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The effect of enriched environment across ages: A study of anhedonia and BDNF gene induction

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Abstract

Enriched environment treatment (EET) is a potential intervention for depression by inducing brain-derived neurotrophic factor (BDNF). However, its *age dependency* remains unclear. We recently found that EET during early-life development (ED) was effective in increasing exploratory activity and anti-despair behavior, particularly in promoter IV-driven BDNF deficient mice (KIV), with the largest BDNF protein induction in the hippocampus and frontal cortex. Here, we further determined age dependency of EET effects on anhedonia and promoter-specific BDNF transcription, by using the sucrose preference test and qRT-PCR. Wild-type (WT) and KIV mice received two months of EET during ED, young-adulthood and old-adulthood (0–2, 2–4, and 12–14 months, respectively). All KIV groups showed reduced sucrose preference, which EET equally reversed regardless of age. EET increased hippocampal BDNF mRNA levels for all ages and genotypes, but increased frontal cortex BDNF mRNA levels only in ED KIV and old WT mice. Transcription by promoters I and IV was age-dependent in the hippocampus of WT mice: more effective induction of exon IV or I during ED or old-adulthood, respectively. Transcription by almost all 9 promoters was age-specific in the frontal cortex, mostly observed in ED KIV mice. After discontinuance of EET, the EET effects on anti-anhedonia and BDNF transcription in both regions persisted only in ED KIV mice. These results suggested that EET was equally effective in reversing anhedonia and inducing hippocampal BDNF transcription, but was more effective during ED in inducing frontal cortex BDNF transcription and for lasting anti-anhedonic and BDNF effects particularly in promoter IV-BDNF deficiency.

Keywords

enriched environment; across ages; anhedonia; brain-derived neurotrophic factor (BDNF); gene expression; promoter IV; early life

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Supplementary information is available.

Introduction

Enriched environment treatment (EET), which combines physical exercise, mental stimulation, and social interaction (Hebb, 1947, Rosenzweig, 1966, Segovia *et al.*, 2009, Van Praag *et al.*, 2000), is a potential intervention for depression (Dimeo *et al.*, 2001, Greenwood *et al.*, 2003, Martinsen, 1990). EET induces expression of brain-derived neurotrophic factor (BDNF) (Bennett *et al.*, 1969, Greenough & Volkmar, 1973, Jha *et al.*, 2011, Kempermann *et al.*, 1997, Molteni *et al.*, 2002, Van Praag *et al.*, 1999), a critical neuronal growth factor implicated in the pathophysiology of depression (Boulle *et al.*, 2012, Castren, 2005, Chourbaji *et al.*, 2011, Duman & Monteggia, 2006, Sakata, 2011, Sakata, 2014). Decreased BDNF expression has been observed in the hippocampus and prefrontal cortex of depressed humans (Dwivedi *et al.*, 2003) and stressed animals (Roth *et al.*, 2009, Smith *et al.*, 1995, Tsankova *et al.*, 2006). In particular, inactivation of promoter IV via epigenetic modification is linked to depression (Hing *et al.*, 2012, Keller *et al.*, 2010) and stress (Fuchikami *et al.*, 2009, Roth *et al.*, 2011, Tsankova *et al.*, 2006). Promoter IV-driven BDNF deficiency leads to depression-like behavior in mice (Sakata *et al.*, 2010). We previously found that EET was more effective than treatment with four classes of antidepressant drugs in reversing the depression-like behavior caused by the promoter IV-BDNF deficiency in young-adult mice (Jha *et al.*, 2011, Sakata *et al.*, 2013b). While 9 promoters control BDNF gene expression in both humans and rodents (Aid *et al.*, 2007, Liu *et al.*, 2005), EET induces BDNF expression driven by promoters I, II, and III (Adlard *et al.*, 2005, Jha *et al.*, 2011, Russo-Neustadt *et al.*, 2000, Zajac *et al.*, 2010) with epigenetic modification at promoters II, IV, and VI (Gomez-Pinilla *et al.*, 2010, Kuzumaki *et al.*, 2011).

Despite the well-established BDNF effects of EET, what remains unclear is its age dependency, particularly for effectiveness against depression. Does EET affect depression differently across ages? This information is important in developing effective strategies for prevention and treatment of depression. We recently examined EET effects across three distinctive life stages in mice: early-life development, young-adulthood, and old-adulthood. We found that EET effects in increasing exploratory activity and anti-despair behavior were largest and long-lasting when EET was provided during early-life development, particularly in promoter IV-BDNF deficient mice (Jha *et al.*, 2016). BDNF protein induction in the hippocampus and frontal cortex was also the largest after early-life EET, while the EET effects were limited in old-adult mice (Jha *et al.*, 2016). These results led to our hypothesis that EET during early-life development provides maximum and lasting effects in antidepressive behavior and BDNF induction. In continuation of this study, we further tested our hypothesis by investigating age dependency of EET effects on anhedonia, one core depression-related phenotype. We also determined long-lasting transcriptional changes driven by each BDNF promoter, which may account for lasting BDNF protein induction after discontinuance of EET.

Materials and Methods

Animals

Wild-type (WT) and knock-in BDNF-promoter IV (KIV) mice were used to assess EET effects in normal and depressed conditions (Sakata *et al.*, 2010), respectively. The generation

of KIV mice has been described previously (Sakata *et al.*, 2009) and followed the breeding guidelines recommended (Crusio *et al.,* 2009). Briefly, KIV mice lack promoter IV-driven BDNF expression by insertion of a green fluorescent protein (GFP) gene, but retain 8 other promoters and the BDNF protein-coding region (Sakata *et al.*, 2009). KIV mice were generated from 129cX/Sv × 129X1/SvJ ES cells and then crossed to C57BL/6J females for more than 12 generations. Heterozygous mice were bred to produce WT and KIV littermates of the same genetic background. Offspring from these littermates were used.

EET effects were examined at three life stages: early-life development (ED: 0–2 months), young-adult (YA: 2–4 months), and old-adult (OA: 12–14 months); these life stages represent development before reproductive maturity, after sexual maturity, and middle-age in mice (Flurkely *et al.*, 2007, Jax). Male and female mice were used to examine sex-specific effects.

