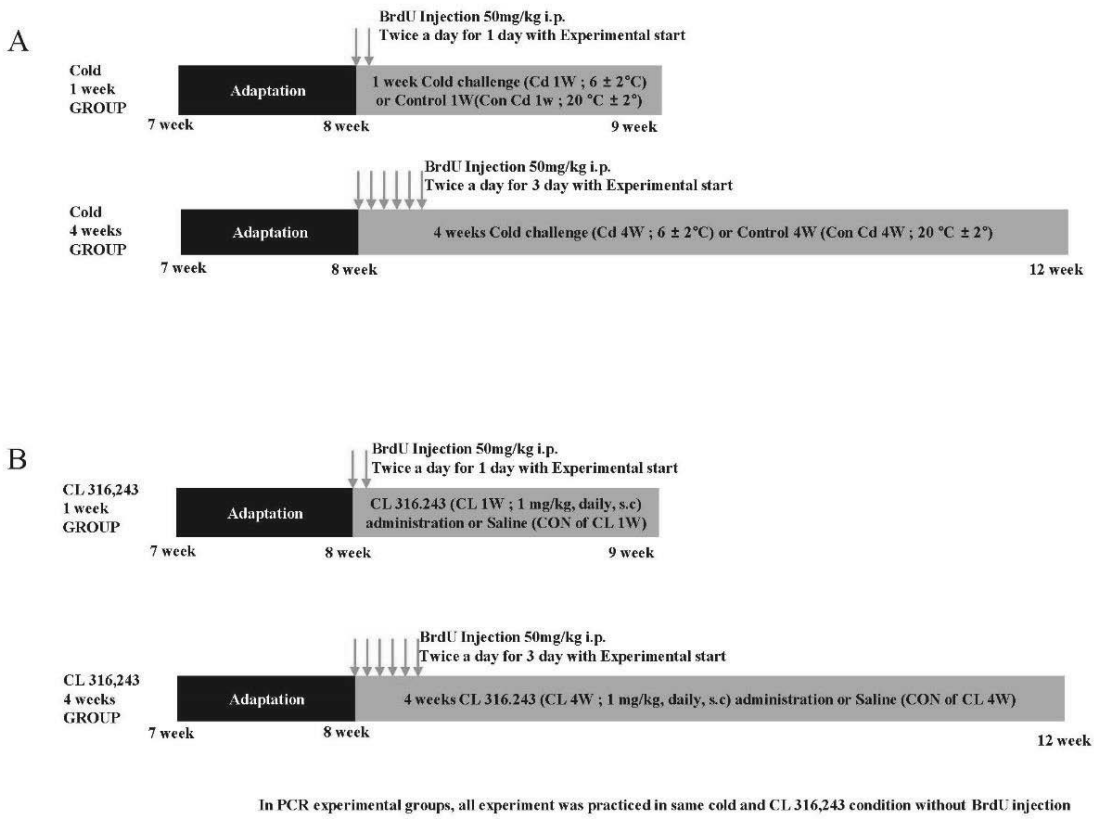

Figure. 1. Schematic demonstration of the experimental design in the present study.

Tissue Preparation

Animals (n = 5 in each group) were anesthetized by intraperitoneal injection of 1 g/kg urethane (Sigma-Aldrich), and perfused transcardially with 0.1 M phosphate-buffered saline (PBS; pH 7.4), followed by 4% paraformaldehyde in 0.1 M PBS. The brains were dissected out and post fixed in the same fixative for 12 h. The brain tissues were cryoprotected by infiltration with 30% sucrose overnight. Thirty micrometer thick brain sections were serially sectioned in the coronal plane using a cryostat (Leica, Wetzlar, Germany) and collected in six-well plates containing PBS at -20°C for further processing.

Mouse BAT, iWAT, and epididymal WAT (eWAT) were dissected and fixed for 48 h in 4% paraformaldehyde. The fixed adipose tissues were dehydrated with graded concentrations of alcohol for embedding in paraffin. Paraffin-embedded tissues were sectioned on a microtome (Leica, Wetzlar, Germany) into 3 μm coronal sections and mounted onto silane-coated slides.

Hematoxylin and eosin staining

Paraffin sections were dewaxed with xylene, hydrated with alcohol, immersed in Mayer's hematoxylin for 5 min, and washed in running tap water for 3 min. Sections were immersed in eosin for 30 s with agitation, washed excess dye, and differentiated the sections in running tap water for 30 s. Thereafter, the sections were dehydrated with serial alcohols, cleared with xylene, and mounted in Canada balsam (Kanto Chemical, Tokyo, Japan).

Immunohistochemistry

To obtain accurate data for immunohistochemistry, the free-floating sections from all animals were processed carefully under the same conditions. For each animal, tissue sections were selected between 1.46 mm and 2.46 mm posterior to the bregma by referring to the mouse atlas by Franklin and Paxinos (Franklin and Paxinos, 2013). Five sections, 180 μm apart, were sequentially treated with 0.3% hydrogen peroxide in PBS for 30 min and 10% normal goat or rabbit serum in 0.05 M PBS for 30 min. They were then incubated with a rabbit anti-Ki67 antibody (1:1,000; Abcam, Cambridge, UK) or goat anti-doublecortin (DCX) antibody (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) overnight at 25°C and subsequently treated with either a biotinylated goat anti-rabbit IgG or rabbit anti-goat IgG, and a streptavidin-peroxidase complex (1:200; Vector Labs, Burlingame, CA, USA). Sections were visualized by reaction with 3,3′-diaminobenzidine tetrachloride (Sigma) in 0.1 M Tris-HCl buffer (pH 7.2) and dehydrated and mounted in Canada balsam (Kanto Chemical) onto gelatin-coated slides.

Immunofluorescence

For TBR-2 immunofluorescence, sections were incubated with a rabbit anti-TBR-2 antibody (1:200; Millipore, Temecula, CA, USA) for 2 h at 25°C, followed by overnight incubation at 4°C. After washing with PBS, the sections were subsequently incubated with Cy3-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch, PA, USA) for 2 h. After, slides were mounted with DAPI-containing mounting medium (Vector Labs, Burlingame, CA, USA) for nuclei staining.

For BrdU and neuronal nuclei (NeuN) double immunofluorescence, the sections were treated with 2 M HCl for 30 min at 37°C and were incubated with a mixture of mouse anti-NeuN (1:1000; Millipore) and rat anti-BrdU (1:200; Abd Serotec, Bio-Rad Laboratories, Inc., Grand Island, NY, USA), or a mixture of goat anti-DCX (1:25; Santa Cruz Biotechnology) and rat anti-BrdU (1:200; Abd Serotec) for 2 h at 25°C, followed by overnight incubation at 4°C. After washing with PBS, the sections were subsequently incubated in a mixture of FITC-conjugated goat anti-mouse IgG (1:100; Jackson ImmunoResearch) and Cy3-conjugated goat anti-rat IgG (1:100; Jackson ImmunoResearch), or FITC-conjugated rabbit anti-goat IgG (1:100; Vector Labs) and Cy3-conjugated rabbit anti-rat IgG (1:100; Vector Labs) for 2 h. Thereafter, the sections were mounted in a DAPI-containing mounting medium (Vector Labs, Burlingame, CA, USA) onto gelatin-coated slides for nuclei staining.

For UCP-1 immunofluorescence in the fat tissues, the sections were hydrated and treated with citrate buffer for 30 min at 37°C. They were then incubated with a rabbit anti-UCP-1 (1:1000; Millipore) for 2 h at 25°C, followed by overnight incubation at 4°C. After washing with PBS, the sections were subsequently incubated with Cy3-conjugated goat anti-rabbit IgG (1:100; Jackson ImmunoResearch) for 2 h. Thereafter, the slides were mounted with a DAPI-containing mounting medium (Vector Labs, Burlingame, CA, USA) for nuclei staining.

Microscopic analysis

Two independent, blinded investigators counted the number of TBR-2, Ki67, DCX,

Ki67/BrdU, DCX/BrdU, or BrdU/NeuN-labeled cells in the DG at 400× magnification under a light microscope (BX51, Olympus, Tokyo, Japan). All TBR-2, Ki67, DCX, Ki67/BrdU, DCX/BrdU, or BrdU/NeuN-labeled cells were counted bilaterally in five sections (180 μm apart from each other) across the entire DG between 1.46 mm and 2.46 mm posterior to the bregma, by referring to the mouse atlas by Franklin and Paxinos (Franklin and Paxinos, 2013).

Quantitative real-time PCR

Animals (n = 5 in each group) were euthanized by an intraperitoneal injection of 2 g/kg urethane (Sigma-Aldrich), and the dorsal hippocampus was quickly dissected from the brain. Total RNA from the dorsal hippocampus was extracted using an RNA purification system (Invitrogen), following the manufacturer's protocol. mRNA was reverse-transcribed using AccuPower CycleScript RT PreMix® (Bioneer, Daejeon, Korea), and a quantitative PCR (qPCR) was performed using SYBR Green and an ABI StepOne Real-Time PCR® instrument (Applied Biosystems, Cheshire, UK). Target gene expression was normalized to that of the control gene (36B4), and relative expression was quantified by the comparative Ct method (ΔΔCt). The primers used for PCR are listed in Table 1. To identify changes in gene expression responding to cold challenge and CL 316,243 administration, the fold change method (1.3 fold as a cutoff value) was used. Genes with fold changes of ≥1.3 were thought to be significantly disturbed in response to cold challenge and CL 316,243 administration and were selected for analysis.

Table 1. Primers used for qRT-PCR analysis

		SEQUENCE (5' --> 3')		SEQUENCE (5' --> 3')
Ache	F	CTCCCTGGTATCCCCTGCATA	R	GGATGCCCAGAAAAGCTGAGA
Ascl-1	F	GCAACCGGGTCAAGTTGGT	R	GTCGTTGGAGTAGTTGGGGG
Adrb1	F	CTCATCGTGGTGGGTAACGTG	R	ACACACAGCACATCTACCGAA
Adrb2	F	GGGAACGACAGCGACTTCTT	R	GCCAGGACGATAACCGACAT
Adrb3	F	GGCCCTCTCTAGTTCCCAG	R	TAGCCATCAAACCTGTTGAGC
Bdnf	F	TCATACTTCGGTTGCATGAAGG	R	AGACCTCTCGAACCTGCCC
Bmp2	F	GGGACCCGCTGTCTTCTAGT	R	TCAACTCAAATTCGCTGAGGAC
Bmp4	F	TTCCTGGTAACCGAATGCTGA	R	CCTGAATCTCGGCGACTTTTT
bmp-8	F	ACATGCAGCGTGAAATCCTG	R	GCGTGGTATAGGTCCAACATGA
Creb1	F	AGCAGCTCATGCAACATCATC	R	AGTCCTTACAGGAAGACTGAACT
Chrm2	F	TGGTTTGGCTATTACCAGTCCT	R	CTGAAGGTGGCGGTTGACTT
Drd2	F	ACCTGTCCTGGTACGATGATG	R	GCATGGCATAGTAGTTGTAGTGG
Erbb2	F	GAGACAGAGCTAAGGAAGCTGA	R	ACGGGGATTTTCACGTTCTCC
Fgf2	F	GCGACCCACACGTCAAACTA	R	TCCCTTGATAGACACAACTCCTC
Grin1	F	AGAGCCCGACCCTAAAAAGAA	R	CCCTCCTCCCTCTCAATAGC
Hdac4	F	CTGCAAGTGGCCCCTACAG	R	CTGCTCATGTTGACGCTGGA
Hes1	F	CCAGCCAGTGTCAACACGA	R	AATGCCGGGAGCTATCTTTCT
Heyl	F	CAGCCCTTCGCAGATGCAA	R	CCAATCGTCGCAATTCAGAAAG
Mdk	F	GAAGAAGGCGCGGTACAATG	R	GAGGTGCAGGGCTTAGTCA
Neurod1	F	ATGACCAAATCATACAGCGAGAG	R	TCTGCCTCGTGTTCCTCGT
Neurog1	F	CCAGCGACACTGAGTCCTG	R	CGGGCCATAGGTGAAGTCTT
Neurog2	F	AACTCCACGTCCCCATACAG	R	GAGGCGCATAACGATGCTTCT
Notch1	F	GATGGCCTCAATGGGTACAAG	R	TCGTTGTTGTTGATGTCACAGT
Nrcam	F	GGAAGTCGAACACCTTCAGAC	R	AGTTCATTGCGTTGACAGGAG
Nrg1	F	ATGGAGATTTATCCCCCAGACA	R	GTTGAGGCACCCTCTGAGAC
Ntf3	F	GGAGTTTGCCGGAAGACTCTC	R	GGGTGCTCTGGTAATTTTCCTTA
Olig2	F	TCCCCAGAACCCGATGATCTT	R	CGTGGACGAGGACACAGTC
Pax3	F	CCGGGGCAGAATTACCCAC	R	GCCGTTGATAAATACTCCTCCG
Pax5	F	CCATCAGGACAGGACATGGAG	R	GGCAAGTTCCACTATCCTTTGG
Pax6	F	TACCAGTGTCTACCAGCCAAT	R	TGCACGAGTATGAGGAGGTCT
Pou4f1	F	CGCGCAGCGTGAGAAAATG	R	CGGGGTTGTACGGCAAAATAG
S100b	F	TGGTTGCCCTCATTGATGTCT	R	CCCATCCCCATCTTCGTCC
Sod1	F	AACCAGTTGTGTTGTCAGGAC	R	CCACCATGTTTCTTAGAGTGAGG
Sox2	F	GCGGAGTGGAAACTTTTGTCC	R	CGGGAAGCGTGTACTTATCCTT
Tgfb1	F	CTCCCGTGGCTTCTAGTGC	R	GCCTTAGTTTGGACAGGATCTG
36B4	F	CGACCTGGAAGTCCAACTAC	R	ATCTGCTGCATCTGCTTG

Statistical analysis

Statistical analyses were performed using SPSS version 20.1 (IBM Corporation, Armonk, NY, USA). Experimental groups were compared using Student's t-test.

RESULTS

Effects of cold challenge on body weight and body weight gain

At 8 weeks of age, the animals in each group showed similar body weight (Fig. 2A and 2B). Body weight tended to increase with age in all groups, but body weight showed less increase in Cd 1W and Cd 4W groups compared to that in each control group, respectively; however, statistical significance was not detected between groups (Fig. 2A and 2B). In contrast, the body weight gain of Cd 1W and Cd 4W groups were significantly less than those in their respective control groups (Fig. 2C and 2D).

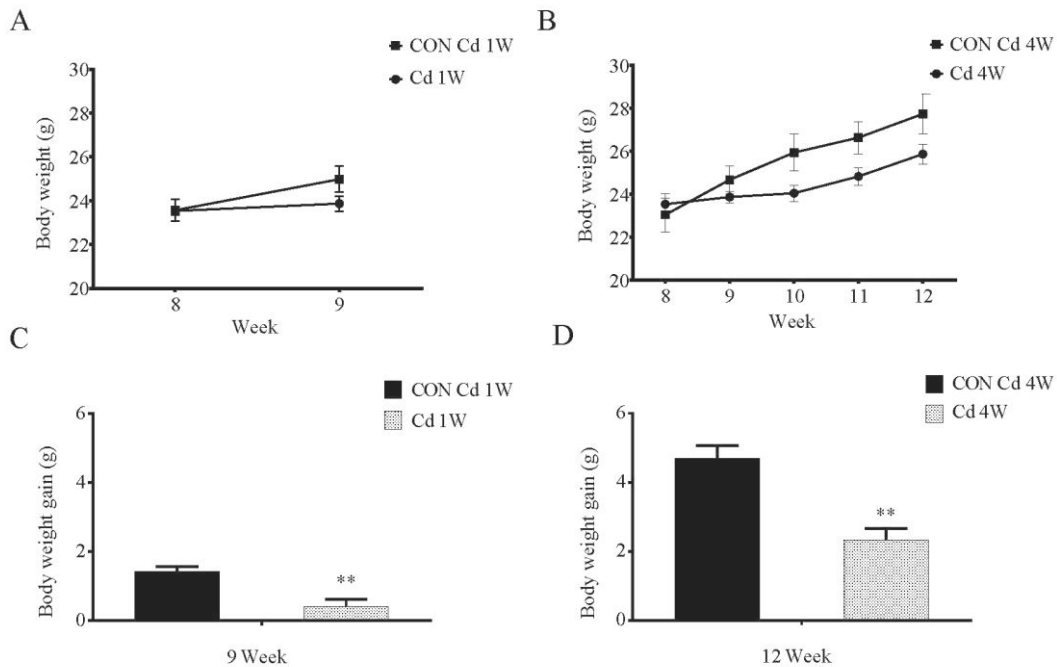

Figure 2. Effects of cold challenge on body weight and body weight gain during experimental periods.

Body weight (A and B) and body weight gain (C and D) after cold challenge for 1 week (A and C) and 4 weeks (B and D) (n = 5 per group). ** indicates a significant difference compared to control group ($p < 0.01$). Data are shown as mean ± standard error of means (SEM).

Effects of cold challenge for 1 week and 4 weeks on beigeing morphological changes in BAT, iWAT, and eWAT

For histological analysis of morphological changes of BAT, iWAT, and eWAT, hematoxylin and eosin staining were performed. In Cd 1W and Cd 4W groups, BAT showed decreases in lipid accumulation compared to each control group, respectively (Fig. 3). In addition, iWAT in Cd 1W and Cd 4W groups showed beigeing morphology with decreased lipid accumulation compared to respective control groups. However, eWAT did not show remarkable morphological changes of beigeing, although lipid accumulation was decreased in eWAT of Cd 1W and Cd 4W groups (Fig. 3).

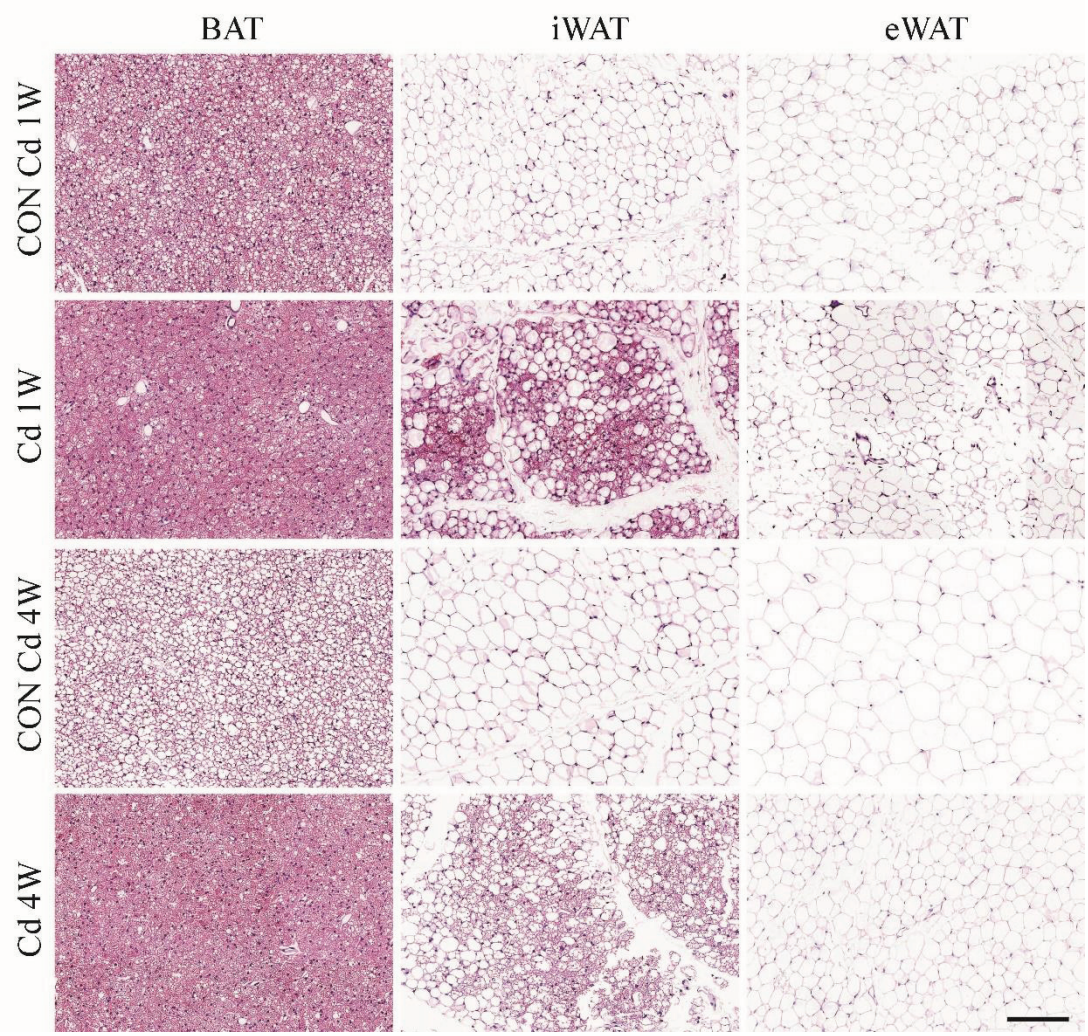

Figure 3. Effects of cold challenge for 1 week and 4 weeks on the morphology of BAT, iWAT, and eWAT.

