The Effects of Enriched Environment on Paraventricular Hypothalamic Brain-derived Neurotrophic Factor Expression

An Undergraduate Honors Research Thesis

Presented in partial fulfillment of the requirements for graduation *with Honors Research Distinction in Neuroscience* in the undergraduate colleges of The Ohio State University

By

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ABSTRACT

The regulation of energy homeostasis involves a balance between food intake and energy expenditure. The hypothalamus is a crucial regulator for energy balance and consists of several discrete nuclei, including the arcuate (ARC), ventromedial (VMH), dorsomedial (DMH), and paraventricular (PVH) hypothalamus. Brain-derived neurotrophic factor (BDNF) expressed in hypothalamic nuclei is significant in regulating energy homeostasis. Our studies demonstrated that an enriched environment (EE), a complex housing with social and physical stimulation, upregulated BDNF expression in the ARC and VMH/DMH leading to an anti-obesity and anti-tumor phenotype. Moreover, the EE anti-obesity phenotype can be mimicked by overexpression of ARC and VMH/DMH *Bdnf*. While studies have shown diet- and genetic-induced obesity can be reversed by transferring the *Bdnf* gene to ARC and VMH/DMH, the roles of other nuclei in mediating the EE phenotype is not known. Research has shown that PVH BDNF has a strong and lasting impact on suppressing food intake and increasing energy expenditure, and the deletion of *Bdnf* in the PVH leads to hyperphagia and severe obesity. Thus, we hypothesized PVH *Bdnf* is upregulated by EE. Our results did not demonstrate significant upregulation of *Bdnf* expression in PVH or ARC after EE exposure. However, we observed a markedly increase of *Vgf* (non-acronymic) expression in PVH of EE mice, suggesting *Vgf* can be our next target gene of interest to study EE's anti-obesity effects.

INTRODUCTION

Obesity is one of the leading health problems in the U.S., with a prevalence of more than 78.6 million adults and more than one-third of children and adolescents (Ogden, Carroll, Kit, & Flegal, 2014; Ogden et al., 2016). Obesity is associated with increased risk of cardiovascular diseases, depression and cancers (Skinner, Perrin, Moss, & Skelton, 2015; Wong, Janssen, & Ross, 2003). The economic burden of obesity is greater than one-fifth of all annual medical expenses, with a $14.1 billion obese-related medical expenditure simply on children and

adolescents (Hammond & Levine, 2010; Trasande & Chatterjee, 2009). Therefore, finding effective treatments for obesity is in urgent need. Obesity is generally resulted from excess energy intake or few energy expenditures. The energy homeostasis is controlled by genetic and environmental factors. One of the genetic components that control energy balance is the hypothalamus. The hypothalamus is a crucial regulator for energy balance. Several discrete nuclei in the hypothalamus are involved in energy balance control circuits: arcuate nucleus (ARC), ventromedial hypothalamus (VMH), dorsomedial hypothalamus (DMH), and paraventricular hypothalamus (PVH). Moreover, many studies found that brain-derived neurotrophic factor (BDNF), a widely-expressed member of the neurotrophin family related to growth factors in the brain, is highly involved in hypothalamic energy control circuits. The deletion of *Bdnf* and its receptor tyrosine kinase receptor B (trkB/Ntrk2) in the hypothalamus can induce severe obesity phenotypes (Gray et al., 2006). Studies also demonstrated that *Bdnf* knockout in hypothalamic nuclei can induce obesity phenotypes, whereas *Bdnf* overexpression can rescue the mutant obese phenotype (Abizaid, Gao, & Horvath, 2006; Liao et al., 2012; Xu et al., 2003). Nevertheless, our group is approaching this association between BDNF and hypothalamic energy homeostasis circuits from the environmental aspect. Our study showed that an enriched environment (EE), -a complex housing supplemented with physical, social, and cognitive stimuli, induced resistance to diet-induced obesity and improved metabolism in obese mice (Cao et al., 2011). EE upregulates *Bdnf* expression in ARC, DMH, and VMH; overexpression of *Bdnf* in these nuclei mimics the EE anti-obesity and anti-cancer phenotype (Cao et al., 2009; 2010; Xiao et al., 2016). However, *Bdnf* expression in other hypothalamic nuclei and their association to EE effects are poorly investigated. A recent study showed *Bdnf* deletion in PVH induced hyperphagia and resulted in severe obesity (An, Liao, Kinney, Sahibzada, & Xu, 2015), suggesting PVH can be our candidate for obesity therapeutic target in EE. Thus, in this study we hypothesized that EE upregulates *Bdnf* expression in PVH.