A cohort of mice was used to assess EET effects on anhedonia (about 360 mice: N=15 mice per group × 2 genotypes × 2 sexes × 2 treatment conditions × 3 life stages). Another cohort of mice was used to assess EET effects on BDNF gene transcription (about 192 mice: N=8 mice of 4 males and 4 females per group × 2 genotypes × 2 treatment conditions × 2 time points × 3 life stages). The sample size was based upon previous experiments and power analyses. The same animals in our previous study (Jha *et al.*, 2016) were used in this study. Mice were group-housed with standard bedding in a climate-controlled vivarium on a normal 12-hour dark/light cycle with *ad libitum* access to food and water. All animal experiments were approved by the University of Tennessee Laboratory Animal Care and Use Committee.

Treatments

Age- and sex- matched mice were randomly assigned to standard condition treatment (SCT) or enriched environment treatment (EET), as described previously (Jha *et al.*, 2011, Jha *et al.*, 2016). Briefly, SCT consisted of a small 27×16×12 cm cage containing 2–5 mice, group-housed to avoid isolation stress. EET consisted of a larger 44×22×16 cm cage, containing one plastic running wheel per 5 mice to allow for physical activity, a variety of toys to increase perception and mental exercise, and 5–10 company mice with nesting material to increase social interaction. Toys were replaced weekly, and mice in EET cages were given bacon-flavored Rodent Foraging Crumbles (Bio-Serv, Frenchtown, NJ) to encourage exploration in the enriched environment.

The treatment period of two-months was used to encompass mouse development from birth to reproductive maturity. ED-EET mice were born and raised in an EET cage, then weaned to another EET cage at 3 weeks of age until 2 months of age. YA-EET and OA-EET mice started treatment at 2 months or 12 months of age, respectively. After treatment, EET mice were then placed in standard condition (EET-SCT), while SCT mice remained in standard condition (SCT-SCT), for 1 additional month to determine, if any, the persisting effects of EET (see Fig. 1a for research design).

Sucrose Preference Test

This test was used to assess mouse anhedonia (Pothion *et al.*, 2004, Sakata *et al.*, 2010) after the initial two months of SCT or EET and, again, after one month of SCT (at T_1 and T_2 in Fig. 1a). Mice were habituated to two water bottles for 4 days and then water-deprived overnight (17:00–9:00 h). During a 3 h session (9:00–12:00 h), mice were housed individually and given access to one bottle of regular water and one bottle of preferable 1% sucrose solution. Liquid consumption was measured by weighing each bottle before and after consumption, and sucrose preference was calculated as total sucrose water intake/total (sucrose + water) intake (Willner *et al.*, 1987).

RNA Extraction and qRT-PCR by BioMark

qRT-PCR was used to measure total BDNF mRNA levels and to identify the involved promoters by detecting each transcript driven by 9 BDNF promoters. Hippocampus and frontal cortex tissues were collected between 14:00–17:00 h from the 4 treatment groups (SCT/EET at T_1 and SCT-SCT/EET-SCT at T_2, see Fig. 1a) and stored at −80°C until processed. Each sample was homogenized by pipet and QIAshredder (Qiagen, Valencia, CA). Total RNA was extracted using an RNeasy Kit with on-column DNase (Qiagen) and quantified using the NanoDrop spectrophotometer (Agilent Technologies, Santa Clara, CA). One μg of total RNA was converted to cDNA (First Strand cDNA Synthesis Kit, Roche Applied Science, Indianapolis, IN). qRT-PCR was performed using BioMark, as described previously (Reiner *et al.*, 2012, Sakata & Duke, 2014, Sakata & Overacre, 2017). Briefly, 25 ng of cDNA was preamplified with pooled primers (200 nM) and PreAmp Master Mix (Applied Biosystems, Carlsbad, CA) for 14 cycles of 95°C for 15 s and 60°C for 4 min. Then diluted reactions (1:5) with TE buffer (1 mM Tris-HCl pH 8 and 0.1 mM EDTA) were used for qPCR in a M96.96 dynamic array chip (Fluidigm, South San Francisco, CA). Each inlet contained 5 μL of assay mix [1 μM of forward primer, 1 μM of reverse primer, 1 μM of UPL probe, and 1× assay reagent (Fluidigm, PN85000736)] or 5 μL of sample mix [2.25 μL of preamplified sample, 2.5 μL of 2× Kapa Probe Fast qPCR Master Mix (Kapa Biosystems, Wilmington, MA) and 0.25 μL of 20× Sample Loading Solution (Fluidigm, PN85000735)]. The thermal cycle consisted of 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Cycle threshold (Ct) values were obtained by BioMark Gene Expression Data Analysis software after automatic inspection for quality. The average Ct values of cyclophilin D and HGPRT were used for reference because these genes previously showed the least variation of expression in the hippocampus and frontal cortex among six tested housekeeping genes (cyclophilin D, HGPRT, TBP, β-actin, β-tubulin, and S19). Relative gene expression values were determined by using the $2^{-\Delta\Delta Ct}$ method of Livak and Schmittgen (Livak & Schmittgen, 2001). Gene expression values were normalized to the average values of gene expression in control mice (e.g., respective SCT groups). Information on primers and probes is presented in Supplementary Table 1.

Statistical Analysis

The D'Agostino-Pearson omnibus normality tests or Shapiro-Wilk tests were performed to analyze the distribution of data for sucrose preference or gene expression, respectively, in each genotype and each treatment condition. All the data were normally distributed

($P>0.05$). Two-way analyses of variance (ANOVA) with *post hoc* Bonferroni multiple comparisons were computed to determine two effects (e.g., effects of EET and age) unless specified. One-way ANOVA was used on three data groups (ED, YA, vs. OA). Student's *t*-tests were performed on the two specific data groups (e.g., KIV-EET vs. WT-SCT). Data are presented as means ± SEM with statistical significance set at $P<0.05$*, $P<0.01$**, and $P<0.005$***.