Representative images of hematoxylin and eosin staining of BAT, iWAT, and eWAT, after 1 week and 4 weeks cold challenge. Scale bar = 100 μm.

Effects of cold challenge for 1 week and 4 weeks on UCP-1 expression in BAT, iWAT, and eWAT

For confirmation of beigeing effects after cold challenge in BAT, iWAT, and eWAT, UCP-1 immunofluorescent staining was conducted after cold challenge for 1 and 4 weeks. In CON Cd 1W and CON Cd 4W groups, UCP-1 immunoreactivity was observed in BAT and was similarly detected in BAT of Cd 1W and Cd 4W groups. In iWAT, UCP-1 immunoreactivity in the CON Cd 1W and CON Cd 4W groups was diffusely found in the cytoplasm of adipocytes, and this distribution pattern was similarly observed in eWAT of Cd 1W and Cd 4W groups. However, in the Cd 1W and Cd 4W groups, UCP-1 immunoreactivity was aggregated and prominent in iWAT and showed similar morphology to BeAT (Fig. 4).

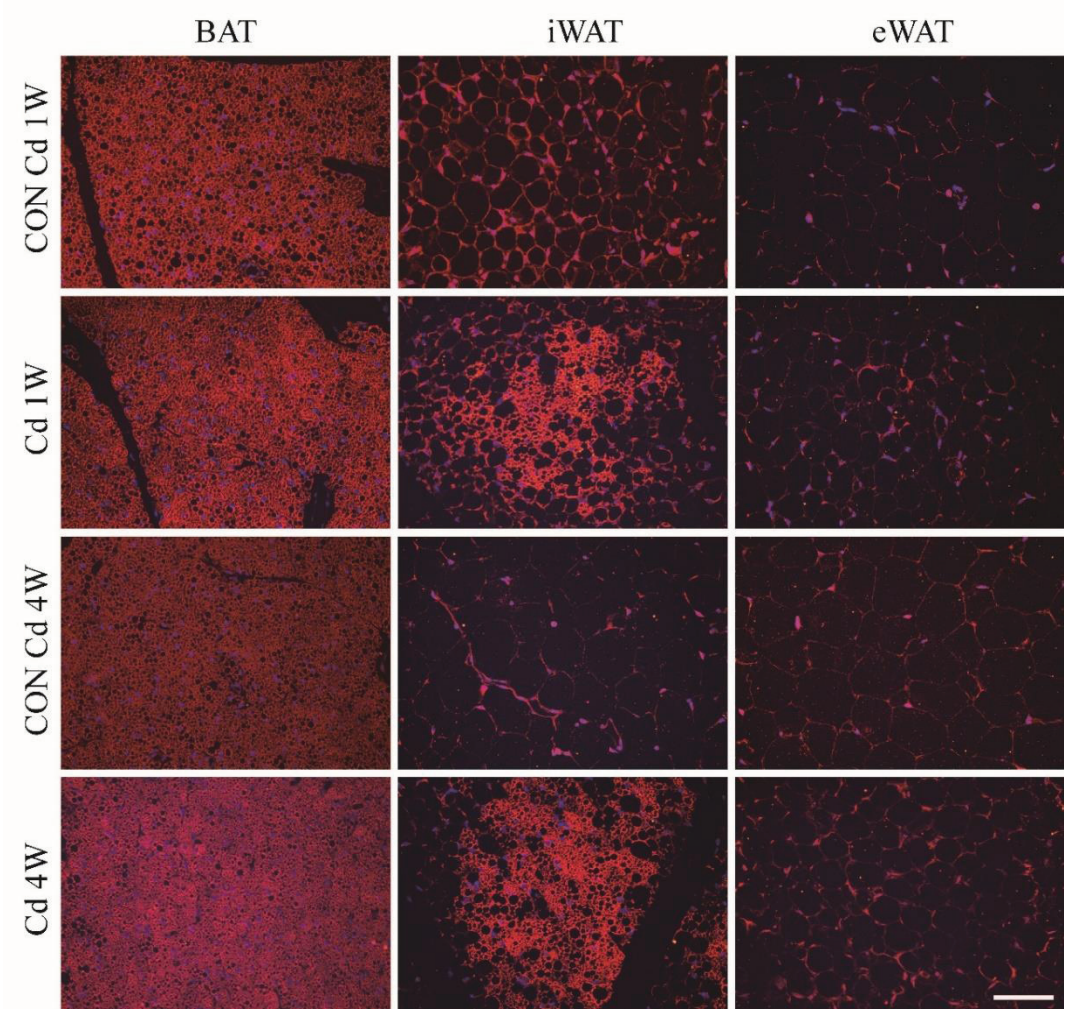

Figure 4. Effects of cold challenge for 1 and 4 weeks on UCP-1 immunofluorescence (red) in BAT, iWAT, and eWAT.

Cold challenge for 1 week and 4 weeks remarkably increased UCP-1 expression in BAT and iWAT, not in eWAT. Scale bar = 100 μm.

Cold challenge for 1 week and 4 weeks increased proliferative NSCs in the hippocampus

We observed proliferative NSCs with Ki-67 immunohistochemical staining in the SGZ of the DG. In all groups, Ki-67 immunoreactivity was found in the SGZ of the DG. In the Cd 1W group, the population of Ki-67-positive cells was significantly increased in the DG compared with the CON Cd 1W group (Fig. 5A). The mean number of Ki-67 positive cells were 52.00 ± 2.30 in CON Cd 1W mice and 79.00 ± 4.52 in Cd 1W mice (Fig. 5B). In addition, cold challenge increased the population of Ki-67 positive cells in the Cd 4W group compared with CON Cd 4W group (Fig. 5C). The mean number of Ki-67 positive cells was 31.00 ± 2.25 and 47.00 ± 1.05 in CON Cd 4W and Cd 4W mice, respectively (Fig. 5D). The increase in percentage of Ki-67 positive cells was 151.92% in the Cd 1W group compared with the CON Cd 1W group, and 151.61% in the Cd 4W group compared with the CON Cd 4W group (Fig. 5B and 5D).

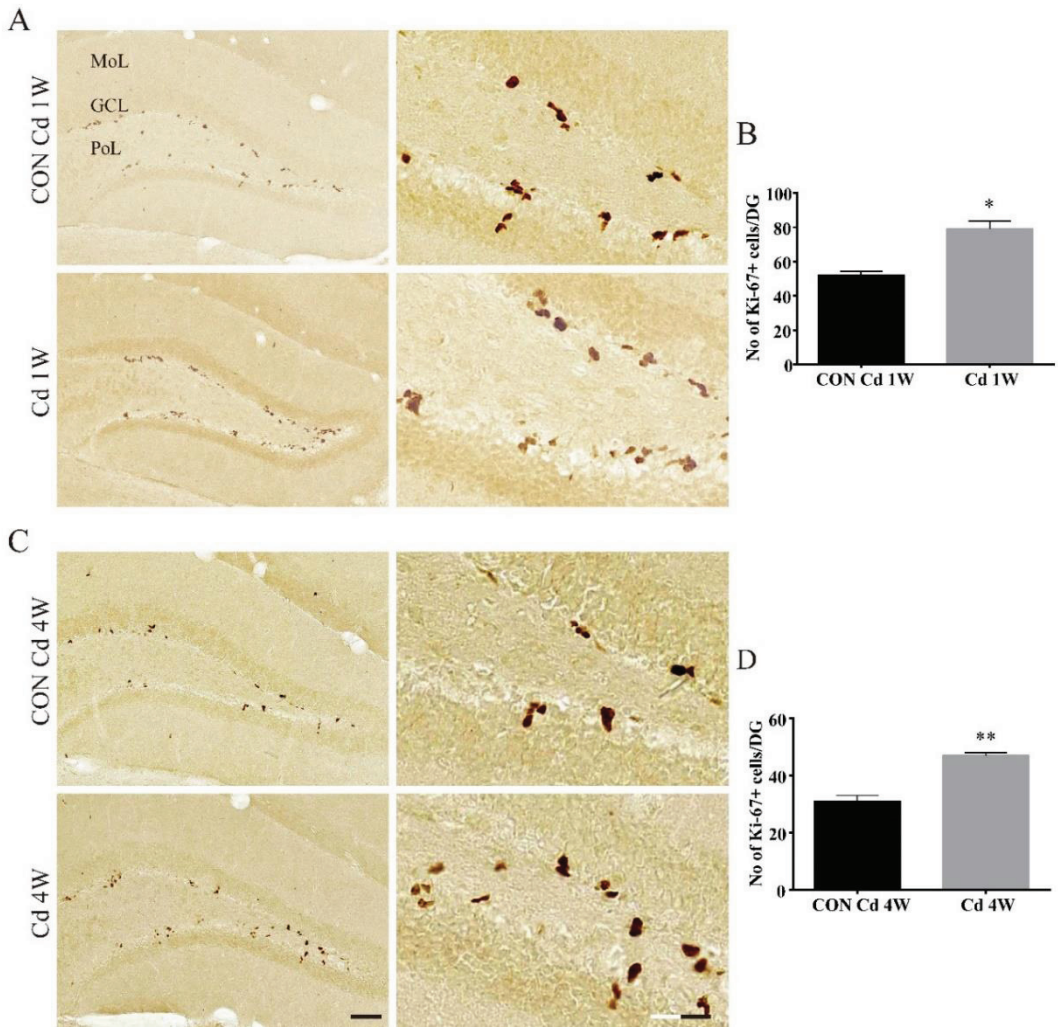

Figure 5. Effects of cold challenge for 1 week and 4 weeks on the Ki-67 positive cells in the DG.

Ki-67 positive cells were abundant in the Cd 1W (A) and Cd 4W (C) groups compared to that in the CON Cd 1W (A) and CON Cd 4W (C) groups, respectively. GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of Ki-67 positive cells per section in the CON Cd 1W and Cd 1W groups (B), as well as the CON

Cd 4W and Cd 4W groups (D) (n = 5 per group); * indicates a significant difference compare to CON ($p < 0.05$); and ** indicates a significant difference compare to CON group ($p < 0.01$). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

Cold challenge for 1 week and 4 weeks increased NPCs in the hippocampus

To analyze correlation between cold challenge and NPCs, population changes of NPCs were observed in the hippocampus with TBR-2. In all groups, TBR-2 immunoreactivity was mainly detected in the SGZ of the DG (Fig. 6A). The mean number of TBR-2 positive cells was significantly increased in the Cd 1W group (44.40 ± 4.11; Fig. 6B) compared to the CON Cd 1W group (26.20 ± 1.53; Fig. 6B). Additionally, there were significantly higher populations of TBR-2 positive cells in the Cd 4W group compared with the CON Cd 4W group (Fig. 6C). The mean number of TBR-2 positive cells was 21.00 ± 1.67 in CON Cd 4W mice and 29.20 ± 1.88 in Cd 4W mice (Fig. 6D). The increase in percentage of TBR-2 positive cells was 151.92% in the Cd 1W group compared with the CON Cd 1W group, and 139.04% in the Cd 4W group compared with the CON Cd 4W group (Fig. 6B and 6D).

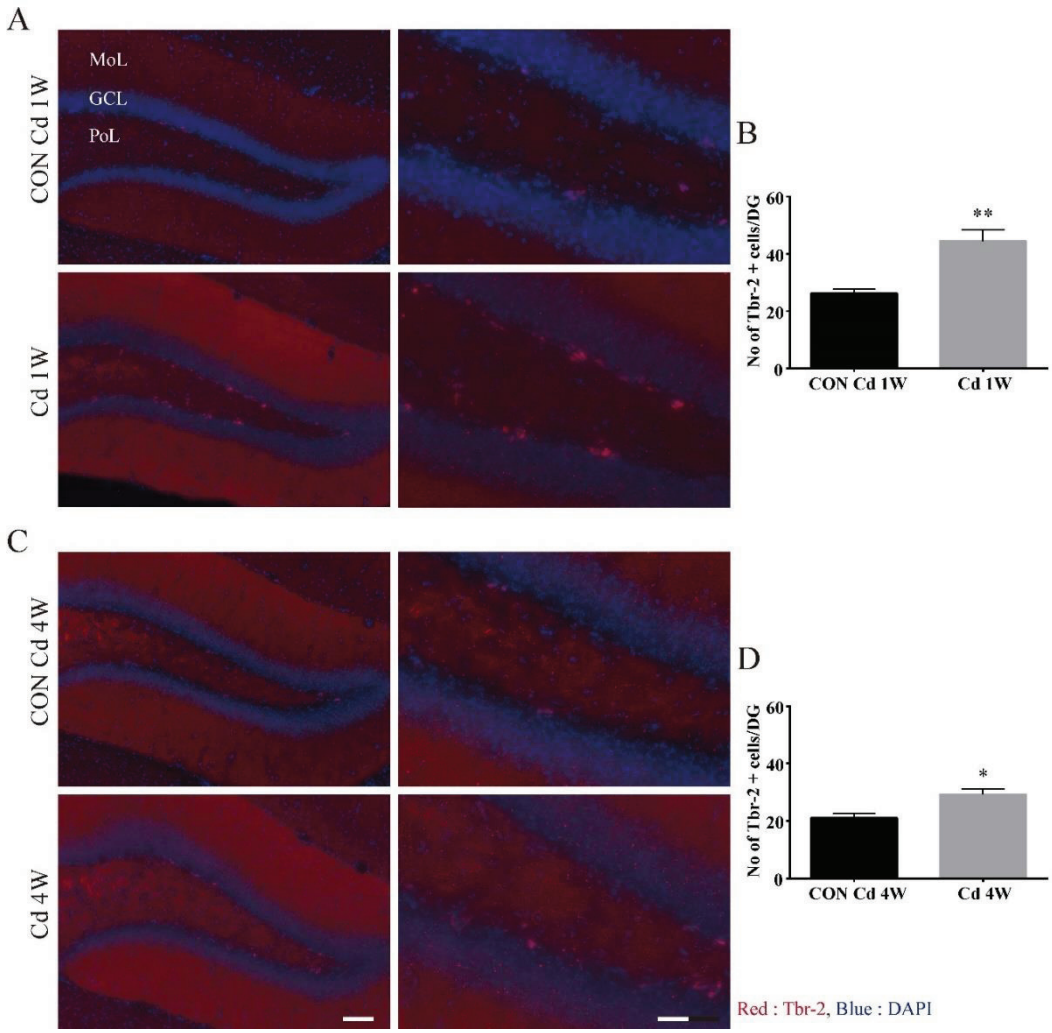

Figure 6. Effects of cold challenge for 1 week and 4 weeks on the Tbr-2 positive cells in the DG.

TBR-2 positive cells are abundant in the DG of Cd 1W (A) and Cd 4W (C) groups compared to that in the Con Cd 1W (A) and Con Cd 4W (C) groups. GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of TBR-2-positive cells per section in CON Cd 1W group and Cd 1W (B) as well as CON Cd 4W and

Cd 4W groups (D) (n = 5 per group); * indicates a significant difference between the CON group ($p < 0.05$); and ** indicates a significant difference between the CON group ($p < 0.01$). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

Cold challenge for 1 week and 4 weeks increased neuroblasts in the hippocampus

To observe differentiated neuroblasts among NSCs, DCX immunohistochemical staining was conducted. In all groups, the cytoplasm of DCX immunoreactive neuroblasts were observed in the SGZ of the DG, and their dendrites extended into the molecular layer of the DG. The Cd 1W group had higher number of DCX immunoreactive neuroblasts and more complex DCX immunoreactive fibers compared with the CON Cd 1W group (Fig. 7A). The mean number of DCX immunoreactive neuroblasts was 162.00 ± 7.07 and 216.40 ± 5.59 in CON Cd 1W and Cd 1W mice, respectively (Fig. 7I). Similarly, the Cd 4W group showed a higher population of DCX immunoreactive neuroblasts in the DG compared with the CON Cd 4W group (Fig. 7C). The mean number of DCX immunoreactive neuroblasts was 129.40 ± 3.75 and 174.00 ± 4.76 in CON Cd 4W and Cd 4W mice, respectively (Fig. 7D). The increase in percentage of DCX immunoreactive neuroblasts was 133.58% in the Cd 1W group compared with the CON Cd 1W group, and 134.47% in the Cd 4W group compared with the CON Cd 4W group (Fig. 7B and 7D).

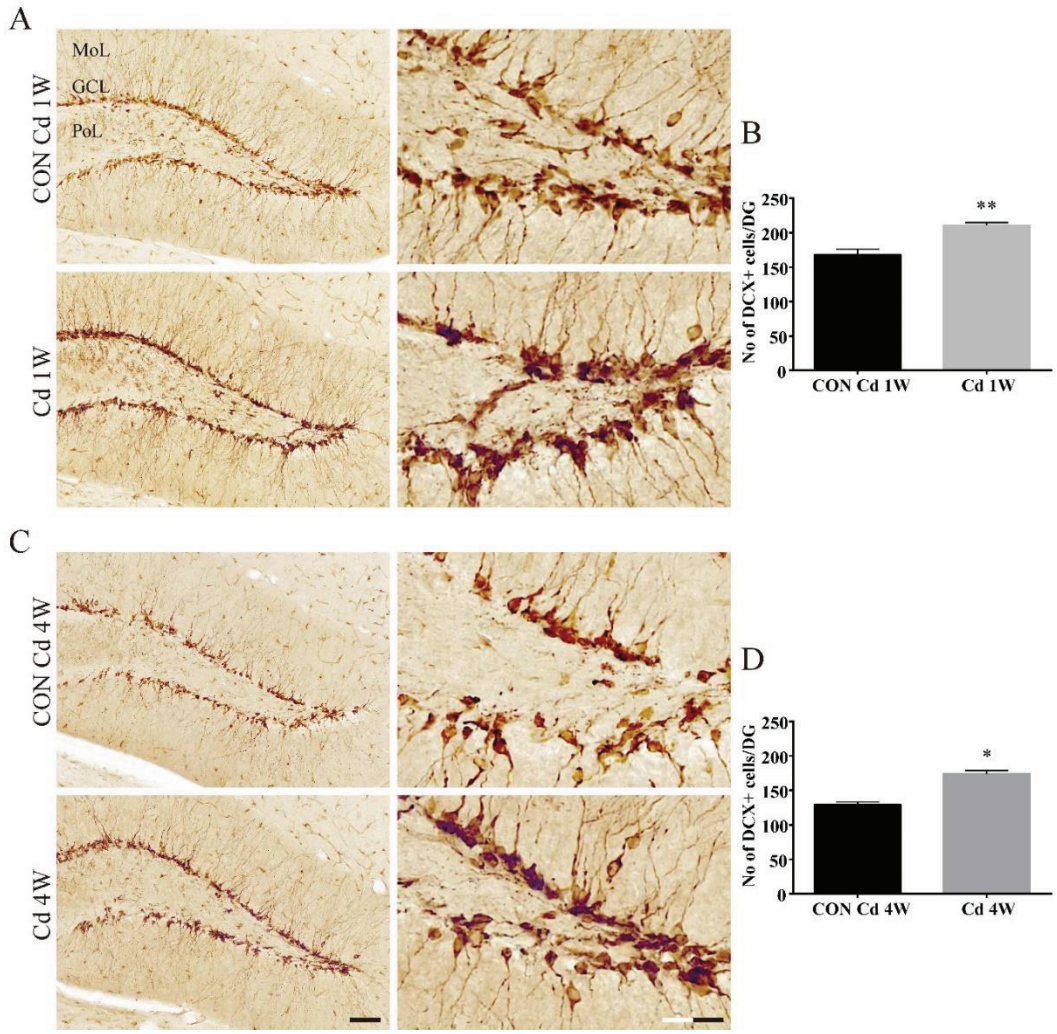

Figure 7. Effects of cold challenge for 1 week and 4 weeks on the DCX immunoreactive neuroblasts in the DG.