METHODS

Environmental Enrichment.

Male 3-week-old C57BL/6 mice (from Charles River) were randomly assigned to control housing (HC) and enriched environment (EE) (*n*=10 per group). Ten mice were housed in two medium enriched bins of 73 cm × 41 cm × 46 cm (*n*=5 per bin). Enriched bins were supplemented with running wheels, tunnels, igloos, huts, and nesting material in addition to standard lab chow and water (Andrew M Slater, 2015) (Figure 1). Control mice were housed under standard laboratory conditions with five mice per cage. Food and water were changed weekly and provided to all mice *ad libitum*. Housing cages and static wires in both control and enrichment groups were cleaned every week. Rearrangement of enrichment devices were conducted weekly and all toys were cleaned each week. Measurement of body weight and food intake was recorded every week. All mice were housed in their respective environments for four weeks before being sacrificed.

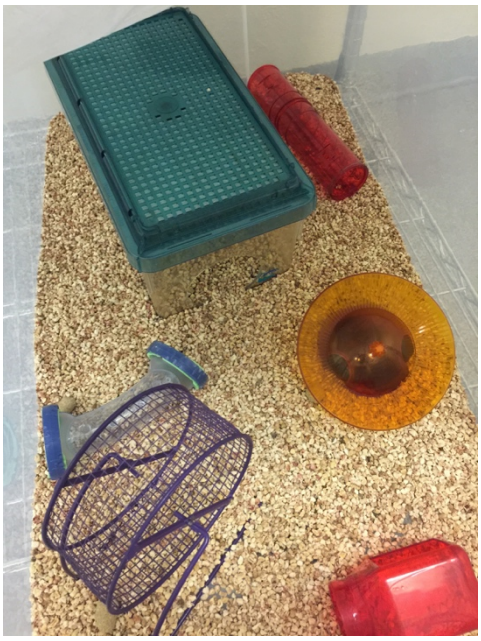

Figure 1. Top-down view of EE

Cryosectioning.

Mice were perfused with 4% paraformaldehyde (Sigma). Fixed brains were kept in phosphate-buffered saline (PBS) overnight at 4 ºC and were subsequently switched to PBS with 30% sucrose. Brains were placed in whole brain cryomolds filled with optimal cutting temperature (OCT) compound. Cryomolds were placed in dry ice until frozen, at which point brains were removed from the mold and sectioned on a ThermoFisher cryostat at -20 °C into 15 *μ*m slices. Sections were collected on slides and stored at -80 °C until staining.

Histology and Microscopy.

Slides were dried at room temperature for two hours before staining. The section was treated with xylene for 5 min, dehydrated with 95% ethanol, then 70%, washed with deionized water, treated with cresyl violet solution for 10 min (at 60°C), washed with distilled water, rehydrated with 70% ethanol, 95%, then 100%, treated with xylene for 5 min, and coverslipped using cytoseal. A 10x imaging of the section was captured on a Zeiss Axio Imager M1 microscope.

Tissue Collection.

Brain and adipose tissue were collected four weeks post housing. PVH and ARC nuclei were microdissected immediately upon isolation from skulls. The following adipose tissue depots were collected and weighed: brown adipose tissue (BAT), epididymal adipose tissue (EAT), inguinal adipose tissue (IAT), and retroperitoneal adipose tissue (RAT).

Hypothalamic Microdissection.

Brains were quickly isolated on ice. The slices containing PVH and ARC were sectioned from three 1-mm-thick coronal sections (-0.58 to -3.58 mm from bregma) with a mouse brain matrix. PVH and ARC were both microdissected with a 30° microsurgical knife (OASIS) under a dissection scope. PVH was dissected from an inverted triangular section 0.75 mm downwards from the ventral tip of the third ventricle and 0.75 mm bilateral from midline in the first slice. ARC was dissected from a rectangular section on the second and third slices. The height of the rectangular section was 0.50 mm upwards from the dorsal edge of hypothalamus, whereas the width was 0.75 mm and 0.50 mm bilateral from midline respectively. In the second slice, it contained a small portion of VMH, but this would be addressed as ARC as the majority was from ARC nucleus. Tissues were snap-frozen on dry ice and stored at -80 °C until further analysis.