Results

1. Genetic and environmental effects on anhedonia

1-1. Genotype effects—We first determined whether promoter IV-BDNF deficiency affected sucrose preference differently across life stages. When compared to WT mice in SCT, all KIV mice in SCT showed significantly reduced sucrose preference (WT vs. KIV: $P<0.05$ for each, detailed statistics are presented in Supplementary Table 2, Fig. 1b). No age effects were observed in the reduced sucrose preference of KIV mice of either sex (ED, YA, and OA: $F_{\text{male (2,28)}}=0.3$, $P>0.05$, $F_{\text{female (2,28)}}=0.4$, $P>0.05$, one-way ANOVA, Supplemental Fig. 1a), indicating that promoter IV-BDNF deficiency universally caused anhedonia.

1-2. EET effects—Next, we asked whether EET effects on anhedonia are age-dependent. EET across all ages significantly increased sucrose preference of KIV mice for both sexes (KIV, SCT vs. EET: $P<0.05$ for each, see Supplementary Table 2 for statistical details), while normalizing its reduction to the levels of WT mice in SCT (WT-SCT vs. KIV-EET: $P>0.05$, Student-*t* test, Fig. 1b). No age-specific effects were observed in the EET effects for either sex ($F_{\text{male(2,84)}}=2.1$, $P>0.05$, $F_{\text{female(2,83)}}=0.04$, $P>0.05$, Supplementary Fig. 1b). EET did not change the sucrose preference in WT mice at any life stage (WT, SCT vs. EET: $P>0.05$ at any ages, Fig. 1b). These results indicated that EET reversed anhedonia caused by BDNF deficiency, regardless of age and sex, but did not increase sucrose preference in normal conditions.

1-3. Lasting EET effects—We then investigated whether the EET effects in reversing anhedonia would persist without EET and whether the effects differ across ages. One month after EET discontinuance, all KIV groups with EET presented similar sucrose preference levels as WT mice (KIV EET-SCT vs. WT SCT-SCT: $P>0.05$ at any ages). Significant EET-induced increases were observed only in ED KIV males and females and YA KIV males (KIV SCT-SCT vs. EET-SCT: $P<0.05$ for each, Fig. 1c). These results indicated that EET effects in reversing anhedonia persisted, regardless of age and sex, with significant effects when EET was provided in early life.

2. Total BDNF mRNA levels across ages

We next examined the age-dependent effects of promoter IV-BDNF deficiency and EET on BDNF gene transcription. Total BDNF mRNA levels were measured by the levels of exon IXc, the common BDNF protein-coding region shared by transcripts driven by all 9 BDNF promoters (Aid *et al.*, 2007, Liu *et al.*, 2005).

2-1. Basal levels—Age effects on basal BDNF levels without EET were examined by comparing SCT groups at ED, YA, and OA. Only WT frontal cortex showed age-dependent decline of basal BDNF levels (WT-SCT, OA vs. ED or YA: $P<0.05$, Fig. 2a). KIV mice presented significant reductions in total BDNF mRNA levels in both frontal cortex and hippocampus, regardless of age, when compared to WT mice (WT-SCT vs. KIV-SCT: $P<0.001$ for each, Fig. 2a).

2-2. EET effects—In the hippocampus, EET significantly increased total BDNF mRNA levels in all age groups for both genotypes (SCT vs. EET: at least $P<0.05$, Fig. 2b left). No significant effects of aging and genotype were observed ($P>0.05$, Supplementary Table 2, 3). By contrast, in the frontal cortex, significant effects of age and genotype were observed ($F_{age(2,42)}=4.4$, $P<0.05$; $F_{genotype(1,42)}=9.2$, $P<0.01$), where EET increased total BDNF mRNA levels only in ED KIV mice and OA WT mice (SCT vs. EET: $P<0.005$ for each, Fig. 2b right).

2-3. Lasting EET effects—One month after EET discontinuance, treatment × age interactions were observed for both regions ($F_{hippocampus(2,38)}=6.28$, $P<0.01$; $F_{frontal\_cortex(1,42)}=3.6$, $P<0.05$) where only ED KIV mice showed significant BDNF increases (KIV, EET-SCT vs. SCT-SCT: $P<0.05$, Fig. 2c). The results indicated that BDNF gene induction persisted only with early-life EET in promoter IV-BDNF deficiency, possibly due to lasting compensation of other BDNF promoter activity for the promoter IV defect.

3. EET activation of BDNF promoters across ages

We further examined which promoters contributed to EET-induced BDNF transcription. We examined activity of 9 promoters by measuring mRNA levels of the immediately downstream BDNF exons I-IXa (Aid *et al.*, 2007, Liu *et al.*, 2005).

3-1. Basal levels of each BDNF exon—WT mice showed age-dependent decline of all BDNF exons except for exon V in the hippocampus, (ED vs. OA: at least $P<0.05$ for each, Supplementary Fig. 2 left). WT mice showed age-dependent decline of only exon I in the frontal cortex (OA vs. ED: $P<0.005$, Supplementary Fig. 2 right), which likely accounted for the age-dependent decline of total BDNF mRNA levels (exon IXc, Fig. 2 right). KIV mice showed age-dependent decline of only exons I in both hippocampus and frontal cortex (YA vs. OA: at least $P<0.05$, Supplementary Fig. 2). KIV mice, when compared to WT mice, showed reduced levels of almost all exons in both regions in all age groups (except VI in YA groups) (WT vs KIV: $P<0.05$, Supplementary Fig. 2), which reproduced previous findings in YA KIV mice (Martinowich *et al.*, 2011, Maynard *et al.*, 2016). Promoter IV-BDNF deficiency may reduce other promoter activity, regardless of age.

3-2. EET effects across ages—In the hippocampus, both WT and KIV mice showed significant EET effects for almost all exons (at least $P<0.05$, except for exon V in KIV mice, Supplementary Table 2). EET significantly increased levels of exons IIa, IIb, IIc, III, VI, VIIa, VIII, and IXa (EET vs. SCT: at least $P<0.05$) without any age or genotype effects (among ED, YA and OA or WT vs. KIV: $P>0.05$, Fig. 3 left), which indicated universal EET activation of the respective promoters. By contrast, EET effects on exons I and IV were age-

and genotype-specific; EET increased levels of exon I only in OA WT mice and exon IV only in ED WT mice (EET vs. SCT: $P<0.005$ for each, Fig. 3 left) with significant genotype differences (WT-EET vs. KIV-EET: at least $P<0.01$). The results indicated that EET activated promoter IV more effectively during early-life development than adulthood, but activated promoter I more effectively in aged stages in the hippocampus of normal mice.