In the Cd 1W (A) and Cd 4W (C) groups, DCX immunoreactive neuroblasts were numerous and had complex dendrites compared to that in the CON Cd 1W (A) and CON Cd 4W (C) groups, respectively. GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of DCX immunoreactive neuroblasts per section

in CON Cd 1W, Cd 1W (B), CON Cd 4W and Cd 4W groups (D) (n = 5 per group); * indicates a significant difference compared to CON ($p < 0.05$); and ** indicates a significant difference compared to CON group ($p < 0.01$). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

Cold challenge for 1 week increased integration of newborn into neuroblasts

To elucidate the integration of newly generated cells into neuroblasts, BrdU and DCX double immunofluorescent staining was conducted in the DG. In all groups, BrdU and DCX double positive cells were observed in the SGZ and GCL of the DG. There were different population levels of BrdU and DCX double positive cells in the CdW1 and CON CdW1 groups (Fig. 8C). Mean number of BrdU and DCX double positive cells were 26.20 ± 1.52 and 46.40 ± 2.91 in CON CdW1 and CdW1 mice, respectively (Fig. 8D).

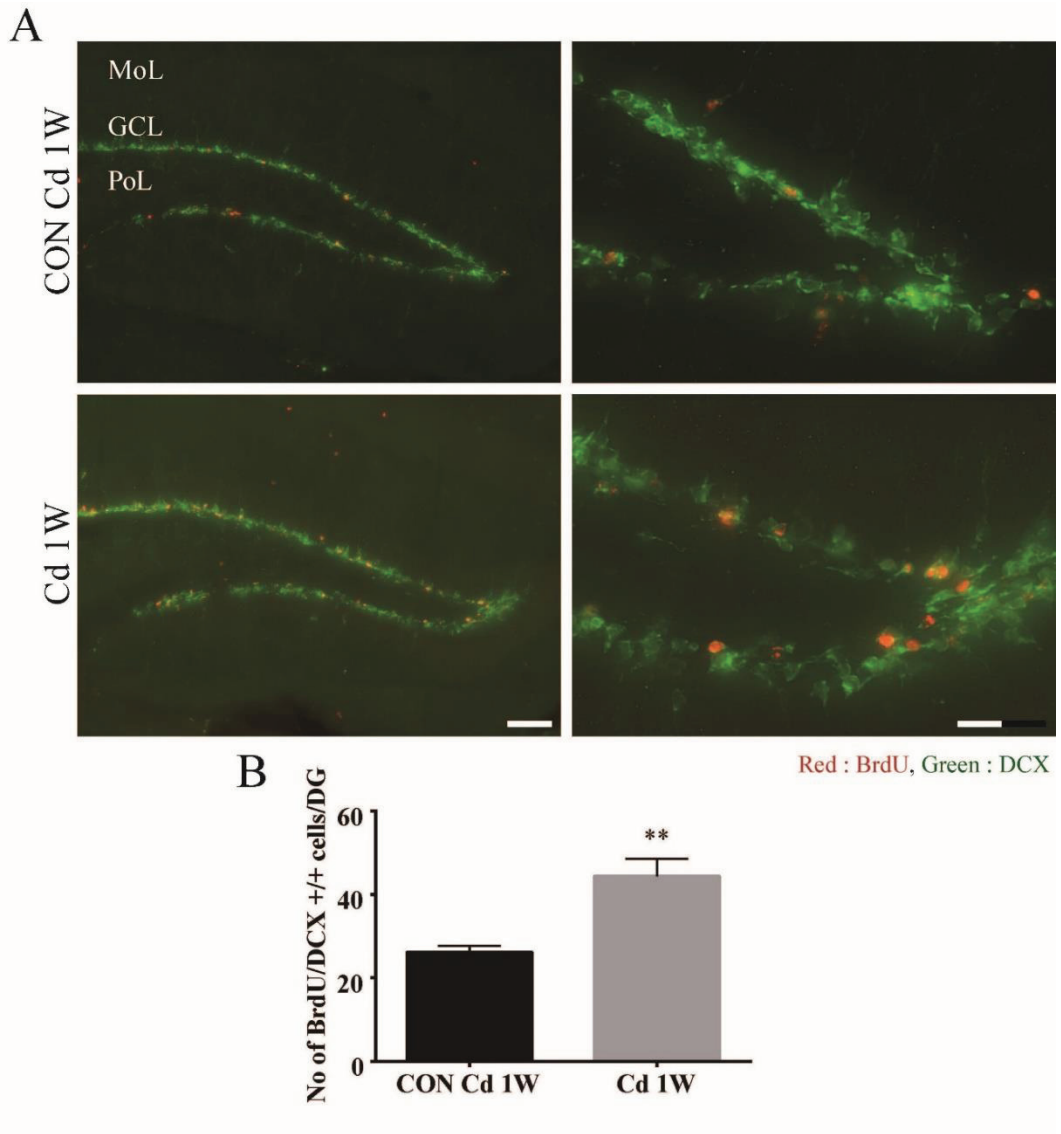

Figure 8. Effects of cold challenge for 1 week on the BrdU (red) and DCX (green) double immunofluorescent neuroblasts in the DG.

BrdU and DCX double positive cells were abundant in the Cd 1W group compared to that in the CON Cd 1W group (A). GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of BrdU/DCX positive cells per section in CON

Cd 1W and Cd 1W groups (B) (n = 5 per group); ** indicates a significant difference compared to CON group ($p < 0.01$). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

Cold challenge for 4 weeks increased integration of newborn cells into mature neurons

To elucidate the integration of newly generated cells into mature neurons, BrdU and NeuN double immunofluorescent staining was conducted. BrdU and NeuN double positive cells were mainly observed in the GCL of the DG (Fig. 9A). Cold challenge for 4 weeks significantly increased the number of BrdU and NeuN double positive cells in the DG of the Cd 4W group compared to that of the CON Cd 4W group. Mean number of BrdU and NeuN double positive cells were 12.20 ± 0.66 and 21.00 ± 2.19 in CON Cd 4W and Cd 4W mice, respectively (Fig. 9B). The increase in the percentage of BrdU and NeuN double-positive cells in the Cd 4W group compared with CON Cd 4W group was 172.13%.

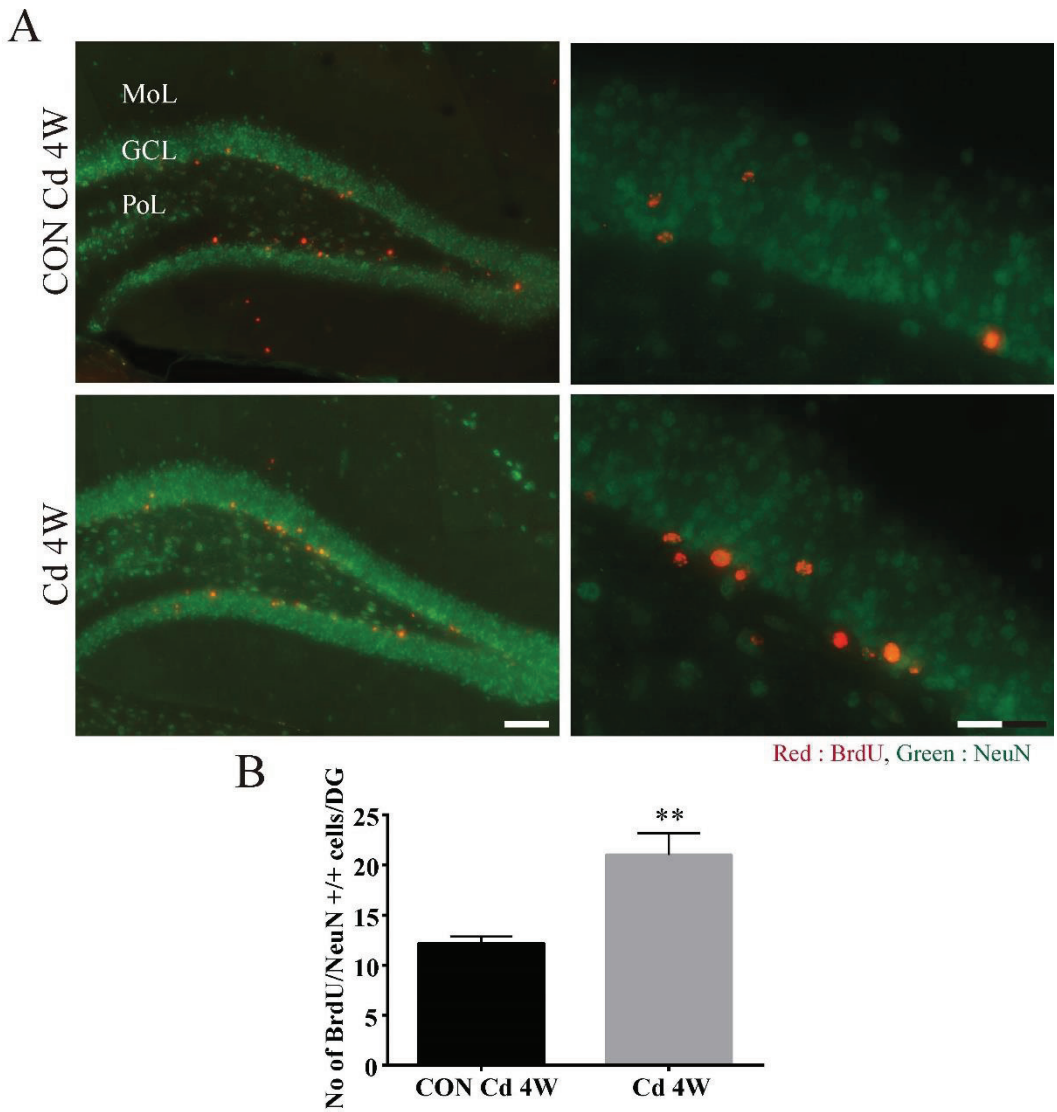

Figure 9. Effects of cold challenge for 4 weeks on the BrdU (red) and NeuN (green) double immunofluorescent mature neurons in the DG. BrdU and NeuN double positive cells were abundantly observed in the Cd 4W group compared to that in the CON Cd 4W group (A). GCL, granule cell layer; MoL, molecular layer; PoL, polymorphic layer.

Quantitative analysis of BrdU and NeuN double positive cells per section in CON Cd 4W and Cd 4W group (B) (n = 5 per group); ** indicates a significant difference compared to CON group ($p < 0.01$). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

Cold challenge for 1 week changes AHN-related gene expression in hippocampus

To identify the effects of cold challenge for 1 week on AHN-related gene expression, real-time PCR was conducted for AHN-related genes. Cold challenge for 1 week increased AHN-related gene expression levels in hippocampal homogenates compared to that in the control group. Cold challenge for 1 week elevated the following genes: early growth response 1 (Egr1) for immediate early response; Achaete-scute homolog 1 (Ascl1) and dopamine receptor D2 (Drd2) for neuronal migration; Ascl1, BDNF, Hes family bHLH transcription factor 1 (Hes1), and neurogenin 2 (Neurog2) for neuronal differentiation; histone deacetylase 4 (Hdac4) and Midkine (Mdk) for cell fate determination and other regulators of cell differentiation; adenosine A1 receptor (Adora1), apolipoprotein E (Apoe), BDNF, Drd2, cAMP responsive element binding protein 1 (Creb1), and fibroblast growth factor 2 (Fgf2) for regulation of synaptic plasticity and synaptic transmission; and amyloid beta A4 precursor protein-binding family B member 1 (Apbb1), Drd2, Erb-B2 receptor tyrosine kinase 2 (Erbb2), and Notch1 for synaptogenesis and axonogenesis (Fig. 10A-10F). A list of mRNAs changed is summarized in Figure 13.

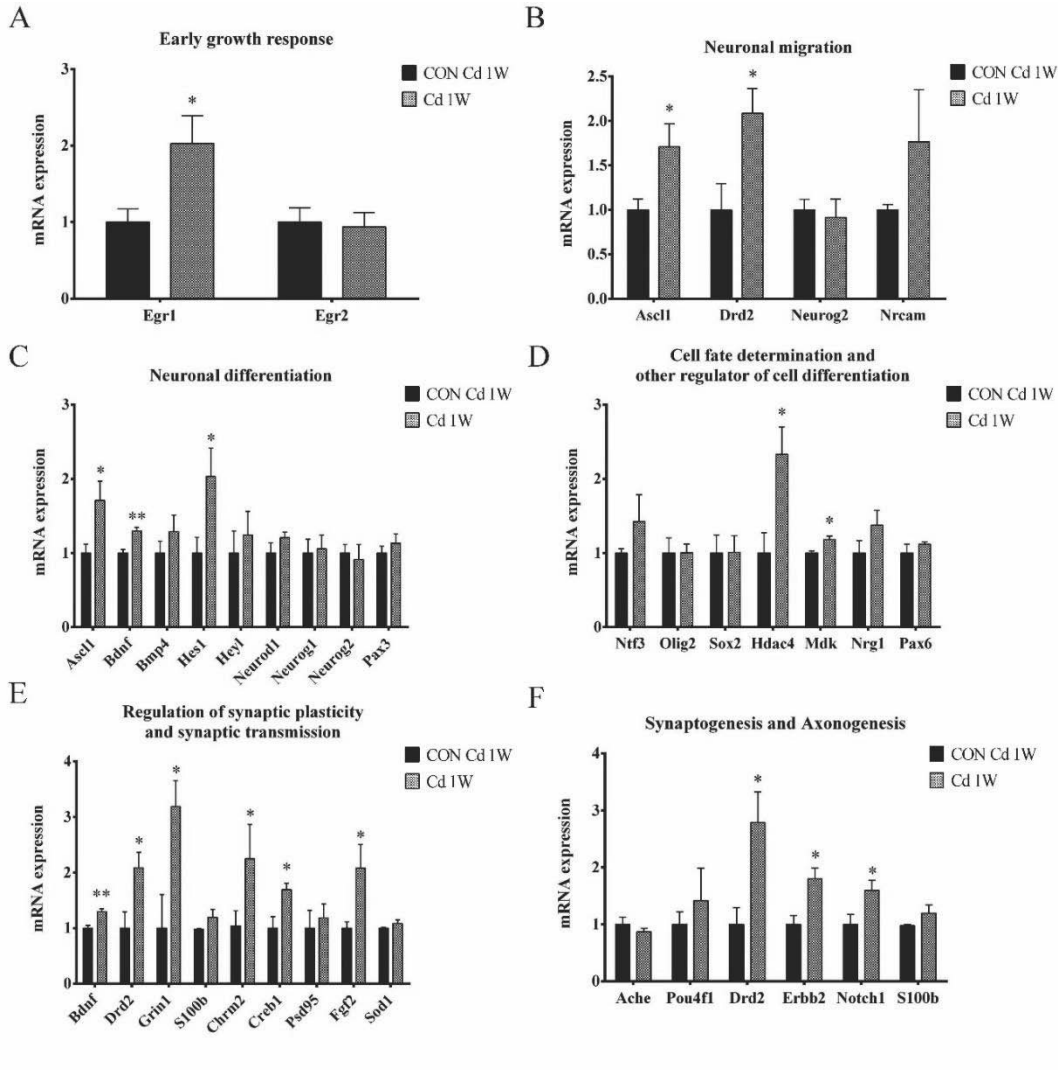

Figure 10. Effects of cold challenge for 1 week on the changes of mRNA expression levels related to AHN.

Cold challenge for 1 week increased several genes related to (A) early growth response, (B) neuronal migration, (C) neuronal differentiation, (D) cell fate determination and other regulator of cell differentiation, (E) regulation of synaptic plasticity and synaptic

transmission, and (F) synaptogenesis and axonogenesis. * indicates a significant difference compared to CON ($p < 0.05$); ** indicates a significant difference compared to CON group ($p < 0.01$). Data are shown as mean ± SEM.

Cold challenge for 4 weeks changes AHN-related gene expression in hippocampus

To identify the effects of cold challenge for 4 weeks on AHN-related gene expression, real-time PCR was conducted for AHN-related genes. Cold challenge for 4 weeks increased AHN-related gene expression levels compared to that in the CON Cd 4W group. Cold challenge for 4 weeks elevated gene expressions in the following: Egr1; Ascl1 and Drd2; Ascl1, BDNF, Hairy/enhancer-of-split related with YRPW motif-like protein (Heyl), neurogenic differentiation 1 (Neurod1) and paired box gene 3 (Pax3) for neuronal differentiation; neurotrophin 3 (Ntf3), Hdac4, and Neuregulin 1 (Nrg1) for cell fate determination and other regulators of cell differentiation; Adora1, Apoe, BDNF, Drd2, Grin1, and cholinergic receptor muscarinic 2 (Chrm2) for regulation of synaptic plasticity and synaptic transmission; and Apbb1, Drd2, Erbb2, and Notch1 (Fig. 11A-11F). A list of mRNAs changed is summarized in Figure 13.

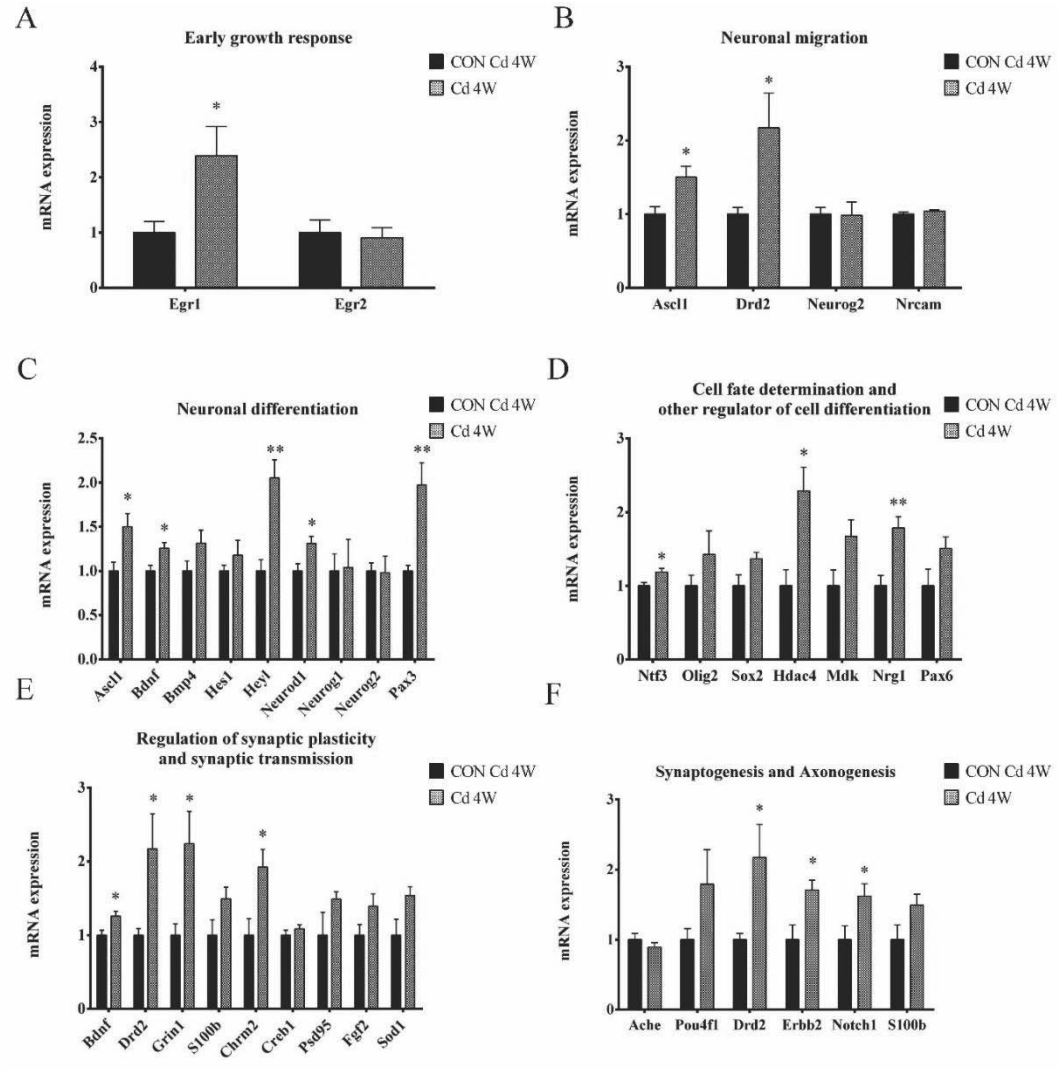

Figure 11. Effects of cold challenge for 4 weeks on the changes of mRNA expression levels related to AHN.

Cold challenge for 4 week increases several genes related to (A) early growth response, (B) neuronal migration, (C) neuronal differentiation, (D) cell fate determination and other regulator of cell differentiation, (E) regulation of synaptic plasticity and synaptic

transmission, and (F) synaptogenesis and axonogenesis. * indicates a significant difference compared to CON ($p < 0.05$); and ** indicates a significant difference compared to CON group ($p < 0.01$). Data are shown as mean ± SEM.