RNA Isolation and cDNA Synthesis.

QIAGEN RNeasy Micro Kit was used to extract RNA from PVH and ARC of selected HC and EE mice (*n*=5 per group). All procedures were conducted according to the manufacturer's protocol. The extracted RNA was eluted with 28 *μ*L RNase-free water to attain a 26 *μ*L RNA sample. A spectrophotometer (NanoDrop1000) was used to determine RNA quantity and quality. All RNA samples were used with 0.1 *μ*g for cDNA synthesis with TaqMan Reverse Transcription Reagent (Applied Biosystems).

Real-time Quantitative Polymerase Chain Reaction (qPCR).

Real-time qPCR was carried out using StepOnePlus System (Applied Biosystem) with the Power SYBR Green PCR Master Mix (Applied Biosystems). Primers were designed to detect the following genes in the PVH cDNA: *Bdnf, Crh, Crhr1, cFos, Ghrh, Mc4r, Ntrk2, Oxt, Sst, Th, Trh, Vgf*. The following genes were detected in the ARC cDNA: *AgRP, Bdnf, Npy, Ntrk2, Vgf*. Sequences of primers are available upon requests. Data were calibrated to endogenous control *Hprt1* and the relative gene expression was quantified using the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis.

Data were expressed as means ± SEM. We used Student's *t test* (two tails, unpaired) to analyze the food intake, body weight, adiposity and gene expression in HC and EE group, as well as in the relative gene expression in PVH and ARC, with a *p*-value < 0.05 considered statistically significant.

RESULTS

PVH and ARC Microdissection.

Our previous study used laser capture microdissection (LCM) for hypothalamic nuclei dissection (Cao et al., 2010; 2011). However, LCM required highly-skilled operators and is expensive in training and operating. For this study, we used microsurgical knife to dissect PVH and ARC. Firstly, we tested our accuracy of dissecting PVH and ARC with a mouse brain matrix and a microsurgical knife. We conducted Nissl-stained histology to learn the location of PVH in the hypothalamus on a separate group of 8- or 9-week-old spare mice. All mice brain histology sections were collected using a cryostat. The histology showed a dense nuclei population located bilaterally adjacent to the ventral tip of the third ventricle which is one of the landmark for PVH (Figure 2A), suggesting our result is consistent with the staining presented in the mice *stereotaxic atlas*. Thus, we were confident of dissecting the correct slice containing PVH.

Secondly, to test the accuracy of our microdissection of PVH and ARC with stab knife,

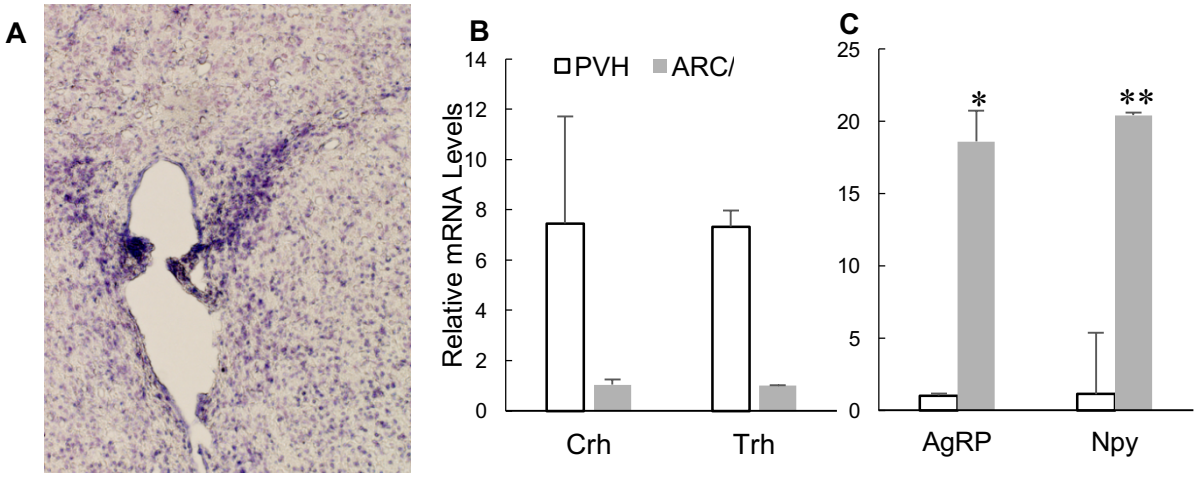

Figure 2. Mice Brain Section Histology and Gene Expression Levels of PVH and ARC in Spare Mice.
(A) Representative Nissl-stained histology of mice brain section.
(B) Relative CRH and TRH expression levels in PVH and ARC (n=3).
(C) Relative AGRP and NPY expression levels in PVH and ARC (n=3; *p < 0.05; **p <0.01)
Values are mean ± SEM.