In the frontal cortex, EET significantly increased levels of almost all exons (I, IIb, IIc, III, IV, VI, VIIa, and IXa) only in ED KIV mice (SCT vs. EET: $P<0.05$), where significant age effects were observed (KIV, ED vs. OA: $P<0.05$, Fig. 3 right). One exception was that EET increased levels of exons I in the OA WT group (SCT vs. EET: $P<0.05$) with a significant age effect (WT, ED vs. OA: $P<0.01$, Fig. 3 right). These results indicated that EET activated various BDNF promoters in the frontal cortex more effectively when provided during ED than in adulthood, only in promoter IV-BDNF deficient condition, while EET may compensate for age-dependent decline in total BDNF mRNA levels in normal aging by activating promoter I.

3-3. Lasting BDNF promoter activation by EET—Interestingly, after one month discontinuance of EET, only ED KIV mice showed significant increases of promoter-specific transcriptions of BDNF, exons IIc, VIIa, VIII, IXa in the hippocampus and exons I, IIb, VIIa, and VIII in the frontal cortex (EET-SCT vs. SCT-SCT: $P<0.05$), where significant age were observed (ED vs. YA/OA: at least $P<0.05$, Fig. 4). These results indicated sustained activity of several promoters after EET, only when EET was provided during early life, specifically in the promoter IV-deficient condition.

Discussion

Results of the present study demonstrated four major findings: 1) BDNF deficiency commonly led to anhedonia, normalized by EET, regardless of age and sex; 2) EET increased BDNF mRNA levels regardless of age and genotypes in the hippocampus but only during KIV ED and WT OA in the frontal cortex; 3) almost all BDNF promoters (I-IX) contributed to BDNF gene induction by EET for both brain regions, where age- and genotype-dependency was observed for promoters I and IV in the hippocampus and almost all promoters in the frontal cortex; and 4) the EET effects in increasing sucrose preference and promoter-specific BDNF transcription persisted only in KIV mice when EET was provided during early life (Fig. 5). Altogether, these findings partially supported our hypotheses: EET was equally effective in reversing anhedonia and inducing hippocampal BDNF levels, regardless of age, but was more effective during early life in inducing frontal cortex BDNF levels and for lasting anti-anhedonic and BDNF effects, particularly in promoter IV-BDNF deficiency. To our knowledge, this is the first study that showed effects of BDNF deficiency and EET on anhedonia and promoter-specific BDNF transcription across ages; many other studies, including ours, have shown these effects mostly in one age stage.

1. Anhedonia

Previous studies have shown that BDNF deficiency reduces sucrose preference in young-adult KIV males (Jha *et al.*, 2011, Sakata *et al.*, 2010) and CaMKII promoter-controlled

BDNF knockout females (Monteggia *et al.*, 2007). This study expanded these findings across ages for both males and females. KIV mice also show reduced exploratory activity and increased stress-induced despair at any age and sex (Jha *et al.*, 2016). Together, our results suggest that promoter IV-BDNF deficiency commonly causes depression-like behavior, regardless of age and sex.

Our findings of no sex difference in KIV (promoter IV-knockin) mice contrast with sex difference in depression-related behavior in BDNF-coding-region knockout mice (+/− and conditional) (Chourbaji *et al.*, 2008, Monteggia *et al.*, 2007). These knockouts retain promoter IV-driven BDNF expression: half in BDNF+/− mice and intact in regions/cells unaffected by the exogenous promoters (e.g., CaMKII). The BDNF loss in the knockouts may be insufficient to cause anhedonia, but is prone to do so when promoter IV-driven BDNF levels are further reduced under stress (Roth *et al.*, 2009, Tsankova *et al.*, 2006) and females may be more susceptible to stress (Autry *et al.*, 2009). BDNF deficiency in the endogenous-promoter IV-controlled regions/cells (e.g., hippocampal CA1 and prefrontal cortex pyramidal cells) is likely critical to cause depression-related behavior [detailed discussion in (Sakata *et al.*, 2010)].

In humans, depression affects anyone, although the prevalence of depression is sex- and age-dependent: depression is more common among females (5.1%) than males (3.6%), particularly around 60 years of age and in puberty-related teenage children (Who, 2017). Chronic mild stress has been shown to decrease sucrose preference in adult, but not juvenile, rats (Toth *et al.*, 2008). It is possible that the susceptibility to chronic stress, which decreases BDNF expression, may be age- and sex-dependent, whereas BDNF deficiency commonly causes depressive behavior. No age-related changes in sucrose preference were observed in normal WT mice in this study, similar to a previous finding by Tordoff *et al.* (2007), while age-dependent declines have been reported in >17 months-old rodents (Inui-Yamamoto *et al.*, 2017, Malatynska *et al.*, 2012).

In this study, EET reversed reduced sucrose preference caused by promoter IV-BDNF deficiency, regardless of age. This universal effect of EET in normalizing anhedonia was in contrast to our previous findings of an age-dependent effect of EET in increasing exploratory activity and decreasing stress-induced despair (Jha *et al.*, 2016). These findings together suggest that age dependency of EET efficacy differs among the types of depressive behavior.

Our results showed no sex difference in the EET effects on sucrose preference at any life stage. These results were similar to no sex difference in EET effects on exploratory activity and stress-induced despair (Chourbaji *et al.*, 2008, Jha *et al.*, 2016). These findings together suggest that EET is equally effective in both sexes for reducing depressive behavior caused by BDNF deficiency.

Interestingly, the significant increases in sucrose preference persisted only with early-life EET, similar to the lasting increases in exploratory activity and stress resilience with early-life EET (Jha *et al.*, 2016). These findings together suggest that EET should be started in early life for prevention of depressive behavior later in life.