Cold challenge for 1 week and 4 weeks changes AHN-related signal transduction gene and β-adrenergic receptor expression in hippocampus

To elucidate the effects of cold challenge for 1 and 4 weeks on AHN-related signal transduction gene and β-adrenergic receptor expression, real-time PCR was conducted. AHN-related signal transduction genes were categorized into three subgroups: notch, transforming growth factor-β (Tgf-β), and G protein-coupled receptor (GPCR) signaling. Cold challenge for 1 week increased gene expressions of the following: Ascl1, Hes1, and Notch1 expression for notch signaling; Tgf-β in Tgf-β signaling; and Beta1-adrenergic receptor (Adbr1) for β-adrenergic receptor expression. Adora1, Chrm2, and Drd2 for GPCR signaling showed increased patterns in Cd 1W and Cd 4W groups compared to control groups, respectively. However, statistical significance was not detected between CON Cd 1W and Cd 1W groups or between CON Cd 4W and Cd 4W groups (Fig. 12A and 12B).

Cold challenge for 4 weeks increased the following: Ascl1, Heyl, Notch1, and Nrg1 expression for notch signaling; Adora1, Chrm2, and Drd2 in GPCR signaling; and Adbr1 for β-adrenergic receptor expression compared to that of the CON Cd 4W group (Fig. 12C and 12D). A list of mRNAs changed is summarized in Figure 13.

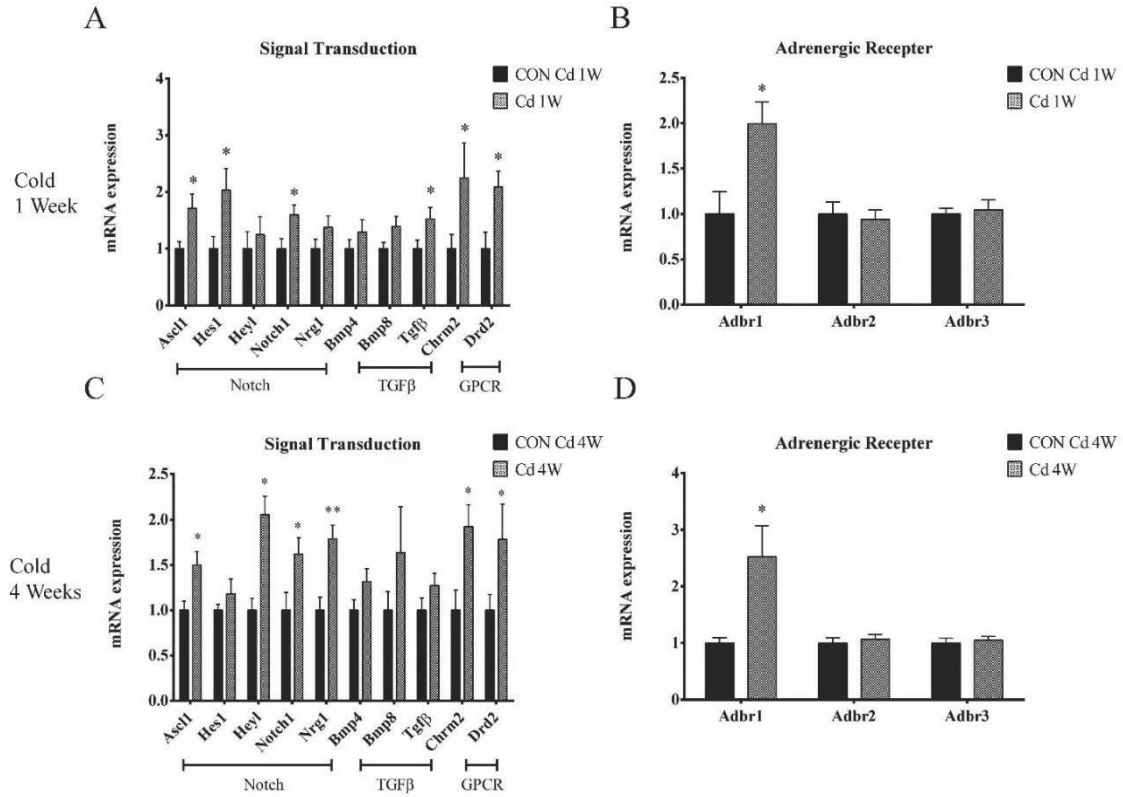

Figure 12. Effects of cold challenge for 1 week and 4 weeks on the changes of mRNA expression levels related AHN-related signal transduction and β-adrenergic receptor. Cold challenge for 1 week (A and B) and 4 weeks (C and D) increased several mRNA expressions related to signaling transduction (A and C)and β-adrenergic beta receptors (B and D).

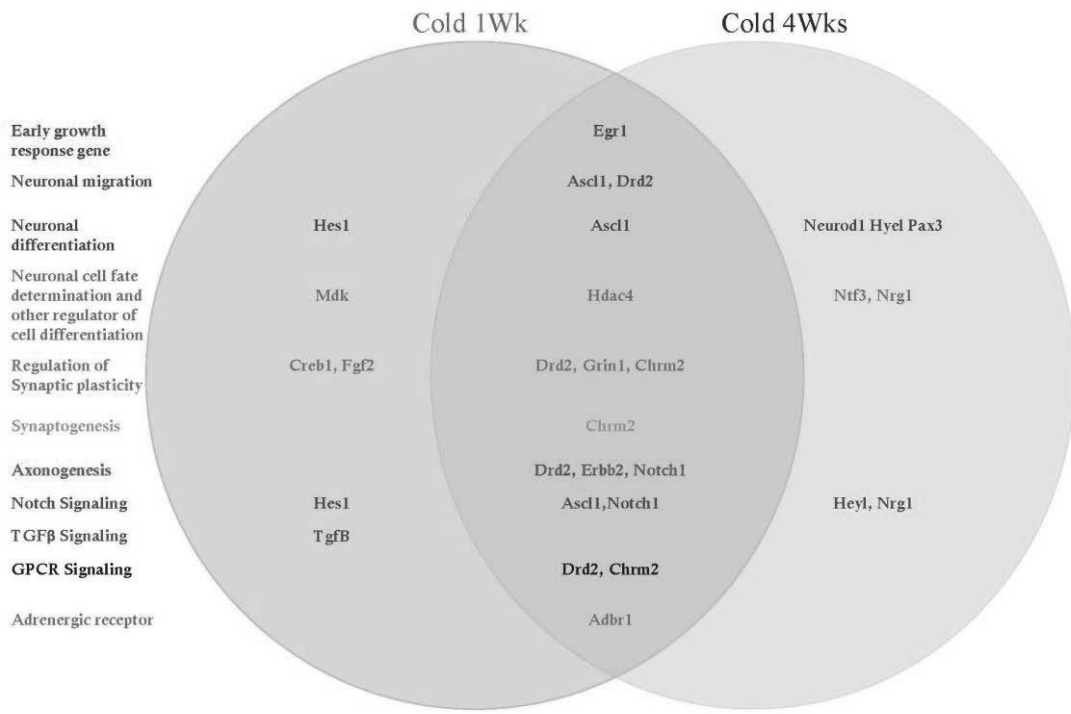

Figure 13. List of mRNAs changed significantly after cold challenge for 1 week and 4 weeks in the hippocampus.

Effects of CL 316,243 administrations on body weight and body weight gain

In all groups, body weight was similarly observed at 8 weeks of age (Fig. 14A and 14B). Body weight increased with age, but there were differences in body weight and body weight gain between the CON CL and CL groups. Treatment of CL 316,243 for 1 and 4 weeks showed less increases in body weight and body weight gain compared with respective control groups. Body weight gain showed significant differences between CON CL and CL groups with 1 and 4 weeks of treatment, although statistical significance was not detected (Fig. 14C and 14D).

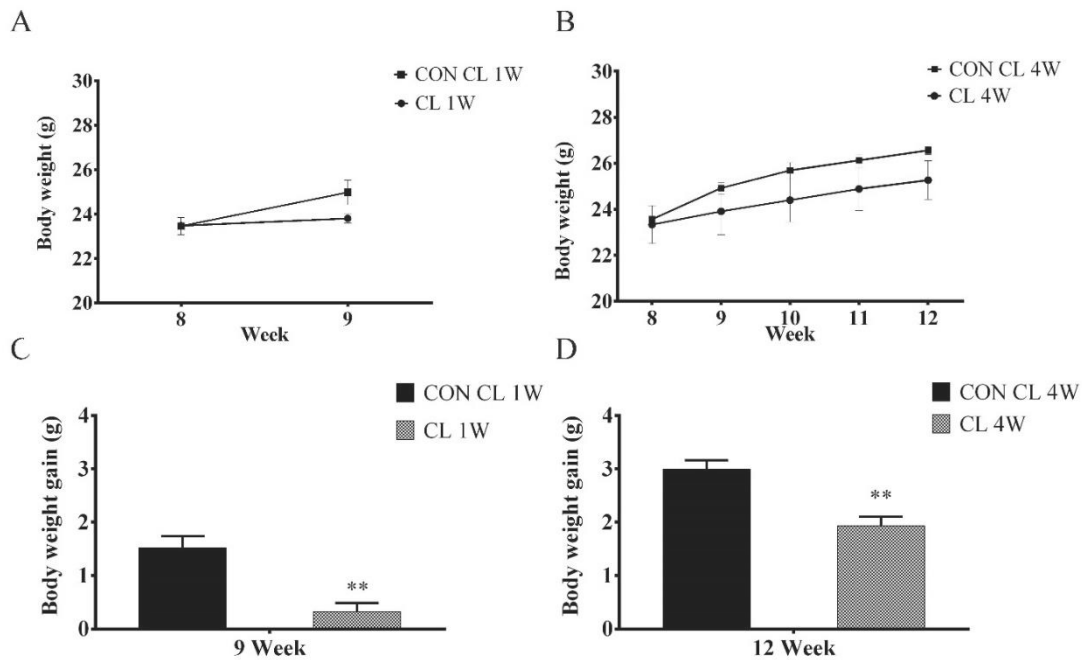

Figure 14. Effects of CL 316,243 on body weight and body weight gains during experimental periods.

Body weight (A and B) and body weight gain (C and D) was changed with age and CL 316,243 treatment, but the body weight and body weight gain were less increased in the CL group compared to those in the CON CL group (n = 5 per group). ** indicates a significant difference compared to control group ($p < 0.01$). Data are shown as mean ± SEM.

Effects of CL 316,243 for 1 week and 4 weeks on beigeing morphological changes in BAT, iWAT and eWAT

To elucidate the morphological changes of BAT, iWAT, and eWAT after CL 316,243 treatment, hematoxylin and eosin staining was performed. In CL 1W and CL 4W groups, lipid accumulation was decreased in BAT compared to control groups (Fig. 15). In addition, in CL 1W and CL 4W groups, iWAT and eWAT showed decreases in lipid accumulation, as well as beigeing morphological changes compared to their respective control groups (Fig. 15).

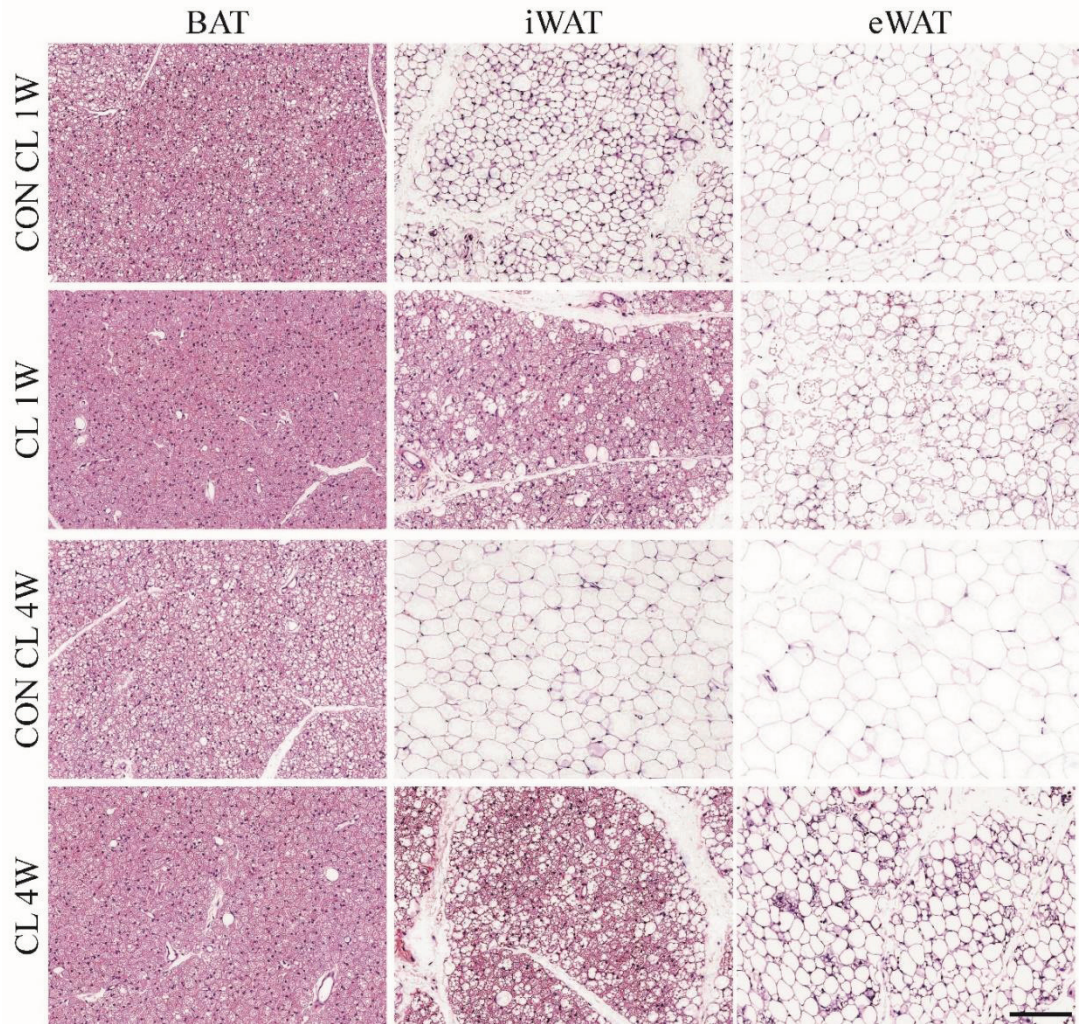

Figure 15. Effects of CL 316,243 for 1 week and 4 weeks on the morphology of BAT, iWAT, and eWAT.

In CL 1W and CL 4W groups, lipid accumulation is increased in BAT compared to that in the CON CL 1W and CON CL 4W group, respectively. In addition, beigeing adipocytes were found in iWAT and eWAT of CL 1W and CL 4W groups. Scale bar = 100 μm.

Effects of CL 316,243 for 1 week and 4 weeks on UCP-1 expression in BAT, iWAT and eWAT

To confirm the beigeing effects of CL 316,243 on BAT, iWAT, and eWAT, UCP-1 immunofluorescent staining was conducted after CL 316,243 treatment for 1 and 4 weeks. In CL 1W and CL 4W groups, UCP-1 immunoreactivity was strongly detected in BAT compared to those in the control groups, respectively. UCP-1 immunoreactivity in iWAT and eWAT of CON CL 1W and CON CL 4W groups were detected in the cytoplasm of adipocytes, which have a small cytoplasm in the periphery. However, in the CL 1W and CL 4W groups, UCP-1 immunoreactive structures were abundantly observed in iWAT and eWAT compared to those in the controls groups. UCP-1 immunoreactivity was most abundant in the iWAT of CL 1W and CL 4W groups compared to other control groups, or eWAT and BAT (Fig. 16).

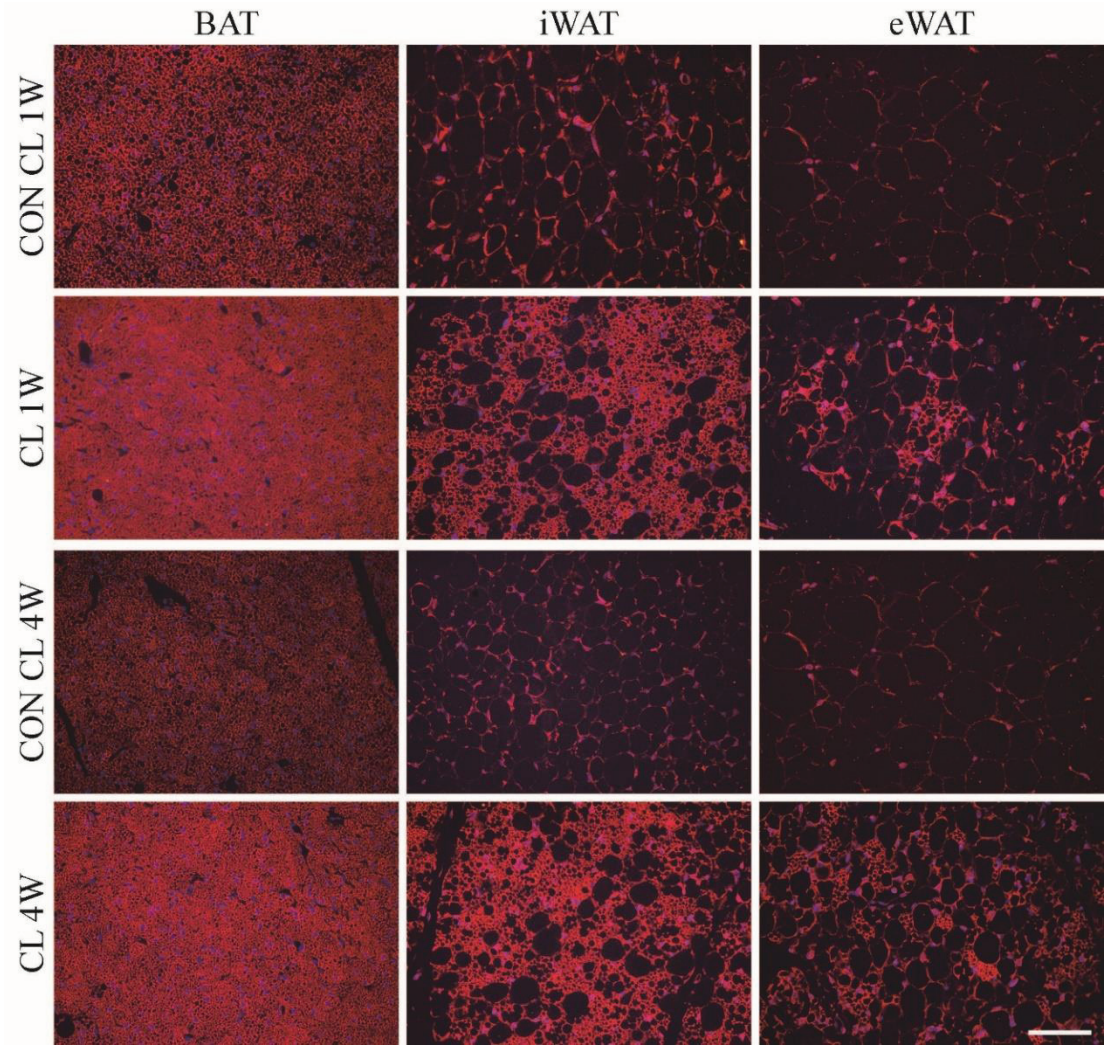

Figure 16. Effects of CL 316,243 administration for 1 week and 4 weeks on the UCP-1 immunofluorescence (red) in BAT, iWAT, and eWAT.

In the CL 1W and CL 4W groups, UCP-1 immunoreactive structures were prominently found in BAT, iWAT, and eWAT compared to those in the CON CL 1W and CON CL 4W groups, repsectively. Scale bar = 100 μm.

CL 316,243 treatment for 1 week and 4 weeks have no effects on proliferative NSCs in the hippocampus

To analyze total proliferative NSCs, positive cells were observed in the hippocampus. In all groups, Ki-67 positive cells were found in the SGZ of the DG, and there were no different populations of Ki-67 positive cells between CON CL 1W and CL 1W groups (Fig. 17A). The mean number of Ki-67 positive cells was 43.60 ± 0.93 in CON CL 1W mice and 45.40 ± 1.57 in Cd 1W mice (Fig. 17B). In addition, the number of Ki-67 positive cells were similarly observed in CON CL 4W and CL 4W groups (Fig. 17C). The mean number of Ki-67 positive cells was 28.60 ± 2.25 in CON CL 4W mice and 30.40 ± 1.03 in CL 4W mice (Fig. 17D).