we ran real-time qPCR to examine the relative gene expressions in these two regions. Two of the major neuropeptidergic populations in PVH are corticotrophin-releasing hormone (CRH) and

thyrotropin-releasing hormone (TRH) (Biag et al., 2011). CRH and TRH were used as gene markers for PVH, and we expected higher *Crh* and *Trh* expressions in PVH than in ARC. The gene markers we used for ARC were neuropeptide Y (NPY) and agouti-gene related protein (AGRP). NPY mRNA has the highest expression in ARC and *Agrp* is almost exclusively expressed in ARC (Chronwall, Chase, & O'Donohue, 1984; Parker & Bloom, 2012). Our results showed that expression of *Agrp* and *Npy* were drastically higher in ARC than in PVH (Figure 2B; **p* <0.05; ***p*<0.01). The *Crh* and *Trh* expressions in PVH were approximately 6-fold higher than in ARC (Figure 2C). The qPCR results indicated that using microdissection stab knife could give us enough accuracy for our further analysis, and therefore we could start our enrichment

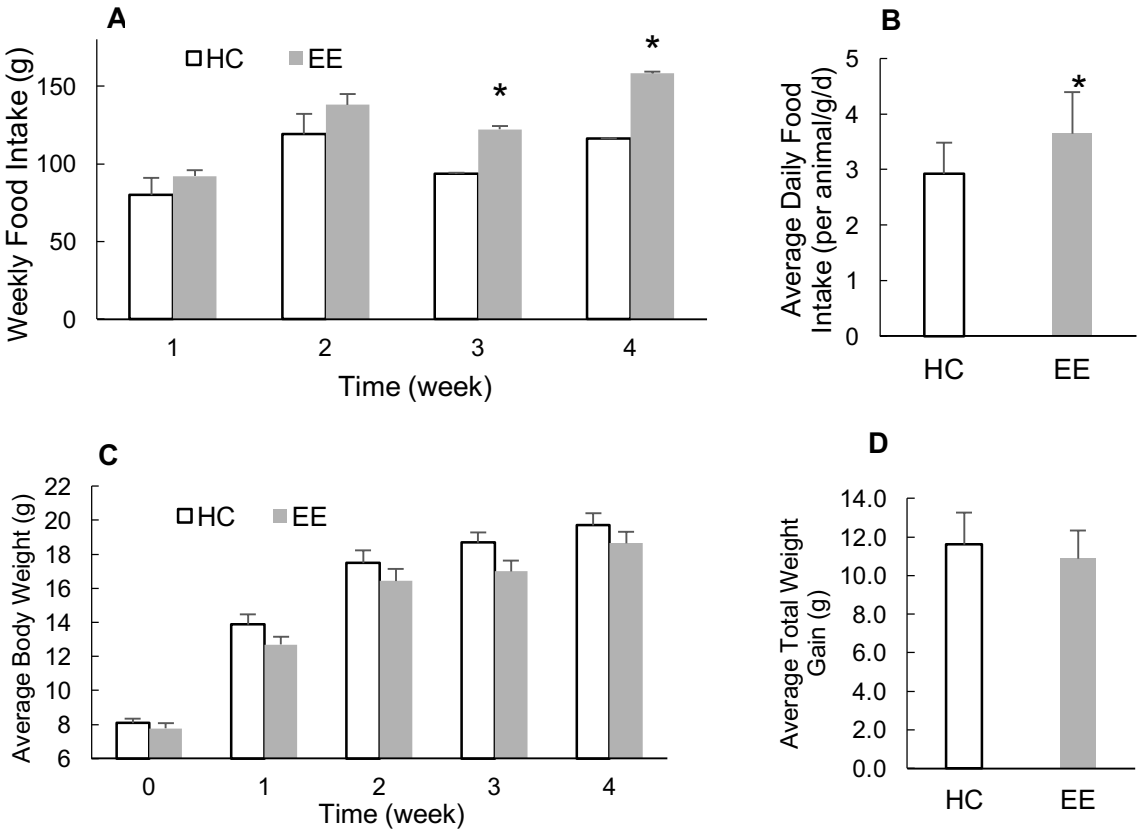

Figure 3. EE Increased Food Intakes Without Changing the Body Weight. (A). EE increases food intake at 3 weeks and 4 weeks (**p* value< 0.05)**.** (B). Average food intake of per animal per day. (* *p* value < 0.05). (C). Average body weight showed no change in EE. (D) The average of the total weight gains in HC and EE mice.
Values are mean ± SEM. *n*=10 per group.

experiment confidently.