2. BDNF gene induction by EET across ages

Previously, we had shown that EET-induced levels of BDNF protein were larger in ED than in adult groups (Jha *et al.*, 2016). We hypothesized that this age dependency might be due to more flexible transcriptional regulations earlier in life, which can last longer such as by epigenetic changes. Thus, this study further clarified EET effects on BDNF gene transcription across ages. Our results demonstrated that EET induction of total BDNF mRNA was observed regardless of ages and genotypes in the hippocampus, but only in ED KIV mice and OA WT mice in the frontal cortex. These results suggest that the age dependency of EET effects in increasing BDNF transcription differs among the brain regions. Early life is likely critical for EET to induce BDNF transcription in the frontal cortex particularly in promoter IV-BDNF deficiency. BDNF induction in the OA WT frontal cortex was likely due to the relatively reduced basal levels of BDNF in OA WT mice, suggesting a beneficial effect of EET on normal aging.

Our current results regarding BDNF mRNA levels contained a few discrepancies when compared to our previous results of BDNF protein levels (Jha *et al.*, 2016). First, EET induction of BDNF protein in the hippocampus was more prominent at early life than at old-adulthood (Jha *et al.*, 2016), but that of BDNF mRNA was relatively similar across ages in this study. One possibility is that EET also increases translation of the BDNF transcripts and/or decreases degradation of BDNF protein, and that the post-transcriptional effects in the hippocampus may be larger during early-life development than at old-adulthood. Second, basal levels of BDNF mRNA declined age-dependently in the frontal cortex of WT mice, but those of BDNF protein were similar across ages in our previous study (Jha *et al.*, 2016). The basal levels of BDNF protein may be protected by homeostatic post-translational regulation, such as reduced degradation of BDNF protein at aged stages.

3. Anhedonia related to hippocampal BDNF levels

EET effects in reversing reduced sucrose preference and inducing hippocampal BDNF mRNA were similarly observed across age, whereas EET effects in inducing frontal cortex BDNF mRNA were limited only at ED in KIV mice. These results suggest that BDNF induction by EET in the hippocampus, rather than the frontal cortex, is critical for reducing anhedonia. Taliaz *et al.* (2010) have shown evidence of hippocampal BDNF directly controlling anhedonia: hippocampus-specific BDNF knockdown reduced sucrose preference while its over-expression normalized a reduction of sucrose preference caused by chronic mild stress in rats. Our result showed increases in hippocampal BDNF but no change in sucrose preference in WT mice, which was also consistent with their findings in normal adult rats (Taliaz *et al.*, 2011).

4. BDNF promoter activity

This study demonstrated that almost all promoters (I-IX), rather than a limited number of promoters, contributed to the BDNF transcription induced by EET in both hippocampus and frontal cortex. These results correspond with the previous studies showing that EET drives multiple BDNF promoters in the hippocampus of YA animals (I, II, III, IV and V) (Adlard *et al.*, 2004, Jha *et al.*, 2011, Russo-Neustadt *et al.*, 2000, Zajac *et al.*, 2010), and expanded similar findings in earlier (ED) and later (OA) in life and in the frontal cortex. Our novel

findings were the age-dependent responses of promoters I and IV to EET in the hippocampus and almost all promoters in the frontal cortex. It should be noted that promoters I and IV are activity-dependent, i.e., activated by calcium influx in depolarized neurons (Shieh *et al.*, 1998, Tabuchi *et al.*, 2000, Tao *et al.*, 1998). While our present study measured static levels of BDNF transcripts without neuronal activity (e.g., by learning), EET may also affect neuronal activity-induced BDNF transcription driven by these promoters at specific life stages.

The present study showed that exon I was more effectively induced by EET in OA groups rather than in ED groups in both hippocampus and frontal cortex of WT mice. This result is in accordance with the recent finding by Neidl *et al.* (2016) that EET in 18–24 month old normal rats significantly increases BDNF transcription by promoter I but not by promoter IV or VI. Promoter I activation by EET involves increases in histone 3 acetylation on a proximal nuclear factor κB site in the promoter region (Neidl *et al.*, 2016). Promoter I can also be activated by inhibiting histone 3 trimethylation (H3K9me) (Snigdha *et al.*, 2016). The current study showed no promoter I activation (exon I) by EET in old-adult KIV mice. This result suggests that the system of nuclear factor κB site acetylation or demethylation at H3K9 to activate promoter I may be defective or interacted by promoter IV-BDNF deficiency.

The results of age-dependency on EET activation of specific BDNF promoters also suggest that EET may affect different types of behavior at different ages because of subregion-specific controls of BDNF promoters. Promoter I is more active in the dentate gyrus, while promoter IV is more active in the CA1 region of the hippocampus (Metsis *et al.*, 1993, Sakata, 2011, Zafra *et al.*, 1990). Therefore, EET activation of promoter I at OA or promoter IV at ED may increase BDNF levels in the dentate gyrus or the CA1, respectively, which can lead to different types of neural effects (e.g., neurogenesis, network activity) and behavior (e.g., pattern separation, learning and memory) (Buzsáki, 2006, Deng *et al.*, 2010, Kempermann *et al.*, 1997, Sakata *et al.*, 2013a, Santarelli *et al.*, 2003).

In the frontal cortex, EET-induced activity of almost all promoters was age-dependent, observed mostly in ED KIV mice. These results agree with the largest induction of total BDNF mRNA levels and BDNF protein levels in ED KIV mice in this study and in our previous study (Jha *et al.*, 2016). Defective promoter IV-driven BDNF transcription has been observed in the frontal cortex under early-life maltreatment (Roth *et al.*, 2009). Our findings suggest that EET implemented at early life, but not at the later time, is critical to compensate for promoter IV-BDNF deficiency by activating alternative promoters in the frontal cortex.

Furthermore, our results demonstrated that EET effects on promoter-specific BDNF induction in both the hippocampus and frontal cortex were mostly sustained only at early-life development in promoter IV-BDNF deficiency. This result highlights early life as a critical period to induce lasting BDNF transcription possibly due to the homeostatic compensational effects in promoter IV defect, while the EET effects may already be maximal in the normal condition (ceiling effects).

5. Why is EET at early-life development more effective than EET at later life in inducing lasting BDNF transcription in BDNF deficiency?