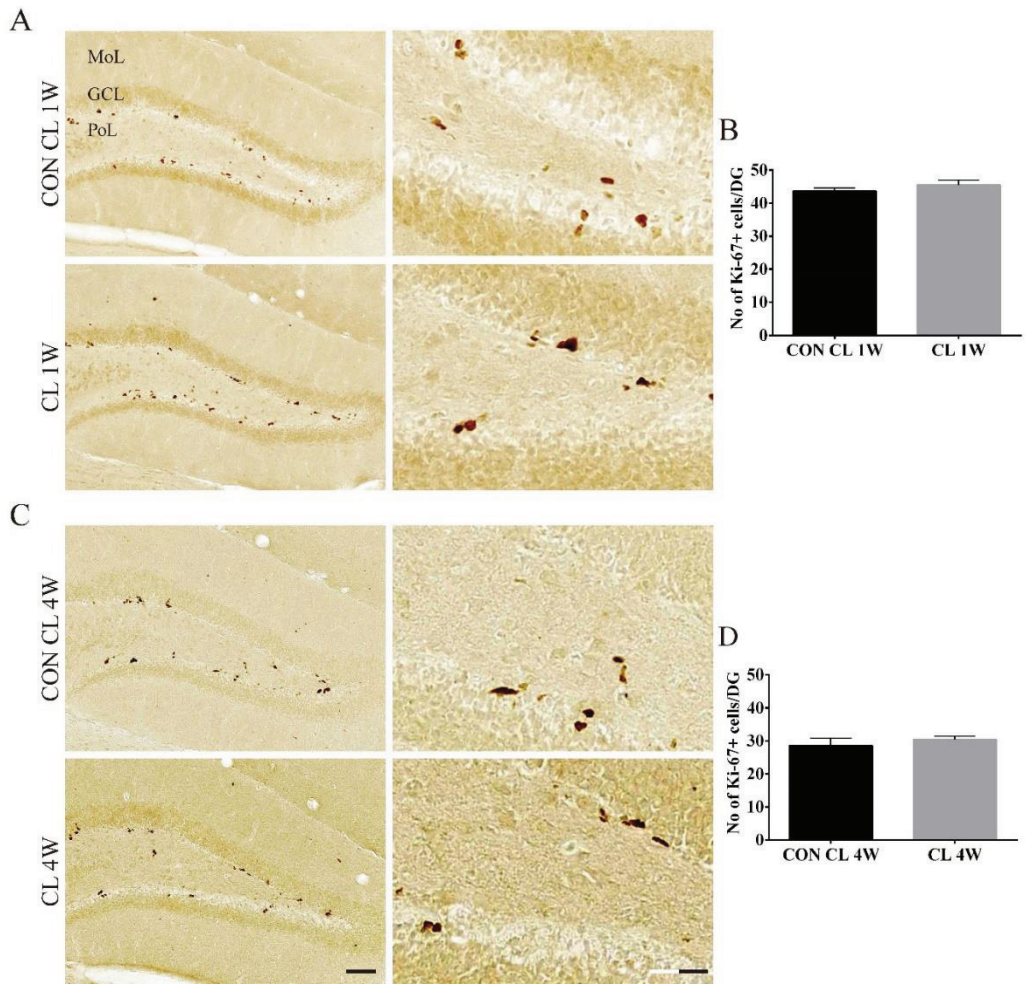

Figure 17. Effects of CL 316,243 administration for 1 and 4 weeks on the Ki-67 positive cells in the DG.

The number and distribution pattern of Ki-67 positive are similarly observed between CON CL 1W and CL 1W groups (A) or between CON CL 4W and CL 4W groups (C). GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of Ki-67 positive cells per section in CON CL 1W, CL 1W (B), CON CL 4W and CL 4W

groups (D) (n = 5 per group). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

CL 316,243 treatment for 1 and 4 weeks have no effects on NPCs

To analyze the correlation between CL 316,243 and NPCs, population changes of NPCs were observed in the hippocampus with TBR-2. In all groups, TBR-2 immunoreactivity was mainly found in the SGZ of the DG (Fig. 18A and 18C). TBR-2 immunoreactivity in the CL 1W and CL 4W groups did not show any different population levels compared to respective control groups. The mean number of TBR-2 positive cells was 26.00 ± 1.79 in CON CL 1W and 25.80 ± 1.59 in the CL 1W mice (Fig. 18B). In addition, the mean number of TBR-2 positive cells were 21.20 ± 1.52 and 21.20 ± 1.69 in CON CL 4W and CL 4W mice, respectively (Fig. 18D).

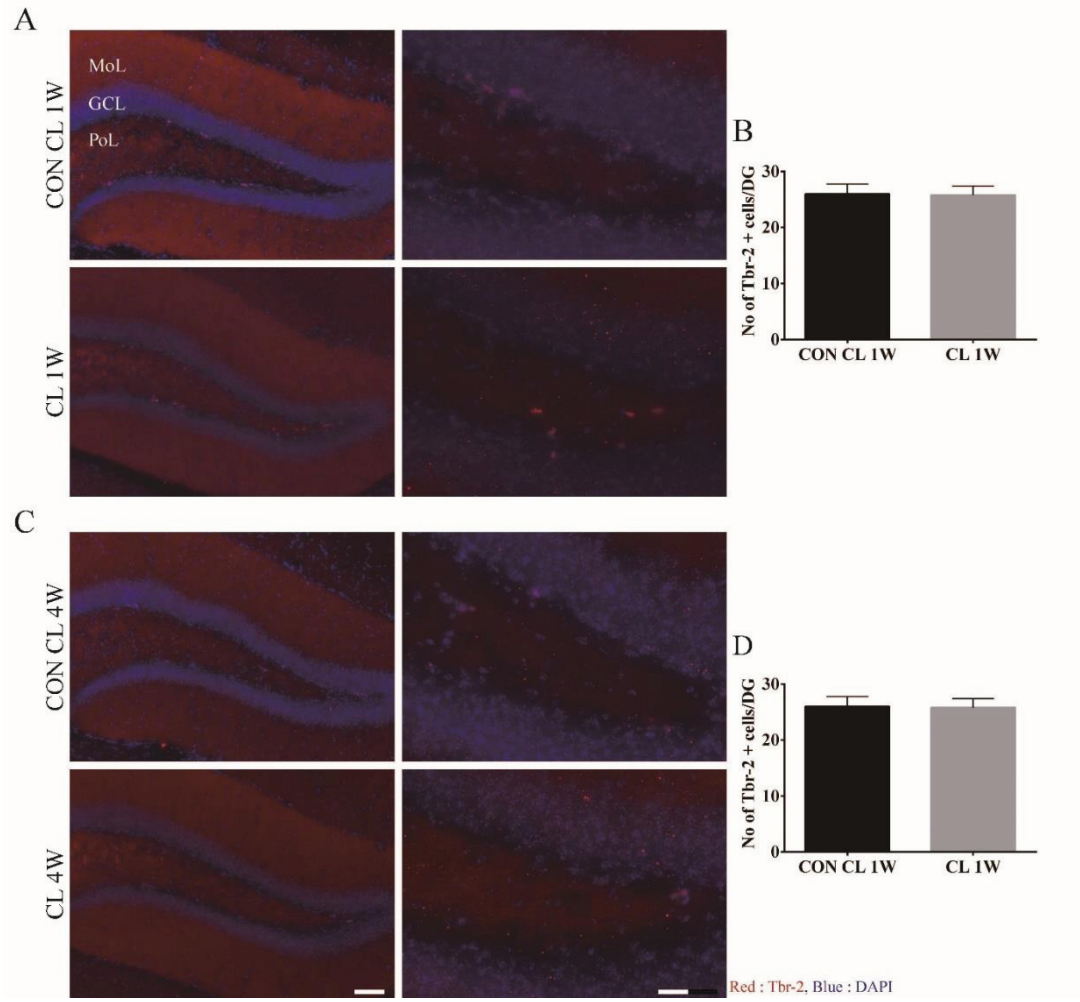

Figure 18. Effects of CL 316,243 treatment for 1 week and 4 weeks on the TBR-2 positive cells in the DG.

TBR-2 immunoreactive cells were detected in the DG with similar numbers between CON CL 1W and CL 1W groups (A), as well as CON CL 4W and CL 4W groups (C). GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of TBR-2-positive cells per section in CON CL 1W group, CL 1W (B), CON CL 4W and CL

4W groups (D) (n = 5 per group). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

CL 316,243 treatment for 1 week and 4 weeks have no effects on differentiated neuroblast

To observe differentiated neuroblasts among NSCs, DCX immunohistochemical staining was conducted. In all groups, DCX immunoreactive neuroblasts were found in the DG. There were no changes of DCX immunoreactive cell populations between CON CL 1W and CL 1W groups (Fig. 19A). The mean number of DCX immunoreactive neuroblasts were 154.00 ± 5.71 in CON CL 1W mice and 148.20 ± 2.41 in CL 1W mice (Fig. 19B). In addition, the number and distribution pattern of DCX immunoreactive neuroblasts were similar in the CON CL 4W and CL 4W groups. The mean number of DCX immunoreactive neuroblasts was 121.00 ± 4.71 in CON CL 4W mice and 126.40 ± 3.61 in CL 4W mice (Fig. 19D).

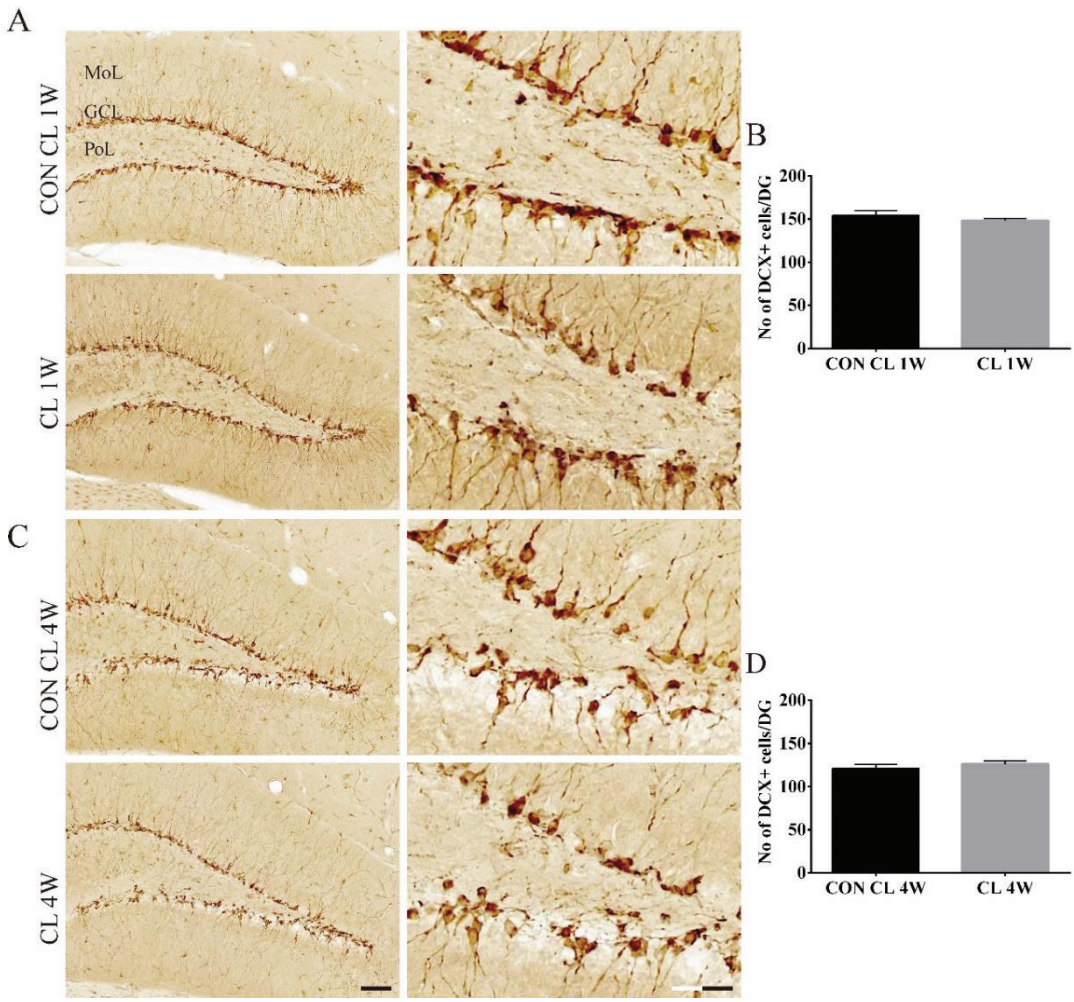

Figure 19. Effects of CL 316,243 for 1 week and 4 weeks on the DCX immunoreactive neuroblasts in the DG.

DCX immunoreactive neuroblasts were found in the SGZ of DG and the distrubition pattern and number of DCX immunoreactive neuroblasts were similarly observed in CON CL 1W and CL 1W groups (A) as well as CON CL 4W and CL 4W groups (C). GCL; granule cell layer, MoL; molecular layer, PoL; polymorphic layer. Quantitative analysis of

DCX immunoreactive neuroblasts per section in CON CL 1W, CL 1W (B), CON CL 4W and CL 4W groups (D) (n = 5 per group). Data are shown as mean ± SEM. Scale bar, ▬ = 100 μm, ▬ = 200 μm.

CL 316,243 treatment for 1 week has no effects on integration of newborn cells into neuroblasts

To elucidate the integration of newly generated cells into neuroblasts, BrdU and DCX double immunofluorescent staining was conducted in the DG. In all groups, BrdU and DCX double-positive cells were observed in the SGZ and GCL of the DG. No remarkable changes were observed in the populations of BrdU and DCX double positive cells between CON CL 1W and CL 1W groups (Fig. 20A). Mean numbers of BrdU and DCX double positive cells were 26.50 ± 2.64 in CON CL 1W mice and 26.00 ± 2.70 in CL 1W mice (Fig. 20B).

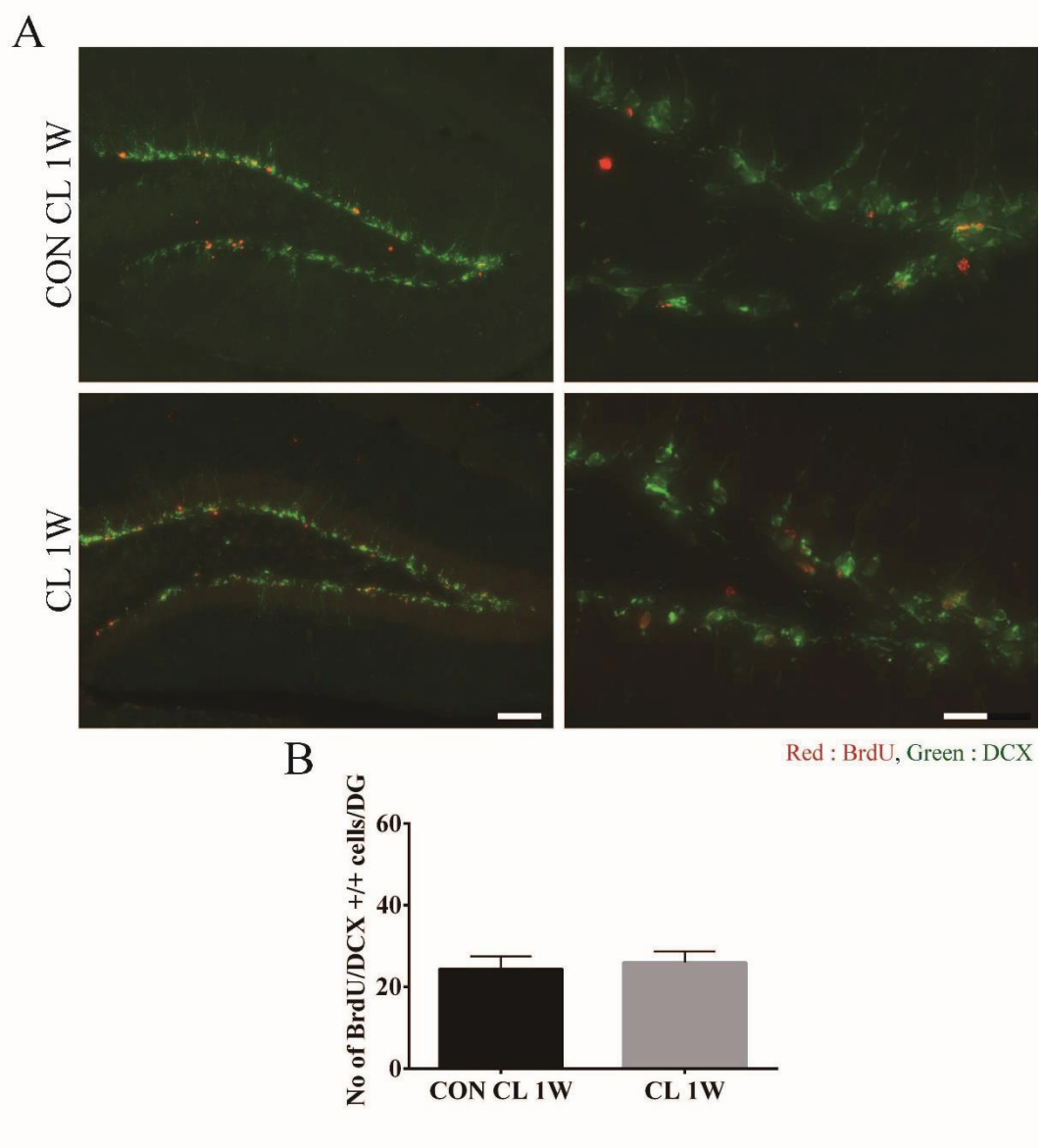

Figure 20. Effects of CL 316,243 for 1 week on the BrdU (red) and DCX (green) double immunofluorescent neuroblasts in the DG. There were no significant differences on the distribution pattern and number of BrdU and DCX double positive cells between the CON CL 1W and CL 1W groups in the DG. GCL; granule cell layer, MoL; molecular layer, PoL;

polymorphic layer. Quantitative analysis of BrdU and DCX positive cells per section in CON CL 1W and CL 1W groups (B) (n = 5 per group). Data are shown as mean ± SEM. Scale bar, ——— = 100 μm, ——— = 200 μm.

CL 316,243 treatment for 4 weeks has no effects on integration of newborn cells into mature neurons

To elucidate the effect of CL 316,243 treatment for 4 weeks on integration into mature neurons, BrdU and NeuN double immunofluorescent staining was conducted. In all groups, BrdU and NeuN double positive cells were detected in the GCL of the DG. There were no differences in populations of NeuN and BrdU double positive cells between CON CL 4W and CL 4W groups (Fig. 21A). Mean numbers of BrdU and NeuN double positive cells were 12.60 ± 1.29 in CON CL 4W mice and 12.60 ± 0.93 in CL 4W mice (Fig. 21B).