EE Increases Food Intake without Changing the Body Weight.

Twenty 3-week-old male C57BL/6 mice were randomized into control housing cages (HC) or mid-size enriched bins (EE) (*n*=5 per cage) for four weeks. Standard laboratory chow and water were provided to all mice *ad libitum*. Food intake was recorded from four cages weekly. At 3 weeks and 4 weeks, EE groups showed significantly increased food consumption compared to HC groups (Figure 3A; $p < 0.05$). The average daily food intake for each EE mice was higher compared to their HC littermates (Figure 3B; $p < 0.05$). Nevertheless, EE mice did not show difference in either weekly body weight or total weight gain than HC group (Figure 3C & 3D). The results suggested that EE induced more energy expenditure but not suppressed food intake.

EE Decreases Adiposity in EE Mice.

All mice were sacrificed after four-week's exposure to their respective housing environment. The adipose and brain tissues of the mice were collected. We measured the weight of the adipose tissue, and calibrated fat pad mass to body weight. Our results demonstrated that EE decreased adiposity, with a drastic reduction in BAT ($p<0.05$), EAT and RAT ($p<0.01$) (Figure 4). Thus, we can confirm that EE reduced adiposity even

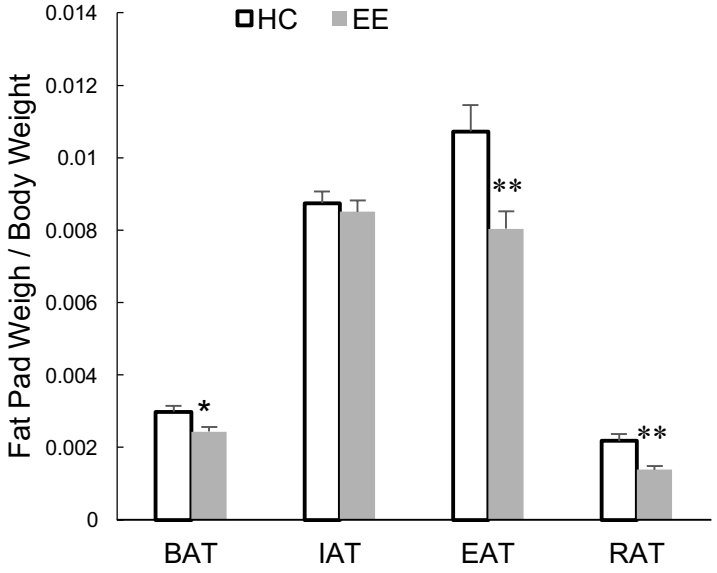

Figure 4. EE Decreases Adiposity in Mice.
The average of fat pad weight calibrated to body weight.
*$p < 0.05$; **$p < 0.01$
n=10 per group

with an increasing food intake. This result is consistent with our previous study, in which large-size enriched bins were used (Cao et al., 2011), suggesting a mid-size enrichment bin can induce EE phenotype.

ARC and PVH Gene Expression Profile

In our previous studies, EE induced upregulation of *Bdnf* expression in ARC and VMH/DMH (Cao et al., 2010; 2011). In this study, we examined the relative expression of *Bdnf* and its receptor *Ntrk2* together with genes expressed in ARC and PVH. One of the gene we examined in both ARC and PVH was *Vgf*, a nerve growth factor-inducible transcript. Our recent study suggested that VGF was acting downstream of hypothalamic BDNF and was involved in