The exact reason is unknown, but one possibility is due to epigenetic programming (e.g., DNA and histone modifications) which is more drastic, flexible (Lister *et al.*, 2013), and long-lasting (Champagne & Curley, 2009, Lister *et al.*, 2013, Meaney & Szyf, 2005, Weaver *et al.*, 2004) during early-life development than in adulthood. Indeed, a dynamic demethylation at BDNF promoters I, II, IV, and VI has been reported from embryonic pallium to the neonate hippocampus at postnatal day 7 (Dennis & Levitt, 2005). The dynamic epigenetic programming may change BDNF transcriptional regulation and allow animals to adapt to the exposed environment by changing neuronal plasticity (Arai *et al.*, 2009).

Additionally, early-life EET involves mother-pup/infant social enrichment. The importance of mother-infant relations for lasting physiological changes has been proposed for over 60 years in both human (Johnson *et al.*, 1992, Winick *et al.*, 1975) and animal models (Levine, 1957, Meaney & Szyf, 2005, Weininger, 1954). Interestingly, mother-pup social enrichment is increased by their brief (~15 min) separation, which can occur as mothers engage in EET (e.g., running). The brief separation increases the licking and grooming of pups upon reunion (Liu *et al.*, 1997), and gentle touching increases the resistance of pups to physiological stress later in adulthood (Levine, 1957, Liu *et al.*, 1997). The offspring of mothers exhibiting high levels of pup licking present increased BDNF expression in the hippocampus at postnatal day 8 (Liu *et al.*, 2000), which likely involves epigenetic controls. Indeed, early social enrichments by communal nesting (where several mothers keep their litters together and share caregiving behavior) turns the epigenetic structure of the BDNF gene into a more active state in 5 months-old mice, increasing acetylated form of histone 3 at BDNF promoters I, IV, VI and VII in the hippocampus (Branchi *et al.*, 2011). The early-life EET used in the present study includes social enrichment by communal nesting, in addition to physical exercise (running wheels) and cognitive stimulation (toys), and activated almost all BDNF promoters in both hippocampus and frontal cortex of KIV mice. It is possible that the mother-pup interaction provides additional epigenetic modifications on these promoters (I, IV, VI, VII) to complement the epigenetic changes induced by exercise [I, II, III, and IV (Adlard *et al.*, 2005, Adlard *et al.*, 2004, Gomez-Pinilla *et al.*, 2011, Jha *et al.*, 2011, Russo-Neustadt *et al.*, 2000, Zajac *et al.*, 2010)] and cognitive stimulation [IV (Bredy *et al.*, 2007, Lubin *et al.*, 2008, Takei *et al.*, 2011)]. Epigenetic changes in these multiple BDNF promoters may last long to more effectively compensate for one promoter defect. Detailed epigenetic mechanisms involved in EET need to be further elucidated in the future. Targeting such mechanisms (e.g., epigenetic therapy on the promoter sites) that enable lasting BDNF transcription may become an alternative strategy to mimic lasting antidepressive and neurotrophic effects of EET, particularly for subjects with BDNF promoter IV defect.

6. Early-life EET as intervention for anhedonia and BDNF deficiency

Our data showed larger, long-lasting effects of early-life EET on anhedonia and BDNF induction in KIV mice, suggesting that EET was more effective in reversing the defects in behavior and BDNF levels, rather than in increasing these beyond the normal state. In real

life, EET may be more effective for people in adverse environments (e.g., isolation and stress), rather than enhancing normal environments. Our findings also provide an important notion of when to implement EET for its maximal and lasting effects. While promoter IV-BDNF deficiency is implicated in depression (Dwivedi *et al.*, 2003, Hing *et al.*, 2012, Keller *et al.*, 2010), epigenetic inactivation of promoter IV occurs by exposure to toxic environments, such as perinatal methylmercury (Onishchenko *et al.*, 2008), early-life maltreatment (Roth *et al.*, 2009), chronic social stress (Tsankova *et al.*, 2006), and maternal low-protein diet (Marwarha *et al.*, 2017). Defective promoter IV-driven BDNF transcription can transmit across generations via abnormal maternal behavior and depression (Roth *et al.*, 2009). To break this vicious cycle of promoter IV defect and mother-infant maltreatment, early-life EET is likely critical and can reduce risk of depression later in life. Early-detection of BDNF deficiency, such as by measuring promoter IV inactivity and related biomarkers, would aid in initiating early-life EET.

7. Limitation and future directions

The EET in this study combined physical, mental, and social activity, so it is unclear which components are responsible for the anti-anhedonic effects and BDNF induction. The EET also covered birth through maturation, so the precise critical period (e.g., gestation, before weaning, childhood, or adolescence) for the most effective EET also remains unclear. Future studies can determine a more precise period in early life when EET provides the largest effects. Our hypothesis is that EET is most effective when neurons rapidly proliferate, migrate, and mature and before neurons and synapses start to decrease by apoptosis and pruning. This study only measured static levels of BDNF transcription. It will be of interest to elucidate how EET affects BDNF transcriptional activity upon stimuli (new environments, learning training, etc.) and how this affects the development of neuronal network connectivity and more broad behavioral phenotypes related to depression (e.g., flexible learning, anxiety, susceptibility to stress and drug addiction, etc.). Although KIV mice have been extensively backcrossed (>12 times) to C57BL/6, 129Sv genes in the close vicinity of the BDNF gene may remain in KIV mice and might have affected the EET responsivity.