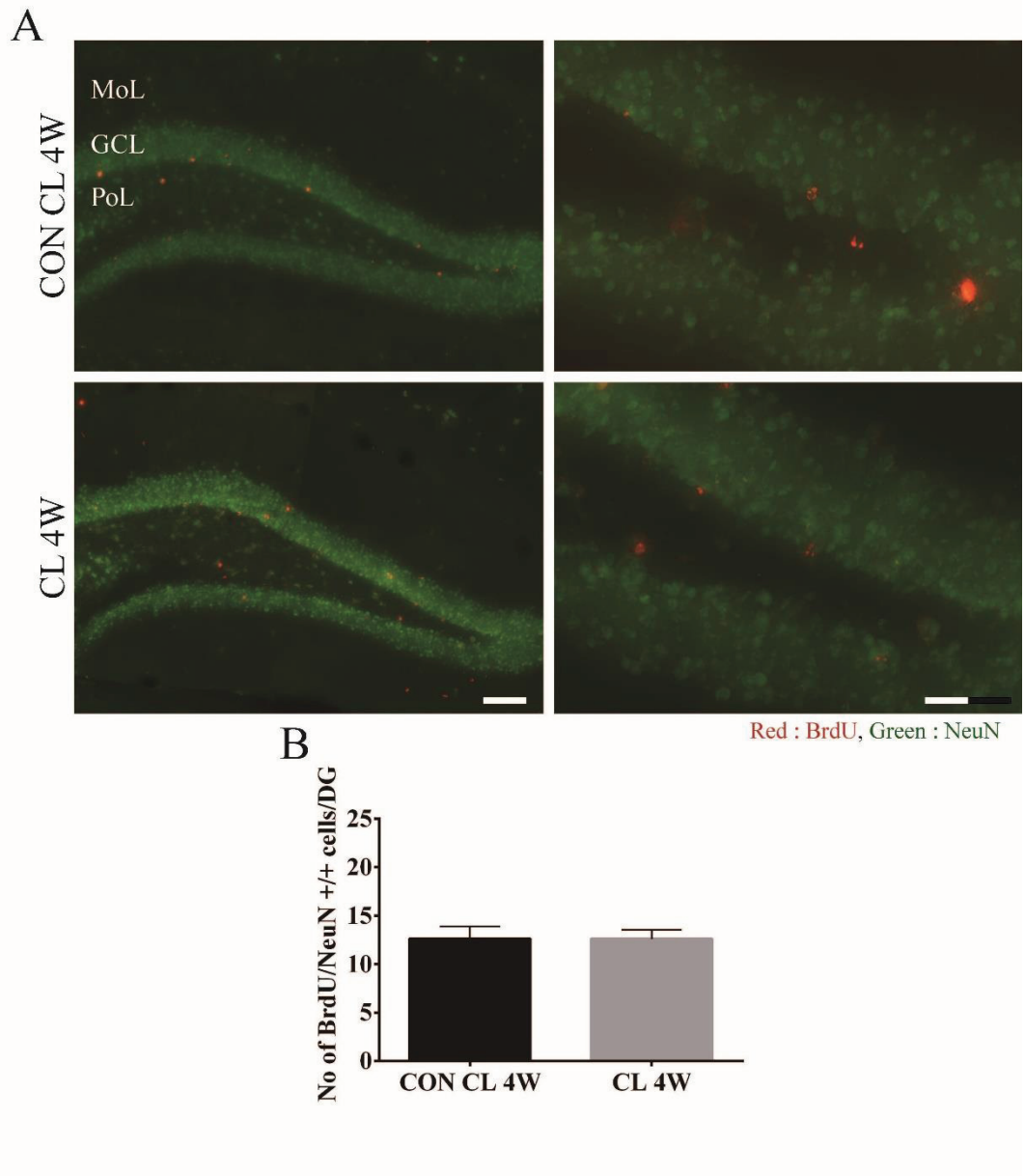

Figure 21. Effects of CL 316,243 treatment for 4 week on the BrdU (red) and NeuN (green) double immunofluorescent mature neurons in the DG. BrdU and NeuN double positive cells were similarly detected in the DG between CON CL 4W and CL 4W group

(A). GCL, granule cell layer; MoL, molecular layer; PoL, polymorphic layer. Quantitative analysis of BrdU and NeuN double positive cells per section in CON CL 4W group, CL 4W (B) (n = 5 per group). Data are shown as mean ± SEM. Scale bar, — = 100 μm, — = 200 μm.

CL 316,243 treatment for 1 week has no effects on AHN-related gene expression in hippocampus

To identify the effect of CL 316,243 treatment for 1 week on AHN-related gene expression, real-time PCR was conducted. CL 316,243 treatment for 1 week did not show any remarkable changes in mRNA levels associated with Egr1, neuronal migration, neuronal differentiation, cell fate determination and other regulators of cell differentiation, regulation of synaptic plasticity and synaptic transmission, or synaptogenesis and axonogenesis (Fig. 22A-22F).

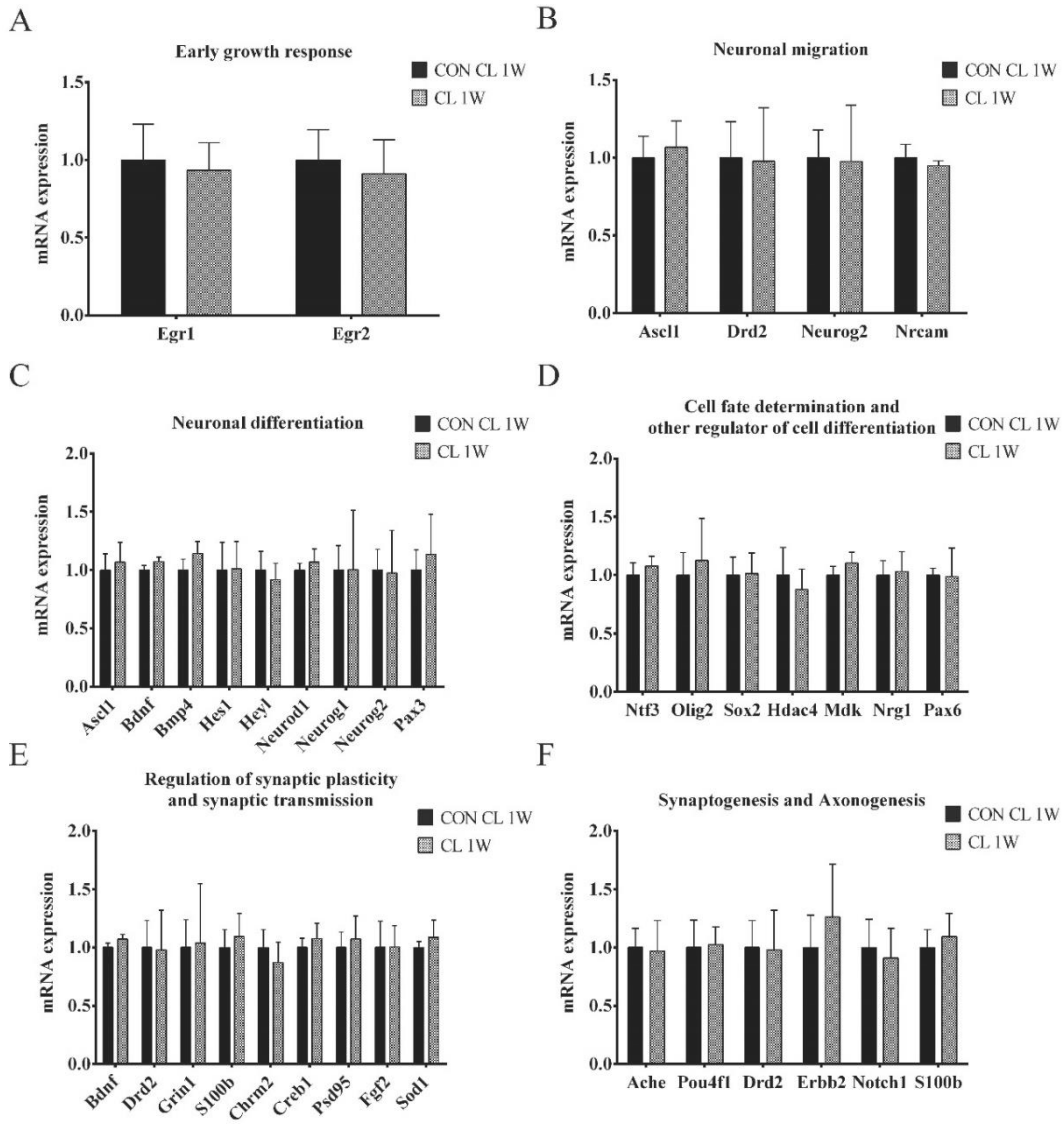

Figure 22. Effects of CL 316,243 treatment for 1 week on the changes of mRNA expression levels related to AHN. There were no significant differences to gene expression levels of (A) early growth response, (B) neuronal migration, (C) neuronal differentiation, (D) cell fate determination and other regulators of cell differentiation, (E)

regulation of synaptic plasticity and synaptic transmission, and (F) synaptogenesis and axonogenesis.

CL 316,243 treatment for 4 weeks changes AHN-related gene expression in hippocampus

To identify the effect of cold challenge for 4 weeks on AHN-related gene expression, real-time PCR was conducted. CL 316,243 treatment for 4 weeks had no effect on mRNA levels associated with Egr1, neuronal migration, neuronal differentiation, cell fate determination and other regulators of cell differentiation, regulation of synaptic plasticity and synaptic transmission, or synaptogenesis and axonogenesis (Fig. 23A-23F).

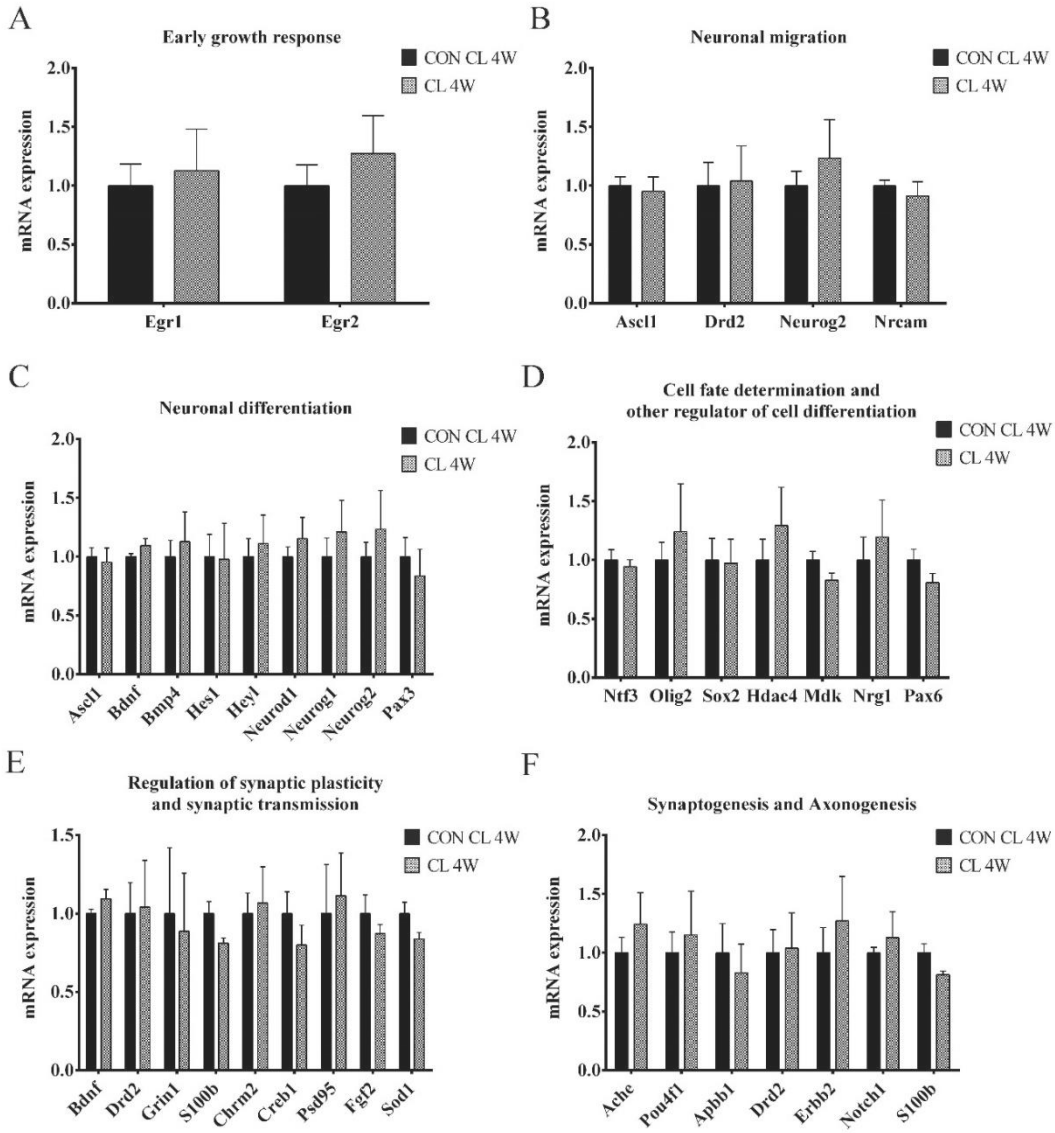

Figure 23. Effects of CL 316,243 treatment for 4 week on the changes of mRNA expression levels related to AHN.

There were no remarkable changes to mRNA expression levles of (A) early growth response, (B) neuronal migration, (C) neuronal differentiation, (D) cell fate determination

and other regulator of cell differentiation, (E) regulation of synaptic plasticity and synaptic transmission, and (F) synaptogenesis and axonogenesis.

CL 316,243 treatment for 1 week and 4 weeks changes AHN-related signal transduction gene and β-adrenergic receptor expression in hippocampus

To elucidate the effect of CL 316,243 treatment for 1 and 4 weeks on AHN-related signal gene transduction and β-adrenergic receptor expression, real-time PCR was conducted. AHN-related signal transduction genes were categorized into three subgroups, as follows; notch, Tgf-β, and GPCR signaling.

CL 316,243 treatment for 1 and 4 weeks did not show any significant changes in notch, Tgf-β, or GPCR signaling, as well as adrenergic receptors 1, 2, or 3 expression in the hippocampus (Fig. 24).

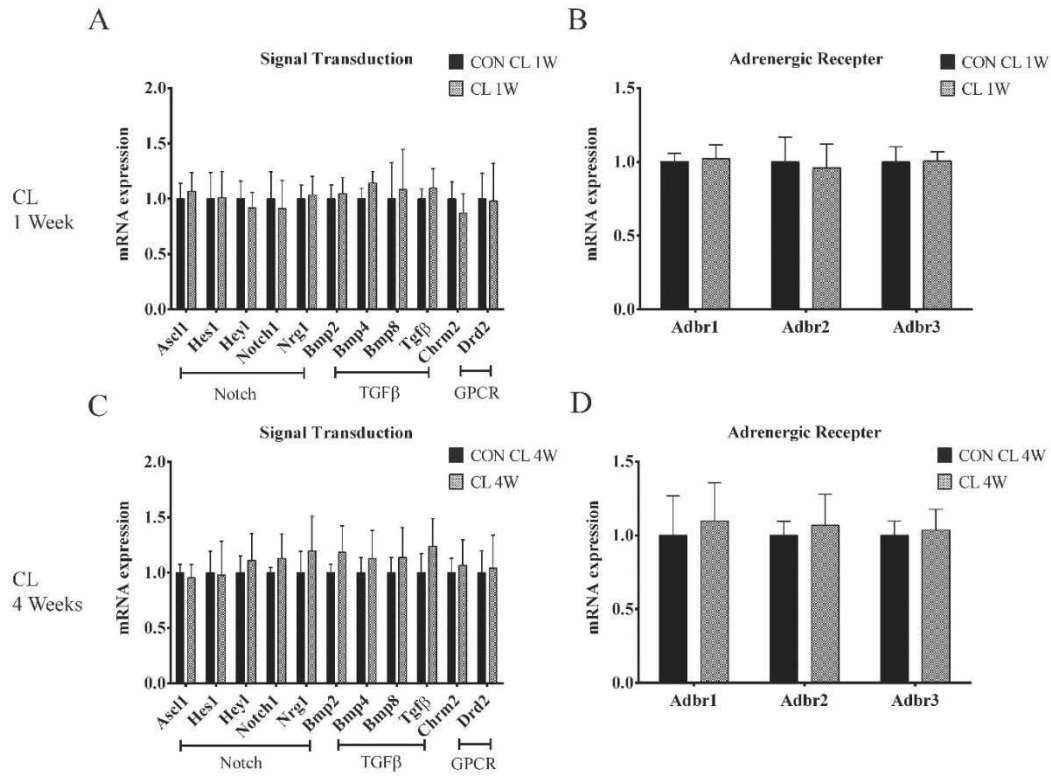

Figure 24. Effects of CL 316,243 treatment for 1 week and 4 weeks on the changes of mRNA expression levels related to AHN-related signal transduction and β-adrenergic receptor.

There were no significant changes to the mRNA expression levels of (A and C) signaling transduction, (B and D) β-adrenergic beta receptors after CL 316,243 treatment for 1 week (A and B) and 4 weeks (C and D).

DISCUSSION

Many studies have found that cold challenge and CL 316,243 offer beigeing effects, metabolic improvement, and therapeutic potentials for metabolic disorders (Labbe *et al.*, 2016; Mirbolooki *et al.*, 2011). To observe the effects of cold challenge and CL 316,243 treatment on physiological parameters, body weight and body weight gain was assessed. In control groups, body weight steadily increased with age during experimental periods. However, cold challenge and CL 316,243 treatment for 1 and 4 weeks significantly decreased body weight gain compared to those in respective control groups. In addition, cold challenge and CL 316,243 showed beigeing effects in BAT and iWAT, but CL 316,243 administration showed more potent beigeing effects than cold challenge, including in eWAT, based on hematoxylin and eosin staining. Beigeing effects were also confirmed by UCP-1 immunofluorescent staining; UCP1 plays a key role in thermogenesis by BAT-mediated adaptive non-shivering (Kopecky *et al.*, 1995) and the activation of UCP-1 in WAT can promote beigeing to prevent diet-induced obesity (Dempersmier *et al.*, 2015; Schulz and Tseng, 2013). UCP-1 immunofluorescence was largely observed in BAT and iWAT after cold challenge and CL 316,243 treatment for 1 and 4 weeks compared to those in the respective control groups. Similarly, UCP-1 immunoreactive structures were more abundantly detected in the CL 316,243 treated groups compared to that in the cold challenge group. These results suggest that both cold challenge and CL 316,243 treatment decreases body weight gain and induces beigeing effects in BAT and iWAT.

Cold challenge showed metabolic improvement by activating thermogenesis in adipose tissue. However, in the hippocampus, cold challenge is poorly understood and controversial results have been reported. For example, cold challenge showed improvement in spatial learning scores and behavioral tests, but paradoxically with LTP impairment (Elmarzouki *et al.*, 2014). As a stress factor, cold challenge in the 0–4°C condition for 30 days increased oxidative stress with increased c-fos expression and estrogen in the hippocampus (Qu *et al.*, 2017).

AHN is not regulated simply by gene changes or one simple pathway; it consists of many neurogenic pathways and sub-regulating pathways under complex genetic controls. The AHN process contains specific stages from NSCs to mature neurons and the process of each stage requires metabolic mechanism identification (Knobloch and Jessberger, 2017). The purpose of this study was to investigate the relationship between cold challenge and changes in AHN and its related genes. Many studies have demonstrated that cold challenge improves metabolic phenotypes with beigeing effects (Harms and Seale, 2013; Jeremic *et al.*, 2017) and the present study confirmed cold challenge increased beigeing effects in iWAT. In addition, cold challenge increased NPCs, proliferative NSCs, differentiated neuroblasts, and surviving mature neurons with increased mRNA expression related to AHN. In the highly complex AHN process, cold challenge enhanced phenotypes of AHN and related mRNA expression. Contrary to enhanced AHN after cold challenge, CL 316,243 treatment for 1 and 4 weeks had no effect on NPCs, proliferative NSCs, neuroblasts, lineage of NSCs, and surviving mature neurons.

In the present study, cold challenge for 1 and 4 weeks increased many AHN-related genes in the hippocampus. For example, Egr1, also known as Zif/268, was increased after cold challenge. Egr1 has a role in the maturation and survival of newborn neurons, along with hippocampal functional maturation, such as LTP and long-term memory formation (Richardson *et al.*, 1992; Veyrac *et al.*, 2013). Cold challenge also increased AHN-related genes, such as Ascl1 and Drd2, which are closely related to neuronal migration, differentiation, and cell fate determination. Ascl1 is pro-neural transcription factor that activates proliferation and differentiation of NPCs and promotes local chromatin accessibility during neurogenesis (Raposo *et al.*, 2015). In addition, Ascl1-expressed lineage of NSCs has long-term neurogenic potential in the SGZ of the adult mouse brain (Kim *et al.*, 2011). In the present study, Drd2 expression was increased after cold challenge. Drd1 and Drd2 are expressed in mouse hippocampal neurons (Gangarossa *et al.*, 2012); the dopaminergic pathways in hippocampal function are well known in the novel information process and synaptic plasticity (Lisman and Grace, 2005; Morice *et al.*, 2007). Drd2 is expressed in GABAergic interneurons in the dorsal hippocampus (Puighermanal *et al.*, 2015), which has regulatory function in the AHN (Ge *et al.*, 2007; Pallotto and Deprez, 2014). In addition, Drd2 could control modulation of mossy cells in the DG (Etter and Krezel, 2014). In the present study, cold challenge increased Drd2 mRNA expression in the hippocampus, and this may be a possible mechanism of cold challenge that promotes AHN via the activation of Drd2 and the GABAergic system in the DG.

HDAC4 is a member of class IIa in the HDAC family (Bertos *et al.*, 2001); HDAC4

lacking mouse brains show abnormalities in the hippocampus, and as it regulates neuronal cell fate determination (Majdzadeh *et al.*, 2008). HDAC4 is one of major components of synaptic plasticity, with neuronal chromatin and transcriptional factors as its functions in the hippocampus (Sando *et al.*, 2012). In addition, HDAC4 is a positive regulator of hippocampus-dependent learning and memory and behavioral results (Kim *et al.*, 2012). In the present study, cold challenge increased mRNA expression of HDAC4, and these results showed that cold challenge caused HDAC4-related enhancement of AHN and hippocampal function.