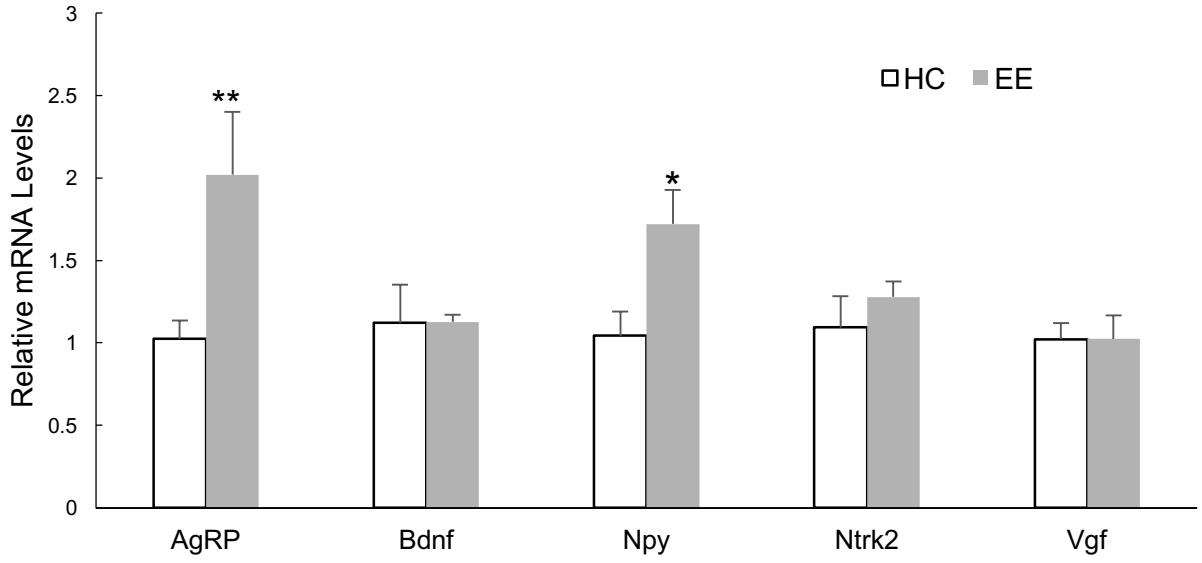

Figure 5. Gene Expression Profile in ARC.
Values are mean ± SEM.
*p <0.05; **p <0.07 (n=5 per group).

the melanocortin central pathway (Foglesong et al., 2016). The melanocortin pathway integrates central hormonal and neuronal signals to control energy balance (Balthasar et al., 2005; Garfield, Lam, Marston, Przydzial, & Heisler, 2009). One of the melanocortin receptor, melanocortin receptor 4 (*Mc4r*), is highly expressed in PVH; PVH receives direct melanocortin

input and is downstream of ARC nucleus (Sutton, Myers, & Olson, 2016). Thus, *Vgf* expression in ARC and PVH may reveal some unknown association along the melanocortin pathway.

AGRP and NPY were used as gene markers for ARC, both of which are neuropeptides involved in promoting feeding behavior (Krashes et al., 2014; Morton, Meek, & Schwartz, 2014). In ARC gene profile, an upregulation of *Npy* (p <0.05) and a trend of increasing *Agrp* (p <0.07) were observed (Figure 5), which was consistent with the observed increasing food intake in EE mice. Nevertheless, the upregulation of *Bdnf* expression in ARC was not observed, and no change was found in *Vgf* expression either (Figure 5).

We examined the expression of *Bdnf* and its receptor *Ntrk2* with some genes of interests in PVH, including *Crh* and one of its receptor *Chrh1*, *c-Fos*, *Trh*, growth hormone-releasing hormone (*Ghrh*), *Mc4r*, oxytocin (*Oxt*), somatostatin (*Sst*), tyrosine hydroxylase (*Th*) (An et al., 2015; Bali & Kovacs, 2003). Our qPCR showed no change of PVH *Bdnf* expression in EE mice.