8. Conclusion

The present study aimed to identify the life period when EET is most effective for anhedonia and BDNF gene induction. Our results demonstrated that EET was equally effective in reversing anhedonia and inducing hippocampal BDNF transcription, regardless of age and BDNF deficient conditions, but was more effective during ED in inducing frontal cortex BDNF transcription, specifically in promoter IV-BDNF deficiency. Almost all BDNF promoters contributed to BDNF gene induction, where promoters I and IV in the WT hippocampus and almost all promoters in the KIV frontal cortex showed age and genotype dependency. Most promoter activity and anti-anhedonic effects persisted when EET was provided during early life, particularly in promoter IV-BDNF deficiency. The results suggest that EET is a universal intervention against anhedonia across ages and that early-life EET is a strategy for lasting intervention against anhedonia and BDNF deficiency.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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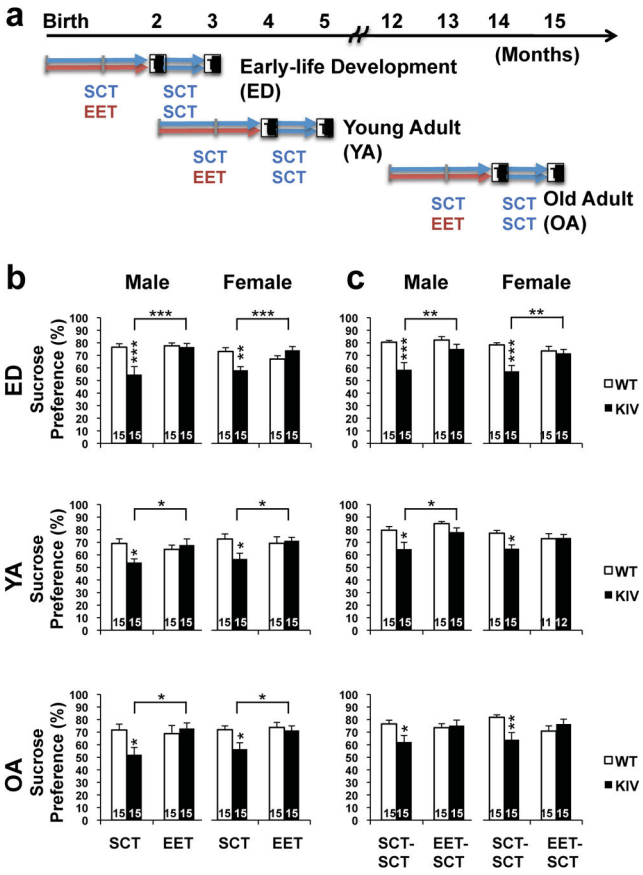

Fig. 1.

a. Research design WT and KIV mice received 2 months of enriched environment treatment (EET; red arrows) or standard condition treatment (SCT: blue arrows) from birth (early-life development: ED), 2 months of age (young-adult: YA), or 12 months of age (old-adult: OA), then received 1 month of SCT. Anhedonia-like behavior and BDNF transcript levels were measured after 2 months of EET (test 1: T_1) and consequent 1 month of SCT (test 2: T_2). Reused from Jha et al. Transl. Psychiatry 2016. **b and c. Effects of BDNF deficiency and EET on sucrose preference across ages** tested after EET (b, SCT/EET, at T_1), then after 1 month of SCT (c, SCT-SCT/EET-SCT, at T_2). Asterisks on the bars show a significant difference between genotypes. Note that all KIV groups showed reduced sucrose preference and that EET reversed it regardless of age and sex, which lasted 1 month after discontinuance with significant increases in KIV ED males and females and YA males. N=11–15 per group (shown at the bar). *P<0.05, **P<0.01, ***P<0.005.

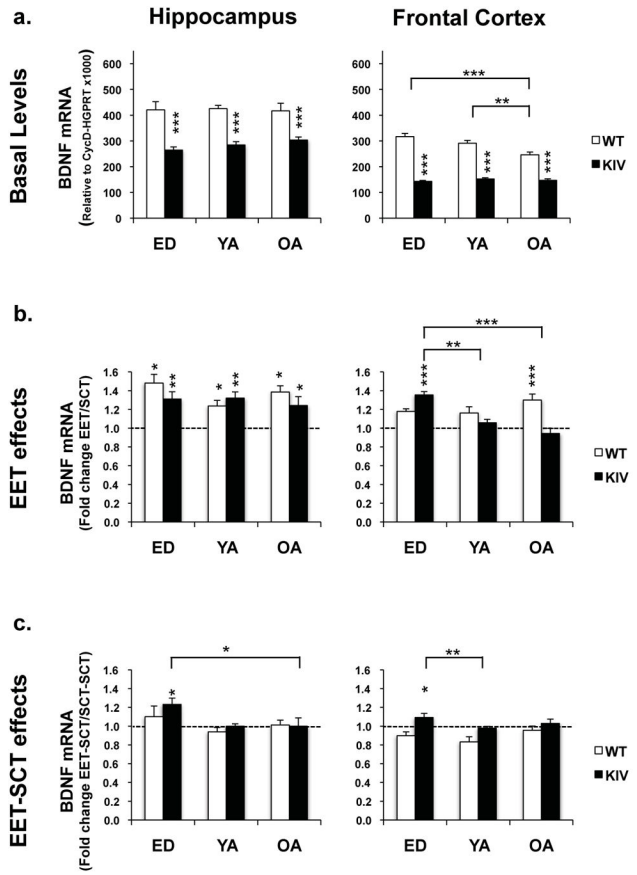

Fig. 2.

Total BDNF mRNA levels (the protein-coding exon IXc) in the hippocampus (left) and frontal cortex (right) at early-life development (ED), young-adult (YA), and old-adult (OA). **a. Basal levels** in standard conditions. Asterisks on the columns show significant differences between genotypes. N=14–16 per group. **b. EET effects** shown by fold changes of BDNF levels in mice with EET divided by those with SCT. Asterisks on the columns show significant BDNF induction in EET group compared to SCT group. N=6–8 per group. **c. Lasting effects of EET** shown by fold changes of BDNF levels in mice with EET and consequent SCT (EET-SCT) divided by levels in SCT mice (SCT-SCT). N=6–8 per group. * $P<0.05$; ** $P<0.01$; *** $P<0.005$.