Genes related to synaptic plasticity, Drd2, Grin1, and Chrm2, were increased after cold challenge. Dopaminergic pathway signaling regulates the information process and synaptic plasticity in the hippocampus (Lisman and Grace, 2005; Morice *et al.*, 2007). Glutamate ionotropic receptor N-methyl-D-aspartate type subunit 1 (Grin1) transgenic mice showed that Grin1 is an essential factor in the acquisition of spatial memories (Tsien *et al.*, 1996). Cholinergic projections play key roles in proliferation and differentiation of NSCs at neurogenic niches in the hippocampus (Asrican *et al.*, 2016). The Chrm2 gene is thought to play a role in cognitive processes (Comings *et al.*, 2003). Muscarinic acetylcholine receptors activate signaling pathways for modulating synaptic plasticity from regulation (Levey *et al.*, 1991; Volpicelli and Levey, 2004). Cold challenge exhibited increases in the expression of Drd2, Grin1, and Chrm2, and showing that cold challenge has effects on synaptic plasticity.

In signal transduction, cold challenge increased genes categorized in notch and GPCR signaling. Notch1 as a postsynaptic receptor has functional interactions with N-methyl-D-aspartate receptors, and is an important regulator of synaptic plasticity and memory formation (Brai *et al.*, 2015). In addition, Ascl1 has a strong correlation with the notch signaling pathway (Raposo *et al.*, 2015). This increased Notch1 and Ascl1 in notch signaling showed that cold challenge has a relationship with the notch signaling pathway. GPCR signaling underlies the basic physiology in signal transduction and the majority of mammalian GPCRs are related to central nervous system (CNS) activity (Brugarolas *et al.*, 2014). Mechanisms of GPCR signaling are essential to the processes of neurotransmission, cellular growth, proliferation, and differentiation (Luttrell, 2008). In AHN, GPCR signaling is thought to be supportive of the adult neurogenesis process and considered a therapeutic strategy for adult NSCs (Doze and Perez, 2012). The Adbr1, Chrm2, and Drd2 genes belong to family of G protein-coupled family A receptors (Jassal *et al.*, 2010). The increases in Drd2 and Chrm2 show that cold challenge triggers Drd2 and Chrm2 gene activation in the GPCR family A receptor, and the GPCR reaction might contribute to the enhanced AHN process.

Norepinephrine (NE) and the adrenergic signaling pathway play an extremely important role in the regulation of hippocampal function. The NE receptors consist of two subtypes; α- and β-adrenergic receptors (Ahlquist, 1948). he β-adrenergic receptor has very specific effects on synaptic plasticity compared to that of the α-adrenergic receptor (Kemp and Manahan-Vaughan, 2008; Segal *et al.*, 1991). In addition, the synaptic plasticity is highly

sensitive to β-adrenergic control in the DG compared to other hippocampal sub regions (Hagena *et al.*, 2016). Memory consolidation and synaptic plasticity in the hippocampus are controlled by noradrenergic signaling (Hagena *et al.*, 2016). Noradrenergic signaling in the hippocampus activates β1- and β2-adrenergic receptors and facilitates phosphorylation of CREB (Hagena *et al.*, 2016), which increases LTP by β-adrenergic receptor modulation and memory-promoting effects (O'Dell *et al.*, 2015). Activation of β1 adrenergic receptor signaling enhances hippocampal network activity in the CA3, and shows correlation with NE signaling with cognitive process (Jurgens *et al.*, 2005). In the present study, cold challenge for 1 and 4 weeks elevated the expression of Adbr1 mRNA, indicating that this activates β-adrenergic signaling in the hippocampus.

In the brain, NE is contained in two systems; the locus coeruleus (LC)-centered system in the brain and HPA axis to peripheral tissues (Chrousos, 2009). Cold challenge activates and increases expression of tyrosine hydroxylase in the LC (Jedema and Grace, 2003; Richard *et al.*, 1988) and enhances electrophysiological properties, which result in underlying central noradrenergic activation of the CNS (Jedema and Grace, 2003). Cold challenge enhances activation of the LC, where there is abundant NE-containing neurons, and stimulates the hippocampus and related information encoding and processing (Mather *et al.*, 2016). Increased Adbr1 expression in the present study, after cold challenge, can be one of possible mechanisms to enhance AHN after cold challenge. NE depletion from stereotaxic injections of anti-dopamine β-hydroxylase-saporin into the LC showed impairment of AHN and working memory function (Coradazzi *et al.*, 2016). The neuronal

degeneration of the LC is an early and well described aspect of Alzheimer's disease (AD) in human data (German *et al.*, 1992) and an AD mouse model (Heneka *et al.*, 2006).

In the present study, CL 316,243 was only used for inducing beigeing effects in adipose tissue without any central effects, as CL 316,243 has no permeability though the brain-blood barrier (Mirbolooki *et al.*, 2011; Mirbolooki *et al.*, 2015). CL 316,243, a β3 specific agonist, administration for 1 and 4 weeks did not elicit any significant changes to NSCs proliferation, differentiated neuroblasts, and surviving mature neurons. In addition, CL 316,243 treatment did not cause any remarkable changes in the expression of mRNA associated with AHN and signal transduction in the hippocampus, although cold challenge and CL 316,243 administration induced activation of BAT and formation of BeAT, and cold and CL 316,243 act as a similar regulating pathway in the activation of BAT and formation of BeAT (Yao *et al.*, 2017). However, direct injection of a β3-adrenergic receptor agonist into the brain increased proliferating NSCs via NE signaling (Jhaveri, 2010). In addition, intraperitoneal injection of isoproterenol, another type of β-adrenergic receptor agonist, elevated NPCs, and inhibition of β-adrenergic receptor by propranolol decreased NPCs and neuroblasts (Jhaveri *et al.*, 2014). This caused NSCs and lineage of NSCs to be influenced by β-adrenergic receptor agonists and their signaling. However, β3 specific adrenergic agonist CL 316,243 administration via subcutaneous injection had no effects on NSCs and lineage if NSCs. This discrepancy may be closely related to permeability to blood-brain barrier. In human studies, about two-thirds of propranolol in the blood can cross the blood-brain barrier in a single passage through the brain (Olesen *et al.*, 1978), while CL 316,243

stimuli by intravenous injection have no effects on the brain based on a neuroimaging study with positron emission tomography-computed tomography (Mirbolooki *et al.*, 2011). These results suggest that BAT and beigeing adipose tissue induced by CL 316,243 administration have no effects on AHN and its related gene expression. In periphery, CL 316,243 has anti-diabetic effects in genetic diabetes rodent models (Ghorbani *et al.*, 2012; Liu *et al.*, 1998) and causes adipokine normalization in KKAy mice and obese Zucker diabetic fatty rats (Fu *et al.*, 2008; Ghorbani and Himms-Hagen, 1998). Collectively, these results suggest that beigeing in normal animals have no effect on AHN and its related gene expression, although the body weight gain was significantly decreased.

In summary, cold challenge and beigeing induced by CL 316,243 treatment decreases body weight gain and induces the beigeing in BAT and iWAT. In addition, cold challenge facilitates the proliferation of NSCs, NPCs population, and integration into neuroblasts and mature neurons with increased expression of AHN-related genes. However, beigeing induced by CL 316,243 treatment had no effect on AHN and its related gene expression. These results suggest that peripherally induced beigeing has no effect on AHN, and that cold challenge promotes AHN through increasing AHN-related gene expression, not through beigeing effects in BAT and iWAT.

CONCLUSION

AHN is not regulated simply by gene changes or one simple pathway; it consists of many neurogenic pathways and sub-regulating pathways under complex genetic controls. The AHN process undergoes specific stages from NSCs to mature neurons and the process of each stage needs metabolic mechanism identification (Knobloch and Jessberger, 2017). In the highly complex AHN process, cold challenge enhanced phenotypes of AHN and mRNA expression related to AHN. The brain has complex neuronal projections, and the hippocampus also has complex afferent and efferent neuronal projections (Deng *et al.*, 2010). Hippocampus-related neuronal projections are involved in the phenotype of AHN and function of hippocampus (Deng *et al.*, 2010). Cold challenge increased gene related notch, GPCR signaling, and Adbr1 expression. Cold signals also may have identified a neuronal circuit, and these results indicate with enhanced AHN in cold challenge. Singularity of our study was increased gene expression of notch, GPCR, and Adbr1 expression in the hippocampus of Cd 1W and Cd 4W groups.

The purpose of this study was to investigate the relationship of cold challenge and changes in the hippocampus. Many studies have investigated the effects of cold challenge on metabolic changes from beigeing effects, correlation of metabolic improvement, and therapeutic approach (Harms and Seale, 2013; Jeremic *et al.*, 2017). These results showed 1 week of cold challenge increased NPCs, proliferative NSCs, and neuroblasts, and 4 weeks of cold challenge increased lineage of NSCs and surviving mature neurons with increased

mRNA expression related to AHN. However, in the beigeing effect, cold challenge induced beigeing only in iWAT, not in eWAT. BAT and iWAT showed increased expression of UCP-1, however, eWAT did not change UCP-1 expression in 1 and 4 weeks of cold challenge. CL 316,243 showed beigeing effects in iWAT and eWAT; however, chemically induced CL 316,243 beigeing effects did not change the phenotype of AHN. Contrary to enhanced AHN in cold challenge, CL 316,243 did not change the phenotype of AHN and related mRNA expression in 1 and 4 weeks experiments. CL groups showed more potency in UCP-1 expression and beigeing morphological changes in BAT and BeAT in our study. However, CL 316,243 did not change the phenotype of AHN and mRNA expression in the hippocampus. CL 316,243 showed antidiabetic effects in transgenic diabetes laboratory models (Ghorbani *et al.*, 2012; Liu *et al.*, 1998). Effects of CL 316,243 also showed changes in adipokine normalization in KKAy mice and obese Zucker-ZDF rats (Fu *et al.*, 2008; Ghorbani and Himms-Hagen, 1998). Moreover, activated BAT by CL 316,243 has neural feedback to the brain (Ryu *et al.*, 2015). These changes could not change the phenotype of AHN, and showing that CL 316,243 induced changes have limitation on the hippocampus.

Ambient cold challenge in systemic aspects, decreases body weight gain and has beigeing effects. In addition, cold challenge increased phenotypes of AHN and its related mRNA gene expression. Enhanced AHN from cold challenge was independent from peripheral beigeing effects of BAT and formation of BeAT, as beigeing induced by CL 316,243 treatment did not show any significant increases in AHN and its related gene

expression. One possible mechanisms of cold challenge is the elevation of Adbr1 expression in the hippocampus.

REFERENCES

Ahlquist, R.P. (1948). A study of the adrenotropic receptors. Am. J. Physiol. *153*, 586-600.

Asrican, B., Paez-Gonzalez, P., Erb, J., and Kuo, C.T. (2016). Cholinergic circuit control of postnatal neurogenesis. Neurogenesis *3*, e1127310.

Bertos, N.R., Wang, A.H., and Yang, X.J. (2001). Class II histone deacetylases: structure, function, and regulation. Biochem. Cell Biol. *79*, 243-252.

Bondolfi, L., Ermini, F., Long, J.M., Ingram, D.K., and Jucker, M. (2004). Impact of age and caloric restriction on neurogenesis in the dentate gyrus of C57BL/6 mice. Neurobiol. Aging *25*, 333-340.

Brai, E., Marathe, S., Astori, S., Fredj, N.B., Perry, E., Lamy, C., Scotti, A., and Alberi, L. (2015). Notch1 Regulates Hippocampal Plasticity Through Interaction with the Reelin Pathway, Glutamatergic Transmission and CREB Signaling. Front. Cell. Neurosci. *9*, 447.

Brandt, M.D., Maass, A., Kempermann, G., and Storch, A. (2010). Physical exercise increases Notch activity, proliferation and cell cycle exit of type-3 progenitor cells in adult hippocampal neurogenesis. Eur. J. Neurosci. *32*, 1256-1264.

Brugarolas, M., Navarro, G., Martinez-Pinilla, E., Angelats, E., Casado, V., Lanciego, J.L., and Franco, R. (2014). G-protein-coupled receptor heteromers as key players in the molecular architecture of the central nervous system. CNS Neurosci. Ther. *20*, 703-709.

Chrousos, G.P. (2009). Stress and disorders of the stress system. Nat. Rev. Endocrinol. *5*, 374-381.

Comings, D.E., Wu, S., Rostamkhani, M., McGue, M., Lacono, W.G., Cheng, L.S., and MacMurray, J.P. (2003). Role of the cholinergic muscarinic 2 receptor (CHRM2) gene in cognition. Mol. Psychiatry *8*, 10-11.

Coradazzi, M., Gulino, R., Fieramosca, F., Falzacappa, L.V., Riggi, M., and Leanza, G. (2016). Selective noradrenaline depletion impairs working memory and hippocampal neurogenesis. Neurobiol. Aging *48*, 93-102.

Cypess, A.M., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A.B., Kuo, F.C., Palmer, E.L., Tseng, Y.H., Doria, A., Kolodny, G.M., and Kahn, C.R. (2009). Identification and importance of brown adipose tissue in adult humans. N. Engl. J. Med. *360*, 1509-1517.

de Souza, C.J., Hirshman, M.F., and Horton, E.S. (1997). CL-316,243, a beta3-specific adrenoceptor agonist, enhances insulin-stimulated glucose disposal in nonobese rats. Diabetes *46*, 1257-1263.

Dempersmier, J., Sambeat, A., Gulyaeva, O., Paul, S.M., Hudak, C.S., Raposo, H.F., Kwan, H.Y., Kang, C., Wong, R.H., and Sul, H.S. (2015). Cold-inducible Zfp516 activates UCP1 transcription to promote browning of white fat and development of brown fat. Mol. Cell *57*, 235-246.

Deng, W., Aimone, J.B., and Gage, F.H. (2010). New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? Nature reviews. Neuroscience *11*, 339-350.

Dodd, G.T., Decherf, S., Loh, K., Simonds, S.E., Wiede, F., Balland, E., Merry, T.L., Munzberg, H., Zhang, Z.Y., Kahn, B.B., Neel, B.G., Bence, K.K., Andrews, Z.B., Cowley, M.A., and Tiganis, T. (2015). Leptin and insulin act on POMC neurons to promote the browning of white fat. Cell *160*, 88-104.

Doze, V.A. and Perez, D.M. (2012). G-protein-coupled receptors in adult neurogenesis. Pharmacol. Rev. *64*, 645-675.

Elmarzouki, H., Aboussaleh, Y., Bitiktas, S., Suer, C., Artis, A.S., Dolu, N., and Ahami, A. (2014). Effects of cold exposure on behavioral and electrophysiological parameters related with hippocampal function in rats. Front. Cell. Neurosci. *8*, 253.

Etter, G. and Krezel, W. (2014). Dopamine D2 receptor controls hilar mossy cells excitability. Hippocampus *24*, 725-732.

Franklin, K.B.J. and Paxinos, G. (2013). Paxinos and Franklin's The mouse brain in stereotaxic coordinates, Fourth edition. edn (Amsterdam: Academic Press, an imprint of Elsevier).

Fu, L., Isobe, K., Zeng, Q., Suzukawa, K., Takekoshi, K., and Kawakami, Y. (2008). The effects of beta(3)-adrenoceptor agonist CL-316,243 on adiponectin, adiponectin receptors and tumor necrosis factor-alpha expressions in adipose tissues of obese diabetic KKAy mice. Eur. J. Pharmacol. *584*, 202-206.

Gage, F.H. (2000). Mammalian neural stem cells. Science *287*, 1433-1438.

Gage, F.H. (2002). Neurogenesis in the adult brain. J. Neurosci. *22*, 612-613.

Gangarossa, G., Longueville, S., De Bundel, D., Perroy, J., Herve, D., Girault, J.A., and Valjent, E. (2012). Characterization of dopamine D1 and D2 receptor-expressing neurons in the mouse hippocampus. Hippocampus *22*, 2199-2207.

Ge, S., Pradhan, D.A., Ming, G.L., and Song, H. (2007). GABA sets the tempo for activity-dependent adult neurogenesis. Trends Neurosci. *30*, 1-8.

German, D.C., Manaye, K.F., White, C.L., 3rd, Woodward, D.J., McIntire, D.D., Smith, W.K., Kalaria, R.N., and Mann, D.M. (1992). Disease-specific patterns of locus coeruleus cell loss. Ann. Neurol. *32*, 667-676.

Ghorbani, M. and Himms-Hagen, J. (1998). Treatment with CL 316,243, a beta 3-adrenoceptor agonist, reduces serum leptin in rats with diet- or aging-associated obesity, but not in Zucker rats with genetic (fa/fa) obesity. Int. J. Obes. Relat. Metab. Disord. *22*, 63-65.

Ghorbani, M., Shafiee Ardestani, M., Gigloo, S.H., Cohan, R.A., Inanlou, D.N., and Ghorbani, P. (2012). Anti diabetic effect of CL 316,243 (a beta3-adrenergic agonist) by down regulation of tumour necrosis factor (TNF-alpha) expression. PLoS One *7*, e45874.

Gil, A., Olza, J., Gil-Campos, M., Gomez-Llorente, C., and Aguilera, C.M. (2011). Is adipose tissue metabolically different at different sites? Int. J. Pediatr. Obes. *6 Suppl 1*, 13-20.

Gordon, C.J. (2012). Thermal physiology of laboratory mice: Defining thermoneutrality. J. Therm. Biol. *37*, 654-685.

Guerra, C., Koza, R.A., Yamashita, H., Walsh, K., and Kozak, L.P. (1998). Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. J. Clin. Invest. *102*, 412-420.

Hagena, H., Hansen, N., and Manahan-Vaughan, D. (2016). beta-Adrenergic Control of Hippocampal Function: Subserving the Choreography of Synaptic Information Storage and Memory. Cereb. Cortex *26*, 1349-1364.

Harms, M., and Seale, P. (2013). Brown and beige fat: development, function and therapeutic potential. Nat. Med. *19*, 1252-1263.

Heneka, M.T., Ramanathan, M., Jacobs, A.H., Dumitrescu-Ozimek, L., Bilkei-Gorzo, A., Debeir, T., Sastre, M., Galldiks, N., Zimmer, A., Hoehn, M., Heiss, W.D., Klockgether, T., and Staufenbiel, M. (2006). Locus ceruleus degeneration promotes Alzheimer pathogenesis in amyloid precursor protein 23 transgenic mice. J. Neurosci. *26*, 1343-1354.

Ishibashi, J. and Seale, P. (2010). Beige Can Be Slimming. Science *328*, 1113-1114.

Jassal, B., Jupe, S., Caudy, M., Birney, E., Stein, L., Hermjakob, H., and D'Eustachio, P. (2010). The systematic annotation of the three main GPCR families in Reactome. Database *2010*, baq018-baq018.

Jedema, H.P. and Grace, A.A. (2003). Chronic exposure to cold stress alters electrophysiological properties of locus coeruleus neurons recorded in vitro. Neuropsychopharmacology *28*, 63-72.

Jeremic, N., Chaturvedi, P., and Tyagi, S.C. (2017). Browning of White Fat: Novel Insight Into Factors, Mechanisms, and Therapeutics. J. Cell. Physiol. *232*, 61-68.

Jhaveri, D.J. (2010). Norepinephrine directly activates adult hippocampal precursors via β3 adrenergic receptors. *30*, 2795-2806.

Jhaveri, D.J., Nanavaty, I., Prosper, B.W., Marathe, S., Husain, B.F., Kernie, S.G., Bartlett, P.F., and Vaidya, V.A. (2014). Opposing effects of alpha2- and beta-adrenergic receptor stimulation on quiescent neural precursor cell activity and adult hippocampal neurogenesis. PLoS One *9*, e98736.

Jurgens, C.W.D., Rau, K.E., Knudson, C.A., King, J.D., Carr, P.A., Porter, J.E., and Doze, V.A. (2005). β1 Adrenergic Receptor-Mediated Enhancement of Hippocampal CA3 Network Activity. J. Pharmacol. Exp. Ther. *314*, 552-560.

Kajimura, S., Spiegelman, B.M., and Seale, P. (2015). Brown and Beige Fat: Physiological Roles beyond Heat Generation. Cell Metab. *22*, 546-559.

Kemp, A., and Manahan-Vaughan, D. (2008). Beta-adrenoreceptors comprise a critical element in learning-facilitated long-term plasticity. Cereb. Cortex *18*, 1326-1334.

Kempermann, G., Kuhn, H.G., and Gage, F.H. (1997). Genetic influence on neurogenesis in the dentate gyrus of adult mice. Proc. Natl. Acad. Sci. U. S. A. *94*, 10409-10414.

Kim, E.J., Ables, J.L., Dickel, L.K., Eisch, A.J., and Johnson, J.E. (2011). Ascl1 (Mash1) Defines Cells with Long-Term Neurogenic Potential in Subgranular and Subventricular Zones in Adult Mouse Brain. PLoS One *6*, e18472.