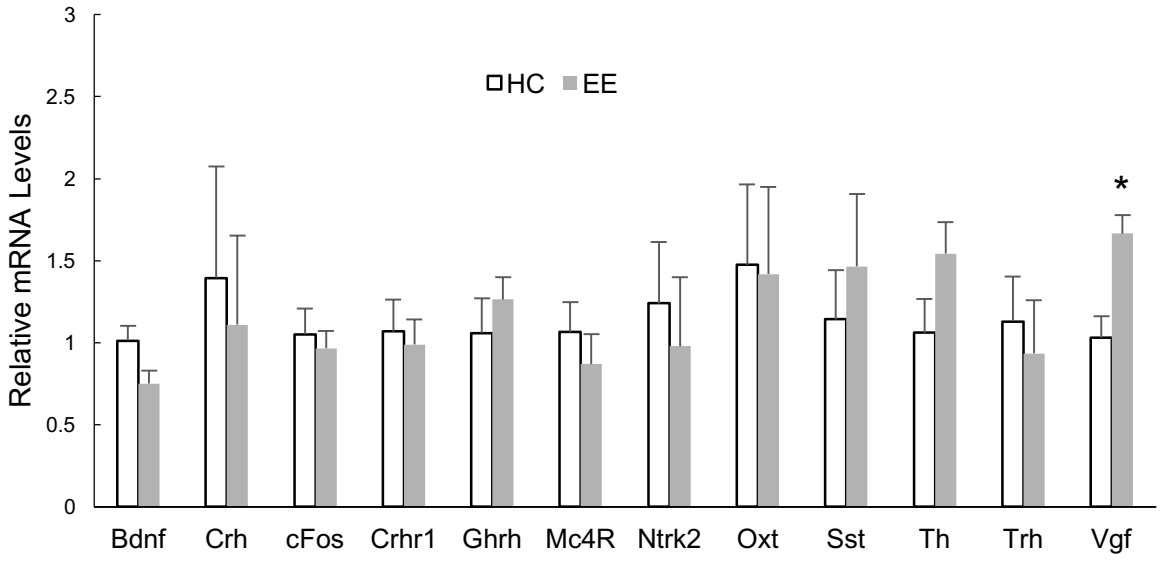

Figure 6. Gene Expression Profile in PVH.
Values are mean ± SEM. *p <0.01.
For *Bdnf*, *Crh*, *Th, Vgf*, *n=4* (HC), *n*=5 (EE)

Among the genes examined, only *Vgf* showed significant upregulation in PVH of EE mice, suggesting PVH VGF might play a role in EE-associated metabolic change.

DISCUSSION

Our results demonstrate that modified enriched environment in mid-size bin markedly decreased adiposity even with increased food intake, and induced upregulation in *Npy* and *Agrp* expression after 4-weeks of EE exposure. This result suggests our mid-size enrichment induced similar EE effects to what we observed in large-size bin with a larger group of mice in our previous study (Cao et al., 2010). However, EE-induced upregulation of *Bdnf* expression in ARC was not observed in this study. One possible explanation is that the mid-size EE setting induced ARC *Bdnf* upregulation at an earlier time point, but this upregulation was not sustained at 4 weeks. Our previous study showed that EE upregulated ARC *Bdnf* at 2 weeks and sustained at 4 weeks, but the upregulation was not significant at 9 weeks (Cao et al., 2010), suggesting *Bdnf* upregulation was an early event responding to EE. Our animal group-size is smaller than the one in the previous study, which could lead to a weaker EE effect. Moreover, compared to our previous study used laser capture microdissection to dissect ARC nuclei, the limited accuracy of the microsurgical knife may also lead to an insignificant result.

The EE effect on PVH gene expression was limited based on our study, and our hypothesized EE-induced PVH *Bdnf* regulation was not observed. One recent study demonstrates that *Bdnf* deletion in anterior PVH leads to hyperphagia, whereas the medial and posterior PVH BDNF is more involved in thermogenesis than food intake (An et al., 2015). Our previous data showed EE led to a slight but significant increase in the body temperature in diet-induced obese mouse model as well as an elevation of adipose thermogenesis (Cao et al., 2011). Therefore, EE might upregulate *Bdnf* in medial/posterior PVH but not in the anterior part. Our dissection was not accurate enough to separate the anterior and posterior PVH, and thus the possible EE-induced upregulation of *Bdnf* in medial/posterior PVH was not observed.

Moreover, our PVH gene profile showed an upregulation of VGF in EE mice. VGF is thought to be downstream of BDNF, which suggests PVH *Bdnf* could be upregulated at an earlier time point.

Our results showed reduced adiposity and upregulated *Npy* and *Agrp* expression in ARC, suggesting an EE anti-obesity effect. In order to test whether PVH and ARC *Bdnf* upregulation occurs at an earlier time point in the mid-size enrichment, we can design an experiment to investigate EE effects at two week's point in mid-size bin. Moreover, we can employ laser caption microdissection to collect anterior and medial/posterior PVH to explore if the *Bdnf* expression levels in the two regions are differentially regulated in EE. Finally, we can examine VGF's sufficiency and necessity in EE effects, and thus to evaluate the potentiality of VGF as a therapeutic gene for obesity.

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