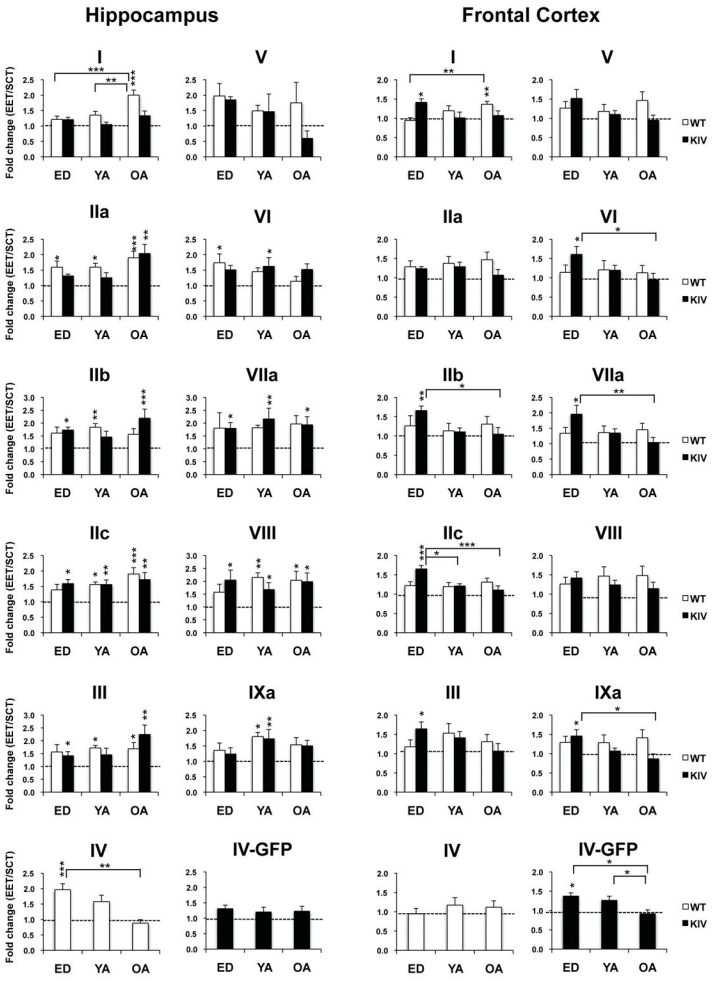

Fig. 3. EET effects on BDNF transcription by 9 promoters across ages

The data show fold changes of exon levels in mice with EET divided by those in mice with SCT in the hippocampus (left) and frontal cortex (right). Asterisks on the columns show significant changes in levels between EET groups vs. SCT groups. ED, early-life development; YA: young-adult; OA: old-adult. N=6–8 per group. **P*<0.05; ***P*<0.01; ****P*<0.005.

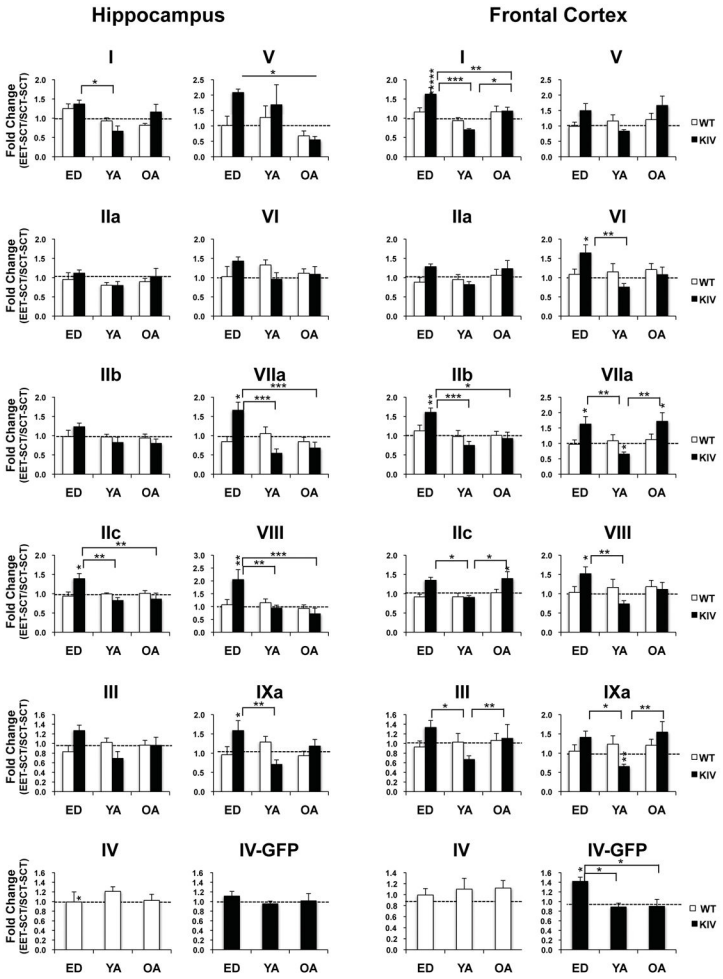

Fig. 4. Lasting effects of EET on promoter-specific BDNF transcription across ages

The data show fold changes of exon levels in mice with EET and subsequent SCT divided by those with SCT-SCT in the hippocampus (left) and frontal cortex (right). Asterisks on the columns show significant changes in levels between EET-SCT vs. SCT-SCT groups. Note the sustained induction of some exons after EET discontinuance particularly in ED KIV mice in both regions. N=6–8 per group.

**P*<0.05; ***P*<0.01; ****P*<0.005.

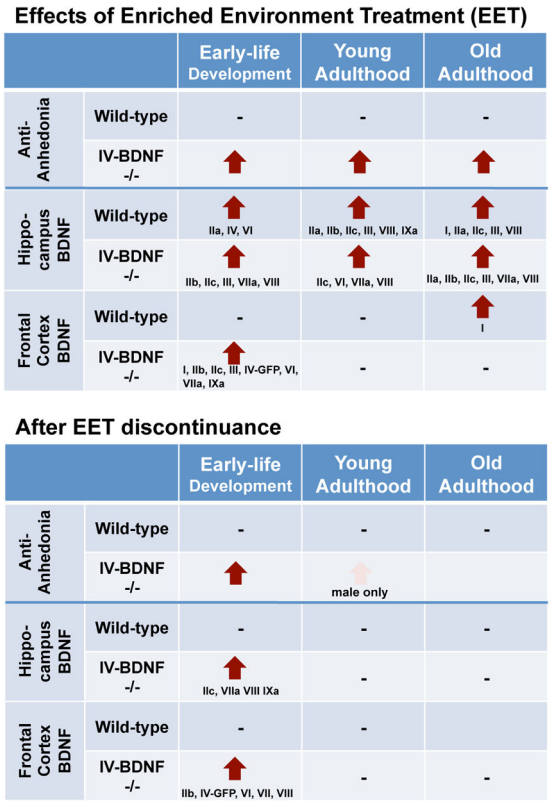

Fig. 5. Effects of EET on anhedonia and BDNF transcription across ages

The arrows show significant increases in sucrose preference and total BDNF mRNA levels. Promoter-specific BDNF transcripts are shown in Roman numbers.