Kim, M.S., Akhtar, M.W., Adachi, M., Mahgoub, M., Bassel-Duby, R., Kavalali, E.T., Olson, E.N., and Monteggia, L.M. (2012). An essential role for histone deacetylase 4 in synaptic plasticity and memory formation. J. Neurosci. *32*, 10879-10886.

Knobloch, M. and Jessberger, S. (2017). Metabolism and neurogenesis. Curr. Opin. Neurobiol. *42*, 45-52.

Kopecky, J., Clarke, G., Enerbäck, S., Spiegelman, B., and Kozak, L.P. (1995). Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. J. Clin. Invest. *96*, 2914-2923.

Kumar, A., Shiloach, J., Betenbaugh, M.J., and Gallagher, E.J. (2015). The beta-3 adrenergic agonist (CL-316,243) restores the expression of down-regulated fatty acid oxidation genes in type 2 diabetic mice. Nutr. Metab. (Lond.) *12*, 8.

Labbe, S.M., Caron, A., Chechi, K., Laplante, M., Lecomte, R., and Richard, D. (2016). Metabolic activity of brown, "beige," and white adipose tissues in response to chronic

adrenergic stimulation in male mice. Am. J. Physiol. Endocrinol. Metab. *311*, E260-268.

Laing, B.T., Do, K., Matsubara, T., Wert, D.W., Avery, M.J., Langdon, E.M., Zheng, D., and Huang, H. (2016). Voluntary exercise improves hypothalamic and metabolic function in obese mice. J. Endocrinol. *229*, 109-122.

Lee, J., Duan, W., and Mattson, M.P. (2002a). Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. J. Neurochem. *82*, 1367-1375.

Lee, J., Seroogy, K.B., and Mattson, M.P. (2002b). Dietary restriction enhances neurotrophin expression and neurogenesis in the hippocampus of adult mice. J. Neurochem. *80*, 539-547.

Lee, P., Smith, S., Linderman, J., Courville, A.B., Brychta, R.J., Dieckmann, W., Werner, C.D., Chen, K.Y., and Celi, F.S. (2014). Temperature-acclimated brown adipose tissue modulates insulin sensitivity in humans. Diabetes *63*, 3686-3698.

Leuner, B., Gould, E., and Shors, T.J. (2002). Is There A Link Between Adult Neurogenesis and Learning? Hippocampus *12*, 578-584.

Levey, A.I., Kitt, C.A., Simonds, W.F., Price, D.L., and Brann, M.R. (1991). Identification and localization of muscarinic acetylcholine receptor proteins in brain with subtype-specific antibodies. J. Neurosci. *11*, 3218-3226.

Lisman, J.E. and Grace, A.A. (2005). The hippocampal-VTA loop: controlling the entry of information into long-term memory. Neuron *46*, 703-713.

Liu, X., Perusse, F., and Bukowiecki, L.J. (1998). Mechanisms of the antidiabetic effects

of the beta 3-adrenergic agonist CL-316243 in obese Zucker-ZDF rats. Am. J. Physiol. *274*, R1212-1219.

Lo K , A. and Sun, L. (2013). Turning WAT into BAT: a review on regulators controlling the browning of white adipocytes. Biosci. Rep. *33*.

Luttrell, L.M. (2008). Reviews in Molecular Biology and Biotechnology: Transmembrane Signaling by G Protein-Coupled Receptors. Mol. Biotechnol. *39*, 239-264.

MacPherson, R.E., Castellani, L., Beaudoin, M.S., and Wright, D.C. (2014). Evidence for fatty acids mediating CL 316,243-induced reductions in blood glucose in mice. Am. J. Physiol. Endocrinol. Metab. *307*, E563-570.

Majdzadeh, N., Wang, L., Morrison, B.E., Bassel-Duby, R., Olson, E.N., and D'Mello, S.R. (2008). HDAC4 inhibits cell-cycle progression and protects neurons from cell death. Dev. Neurobiol. *68*, 1076-1092.

Malendowicz, L.K., Rucinski, M., Belloni, A.S., Ziolkowska, A., and Nussdorfer, G.G. (2007). Leptin and the regulation of the hypothalamic-pituitary-adrenal axis. Int. Rev. Cytol. *263*, 63-102.

Mather, M., Clewett, D., Sakaki, M., and Harley, C.W. (2016). Norepinephrine ignites local hotspots of neuronal excitation: How arousal amplifies selectivity in perception and memory. Behav. Brain Sci. *39*, e200.

Mirbolooki, M.R., Constantinescu, C.C., Pan, M.-L., and Mukherjee, J. (2011). Quantitative assessment of brown adipose tissue metabolic activity and volume using (18)F-FDG PET/CT and β3-adrenergic receptor activation. EJNMMI Research *1*, 30-30.

Mirbolooki, M.R., Schade, K.N., Constantinescu, C.C., Pan, M.-L., and Mukherjee, J.

(2015). Enhancement of (18)F-Fluorodeoxyglucose Metabolism in Rat Brain Frontal Cortex Using a β3 Adrenoceptor Agonist. Synapse (New York, N.Y.) *69*, 96-98.

Morice, E., Billard, J.-M., Denis, C., Mathieu, F., Betancur, C., Epelbaum, J., Giros, B., and Nosten-Bertrand, M. (2007). Parallel loss of hippocampal LTD and cognitive flexibility in a genetic model of hyperdopaminergia. Neuropsychopharmacology *32*, 2108-2116.

Nakamura, K. and Morrison, S.F. (2007). Central efferent pathways mediating skin cooling-evoked sympathetic thermogenesis in brown adipose tissue. Am. J. Physiol. Regul. Integr. Comp. Physiol. *292*, R127-136.

Nicholls, D.G., Bernson, V.S., and Heaton, G.M. (1978). The identification of the component in the inner membrane of brown adipose tissue mitochondria responsible for regulating energy dissipation. Experientia. Suppl. *32*, 89-93.

O'Dell, T.J., Connor, S.A., Guglietta, R., and Nguyen, P.V. (2015). beta-Adrenergic receptor signaling and modulation of long-term potentiation in the mammalian hippocampus. Learn. Mem. *22*, 461-471.

Ohno, H., Shinoda, K., Spiegelman, B.M., and Kajimura, S. (2012). PPARgamma agonists induce a white-to-brown fat conversion through stabilization of PRDM16 protein. Cell Metab. *15*, 395-404.

Olesen, J., Hougard, K., and Hertz, M. (1978). Isoproterenol and propranolol: ability to cross the blood-brain barrier and effects on cerebral circulation in man. Stroke *9*, 344-349.

Ouellet, V., Labbe, S.M., Blondin, D.P., Phoenix, S., Guerin, B., Haman, F., Turcotte, E.E., Richard, D., and Carpentier, A.C. (2012). Brown adipose tissue oxidative metabolism contributes to energy expenditure during acute cold exposure in humans. J. Clin. Invest. *122*, 545-552.

Pallotto, M. and Deprez, F. (2014). Regulation of adult neurogenesis by GABAergic transmission: signaling beyond GABA(A)-receptors. Front. Cell. Neurosci. *8*, 166.

Palmer, T.D., Takahashi, J., and Gage, F.H. (1997). The adult rat hippocampus contains primordial neural stem cells. Mol. Cell. Neurosci. *8*, 389-404.

Park, H.R., Park, M., Choi, J., Park, K.Y., Chung, H.Y., and Lee, J. (2010). A high-fat diet impairs neurogenesis: involvement of lipid peroxidation and brain-derived neurotrophic factor. Neurosci. Lett. *482*, 235-239.

Peng, X.R., Gennemark, P., O'Mahony, G., and Bartesaghi, S. (2015). Unlock the Thermogenic Potential of Adipose Tissue: Pharmacological Modulation and Implications for Treatment of Diabetes and Obesity. Front. Endocrinol. (Lausanne) *6*.

Petrovic, N., Walden, T.B., Shabalina, I.G., Timmons, J.A., Cannon, B., and Nedergaard, J. (2010). Chronic peroxisome proliferator-activated receptor gamma (PPARgamma) activation of epididymally derived white adipocyte cultures reveals a population of thermogenically competent, UCP1-containing adipocytes molecularly distinct from classic brown adipocytes. J. Biol. Chem. *285*, 7153-7164.

Puighermanal, E., Biever, A., Espallergues, J., Gangarossa, G., De Bundel, D., and Valjent, E. (2015). drd2-cre:ribotag mouse line unravels the possible diversity of dopamine d2 receptor-expressing cells of the dorsal mouse hippocampus. Hippocampus *25*, 858-875.

Qu, T.T., Deng, J.X., Li, R.L., Cui, Z.J., Wang, X.Q., Wang, L., and Deng, J.B. (2017). Stress injuries and autophagy in mouse hippocampus after chronic cold exposure. Neural Regen Res *12*, 440-446.

Ramos-Rodriguez, J.J., Molina-Gil, S., Ortiz-Barajas, O., Jimenez-Palomares, M., Perdomo, G., Cozar-Castellano, I., Lechuga-Sancho, A.M., and Garcia-Alloza, M. (2014).

Central proliferation and neurogenesis is impaired in type 2 diabetes and prediabetes animal models. PloS one *9*, e89229.

Raposo, Alexandre A.S.F., Vasconcelos, Francisca F., Drechsel, D., Marie, C., Johnston, C., Dolle, D., Bithell, A., Gillotin, S., van den Berg, Debbie L.C., Ettwiller, L., Flicek, P., Crawford, Gregory E., Parras, Carlos M., Berninger, B., Buckley, Noel J., Guillemot, F., and Castro, Diogo S. (2015). Ascl1 Coordinately Regulates Gene Expression and the Chromatin Landscape during Neurogenesis. Cell Rep. *10*, 1544-1556.

Ravussin, Y., Xiao, C., Gavrilova, O., and Reitman, M.L. (2014). Effect of intermittent cold exposure on brown fat activation, obesity, and energy homeostasis in mice. PLoS One *9*, e85876.

Richard, F., Faucon-Biguet, N., Labatut, R., Rollet, D., Mallet, J., and Buda, M. (1988). Modulation of tyrosine hydroxylase gene expression in rat brain and adrenals by exposure to cold. J. Neurosci. Res. *20*, 32-37.

Richardson, C.L., Tate, W.P., Mason, S.E., Lawlor, P.A., Dragunow, M., and Abraham, W.C. (1992). Correlation between the induction of an immediate early gene, zif/268, and long-term potentiation in the dentate gyrus. Brain Res. *580*, 147-154.

Ruan, H.B., Dietrich, M.O., Liu, Z.W., Zimmer, M.R., Li, M.D., Singh, J.P., Zhang, K., Yin, R., Wu, J., Horvath, T.L., and Yang, X. (2014). O-GlcNAc transferase enables AgRP neurons to suppress browning of white fat. Cell *159*, 306-317.

Ryu, V., Garretson, J.T., Liu, Y., Vaughan, C.H., and Bartness, T.J. (2015). Brown adipose tissue has sympathetic-sensory feedback circuits. J. Neurosci. *35*, 2181-2190.

Sando, R., 3rd, Gounko, N., Pieraut, S., Liao, L., Yates, J., 3rd, and Maximov, A. (2012). HDAC4 governs a transcriptional program essential for synaptic plasticity and memory.

Cell *151*, 821-834.

Schulz, T.J. and Tseng, Y.H. (2013). Brown adipose tissue: development, metabolism and beyond. Biochem. J. *453*, 167-178.

Segal, M., Markram, H., and Richter-Levin, G. (1991). Actions of norepinephrine in the rat hippocampus. Prog. Brain Res. *88*, 323-330.

Shabalina, I.G., Petrovic, N., de Jong, J.M., Kalinovich, A.V., Cannon, B., and Nedergaard, J. (2013). UCP1 in brite/beige adipose tissue mitochondria is functionally thermogenic. Cell Rep. *5*, 1196-1203.

Stangl, D. and Thuret, S. (2009). Impact of diet on adult hippocampal neurogenesis. Genes Nutr. *4*, 271-282.

Tsien, J.Z., Huerta, P.T., and Tonegawa, S. (1996). The essential role of hippocampal CA1 NMDA receptor-dependent synaptic plasticity in spatial memory. Cell *87*, 1327-1338.

van Marken Lichtenbelt, W.D., Vanhommerig, J.W., Smulders, N.M., Drossaerts, J.M., Kemerink, G.J., Bouvy, N.D., Schrauwen, P., and Teule, G.J. (2009). Cold-activated brown adipose tissue in healthy men. N. Engl. J. Med. *360*, 1500-1508.

Veyrac, A., Gros, A., Bruel-Jungerman, E., Rochefort, C., Kleine Borgmann, F.B., Jessberger, S., and Laroche, S. (2013). Zif268/egr1 gene controls the selection, maturation and functional integration of adult hippocampal newborn neurons by learning. Proceedings of the National Academy of Sciences *110*, 7062-7067.

Vivar, C., Potter, M.C., and van Praag, H. (2013). All about running: synaptic plasticity, growth factors and adult hippocampal neurogenesis. Curr. Top. Behav. Neurosci. *15*, 189-210.

Volpicelli, L.A., and Levey, A.I. (2004). Muscarinic acetylcholine receptor subtypes in cerebral cortex and hippocampus. Prog. Brain Res. *145*, 59-66.

Yao, L., Cui, X., Chen, Q., Yang, X., Fang, F., Zhang, J., Liu, G., Jin, W., and Chang, Y. (2017). Cold-Inducible SIRT6 Regulates Thermogenesis of Brown and Beige Fat. Cell Rep. *20*, 641-654.

Yau, S.Y., Lau, B.W., Tong, J.B., Wong, R., Ching, Y.P., Qiu, G., Tang, S.W., Lee, T.M., and So, K.F. (2011). Hippocampal neurogenesis and dendritic plasticity support running-improved spatial learning and depression-like behaviour in stressed rats. PLoS One *6*, e24263.

국문 초록

저온자극에 의한

해마의 신경세포재생에 관한 연구

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환경의 변화로서 저온자극은 대사를 개선시켜주는 요인의 하나로 알려져 있으며, 이러한 기전은 당대사와 지질대사를 통해 이루어지며, 대사 개선은 말초 지방조직에서의 변화에 의해서 유도된다고 알려져 있다. 최근 많은 연구에서 대사의 변화와 신경세포재생(adult hippocampal neurogenesis) 사이의 연관성에 대한 연구가 진행 되었다. 즉, 대사이상(metabolic disorder) 시에 신경세포재생의 저하가 오며, 반대로 대사의 개선 상황에서는 신경세포재생을 증가시키는 것으로 알려졌다. 한편 저온자극은 대사 개선을 유도하는 인자로서 알려져 왔으며, 당대사와 지질대사의 개선을 유도하여, 온몸 대사의 개선을 가져 온다고 알려져 있다. 따라서, 본 연구에서는 저온자극이 해마의 신경세포재생에 영향을 줄 것이라고 생각하고, 저온자극에 의해서 유도되는 갈색화현상(beigeing effect) 및 신경세포재생을 확인하였다.

한편, 지방의 갈색화현상으로 인한 대사 개선이 신경세포재생에 영향을 미치는지 확인하기 위하여 뇌혈관문(blood-brain barrier)을 통과하지 못하며, β3-

교감신경작용제로 알려진 CL 316,243을 투여하여 갈색화현상을 유도하고 신경세포재생을 확인하였다.

본 연구는 저온자극과, 저온자극시 일어나는 지방의 갈색화현상을 유도하는 CL 316,243을 이용하여 연구를 설계하였으며, 1주, 4주간 저온자극 또는 CL 316,243을 투여하여 본 연구를 진행하였다. 저온자극 및 CL 316,243의 투여는 마우스에서 증체량의 유의적인 감소를 유도하는 것을 확인할 수 있었으며, 갈색지방(brown adipose tissue)과 고샅부백색지방(inguinal white adipose tissue)에서의 갈색화현상이 유도됨을 확인할 수 있었으며, 갈색화현상이 저온자극보다는 CL 316,243 투여군에서 보다 현저함을 hematoxylin & eosin 염색을 통해 확인하였으며, 갈색화현상의 표지자로 알려진 uncoupled protein-1의 면역형광염색을 통해 재확인하였다.

신경세포재생에 대한 연구를 통해서는 1주 및 4주의 저온자극은 신경세포재생 과정 중의 세포인 신경선조세포(neural progenitor cells)의 증가를

가져왔으며, 분화한 신경모세포(differentiated neuroblast)의 증가를 확인할 수 있었다. 또한 저온자극이 성숙한 신경세포로의 분화를 촉진하는 것을 확인할 수 있었다. 전령RNA (mRNA) 분석을 통해 1주와 4주의 저온자극이 신경세포재생과 연관된 전령RNA에 미치는 영향을 확인하였다. 저온자극(1주 및 4주)은 신경세포재생과 연관된 전령RNA의 증가를 가져왔으며, 신경세포재생과 연관된 신호전달(signal transduction) 및 베타1아드레날린수용체 전령RNA의 증가를 가져오는 것을 확인할 수 있었다.

그러나, CL 316,243 투여를 통해 갈색화를 유도한 실험군의 경우, 1주 및 4주 투여군 모두에서 신경세포재생과 연관된 신경선조세포와 신경모세포에 아무런 영향을 미치지 못하는 것을 확인하였다. 전령RNA 분석에서도 1주와 4주의 CL 316,243 투여가 신경세포재생과 연관된 전령RNA 발현에 영향이 없었으며, 신경세포재생과 연관된 신호전달 및 베타1아드레날린수용체 전령RNA에도 어떠한 영향을 미치지 못하는 것을 확인할 수 있었다.

위의 결과를 통해 외부자극의 요인으로서, 저온자극이 갈색화현상을 유도하는 것을 확인하였으며, 해마의 신경세포재생에 증가를 가져오는 것을 확인하였다. 그러나 β3-교감신경 작용제로 알려진 CL 316,243의 투여는 갈색화현상을 보다 강하게 유도하나, 해마의 신경세포재생을 유도하지 못함을 확인할 수 있었다. 이러한 결과로 미루어 볼 때, 저온자극에 의한 신경세포재생은 말초에서 유도되는 갈색화현상 때문이라기보다는 베타1아드레날린수용체 전령RNA의 증가 등으로 인하여, 신경세포재생을 촉진시켰을 것으로 사료된다.

주요어: 저온자극, 신경세포재생, 해마, 갈색화현상, 베타아드레날린신호전달

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감사의 글

설레임과 두려움을 안고 시작했던 대학원 과정을 지나, 박사과정을 마치며 지난 시간을 되돌아 봅니다. 작지만 수많은 노력과 시간이 모여 논문이 완성되어 가는 과정은 저에게 학문의 길 뿐 아니라 인격수양의 시간이었습니다. 이렇게 결실을 맺기까지 부족한 저를 지도해주신 윤여성 교수님, 제자에 대한 교수님들의 깊은 애정을 느낄 수 있었고 연구자로서의 태도와 학습방법을 배울 수 있었습니다. 교수님은 제가 할 수 있는 이상의 것을 펼칠 수 있게 해주셨고, 삶의 자세에 대한 가르침을 주셨습니다. 진심으로 감사드립니다. 논문 심사과정에서 위원장을 맡아 주신 성제경 교수님의 세심하며 명철한 제안과 배려는 연구자로서 더욱 깊은 사고를 할 수 있도록 해주었습니다. 깊은 감사를 드립니다. 연구자로서 첫발을 내디딜 수 있도록 정확한 시각과 혜안으로 조언을 아끼지 않으셨던 황인구 교수님께도 감사드립니다. 많은 가르침을 주시고 늘 응원해주시는 오승현 교수님의 지도는 연구에 큰 힘이 되었고 과학적 근거를 통한 연구과정의 지도는 저를

성장시키는 매우 큰 동력이었습니다. 대학원 입학시절부터 많은 조언을 주시고 연구에 대한 세심한 제안과 깊은 관심을 가져 주신 이순신 교수님께도 감사를 드립니다. 학위과정 동안 함께했던 조직학교실 선배님들과 후배님들께도 깊은 감사를 전합니다.

항상 저를 믿어주시고 묵묵히 응원해주시는 사랑하는 부모님께 감사드립니다. 항상 이해해 주시고 믿어주셔서 제가 여기까지 할 수 있었고, 한 발짝 더 나아가겠다는 결심도 할 수 있었습니다. 열심히, 그리고 부모님께서 해주신 은혜에 보답할 수 있는 자랑스러운 아들이 되도록 하겠습니다. 제 소중한 인연, 부인에게도 감사를 전합니다. 항상 변함없는 마음으로 같은 자리를 지켜주셔서 제가 마음 편히 졸업 할 수 있었습니다. 앞으로도 잘 부탁하고 사랑합니다.

마지막으로 지면으로 통해서 일일이 언급을 하지 못했지만 그 동안 저를 아끼고 사랑해주신 모든 분들께 다시 한번 진심으로 감사 드립니다.